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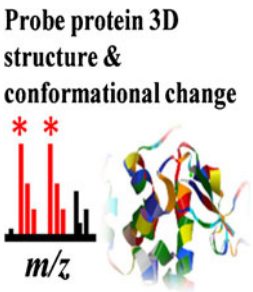
J. Am. Soc. Mass Spectrom. (2016) 27:864–875
DOI: 10.1007/s13361-016-1356-6

RESEARCH ARTICLE

Probing Protein 3D Structures and Conformational Changes Using Electrochemistry-Assisted Isotope Labeling Cross-Linking Mass Spectrometry

Qiuling Zheng,[1] Hao Zhang,[2] Shiyong Wu,[1] Hao Chen[1]

[1]Center for Intelligent Chemical Instrumentation, Department of Chemistry and Biochemistry, Edison Biotechnology Institute, Ohio University, Athens, OH 45701, USA
[2]Department of Chemistry, Washington University, St. Louis, MO 63130, USA



Abstract. This study presents a new chemical cross-linking mass spectrometry (MS) method in combination with electrochemistry and isotope labeling strategy for probing both protein three-dimensional (3D) structures and conformational changes. For the former purpose, the target protein/protein complex is cross-linked with equal mole of premixed light and heavy isotope labeled cross-linkers carrying electrochemically reducible disulfide bonds (i.e., DSP-d_0 and DSP-d_8 in this study, DSP = dithiobis[succinimidyl propionate]), digested and then electrochemically reduced followed with online MS analysis. Cross-links can be quickly identified because of their reduced intensities upon electrolysis and the presence of doublet isotopic peak characteristics. In addition, electroreduction converts cross-links into linear peptides, facilitating MS/MS analysis to gain increased information about their sequences and modification sites. For the latter purpose of probing protein conformational changes, an altered procedure is adopted, in which the protein in two different conformations is cross-linked using DSP-d_0 and DSP-d_8 separately, and then the two protein samples are mixed in 1:1 molar ratio. The merged sample is subjected to digestion and electrochemical mass spectrometric analysis. In such a comparative cross-linking experiment, cross-links could still be rapidly recognized based on their responses to electrolysis. More importantly, the ion intensity ratios of light and heavy isotope labeled cross-links reveal the conformational changes of the protein, as exemplified by examining the effect of Ca^{2+} on calmodulin conformation alternation. This new cross-linking MS method is fast and would have high value in structural biology.

Keywords: Protein 3D structure, Protein conformational change, Isotope-labeling, Chemical cross-linking, Mass spectrometry, Electrochemistry

Received: 18 October 2015/Revised: 25 January 2016/Accepted: 28 January 2016/Published Online: 22 February 2016

Introduction

Cross-linking MS has emerged as a powerful method to provide information of protein 3D structures and to probe protein–protein interactions (PPIs) [1–3]. By introducing a cross-linker with a defined length, functional groups of amino acids within the distance can be permanently modified. The modification sites from cross-linking reactions can be determined by MS, which suggest where spatially defined interactions occur and help build up the distance geometry of a protein complex. Although it is regarded as a low resolution approach compared with X-ray or NMR, chemical cross-linking MS still has significant advantages for protein 3D structural analysis: (1) the size of sample under analysis is theoretically unlimited; (2) MS analysis is generally fast and requires a small amount of sample; and (3) it is possible for measuring in-solution protein 3D structural analysis [1]. However, despite the advances of chemical cross-linking MS, there are still grand challenges hampering this approach. First, due to the complex peptide mixture produced by enzymatic digestion of the cross-linked protein complex, the identification of target cross-link products among the mixture is the bottleneck of this approach and difficulties increase with the protein size [4]. Second, in the presence of cross linkages, cross-link product

Dedicated to Professor R. Graham Cooks on the occasion of his 75th birthday.

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1356-6) contains supplementary material, which is available to authorized users.

Correspondence to: Hao Chen; *e-mail:* chenh2@ohio.edu

ion fragmentation patterns become irregular and unexpected, which makes data analysis challenging [4]. Cleavage of cross-links prior to MS/MS yields linear peptides, which would be much easier to sequence. In order to solve these problems, elegant methodologies have been proposed and reported, which employs cross-linkers carrying isotope [5, 6], fluorescence [7] and affinity tags [8], or cleavable cross-linkers [9–14]. However, new strategies in this regard are still in need.

The combination of electrochemistry (EC) with MS (i.e., EC/MS) started over 40 y ago, for the reason that MS could serve as a sensitive detector for electrolysis and provide molecular weight and structural information about products and intermediates of electrochemical reactions [15, 16]. Major applications of EC/MS include differential electrochemical mass spectrometry (DEMS) study [17], drug metabolism mimicking [18], protein tagging [19], and protein oxidation cleavages [20–22]. In our laboratory, desorption electrospray ionization (DESI) [23] is used as the interface to couple EC with MS to explore EC/MS applications in proteomics and to study protein/peptide electrochemistry [24–33], with focus on protein disulfide bond reduction reactions. Very recently, an innovative EC/DESI-MS coupling, involving a "waterwheel" working electrode, was reported by Zare et al., which enabled the detection of fleeting electrochemically generated intermediates with lifetime down to microseconds [34–36].

Previously, we reported a fast approach [37] using electrochemical MS to analyze chemically cross-linked proteins or protein complexes involving electrochemically reducible cross-linkers such as dithiobis[succinimidyl propionate] (DSP). In the experiment [37], DSP-cross-linked proteins were first subjected to enzymatic digestion and the resulting digest underwent electrolytic reduction and online MS (and MS/MS) analysis. It showed the electrochemical reduction of cross-links gave rise to linear reduced peptides, facilitating the subsequent MS/MS structural analysis. Upon electrolysis, cross-links had a large relative intensity decrease, which helped the quick identification of cross-links in the protein digest. In this study, we further integrate the isotope labeling strategy with electrochemical cross-linking MS to probe both protein 3D structures and conformational changes, using a pair of light and heavy isotope labeled DSP (DSP-d_0 and DSP-d_8, structures shown in Scheme 1a) as cross-linkers. There are a couple of reasons for introducing isotope labeling strategy. First, besides the intensity change upon electroreduction, the appearance of doublet isotopic peaks provides an additional dimension of specificity for the cross-link identification. In particular, under some circumstances where the target protein contains native disulfide bonds, the differentiation of cross-links from peptides carrying native disulfide bonds (from the digestion of the target protein) is possible, as the former carries characteristic doublet peaks resulting from isotope labeling. In addition, the isotope labeling helps to identify reduced peptide products from electrolysis and their cross-linking patterns. Second, a comparative cross-linking experiment [6, 38, 39] can be developed for monitoring protein conformational changes due to a stimulus. In this case, a protein in two different conformations (e.g., calmodulin with or without Ca^{2+} in this study) are cross-linked using DSP-d_0 and DSP-d_8 separately (see illustration in Scheme 2). The resulting cross-linked protein samples are merged in the equal molar ratio (1:1) and subjected to digestion and EC/MS analysis to determine cross-links and their structures. The intensity ratios of cross-link isotopic peaks could reveal the conformational changes of the target protein. In this study, peptide SLIGKV-NH_2, somatostatin-14, α-lactalbumin (α-LA), and calmodulin-mastoparan complexes were chosen as test samples to validate our method. The results show that the isotope-labeling strategy is compatible with electrochemical cross-linking MS and extends the application of our method for probing protein 3D structures, protein–substrate interactions, and protein conformational changes.

