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Quantification of Protein–Protein Interactions with Chemical Cross-Linking and Mass Spectrometry

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Abstract

Chemical cross-linking in combination with mass spectrometry has largely been used to study protein structures and protein–protein interactions. Typically, it is used in a qualitative manner to identify cross-linked sites and provide a low-resolution topological map of the interacting regions of proteins. Here, we investigate the capability of chemical cross-linking to quantify protein–protein interactions using a model system of calmodulin and substrates melittin and mastoparan. Calmodulin is a well-characterized protein which has many substrates. Melittin and mastoparan are two such substrates which bind to calmodulin in 1:1 ratios in the presence of calcium. Both the calmodulin–melittin and calmodulin–mastoparan complexes have had chemical cross-linking strategies successfully applied in the past to investigate topological properties. We utilized an excess of immobilized calmodulin on agarose beads and formed complexes with varying quantities of mastoparan and melittin. Then, we applied disuccinimidyl suberate (DSS) chemical cross-linker, digested and detected cross-links through an LC-MS analytical method. We identified five interpeptide cross-links for calmodulin–melittin and three interpeptide cross-links for calmodulin–mastoparan. Using cross-linking sites of calmodulin–mastoparan, we demonstrated that mastoparan also binds in two orientations to calmodulin. We quantitatively demonstrated that both melittin and mastoparan preferentially bind to calmodulin in a parallel fashion, which is opposite to the preferred binding mode of the majority of known calmodulin binding peptides. We also demonstrated that the relative abundances of cross-linked peptide products quantitatively reflected the abundances of the calmodulin peptide complexes formed.

Keywords

cross-linking; LC-MS; quantification; mass spectrometry; protein-protein interaction; proteomics; FT-ICR MS

INTRODUCTION

Protein-protein interactions comprise a complex and dynamic network called the interactome¹ which mediates cellular functions.² Changes in this protein interaction network reflect changes in the state of the cell that, if mapped and interpreted, could enable significant progress toward the concepts of systems biology.^{3,4} Diseases are often associated with alteration in protein interaction networks that result in cellular dysfunction.^{5,6} Analysis of current protein interaction networks has indicated that functionally essential proteins serve as hubs while, a majority of disease proteins are peripheral ones in the network.⁷ Furthermore, it has been suggested that mediator proteins, which serve as interaction partners for disease proteins, are in fact more important in system-based medicine and drug design than either the disease proteins or hub proteins themselves.⁸ The ability to identify key protein-protein interactions and monitor changes with disease states will allow the development of advanced treatments. Therefore, much effort has been devoted to developing analytical techniques that can enable identification of protein-protein interactions and monitor changes in levels of these interactions. Analytical techniques such as X-ray crystallography and NMR are the gold standard methods and provide detailed, high-resolution atomic structures of protein complexes; however, these methods require large amounts of purified protein and are not applicable in many situations. In complex biological samples, the ability to identify and quantify protein interactions in an unbiased or untargeted fashion does not currently exist.

Chemical cross-linking in combination with mass spectrometric analysis has emerged as a powerful analytical technique that allows one to gain insight into the molecular features of protein-protein interactions.⁹⁻¹² Chemically cross-linking interacting proteins stabilizes transient interactions and allows many types of measurements to be performed. High-accuracy mass measurements provide the identities and the cross-linked amino acid residues of the interacting proteins and yield low-resolution topological maps of the interacting

regions. In this way, cross-linking holds a complementary role to X-ray crystallography and NMR spectroscopy for protein structural characterization. Chemical cross-linking with mass spectrometric analysis has most commonly been used to study interactions in model protein systems. Recently, it was shown that chemical cross-linking in combination with MALDI-TOF MS could be used in a semi-quantitative fashion to rank the binding affinities of protein complexes.¹³ In addition, a few studies have successfully used chemical cross-linking to identify protein interactions in complex systems such as cells or cell lysates.^{14–16}

