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Mapping protein structural changes by quantitative cross-linking

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ABSTRACT

Chemical cross-linking is a promising technology for protein tertiary structure determination. Though the data has low spatial resolution, it is possible to obtain it at physiological conditions on proteins that are not amenable to standard high resolution techniques such as X-ray, NMR analysis and cryo-EM. Here we demonstrate the utilization of isotopically labeled chemical cross-linking to visualize protein conformation rearrangements. Since calmodulin exists in two distinct conformations (calcium-free and calcium-containing forms), we selected this protein for testing the potential and the limits of a new technique. After cross-linking of both calmodulin forms, the calcium-free and calcium-containing forms were mixed together and digested under different conditions and the products of proteolysis were monitored using high resolution mass spectrometry. Finally, the ratios of heavy/light cross-links were calculated by mMass open source platform.

1. Introduction

Chemical cross-linking in combination with mass spectrometry (XL-MS) is an established and powerful tool for the elucidation of three-dimensional protein structures which also provides valuable information about protein–protein and protein–nucleic acid interactions [1–5]. Current cross-linking strategies do not significantly differ from early experiments in which the analysis of products was limited to photometric and electrophoretic methods [6]. The methodology has, however, been significantly improved by the introduction of soft ionization techniques, which enabled the analysis of cross-linked biomolecules by mass spectrometry [7,8].

Current XL-MS includes chemical and photo-crosslinking whose aim is to determine distances in proteins or protein complexes [9–11]. Modified proteins with amino acids connected by cross-linkers are enzymatically digested and the resulting peptide mixture is separated by liquid chromatography and analyzed by mass spectrometer coupled on-line. The initial structural information includes the length of cross-linker spacer and the position of cross-linked amino acids in protein sequence. The cross-links formed provide distance constraints which form a basis for generating three dimensional models [12–15], mapping protein interaction interface [16–19] or refinement of earlier resolved structures [20–23].

Analysis of conformational changes in proteins represents a very challenging task because proteins are not static objects and their structural dynamics has crucial effect on the behavior of biological systems. Until recently XL-MS was used for studying the dynamics of proteins or protein complexes in a qualitative manner by identifying cross-links that are specifically formed only in one conformation or state [24,25]. However, recent publications show that XL-MS analysis using isotope-labeled cross-linkers allows quantification of structural changes and protein interaction dynamics [26,27]. While quantitative proteomics is a well-established tool, quantitative determination of dimension in protein structural analysis by XL-MS is still challenging. Quantification based on the proportional relationship between the sample concentration and the measured intensities of signal is a commonly used method in differential proteomics [28–31]. Isotope labeling for XL-MS quantification was introduced 8 years ago, and included only 180-labeling of cross-linked peptides [32].

Isotope-labeled cross-linkers were introduced at the beginning of the millennium and immediately became a valuable tool for the identification of modified peptides. Only peptides containing the cross-linker will be represented by a specific doublet (light and heavy form) isotopic pattern in a mass spectrum [33–35]. This distinguishing feature is advantage of quantification by isotope-labeled cross-linker over other general differential labeling methods such as iTRAQ [36,37] or TMT [38]. The use of the isotope-labeled cross-linkers is also an alternative technique to SILAC [39] and does not require preparation of labeled proteins.

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In the present work, we used isotope-labeled cross-linkers for quantification of two different conformational states of the model protein, the 17 kDa large calmodulin. The sequence of this protein is highly conserved across many organisms. It is known that calmodulin exists in two structurally different conformations – calcium containing and calcium free forms [40]. Using this simple system we describe the feasibility and limitation of the cross-linking strategy.

The structures of calmodulin forms with and without calcium ions are completely different [40]. Both conformational states have been well characterized by X-ray and NMR spectroscopy [41–43] and calmodulin has been often used for developing and improving cross-linking approaches [44–46]. Different software tools for quantification of the acquired spectra have also been described [26,47]. In this study, we introduce another method to determine the ratios of light and heavy cross-linker in modified peptides – mMass 5.5 software [48–50].

2. Material and methods

2.1. Material

Calmodulin from bovine brain was obtained from Calbiochem (Germany). Cross-linkers disuccinimidyl glutarate (DSGd0/DSGd4) and disuccinimidyl suberate (DSSd0/DSSd4) were purchased from Proteochem (USA). Sequencing grade modified trypsin was obtained from Promega (USA). Water and HPLC solvents were LC/MS grade purity and were obtained from Thermo Scientific (USA). Other chemicals at the highest available purity were purchased from Sigma–Aldrich (USA).

2.2. Sample preparation

Calmodulin was dissolved in 1 mM EGTA to 1 mg/ml concentration and transferred to 10 mM HEPES buffer (pH 7.5) containing 100 mM NaCl and 1 mM EGTA using Micro Bio-Spin™ 6 columns (cut off 6 kDa BioRad, USA). Calmodulin concentration after chromatography was monitored by Bradford assay [51].