Experimental

Chemicals

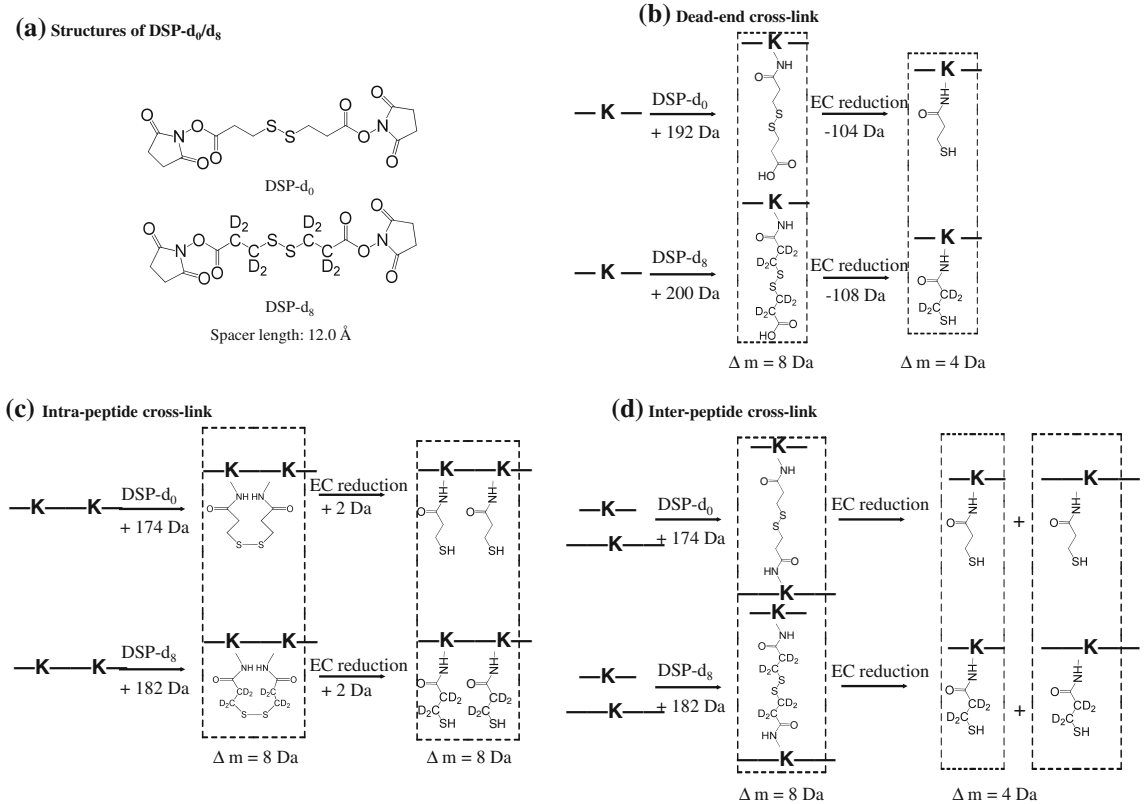
Mastoparan was purchased from American Peptide (Sunnyvale, CA, USA). Phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), SLIGKV-NH_2, α-lactalbumin from bovine milk, and calmodulin bovine were all purchased from Sigma-Aldrich (St. Louis, MO, USA). DSP-d_0 was bought from Thermo Scientific (Grand Island, NY, USA) and DSP-d_8 was purchased from ProteoChem (Loves Park, IL, USA). Deionized water used for sample preparation was obtained from a Nanopure Diamond Barnstead purification system (Barnstead International, Dubuque, IA, USA). HPLC-grade methanol was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid (FA) was purchased from Spectrum Chemical Mfg. Corp (Gardena, CA, USA).

Cross-Linking for Probing Protein 3D-Structures

DSP-d_0 and DSP-d_8 were dissolved in DMSO and premixed in 1:1 molar ratio. Then, the premixed DSP-d_0/d_8 was added to proteins or protein complex samples dissolved in PBS buffer (pH 7.4) at molar ratio of 20:1 (DSP:protein) (except in the case of α-LA, 2.5:1 (DSP:protein) was applied as α-LA formed aggregation when the added DSP was more than 2.5-fold excess), and incubated at room temperature for 1 h to effect the cross-linking reaction. Then, 250 mM NH_4HCO_3 was added to adjust pH to 8 to quench the reaction. Sample desalting was performed by using a 5000 MW cutoff filter and the resulting mixture was redissolved in 25 mM NH_4HCO_3 aqueous solution. Trypsin was added at a protein:enzyme ratio of 25:1 for overnight digestion (17–20 h). C18 ZipTip was used for further desalting and enrichment. The sample was re-dissolved in ACN:H_2O (v/v 60:40) containing 1% FA before EC/MS analysis.

Comparative Cross-Linking for Probing Protein Conformational Changes

Two hundred μL of 40 μM calmodulin was reacted with 16 μL of 10 mM DSP-d_0 dissolved in DMSO. Another 200 μL of 40 μM calmodulin containing 80 μM $CaCl_2$ was reacted with 16 μL of 10 mM DSP-d_8 dissolved in DMSO. Both solutions



Scheme 1. **(a)** Structures of DSP-d_0 and DSP-d_8; **(b)–(d)** possible cross-linking reaction products and their conversion upon electrochemical reduction

were incubated under room temperature for 1 h to effect the cross-linking reaction, and then 250 mM NH_4HCO_3 was added to adjust pH to 8 so that the reaction would be quenched. Two solutions were mixed at protein molar ratio of 1:1 and sample desalting was performed using a 5000 MW cutoff filter. The resulting sample was redissolved in 25 mM NH_4HCO_3 aqueous solution and subjected to overnight trypsin digestion (17–20 h) at a protein:enzyme ratio of 25:1. C18 Ziptip was used for further desalting and enrichment. Sample was redissolved in ACN:H_2O (v/v 60:40) containing 1% FA prior to EC/MS analysis.