Proteins are often involved in complex interaction networks and many have multiple binding partners.¹⁷ The ability to track changes in levels of interactions will enable an improved understanding of the dynamic nature of protein–protein interactions. In this study, we set out to investigate the applicability of chemical cross-linking in conjunction with mass spectrometry to quantify differences between various protein–peptide interaction states using the resulting cross-linked peptide products. To achieve this, we used a model system consisting of calmodulin, melittin, and mastoparan. Calmodulin (CaM) is a small calcium binding protein that is ubiquitously expressed in eukaryotic cells, and has frequently been used in protein structural and binding studies.^{18–20} As a classic example of a hub protein, calmodulin is involved in the regulation of a wide variety of metabolic processes where it binds to a number of enzymatic targets and mediates their activities. Calmodulin undergoes a conformational change upon binding Ca^{2+} and adopts a dumbbell-like structure with two nearly symmetrical domains connected by a flexible hinged helix region. This flexible helix allows calmodulin to bind tightly to a wide range of target proteins by wrapping around them. In addition to binding other proteins, calmodulin is known to tightly bind ($K_d < 10^{-7}$ M) a number of smaller peptides which adopt an amphiphilic α -helical structure.²¹ Two of these are the cytotoxic peptides, melittin ($K_d = 3$ nM)²² and mastoparan ($K_d = 0.3$ nM),²³ isolated from honeybee and wasp venom, respectively. Both bind to calmodulin in a 1:1 fashion and occupy the same binding site, and will therefore competitively form complexes with calmodulin based on their binding affinities and concentrations. The amino acid sequences of CaM, mastoparan, and melittin are shown in Figure 1. Chemical cross-linking and mass spectrometry have been previously employed to map the topology of the calmodulin–melittin complex, confirming that melittin can bind to calmodulin in two different orientations.^{19,20} To date, cross-linking experiments on model systems have been performed with the interacting proteins in solution. For the experiments reported here, we employed calmodulin that was immobilized on a sepharose solid support resin. It is reported by the manufacturer, Agilent, to have a binding affinity of 10^{-9} M for the calmodulin binding peptide (CBP) tag, derived from a C-terminal fragment of muscle myosin light-chain kinase in the presence of Ca^{2+} .²⁴ The use of immobilized calmodulin allowed sample washing steps to extensively remove any nonspecifically bound interacting partners before cross-linking, as well as remove any non-cross-linked partners and unreacted cross-linker before analysis. Here, we demonstrate a novel approach using the quantitative analysis of cross-linked peptide pairs to measure abundance level changes in protein–peptide interactions.

MATERIALS AND METHODS

Materials

Melittin (Me), Mastoparan (Ma), and Angiotensin I (A) were purchased from Sigma-Aldrich (St. Louis, MO). Calmodulin Affinity Resin (CaM) was purchased from Agilent Technologies (Wilmington, DE). Disuccinimidyl suberate (DSS), mass spectrometry-grade Trypsin endoproteinase, and cellulose acetate filter spin cups were purchased from Thermo Scientific Pierce (Rockford, IL). Phenylmethylsulfonyl fluoride (PMSF) was purchased from GBiosciences (Maryland Heights, MO).

Complex Formation Confirmation and Calibration Curve Development

A total of 50 μL of CaM was placed in spin cups and washed with 500 μL of 150 mM NaCl, 10 mM CaCl_2 , and 20 mM HEPES solution (Calmodulin Binding Buffer, CBB) five times. For all elution and washing steps, the centrifuge was set to 2000g for 30 s. The CaM beads were resuspended in 200 μL of CBB. Four such CBB equilibrated CaM mixtures were prepared. Fourteen nanomoles of mastoparan and 1.0 nmol of melittin, 12 nmol of mastoparan and 3.0 nmol of melittin, 10 nmol of mastoparan and 5.0 nmol of melittin, 7.5 nmol of mastoparan and 7.5 nmol of melittin were added to separate CBB equilibrated CaM mixtures. Fifteen nanomoles of Angiotensin I was also added to each reaction mixture. The reaction mixtures were kept in the spin cup and the caps were wrapped in Parafilm to prevent solution leakage through the filter during incubation. The mixture was then incubated with constant agitation at room temperature for 2 h.

Each sample was spun down. The eluent was saved for future analysis. The reaction mixtures were washed with 500 μL of CBB five times and then with 200 μL of 150 mM NaCl, 2 mM CaCl_2 , and 20 mM HEPES solution two times. Finally, the beads were eluted with 30% acetonitrile and 0.3% formic acid in water solution two times, each time using 200 μL of elution solution.