2.3. Calmodulin characterization using ESI-FTICR-MS

One microgram of calmodulin was desalted on protein micro trap column (C4 phase, Michrom Bioresources, USA) according manufacturer instruction, and eluted in 100 µl of 80% acetonitrile/1% acetic acid. Protein was electrosprayed to solariX XR FT-ICR mass spectrometer (Bruker Daltonics, Germany) equipped with 12 T superconducting magnet. The instrument was internally calibrated using Agilent tuning mix (Agilent Technologies, USA). Mass spectra were acquired in the positive mode over the m/z range 245–2500 with 1 M data points transient and 0.4 s ion accumulation, 8 scans were accumulated per spectrum. Data acquisition was performed using solariXControl and interpreted by DataAnalysis 4.1.

2.4. Chemical cross-linking reaction

Calmodulin aliquots in 10 mM HEPES buffer (pH 7.5) containing 100 mM NaCl and 1 mM EGTA for the induction of calcium conformational structural changes were spiked with calcium ions ($CaCl_2$) to produce a 12 mM final concentration of Ca^{2+}. Aliquots intended to represent the calcium-free state were mixed with sodium ions (NaCl) to balance ionic strength to achieve a 136 mM final concentration of Na^{+}. After 30 min incubation at room temperature, aliquots were mixed with the cross-linking reagents. Calmodulin samples representing the calcium-containing state were mixed only with non-deuterated cross-linker (DSGd0 or DSSd0) while calcium-free samples were mixed with deuterated cross-linker (DSGd4 or DSSd4). A ten and thirty-fold molar excess of cross-linker (dissolved in DMSO to 15 mM concentration, final content of DMSO in reaction mixture was 1.3%) compared to the protein was used. Thus the final concentration of calmodulin was 20 µM and concentrations of cross-linking agents were 200 µM/600 µM. The cross-linking reactions were quenched after 2 h of incubation at room temperature by adding ethanolamine to a final concentration of 0.4/1.2 mM. Calcium-containing samples and identical calcium-free samples were mixed in 1:1 ratio. Control samples without cross-linkers, and samples cross-linked with 1:1 mixture DSGd0/DSGd4 or DSSd0/DSSd4 were prepared at the same time, in order to obtain the chemical activities of each cross-linker which could have changed during synthesis, transport, storage and/or preparation of cross-linking agents. Reaction mixtures were split into two identical aliquots. One half of each cross-linked protein sample was analyzed using SDS-PAGE and the other was taken for high-resolution mass spectrometric characterization. Three sets of replicates were prepared and measured.

2.5. Protein electrophoresis

The cross-linking reaction mixture (approximately 10 µg of calmodulin in 30 µl) was mixed with 4×–concentrated LDS sample buffer (Invitrogen) containing 100 mM dithiothreitol as the reducing agent in a 3:1 (v:v) ratio. Samples were incubated for 5 min at 90 °C and then loaded onto a NuPage 4–12% Bis–Tris gel (80.0 × 80.0 × 1.0 mm, 10 wells). Separation was performed in MES running buffer for 35 min at 200 V. After separation, the gels were stained by Coomassie Brilliant Blue R250 and destained by solution containing ethanol, water, and acetic acid in the ratio 55:35:10 [52].

2.6. Enzymatic digestion

For *in-gel* digestion, the calmodulin bands were excised and destained. The gel pieces were covered with trypsin solution (trypsin in 100 mM ethylmorpholine buffer, pH 8.5 with 10% AcN, enzyme:protein ratio 1:20) and incubated at 37 °C overnight.

For *in solution* digestion, the cross-linked protein was digested with trypsin in five different ways:

(A) The cross-linking reaction mixture was diluted 1:1 (protein;buffer) with 100 mM ethylmorpholine buffer (pH 8.5) with 10% AcN, while the pH of the mixture was monitored. Trypsin in 100 mM ethylmorpholine buffer (pH 8.5) with 10% was added to give a final concentration 1:20 (w/w) trypsin to protein and digestion was carried out at 37 °C overnight.

(B) The cross-linking reaction mixture was diluted 1:1 (protein;buffer) with 100 mM ethylmorpholine buffer (pH 8.5) with 20% AcN. Trypsin in 100 mM ethylmorpholine buffer (pH 8.5) with 10% was added to give a final concentration of 1:20 (w/w) trypsin to protein and digestion was carried out at 37 or 55 °C. After a 6-h incubation, trypsin was added to give a final concentration of 1:10, and digestion was allowed to proceed at 37 or 55 °C overnight.

(C) Guanidine was added to the cross-linking reaction mixture to give a final concentration of 6 M and the reactions were carried out for 2 min at 90 °C. After incubation, the guanidine was diluted by 100 mM ethylmorpholine buffer (pH 8.5) with 20% AcN to give a final concentration of 2 M. Trypsin in 100 mM ethylmorpholine buffer (pH 8.5) with 10% was added to give a final concentration of 1:20 (w/w) trypsin:protein, and digestion was carried out at 37 or 55 °C. After a 6-h incubation, trypsin was added to give a