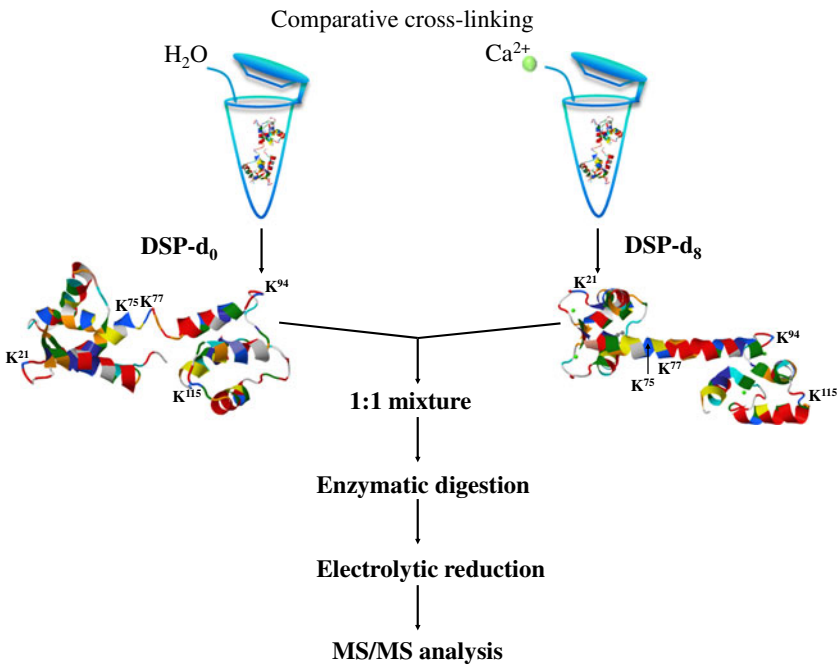
Apparatus

All data were acquired using a Waters Xevo QTOF mass spectrometer (Milford, MA, usa). A μ-PrepCell thin-layer electrochemical flow cell (Antec BV, Leyden, The Netherlands) was coupled with the mass spectrometer by liquid sample DESI (Scheme S-1, Supporting Information), which was previously described in details [27]. The electrochemical flow cell was equipped with a magic diamond electrode (12 mm × 30 mm, Antec BV) as the working electrode (WE), and a Roxy potentiostat (Antec BV) was employed to apply a reduction potential to the WE. A sample syringe was connected with the cell by a piece of PEEK capillary tube. The sample solution was injected at a flow rate of 5 μL/min and flowed through the cell for electroreduction. The reduced species flowed out of the cell via a piece of fused silica capillary and underwent ionization by DESI via the interaction with charged microdroplets from DESI spray. H_2O:MeOH (v/v 50:50) containing 1% FA was used as the DESI spray solvent and injected at a flow rate of 5 μL/min. A high voltage of +5 kV was applied to the DESI spray probe with nebulization gas of N_2 (pressure 170 psi). Collision induced dissociation (CID) was carried out for ion structural analysis, in which a wide mass selection window was used for simultaneous selection of both DSP-d_0 and DSP-d_8 labeled cross-link ions or a pair of the reduced cross-link ions.

Results and Discussion

DSP has specific reactivity towards amines such as lysine residues or N-termini of proteins/peptides. Dead-end, intra-peptide, or inter-peptide cross-links (structures are shown in Scheme 1b–d) could be formed with characteristic mass increment after the DSP reactions with proteins/peptides.

With the addition of 1:1 DSP-d_0 and DSP-d_8, all three types of cross-links would display doublet peaks with mass interval of 8 Da, whereas their reduced products would have distinguishing mass interval changes (Scheme 1b–d). Upon electrolytic reduction, the mass for dead-end cross-links would decrease by 104/108 Da corresponding to the loss of $SCH_2CH_2COOH/SCD_2CD_2COOH$ and the addition of one H atom (Scheme 1b). The electrolytic reduction of intra-peptide cross-links will result in the formation of adjacent reduced



Scheme 2. Comparative cross-linking of calmodulin to probe the effect of Ca^{2+} on its conformational change; the calmodulin structure in the aqueous solution without Ca^{2+} is from PDB: 1CFD and the calmodulin structure in the aqueous solution in the presence of Ca^{2+} is from PDB: 1CLL

products with +2 Da mass shift (Scheme 1c). As the cleavage of intra-peptide disulfide bond would not have any tag loss or chain separation, the mass interval for the doublet reduced peptide peaks would remain 8 Da (Scheme 1c). The inter-peptide cross-links would yield two separate peptide chains due to the cleavage of the linkage disulfide bond originating from DSP, and each pair of product peptide doublet peaks displays 4 Da mass interval. To validate the feasibility of combining electrochemistry and isotope labeling strategy with cross linking MS in this study, peptide SLIGKV-NH_2 was first chosen as a test sample.

Peptide

Figure 1a shows the DESI-MS spectrum of the cross-linked peptide SLIGKV-NH_2. Besides the protonated SLIGKV-NH_2 detected at *m/z* 615.4, the ions of two cross-links, P1 and P2, are observed at *m/z* 789.4/797.4 (DSP-d_0/d_8 labeled species) and *m/z* 807.4/815.4 (Figure 1a), respectively. Compared with the singly charged intact peptide ion, two cross-link ions show obvious doublet peaks with mass interval of 8 Da (e.g., Figure 1a inset shows the zoomed-in P1 ion isotopic peaks). On the basis of the measured masses, types of cross-links are determined rapidly. The 174.0 Da mass difference between the intact peptide (measured mass: 614.4 Da) and P1 (measured mass: 788.4 Da) indicates P1 as an intra-peptide cross-link. Likewise, the measured mass of P2 of 806.4 Da shows a 192 Da mass increment in comparison to the intact peptide, which suggests P2 as a dead-end cross-linking product. Figure 1b shows that, upon electrolytic reduction, two cross-links suffered a large relative intensity drop by –46.1% and –75.6%, respectively (using the intact peptide ion at *m/z* 615.4 as reference, Table S-1, Supporting Information), while new reduced peptide ions appeared at *m/z* 703.4/707.4 and *m/z* 791.4/799.4. The newly generated peptides can be related to their precursors according to their characteristic mass changes. The +2.0 Da mass increment indicates that P1 (788.4 Da) is the precursor of the reduced peptide $S^{\blacktriangle}LIGK^{\blacktriangle}V$-$NH_2$ (790.4 Da, $^{\blacktriangle}$ denotes one reduced tag of $-C(O)CH_2CH_2SH$). Interestingly, the ion of $S^{\blacktriangle}LIGK^{\blacktriangle}V$-$NH_2$ also displays doublet peak characteristics in Figure 1b, indicating that the DSP-d_0 and DSP-d_8 labeled P1 underwent reduction to the same extent. The mass change of –104.0 Da between P2 (806.4 Da) and the reduced peptide $S^{\blacktriangle}LIGKV$-NH_2 (702.4 Da) suggests the former to be the precursor of the latter. As expected, the ion of the reduced peptide $S^{\blacktriangle}LIGKV$-NH_2 also remains doublet peaks in Figure 1b with a mass interval of 4 Da, consistent with the fact that P2 is a dead-end cross-link.

The modification site can be determined using CID MS/MS analysis. Figure 1c shows CID spectrum of the singly charged ion of P1 (*m/z* 789.4/797.4) in which a modified fragment ion $b_5^{\blacksquare}$ ($^{\blacksquare}$ denotes the intra-peptide cross-linking modification tag of $-C(O)CH_2CH_2SSCH_2CH_2C(O)$-) is observed. Owing to the linkage, the missing fragmentation between the first to fifth residues suggests the cross-linking takes place between the N-terminus and the lysine residue. Furthermore, CID of the reduced P1 ion (*m/z* 791.4/799.4) yields fragment ions $b_3^{\blacktriangle}$, $b_4^{\blacktriangle}$, and $b_5^{\blacktriangle\blacktriangle}$, which verifies that the modification positions are located at the N-terminus and the