A 25 cm long C18 column was made in house by packing a fused silica capillary (360 $\mu\text{m} \times 75 \mu\text{m}$) with MAGIC C18AQ 100 $\text{\AA}$ 5 μm beads (Michrom Bioresources, Inc., Auburn, CA). A 2 cm long trap column was prepared similarly by packing a fused silica capillary (360 $\mu\text{m} \times 100 \mu\text{m}$) with MAGIC C18AQ 200 $\text{\AA}$ 5 μm beads. Samples were analyzed using a nanoAcquity LC (Waters Corp., Milford, MA) coupled with a LTQ XL mass spectrometer (Thermo Scientific, Asheville, NC) using normal scan speed. Each sample was analyzed with the following LC conditions: 10 min trapping with a 2 $\mu\text{L}/\text{min}$ flow rate at 3% solvent B; analytical separation using a flow rate of 0.3 $\mu\text{L}/\text{min}$ with a linear gradient from 0 to 60 min 5–60% solvent B, 60–85 min flushing with 80% solvent B, and 85–100 min equilibrating with 5% solvent B (solvent A, 0.1% formic acid in DI water; solvent B, 0.1% formic acid in acetonitrile). A blank injection was run between each sample injection.

XCalibur Qual Browser was used to integrate peak areas for extracted ion chromatograms for mastoparan at the +3 charge state (494.00 m/z) and for melittin at +5 charge state (570.15 m/z). The ratios of the peak areas were plotted against the ratios reacted peptide amounts within each sample using Microsoft Excel (2007).

Quantification of Cross-Linking Products from CaM Complex

Four quantification samples were prepared using the same protocol for preparing the calibration samples. However, after washing with CBB, samples were resuspended in 100 μL of CBB and 2 μL of 100mMDSS in DMSO was added. Sample tube caps were sealed with Parafilm and incubated for 1 h at room temperature. Samples were washed with 500 μL of 0.1% Triton $\times$ 100 five times, then washed with 500 μL of 100 mM NH_4HCO_3 solution 10 times and resuspended in 100 μL of NH_4HCO_3 solution. Fifty microliters of trypsin endoproteinase in NH_4HCO_3 solution (10 ng/ μL) was added to the each sample. Samples were capped and wrapped in Parafilm and incubated for 2 h at 37 $^\circ\text{C}$. One microliter of 100 mM PMSF in DMSO was added to each sample to stop trypsin digestion. Samples were eluted with 30% acetonitrile and 0.3% formic acid in water solution two times, each time using 200 μL of eluting solution. Finally, samples were vacuum concentrated overnight until dry and resuspended in 30 μL of 0.1% formic acid solution.

A two-step analytical strategy was developed. First, samples were analyzed by LC-MS/MS using a nanoAcquity UPLC system coupled to a LTQ-FT Ultra (Thermo Scientific, Asheville, NC). The following LC conditions were used with the same trap column and

analytical column as previously described: 10 min trapping with a 2 $\mu\text{L}/\text{min}$ flow rate at 3% solvent B; analytical separation using a flow rate of 0.3 $\mu\text{L}/\text{min}$ with a linear gradient from 0 to 120 min 5–60% solvent B, 120–135 min flushing with 80% solvent B, and 135–150 min equilibrating with 5% solvent B (solvent A, 0.1% formic acid in DI water; solvent B, 95% acetonitrile and 0.1% formic acid in DI water). A blank injection was run between each sample injection. Tandem mass spectrometric analysis was performed using the LTQ-FT Ultra performing one MS scan in the ICR cell at 25 000 resolving power and five data-dependent MS/MS scans in the ion trap selecting the most intense peaks above 5000 absolute intensity. RAW data files were converted to .mzXML format using ReAdW and the resulting .mzXML files were converted using mzXML2search into .mgf files which were submitted for xQuest analysis (<http://prottools.ethz.ch/orinner/public/htdocs/xquest/>).¹⁴ Parameters used in xQuest which differed from default settings are 1 amino acid minimum peptide length, maximum of 3 missed tryptic cleavages and MS/MS tolerance of 0.5 m/z , and inclusion of the N-terminal residues (G for melittin, and I for mastoparan) as potentially cross-linker reactive. Cross-linked peptide pairs putatively identified by xQuest were then manually validated by comparing the peaks in the raw MS/MS spectra of cross-linked peptide pairs to a list of theoretical masses of fragment ions calculated using Protein XXX (<http://www.gpmaw.com/Downloads/ProteinXXX/proteinxxx.html>).

After conclusively identifying the cross-linked peptide pairs, samples were reanalyzed on the same LC-MS system using the same conditions except this time performing only MS scans in the ICR cell at 25 000 resolving power. This allowed collection of more data points over the chromatographic peak, thereby enabling more accurate quantification. Samples were analyzed in triplicate. The LC-MS data was analyzed with XCalibur by integrating the peak areas of the extracted ion chromatograms for each of the cross-linked peptide pair precursor ions previously identified. Accurate mass (± 0.01 m/z) and retention time values (± 60 s) for each cross-linked peptide pair were inputted to ensure the correct selection of peaks for quantification. Peak detection was accomplished using the Genesis algorithm. Genesis settings included: peak detection limits set to 1.0% of the highest peak detected; peaks signal-to-noise height ratio greater than 3.00, Gaussian smoothing limits to 5 addition points per peak.

For each sample, relative ratios were obtained by taking the sum of the peak areas from each of the identified mastoparan–CaM cross-linked peptide pairs and dividing these values by the sum of the peak areas from the identified melittin–CaM cross-linked peptide pairs. An average for each ratio of mastoparan to melittin was calculated from the triplicate analyses. This average ratio was plotted against the ratio of mastoparan and melittin allowed to react with CaM. The relative standard deviation among the triplicate samples was calculated and added as vertical error bars to each point on the plots.

RESULTS AND DISCUSSION

Binding and Elution of Melittin and Mastoparan with Immobilized Calmodulin

Our analytical strategy consisted of a two pronged approach which is outlined in Figure 2. To evaluate the quantitative nature of complex formation between melittin and mastoparan with calmodulin, we incubated immobilized calmodulin with varying amounts of melittin and mastoparan followed by extensive washing to remove any unbound peptides and finally eluted the bound peptides by denaturing calmodulin with a solution consisting of 0.3% formic acid and 30% acetonitrile. The eluent was analyzed by LC–MS as described in the Materials and Methods section to allow determination of relative amounts of melittin and mastoparan that were bound to calmodulin. Extracted ion chromatograms were generated and the peak areas obtained for the +3 and +4 charge states for mastoparan at 495.00 m/z and 370.9 m/z , respectively, and the +4 and +5 charge states for melittin found at 570.5 m/z

and 712.44 m/z . The areas for the extracted ion chromatograms for the two charges states of mastoparan were summed together and divided by the sum of the areas for the extracted ion chromatograms of the two charge states of melittin. A calibration curve with the log of the ratios of the integrated peak areas of mastoparan to melittin plotted against the log of their concentration ratios during complex formation is shown in Figure 3. The resulting data fit well to a line, with an R^2 value of 0.9755 illustrating an excellent correlation between the amounts of melittin and mastoparan added and their measured chromatographic peak areas. Error bars at each point represent $\pm$ the standard deviation of each measured area ratio resulting from measurements on triplicate preparations of the samples.

Identification and Quantification of Cross-Linking Products

After demonstrating the quantitative nature of complex formation between melittin and mastoparan with calmodulin, we performed experiments to show that cross-linked peptide pairs between the interacting partners also carry quantitative information. Immobilized calmodulin was incubated with varying amounts of melittin and mastoparan and the protein-peptide complexes were chemically cross-linked using the homobifunctional primary amine reactive cross-linker, DSS. DSS was chosen as a cross-linker because it is a commercially available product and is one of the most widely used chemical cross-linking reagents. DSS has a spacer arm of 11.4 Å, but can cross-link sites with apparent distances up to 25 Å likely due to the flexibility of the protein structure.¹¹