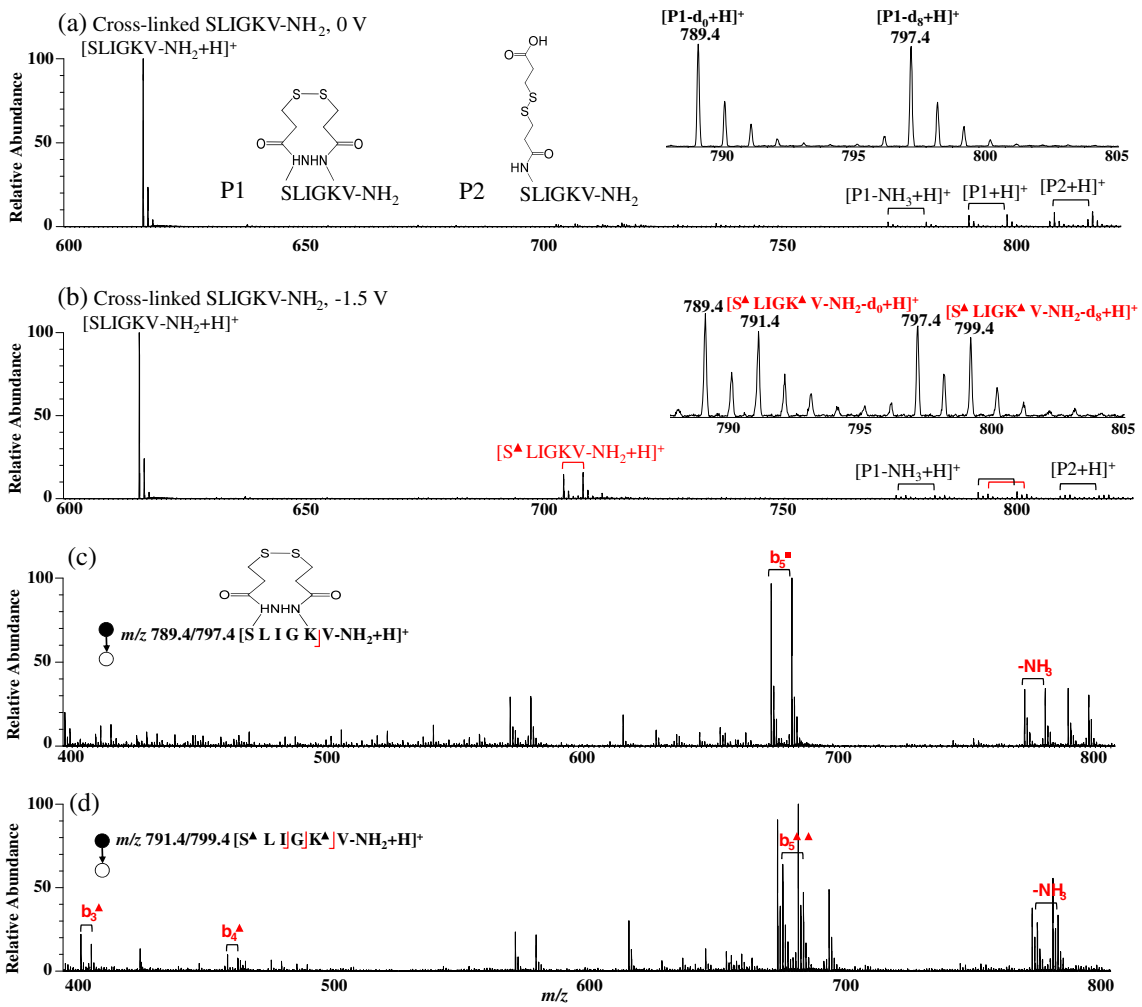


Figure 1. DESI-MS spectra of cross-linked peptide SLIGKV-NH_2 when the cross-linked peptide was flowed through the electrochemical cell with the applied potential of **(a)** 0 V and **(b)** –1.5 V; CID MS/MS spectra of **(c)** $[P1 + H]^+$ (*m/z* 789.4/797.4) and **(d)** the reduced P1 ion $[S^{\blacktriangle}LIGK^{\blacktriangle}V\text{-}NH_2 + H]^+$ (*m/z* 791.4/799.4). Inset in **(a)** shows the structures of P1 and P2, and zoomed-in spectrum of P1 ion; inset in **(b)** shows zoomed-in spectrum of the reduced P1 ion

fifth lysine residue. In addition, the comparison of CID data of the intact and reduced P1 ions shows that the electrolytic reduction helps to obtain more sequence information as more cleavage sites are seen for the reduced P1 ion. Likewise, Figure S-1 (Supporting Information) shows CID MS/MS spectrum of the singly charged P2 ion (*m/z* 807.4/815.4). The observation of a series of modified fragment ions b_2', $b_2\text{-}H_2O'$, b_3', b_4', b_5', and $b_5\text{-}H_2O'$ (' denotes the dead-end modification tag $-C(O)CH_2CH_2SSCH_2CH_2COOH$), which determines the modification position to be on the N-terminus. As SLIGKV-NH_2 is a small peptide, it is reasonable that both of its N-terminus and the fifth lysine residue are solvent accessible and readily modified by DSP.

With the examination of the first simple peptide, it appears that the isotope labeling can be compatible with electrochemical cross-linking MS. The light and heavy isotope labeled cross-links can undergo electroreduction and the resulting reduced peptides are useful for peptide sequencing and determination of the modification sites. With this preliminary test, proteins and protein complexes are further tested.

α-Lactalbumin

Protein α-LA, a protein carrying four native disulfide bonds, was also chosen for cross-linking reaction with a pair of DSP-d_0 and DSP-d_8. Figure 2a shows the MS spectrum of tryptic digest of the cross-linked α-LA, among which 11 unmodified peptide ions are easily determined based on the known sequence of α-LA (shown as peaks 1–5, 7–10, 14, and 15 in Figure 2b).

Upon the electrolytic reduction, six peptide ions $[P4 + 3H]^{3+}$ (*m/z* 600.3), $[P6 + H]^+$ (*m/z* 810.3/818.3), $[P3 + 2H]^{2+}$ (*m/z* 842.8), $[P4 + 2H]^{2+}$ (*m/z* 899.4), $[P5 + 2H]^{2+}$ (*m/z* 1132.5), and $[P7 + H]^+$ (*m/z* 1522.7/1530.7) have large relative intensity decreases (–20.2% to –100%, using the base peak 10 corresponding to the disulfide-free peptide ion $[VGINYW + H]^+$ as reference, Table S-2, Supporting Information), indicating that