The identification of cross-linked peptide pairs by tandem mass spectrometry remains a challenging task. When working with model systems as in this study, having high mass accuracy and using software such as xQuest, are often sufficient to conclusively identify cross-linked peptide pairs. However, the use of stringent search criteria to identify cross-linked pairs becomes increasingly important when working with more complex biological samples. In total, five cross-linked peptide pairs were identified between calmodulin and melittin which are summarized in Table 1 and three cross-linked peptide pairs between calmodulin and mastoparan were identified and are summarized in Table 2. Of the five cross-linked products we identified between calmodulin and melittin, two are identical to those found by Schulz et al., while the other three have not been previously reported. Schulz et al. did not report identification of cross-linked products with K115 of calmodulin since the form of calmodulin they were using contained a trimethylated lysine at position 115.²⁰ *In vivo*, calmodulin is trimethylated on K115 by a specific enzyme, *S*-adenosylmethionine-*N*-methyltransferase. While the exact biological function of the trimethylation of lysine 115 is unknown, it has been suggested that it may play a part in the regulation of the ability of calmodulin to activate various calmodulin-regulated enzymes.²⁵ Studies have reported that *N*-methylation of calmodulin can prevent its degradation by ubiquitin-ATP-dependent proteolysis.²⁶ Additionally, it has been reported that methylation has no effect on the ability of calmodulin to activate cyclic nucleotide phosphodiesterase;²⁷ however, methylation severely impairs calmodulin's ability to activate NAD kinase.²⁸ We were able to identify K115 on the C-terminal domain of calmodulin cross-linked with both the N-terminal amine of melittin and K23 near the C-terminus of melittin, as well as cross-linked with the N-terminal amine of mastoparan. One previous investigation used DSS to cross-link mastoparan to calmodulin; however, no mass spectrometric analysis was performed, and therefore, none of the cross-linked sites were reported.²⁹ In the present work, both K95 and K115 of calmodulin were identified cross-linked to mastoparan and melittin. K22 and K78 of calmodulin were only found cross-linked to melittin, while K76 was exclusively identified as cross-linked to mastoparan.

Previous reports have demonstrated that melittin can bind to calmodulin in two different orientations, namely, in a parallel orientation in which the C-terminus of the peptide interacts with the C-terminal domain of calmodulin (orientation A) or in an antiparallel

orientation in which the C-terminus of the peptide interacts with the N-terminus of calmodulin (orientation B).^{19,20,30} The cross-linked peptide pairs shown in Tables 1 and 2 are separated into their respective orientations. A thermodynamic study reported that 80% of melittin binds in the parallel orientation,³⁰ while a purely qualitative assessment of cross-linking data supported the idea that a majority of melittin binds in orientation A.²⁰ We examined this quantitatively by taking the peak areas for all of the cross-linked peptide pairs representing melittin binding in orientation A and dividing that by the sum of the peak areas for the cross-linked peptide pairs from melittin binding in orientation B. The ratio was calculated to be 4.50 (82%) with a standard deviation of 0.59 which agrees quite well with the previous report of 80% binding in orientation A.³⁰ Performing a similar calculation with the cross-linked peptide pairs from mastoparan resulted in a ratio of A/B orientation of 7.44 (88%) with a standard deviation of 1.54. No prior reports of the preference of mastoparan for binding to calmodulin in orientation A or B exist to allow comparison to our results. Both of these ratios suggest a preference for melittin and mastoparan binding in a parallel fashion with calmodulin and were found to be independent of the respective peptide concentration. There are no existing PDB structures available for the calmodulin–melittin complex or the calmodulin–mastoparan complex to measure distances to compare against our identified cross-linked sites. The solution phase NMR structure for calmodulin in complex with a 26-residue synthetic peptide comprising the CaM binding domain (residues 577–602) of skeletal muscle myosin light chain kinase (PDB = 2BBM) along with the PDB structures formelittin (PDB = 2MLT), and mastoparan (PDB = 2CZP) were used to generate structures for the calmodulin–melittin and calmodulin–mastoparan complexes using the protein docking program Hex (version 6.1). Structures were generated for melittin and mastoparan binding to calmodulin in both orientations and the resulting structures were analyzed with Molsoft ICM Browser (version 3.6-1i) to measure the distances between the identified cross-linked sites. All distances measured between identified cross-linked sites were less than 25 Å which is consistent with the length of DSS and with the binding geometries. The generated structures with the measured distances are provided as Supplemental Figures S1–S4.

As an example of the typical MS and MS/MS data used to identify and quantify a cross-linked peptide pair, the MS and MS/MS spectra for four of the cross-linked peptide pairs representing melittin and mastoparan bound in both orientation A and B are shown in Figures 4–7. Data for the peptide pair (EAFSLFDKDGDTITTK–KR) consisting of residues 11–31 of calmodulin and residues 23–24 of melittin represents melittin bound in orientation A and is shown in Figure 4. The top portion of the figure displays the total ion chromatogram (TIC) with Figure 4a displaying the extracted ion chromatogram (EIC) for this cross-linked peptide pair. The FTICR-MS spectrum of the triply charged precursor ion (m/z 762.3962) is shown in Figure 4b. The annotated MS/MS spectrum shows the b- and y-type fragment ions originating from the calmodulin peptide EAFSLFDKDGDTITTK that are labeled with α while the fragment ions originating from the melittin peptide KR are labeled with β . Fragment ions which contain the mass of the cross-link are designated with a capital Y and B and are shown in red. Data for the peptide pair (HVMTNLGEKLTDEEVDEMIR–GIGAVLK) consisting of residues 108–127 of calmodulin and residues 1–7 of melittin represents melittin bound in orientation B and is shown in Figure 5. Similar data are shown in Figures 6 and 7 for mastoparan bound to calmodulin in orientations A and B, respectively. Annotated tandem mass spectra for all identified cross-linked peptide pairs along with fragment ion mass tables are provided in Supplemental Figures S5–S12. After identifying the major cross-linked products from the two peptides and calmodulin, the integrated peak areas of the extracted ion chromatograms for each of the cross-linked products were summed. This sum was divided by the sum of the integrated peak areas for each of the cross-linked products between melittin and calmodulin for each sample at the following ratios of mastoparan to melittin 1:1, 2:1, 4:1, and 14:1.

Figure 8 shows the log of the ratio of the integrated peak areas for total cross-linked peptide pairs found from CaM–mastoparan to cross-linked peptide pairs found from CaM–melittin plotted versus the log of the ratio of CaM–mastoparan to CaM–melittin complex formed. The plotted data show averages from sample triplicates. Relative standard deviations for each of the ratios were as follows: 4.91%, 9.12%, 9.15%, and 4.44% for the 1:1, 2:1, 4:1, and 14:1 concentration ratios, respectively. The mean overall relative standard deviation for all data was 6.91%. The data fit very well to a line with an R^2 value of 0.9932 and clearly demonstrates that cross-linked peptide pairs carry quantitative information about the abundance of complex formed between calmodulin and its two binding partners.

CONCLUSIONS

In the present study, we demonstrate that chemical cross-linking with mass spectrometry can provide quantitative information about the levels of binding partners in protein–peptide interactions. The use of nano-LC along with high-resolution FTICR-MS analysis allows one to track changes in the relative abundances of cross-linked peptide pairs. Importantly, these data directly relate to the abundances of the various complexes that were present during cross-linking.

It has previously been demonstrated that melittin can bind to calmodulin in two different orientations.²⁰ It was suggested by a qualitative examination of the number of cross-linked products identified that melittin bound to calmodulin primarily in orientation A. The results presented here confirm these previous results as well as show that mastoparan, a shorter amphiphilic peptide, also binds in two orientations. Extraction of quantitative information from cross-linking experiments allows determination of the relative amounts of each peptide binding in a specific orientation.

The results presented here demonstrate the quantitative nature of cross-linking on the model system of CaM with melittin and mastoparan. A similar strategy of quantifying cross-linked peptide pairs should be applicable to interacting proteins in complex biological systems and allow measurement of changes of protein–protein interaction levels *in vivo*. It should be noted that in this study on a model system, a level of optimization is possible that is not available in complex biological samples. For example, the ratios of mastoparan and melittin used in this study were adjusted to aid our ability to detect cross-linked peptide products in the system used here. New developments in cross-linking technology such as the PIR concept should facilitate this type of study in complex biological systems.³¹ As the field of chemical cross-linking and mass spectrometry continues to advance, the ability to generate quantitative information concerning protein–protein interactions will provide a more comprehensive understanding of biomolecular function and protein interaction networks in systems biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CaM					
MDGLDST	RTDQST	SDGSDST	RTDQST	SDGSDST	SDGSDST
MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST
Melittin					
MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST
Mastoparan					
MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST
Mastoparan					
MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST	MDGLDST

Figure 1. Amino acid sequences for CaM, melittin, and mastoparan. The C-terminal amino acid of melittin and mastoparan are amidated. Red print marks cross-link modified residues found.

Figure 2.
General analytical strategy for relative quantification of cross-linked sequences between CaM and melittin and CaM and mastoparan.

Figure 3.