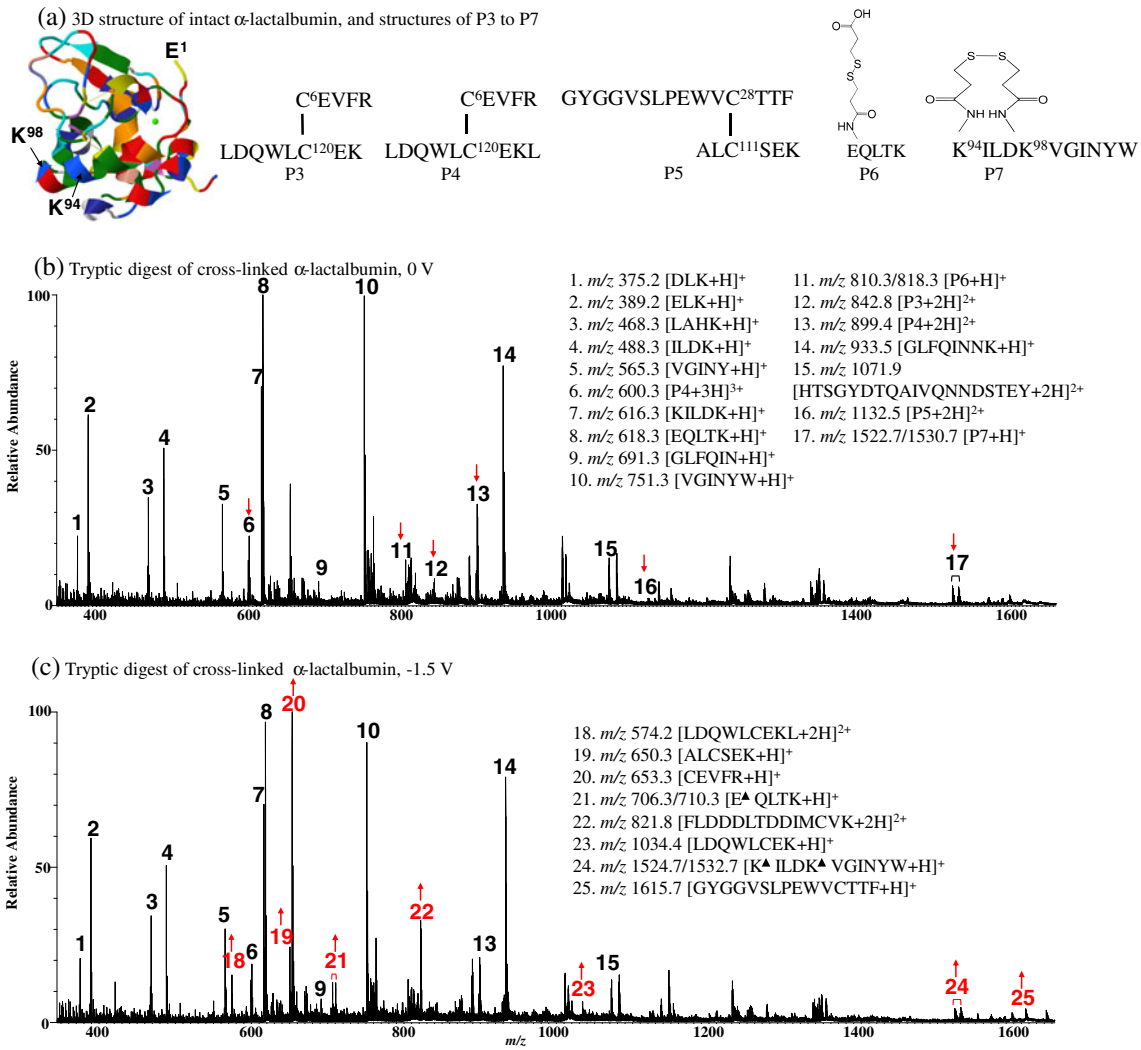


Figure 2. **(a)** 3D structure of α-lactalbumin (α-LA) (PDB 1HFZ), and structures of P3 to P7; DESI-MS spectra of tryptic digest of cross-linked α-LA with an applied potential of **(b)** 0 V; and **(c)** –1.5 V

they are either from cross-links or peptides containing native disulfide bonds. However, P3, P4, and P5 are considered to be native disulfide bond-carrying peptides as their ions have no doublet peaks (zoomed-in P3 ion peak is shown in Figure S-2a, Supporting Information). In contrast, P6 and P7 ions show doublet peaks with 8.0 Da mass intervals, suggesting them as cross-link products (zoomed-in P7 ion is shown in Figure S-2b, Supporting Information).

MS/MS analysis of these peptide ions and their reduced peptide ions provides their structure information. For instance, P3 is determined to be the precursor peptide of CEVFR and LDQWLCEK, which are bridged via a native inter-chain disulfide bond linking the first residue of CEVR and the sixth residue of LDQWLCEK (Figure S-3, see discussion in Supporting Information). After electrolytic reduction, P6 ion has a 100% intensity drop. The cleavage of disulfide bond results in 104/108 Da loss to form a new product ion at *m/z* 706.3/710.3, which indicates P6 as a dead-end cross-link. Upon CID, the new product ion at *m/z* 706.3/710.3 produces modified fragment ions $b_2^{\blacktriangle}$, $b_3^{\blacktriangle}$, and $b_4^{\blacktriangle}$ and unmodified fragment ions y_1, y_2, y_3, and y_4 (Figure S-4, Supporting Information), which determines the reduced peptide sequence to be EQLTK. Therefore, P6 is the DSP-modified EQLTK with the modification position at the first glutamic acid residue of the peptide (i.e., the N-terminus of α-LA). Likewise, the intensity of $[P7 + H]^+$ (*m/z* 1522.7/1530.7) drops by 20.2% upon electrolytic reduction while a newly generated peptide ion at *m/z* 1524.7/1532.7 with 2.0 Da mass increment appears, suggesting that P7 is an intra-peptide cross-link. CID spectrum of $[P7 + H]^+$ (*m/z* 1522.7/1530.7) yields a series of modified fragment ions $b6^{\blacksquare}$, $b7^{\blacksquare}$, $b8^{\blacksquare}$, $b9^{\blacksquare}$, and $b10^{\blacksquare}$ (Figure S-5a, Supporting Information) which indicates the C-terminal sequence to be GINYW (seventh to eleventh residues). Based on this unique partial sequence and the known sequence of α-LA, P7 can be determined as cross-linked KILDKVGINYW with the modification sites located on the first and fifth lysine residues (i.e., the 94th and 98th lysine residues of α-LA). This assignment can be verified by observing the generation of fragment ions $y_8^{\blacktriangle}$, $y_9^{\blacktriangle}$, $b_6^{\blacktriangle\blacktriangle}$, $b_7^{\blacktriangle\blacktriangle}$, $b_8^{\blacktriangle\blacktriangle}$, $b_9^{\blacktriangle\blacktriangle}$, and $b_{10}^{\blacktriangle\blacktriangle}$ from CID spectrum of its reduced product ion $[K^{\blacktriangle}ILDK^{\blacktriangle}VGINYW+H]^+$ (*m/z*

1524.7/1532.7, Figure S-5b, Supporting Information). Note that P7 arose from unspecific cleavage of the C-terminal of tryptophan residue (W) by trypsin in this case, which is possibly caused by the modification of the protein α-LA by DSP. In some cases when the lysine residues are modified, they would not become the cleavage sites for trypsin, which could lead to unspecific cleavages [1, 40–42]. Based on the structural analyses of P6 and P7, it is clear that the 94th and 98th lysine residues among 12 lysine residues of α-LA and *N*-terminus of α-LA are exposed on the surface of the protein and the most solvent accessible. The distance (6.25 Å) between the 94th and 98th lysine residues are also within the length of DSP (space arm 12 Å) so that they can be cross-linked, which is in agreement with previous reports [43, 44].

With the assistance of electrolytic reduction and isotope-labeled cross-linkers, target cross-links are identified from peptide mixture with high confidence, based on relative intensity drop and doublet peak characteristics. Further MS/MS analysis of resulting linear peptide ions help sequence the peptide and map the cross-linking bridges.

Calmodulin–Mastoparan Complex

This method was also applied to probe protein–substrate interaction and the calmodulin–mastoparan complex was chosen as a test sample. In the MS spectrum of trypsin digest of cross-linked calmodulin–mastoparan complex, two unmodified peptide ions $[ALAALAK + H]^+$ and $[ELGTVMR + H]^+$ are easily identified (Figure 3a). Upon electrolytic reduction, three peptide ions, $[P8 + 2H]^{2+}$ (*m/z* 974.1/978.1), $[P9 + 2H]^{2+}$ (*m/z* 1276.1/1280.1), and $[P10 + 2H]^{2+}$ (*m/z* 1510.2/1514.2) are recognized as cross-links due to their large relative intensity drops (–30.9% to –100% using disulfide free peptide ion $[ELGTVMR + H]^+$ as reference, Table S-3, Supporting Information) and their characteristic doublet peaks. Three newly generated peptide ions, $[I^{\blacktriangle}NLK+H]^+$ (*m/z* 575.4/579.4), $[VFDK^{\blacktriangle}DGNGYISAAELR + 2H]^{2+}$ (*m/z* 922.1/924.1), and $[HVMTNLGEK^{\blacktriangle}LTDEEVDEMIR + 2H]^{2+}$ (*m/z* 1224.0/1226.0), are determined as reduced products from cross-links as they are newly generated and also display doublet signals (Figure 3b). Precursors and their reduced products can be linked by their mass relationships and structural analysis can be carried out using tandem MS analysis.