Calibration curve showing ratio of CaM and mastoparan complex formed versus CaM and melittin complex formed by varying mastoparan and melittin quantity in an excess of CaM in 1:1, 2:1, 4:1, 14:1, ratio.

Figure 4.

Melittin to calmodulin cross-linked peptide pair EAFSLFDKDGDTITTK-KR. This cross-link pair demonstrates melittin bound to CaM in orientation A. (a) Total ion chromatogram with extracted ion chromatogram for precursor ion with integrated peak area (b) FT-ICR MS spectrum showing the isotope distribution for the triply charged precursor ion. (b) MS/MS spectrum of cross-linked peptide pair with labeled b- and y-type fragment ions. Fragment ions containing the mass of the cross-linked site are labeled with red colored capital B and Y.

Figure 5.

Melittin to calmodulin cross-linked peptide pair HVMTNLGEKLTDEEVDEMIR–GIGAVLK. This cross-link pair demonstrates melittin bound to CaM in orientation B. (a) Total ion chromatogram with extracted ion chromatogram for precursor ion with integrated peak area (b) FT-ICR MS spectrum showing the isotope distribution for the triply charged precursor ion. (c) MS/MS spectrum of cross-linked peptide pair with labeled b- and y-type fragment ions. Fragment ions containing the mass of the cross-linked site are labeled with red colored capital B and Y.

Figure 6.

Mastoparan to calmodulin cross-linked peptide pair INLKALAALAK-KMK. This cross-link pair demonstrates mastoparan bound to CaM in orientation A. (a) Total ion chromatogram with extracted ion chromatogram for precursor ion with integrated peak area (b) FT-ICR MS spectrum showing the isotope distribution for the quadruply charged precursor ion. (b) MS/MS spectrum of cross-linked peptide pair with labeled b- and y-type fragment ions. Fragment ions containing the mass of the cross-linked site are labeled with red colored capital B and Y.

Figure 7.

Mastoparan to calmodulin cross-linked peptide pair HVMTNLGEKLTDEEVDEMIR–

INLK. This cross-link pair demonstrates mastoparan bound to CaM in orientation B. (a)

Total ion chromatogram with extracted ion chromatogram for precursor ion with integrated

peak area (b) FT-ICR MS spectrum showing the isotope distribution for the quadruply

charged precursor ion. (b) MS/MS spectrum of cross-linked peptide pair with labeled b- and

y-type fragment ions. Fragment ions containing the mass of the cross-linked site are labeled

with red colored capital B and Y.

Quantitation of Mastoparan and Melittin Cross-links

Figure 8.

Graph showing the log of the ratio of the total peak area of CaM and mastoparan complex cross-links to total peak area of CaM and melittin cross-links. The ratios are plotted versus the log of the ratios of mastoparan to melittin which formed complexes with CaM. Error bars show relative standard deviation at each point. Mean relative standard deviation is 6.91%.

Table 1
Identified Cross-Linked Peptide Pairs between Cam and Melittin Using the Cross-Linking Reagent DSS

CaM	modified residue	melittin	modified residue	$[M + H]^+$ calcd	$[M + H]^+$ exp	ppm
Orientation A						
EAFSLFDKDGDTTITK	K22	KR	K23	2285.174	2285.166	3.9
VFDKDGNGYISAAELR	K95	KR	K23	2195.156	2195.146	4.5
HVMTNLGEKLTDEEVDEMIR	K116	KR	K23	2799.413	2799.402	4.1
Orientation B						
HVMTNLGEKLTDEEVDEMIR	K116	GIGAVLK	G1(N-term)	3153.630	3153.617	4.2
MKDTDSEEEIR	K78	KR	K23	1792.879	1792.875	2.3

Table 2
Identified Cross-Linked Peptide Pairs between Cam and Mastoparan Using the Cross-Linking Reagent DSS

CaM	modified residue	mastoparan	modified residue	$[M + H]^+$ calcd	$[M + H]^+$ exp	ppm
Orientation A						
VFDKDGNGYISAAELR	K95	ALAAALAKK	K11	2677.462	2677.456	2.2
KMK	K76	INLKALAAALAK	K4	1669.048	1669.044	2.4
Orientation B						
HVMTNLGEKLTDEEVDEMIR	K116	INLK	I1(N-term)	2983.525	2983.511	4.8