In particular, the two peptide ions, $[I^{\blacktriangle}NLK + H]^+$ (*m/z* 575.4/579.4) and $[HVMTNLGEK^{\blacktriangle}LTDEEVDEMIR + 2H]^{2+}$ (*m/z* 1224.0/1226.0), correspond to two reduced peptides whose mass sum (measured mass: 3020.4 Da) is +2.0 Da greater than the mass of P10 (measured mass: 3018.4 Da). This mass relationship indicates P10 as an inter-peptide cross-link of INLK and HVMTNLGEKLTDEEVDEMIR. Upon CID (Figure S-6b, Supporting Information), $[HVMTNLGEK^{\blacktriangle}LTDEEVDEMIR + 2H]^{2+}$ (*m/z* 1224.0/1226.0) yields fragment ions y_2, y_4, y_5, y_7,

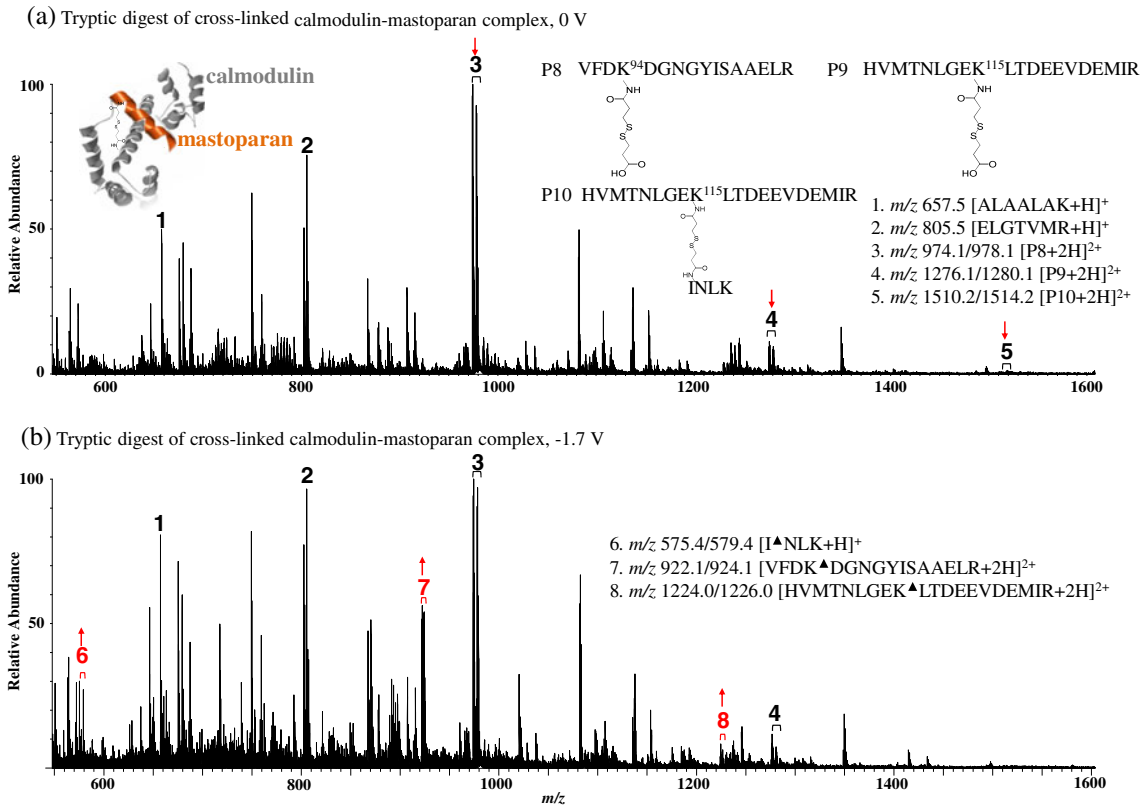


Figure 3. DESI-MS spectra of tryptic digest of cross-linked calmodulin–mastoparan complex with an applied potential of **(a)** 0 V; and **(b)** –1.7 V. Inset in **(a)** illustrates 3D structure of the calmodulin–mastoparan complex

y_8, y_9, y_{10}, y_{11}, $y_{12}^{\blacktriangle}$, $y_{14}^{\blacktriangle}$, $y_{15}^{\blacktriangle}$, a_2, b_2, b_3, b_3-H_2O, b_4, b_4-NH_3, b_5, b_6, and b_8. The unmodified fragment ion y_{11} and the modified fragment ion $y_{12}^{\blacktriangle}$ manifest that the modification position is at the 12th lysine residue of this peptide (i.e., the 115th lysine residue of calmodulin). The most sequence for the reduced peptide HVMTNLGEK▲LTDEEVDEMIR can also be determined based on these observed fragment ions (only four backbone cleavages are missing). CID of $[I^{\blacktriangle}NLK + H]^+$ (*m/z* 575.4/579.4) (Figure S-6c, Supporting Information) gives rise to modified $b_1^{\blacktriangle}$ and $b_2^{\blacktriangle}$, and unmodified y_1, y_2, and y_3, which determinates the peptide sequence to be INLK with the modification position at the first isoleucine of this peptide (i.e., the N terminus of mastoparan). Thus, the most sequence of P10 is covered and the modification sites are determined to be the 115th lysine residue of calmodulin and the N-terminus of mastoparan. These results suggest both the 115th lysine residue of calmodulin and the N-terminus of mastoparan are solvent accessible and close to each other during the formation of complex, which can be linked by DSP. This result is in excellent agreement with the previous report [45]. Clearly, CID of the reduced peptide ions from P10 provides much more sequence information than that of the ion $[P10 + 2H]^{2+}$ (*m/z* 1510.2/1514.2), which only shows the formation of fragment ions $A(y_1)$, $A(y_2)$, $A(y_4)$, $A(y_{11})$, $A(b_5)$, $B/A(y_{14})^{2+}$, and $B/A(y_{16})^{2+}$ (Figure S-6a, Supporting Information).

Likewise, by using tandem MS analysis, structures of P8 and P9 are also determined to be dead-end cross-links (Figures S-7 and 8, see discussion in Supporting Information), indicating that the 94th lysine residue is also solvent accessible [45]. Again, this result is in line with the previous report [45]. These results show that the cross-linking MS is of value in probing the protein–substrate interactions and is compatible with the employment of combined isotope labeling approach and electroreduction.

Probe Protein Conformational Changes

Besides the capability of probing solvent accessibility of protein residues and protein–substrate interactions as shown above, the electrochemistry-assisted isotope-labeled cross-linking MS further provides information about conformational changes of target protein, using a "comparative cross-linking" strategy[38, 39]. In comparative cross-linking experiment, two solutions, one containing the untreated protein and the other containing the stimulated protein, are cross-linked by light and heavy isotope labeled cross-linkers independently, then mixed in 1:1 molar ratio and subjected to enzymatic digestion prior to MS analysis. The intensity ratios of light to heavy isotope labeled cross-links provide direct clues about conformational changes in response to the stimulus. One of the most attractive advantages of comparative cross-linking is that it can deliver quantitative values for structural changes within protein assemblies [38]. Another advantage is that proteins in different conformational states are analyzed within the same solution, which offers structural information from the same measurement and helps reduce time for sample preparation and data analysis. In addition, comparative cross-linking could be performed with all chemical cross-linkers in unlabeled and labeled forms (i.e., ^2D, ^{15}N, or ^{13}C), which is universal and easily accessible [38].

Calmodulin is known as a Ca^{2+} regulated protein, whose 3D structure changes in the presence or absence of Ca^{2+}. DSP-d_0 was reacted with calmodulin in aqueous solution (no Ca^{2+}) whereas DSP-d_8 was reacted with equal molar amount of calmodulin in the presence of Ca^{2+} separately. The resulting two calmodulin samples were mixed together and underwent enzymatic digestion prior to EC/MS analysis (Scheme 2 illustrates the experimental process). With the assistance of electrolytic reduction, cross-links would be identified rapidly, and comparison of isotope-labeled cross-link doublet peaks provides detailed information about conformational change. Upon electrolytic reduction (Figure 4), peptide ions showing doublet peaks, such as $[P12 + 2H]^{2+}$ (*m/z* 828.0/832.0), $[P8 + 2H]^{2+}$ (*m/z* 974.1/978.1), $[P11 + 2H]^{2+}$ (*m/z* 1019.0/1023.0), and $[P9 + 2H]^{2+}$ (*m/z* 1276.1/1280.1), suffer large relative intensity decrement (–22.7% to –100.0%) (using the disulfide-free peptide ion $[LTDEEVDEMIR + 2H]^{2+}$ as a reference, Table S-4, Supporting Information), which suggests P8, P9, P11, and P12 to be cross-links. Note that in this case, the use of intensity drop for finding cross-link peptide is particularly useful as the d_0- and d_8-labeled cross-link ions do not necessarily have the same abundance.

Taking P12 as an example, the intensity ratio of DSP-d_0 and DSP-d_8 labeled P12 cross-links is informative to indicate the conformational change in response to addition of Ca^{2+}. Upon the electrolytic reduction, $[P12 + 2H]^{2+}$ (*m/z* 828.0/832.0) suffers a 100% relative intensity drop, while adjacent peaks at *m/z* 829.0/833.0 appear, due to the formation of reduced peptide product ions. The observation of +2 Da mass shift indicates P12 as an intra-peptide cross-link (Figure 5). CID spectrum of the reduced P12 ion $[K^{\blacktriangle}MK^{\blacktriangle}DTDSEEEIR + 2H]^{2+}$ (*m/z* 829.0/833.0) yields fragment ions y_4, y_6, y_7, y_8, y_9, $y_{10}^{\blacktriangle}$, and $y_{11}^{\blacktriangle}$ (Figure S-9b, Supporting Information), which suggests the modification positions located at first and third lysine residues of P12 (i.e., the 75th and 77th lysine residues of calmodulin).

Interestingly, prior to electroreduction, the zoomed-in spectrum (Figure 5a) shows that DSP-d_8 labeled P12 peak at *m/z* 832.0 (corresponding to the cross-link formed in the presence of Ca^{2+}) has higher intensity compared with DSP-d_0 labeled P12 peak at *m/z* 828.0 (corresponding to the cross-link formed in the absence of Ca^{2+}). The unequal intensity indicates that the addition of Ca^{2+} causes the conformational change so that the 75th and 77th lysine residues of calmodulin become more exposed. This conclusion is verified by examining the zoomed-in spectrum of P12 under reversed reaction conditions (i.e., calmodulin modified with DSP-d_8 in the absence of Ca^{2+} and calmodulin modified with DSP-d_0 in the presence of Ca^{2+} were mixed prior to EC/MS analysis), which shows that the DSP-d_0 labeled product ion at *m/z* 828.0 has higher intensity compared with the DSP-d_8 labeled product ion at *m/z* 832.0 (Figure 5c). Furthermore, in another control experiment, when the cross-linking reactions of calmodulin with DSP-d_0 and DSP-d_8 were

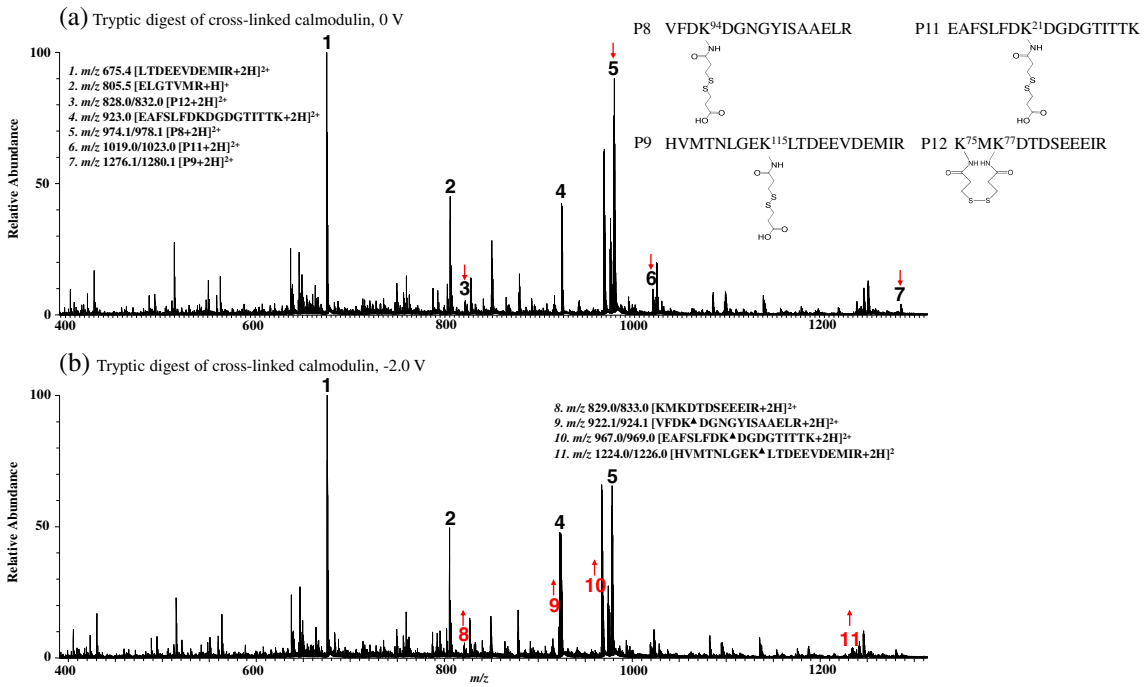


Figure 4. DESI-MS spectra of tryptic digest of a cross-linked calmodulin mixture (calmodulin modified by DSP-d_0 in absence of Ca^{2+} and calmodulin modified by DSP-d_8 in the presence of Ca^{2+}) with an applied potential of **(a)** 0 V; and **(b)** –2.0 V

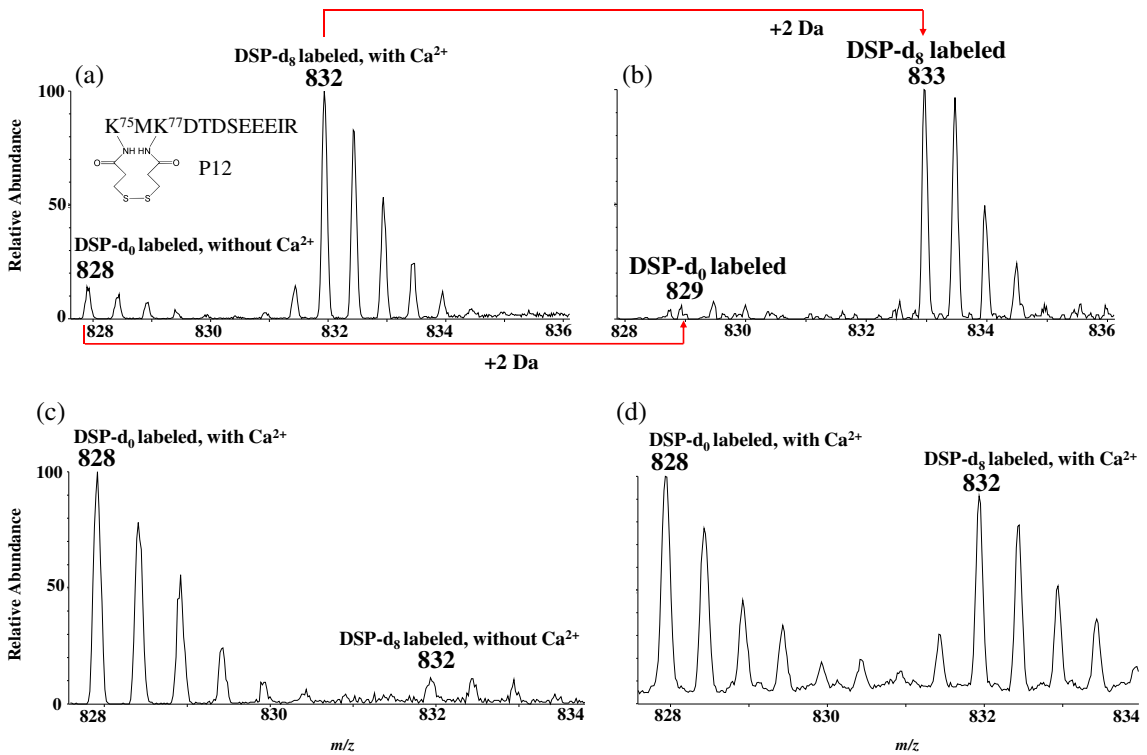
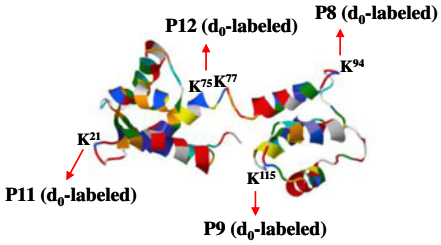
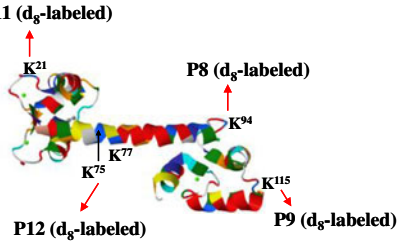
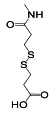
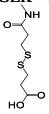
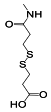


Figure 5. Zoomed-in spectra of **(a)** $[P12 + 2H]^{2+}$ (*m/z* 828.0/832.0) and **(b)** its reduced product ions (*m/z* 829.0/833.0). In this case, the calmodulin modified by DSP-d_0 in the absence of Ca^{2+} and the calmodulin modified by DSP-d_8 in the presence of Ca^{2+} was mixed prior to EC/MS analysis. **(c)** Zoomed-in spectrum of $[P12 + 2H]^{2+}$ (*m/z* 828.0/832.0) when calmodulin modified with DSP-d_8 in the absence of Ca^{2+} and calmodulin modified with DSP-d_0 in the presence of Ca^{2+} were mixed prior to EC/MS analysis and **(d)** Zoomed-in spectrum of $[P12 + 2H]^{2+}$ (*m/z* 828.0/832.0) when the cross-linking reactions of calmodulin with DSP-d_0 and DSP-d_8 were both carried out in the presence of Ca^{2+}

Table 1. The Intensity Ratios of Light and Heavy Isotope Labeled Cross-Link Products When the Calmodulin Modified by DSP-d_0 in the Absence of Ca^{2+} and the Calmodulin Modified by DSP-d_8 in the Presence of Ca^{2+}

	 Calmodulin without Ca^{2+}		 Calmodulin with Ca^{2+}	
Cross-link products	P8 VFDK^{94}DGNGYISAAELR 	P9 HVMTNLGEK^{115}LTDEEVDEMIR 	P11 EAFSLFDK^{21}DGDGTITTK 	P12 K^{75}MK^{77}DTDSEEEIR
Intensity ratio (d_0-labeled: d_8-labeled)	1:2	1:3	1:2	1:7

both carried out in the presence of Ca^{2+}, the doublet peaks of P12 ion display nearly equal intensity (Figure 5d). This result further confirms that the uneven intensity between d_0- and d_8–labeled products observed in the comparative cross-linking experiments (Figure 5a) is the effect of conformational change caused by the addition of Ca^{2+} rather than due to different ionization efficiencies from isotope labeling effect. Likewise, the zoomed-in spectrum of P8 (Figure S-10a, Supporting Information) shows unequal intensity of doublet (*m/z* 974.1/978.1) indicating that the 94th lysine residue becomes more solvent accessible and more reactive towards DSP with adding Ca^{2+}. More informatively, the intensity ratio of light and heavy isotope labeled P12 ion (d_0-labeled:d_8-labeled = 1:7, Figure 5a and Table 1) is much more skewed compared with that of P8 ion (d_0-labeled:d_8-labeled = 1:2, Table 1 and Figure S-10a, Supporting Information), which reveals quantitatively that with addition of Ca^{2+}, the conformational change of the region where K^{75} and K^{77} are located is more dramatic compared with that of K^{94} region.

In addition, by tandem MS analysis, structures of P9 and P11 are determined to be dead-end cross-links as modification positions located at K^{115} and K^{21} of calmodulin, respectively (Figures S-8 and S-11, see discussion in Supporting Information). The intensity ratios of light and heavy isotope labeled P9 (d_0-labeled:d_8-labeled = 1:3, Table 1) and P11 ions (d_0-labeled:d_8-labeled = 1:2, Table 1) are also much less skewed compared with that of P12 ion. The results are in line with the previous literature report that with the addition of Ca^{2+}, both N- and C- termini move further apart so that K^{21}, K^{94}, and K^{115} residues become more solvent accessible. However, with the two termini moving away, the central helix linker (at which P12 is located) becomes more extended so that K^{75} and K^{77} become exposed to a larger extent than those residues K^{21}, K^{94}, and K^{115}. Clearly, the formation of four cross-link products and their distinctive unequal doublet peak intensity ratios provide rich information about the alternation of the calmodulin protein conformation upon adding Ca^{2+}.

Conclusions

This study presents a novel and rapid approach for probing protein/protein complex 3D structure (i.e., residue solvent accessibility analysis) and protein–substrate interactions, involving electrochemical cross-linking MS coupled with isotope-labeling strategy. Owing to the relatively large intensity change before and after electrolytic reduction, the cross-links could be quickly identified. The assistance of isotope-labeling helps confirm the identification and further differentiate the cross-links from those peptides carrying native disulfide bonds as a result of protein digestion. In addition, the electrolytic reduction results in linear peptides, which benefits MS/MS analysis by providing more sequence information and pinpointing the modification sites in comparison to their cross-linked precursors. Furthermore, the electrochemistry and isotope labeling protocol can also be used in the comparative cross-linking MS experiment, for monitoring protein conformational changes based on the intensity ratios of light and heavy isotope labeled cross-link peaks, as exemplified by studying Ca^{2+}-triggered calmodulin conformational change in this study. For future work, more than two isotopic states of cross-linkers could be employed at the same time so that more than two conformational states could be monitored at the same time. One possible pitfall is the overlapping issue of the cross-linking products. However, this can be solved if we could maximize the mass differences among those cross-linkers. As the electrolytic reduction of disulfide bonds only takes seconds and no separation is involved in this study, the presented methodology

should be of high value for structural biology and proteomics applications. One issue in this study is that only few cross-linking products were detected. Such a problem is common in other cross-linking MS reports [45–47]. This is probably due to the fact that only solvent accessible reactive residues are reacted. Future solution would be to use cross-linkers targeting on different residues and cross-linkers with different lengths to produce more cross-links for gaining increased information about protein 3D-structures.

Acknowledgments

This work was supported by NSF IDBR (CHE-1455554), NSF Career Award (CHE-1149367), NSFC (21328502), and Edison Biotechnology Institute Faculty Fellowship. H.Z. also thanks the National Institutes of Health\National Institute of General Medical Sciences (grant # 8 P41GM103422 to Professor Michael L. Gross at Washington University in St. Louis) for support.

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