J. Am. Soc. Mass Spectrom. (2018) 29:207–210
DOI: 10.1007/s13361-017-1812-y

APPLICATION NOTE

Bottom-Up Two-Dimensional Electron-Capture Dissociation Mass Spectrometry of Calmodulin

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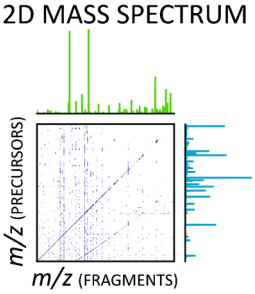

Abstract. Two-dimensional mass spectrometry (2D MS) is a tandem mass spectrometry technique that allows data-independent fragmentation of all precursors in a mixture without previous isolation, through modulation of the ion cyclotron frequency in the ICR-cell prior to fragmentation. Its power as an analytical technique has been proven particularly for proteomics. Recently, a comparison study between 1D and 2D MS has been performed using infrared multiphoton dissociation (IRMPD) on calmodulin (CaM), highlighting the capabilities of the technique in both top-down (TDP) and bottom-up proteomics (BUP). The goal of this work is to expand this study on CaM using electron-capture dissociation (ECD) 2D MS as a single complementary BUP experiment in order to enhance the cleavage coverage of the protein under analysis. By adding the results of the BUP 2D ECD MS to the 2D IRMPD MS analysis of CaM, the total cleavage coverage increased from ~40% to ~68%.

Keywords: Tandem mass spectrometry, Two-dimensional mass spectrometry, Electron capture dissociation, Bottom up proteomics

Received: 25 August 2017/Revised: 26 August 2017/Accepted: 8 September 2017/Published Online: 3 October 2017

Introduction

Two-dimensional (2D) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) is a variant of the well-known FT-ICR MS technique, based on the characterization of analytes via ion motion in a high magnetic field [1, 2]. 2D FT-ICR MS (or 2D MS) allows data-independent fragmentation of all the ions in a mixture by modulating their fragmentation through a series of rf pulses inside the ICR cell without the necessity of previous isolation. The first pulse program for 2D MS was written by Pfandler and others [3–6] in the 1980s based on the proven capacity to excite or de-excite the ions inside an ICR-cell [7]. Due to limited computational capabilities, 2D MS was not implemented until 2010, when the first high resolution 2D IRMPD MS spectrum was acquired [8]. Since then, the interest in developing 2D MS was renewed: a novel processing program with a user-friendly interface was developed, SPIKE [9], which includes sophisticated denoising algorithms to improve the quality of 2D MS spectra [10] such as urQRd [11].

2D MS has been successfully applied to the analysis of small molecules [12] and macromolecules [8, 13], and proved its analytical capabilities in a series of bottom-up proteomics (BUP) studies involving tryptic digests of proteins of increasing size, such as cytochrome *c* [14] and collagen [15]. More recently, 2D MS was developed to perform top-down (TDP) protein analysis, in a study that compared standard BUP and TDP IRMPD MS/MS of calmodulin with 2D IRMPD MS: the study demonstrated that 2D MS is suitable for TDP, with results comparable to 1D MS/MS but obtained with a substantial saving in sample and experimental time [16]. Finally, the versatility of 2D MS improved with the theoretical application of the technique to linear ion traps through SWIM [17–19]. The theory behind 2D MS is still under investigation [20], as well as new applications [21], and developments to reduce processing times and improve resolutions [22].

In this study, the 2D MS work with calmodulin (CaM) is expanded using ECD as fragmentation technique, with the

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13361-017-1812-y) contains supplementary material, which is available to authorized users.

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purpose of increasing the cleavage coverage of the protein in a single 30-min experiment that uses the features of 2D MS.

Experimental

Materials

Bovine calmodulin (CaM), trypsin (TPCK treated from bovine pancreas), and ammonium bicarbonate [ABC, $(NH_4)HCO_3$] were purchased from Sigma Aldrich (Dorset, UK). HPLC grade methanol and formic acid (HAc) were obtained from Fisher Scientific (Loughborough, UK). Water was purified by a Millipore Direct-Q purification system (Merck Millipore, MA, USA).

Sample Preparation

Calmodulin (aqueous solution, 40 µM) was digested with trypsin in ABC 100 mM at an enzyme-to-protein ratio of 1:40. A 3 kDa centrifugal filter device (Amicon Ultra 0.5 mL, Merck Millipore Ltd., Tullagreen, Ireland) was used as a reactor at 37 °C, and the digestion was stopped after 4 h by isolation from trypsin through centrifugation of the peptides through the molecular weight cut-off filter. The tryptic-digest was diluted to an end concentration of ~10 µM using a 75:25 water/methanol (v/v) solution and acidified with 0.3% formic acid (v/v).

Methods

FT-ICR MS was performed on a 12 T Bruker SolariX FT-ICR Mass Spectrometer (Bruker Daltonics GmbH, Bremen, Germany) equipped with a custom nano-electrospray source (nESI). The experiment used 10 µL of sample (~10 µM). The pulse program used for the 2D MS analysis was optimzed in order to obtain the highest fragmentation efficiency for ECD according to previous studies [23]. ECD was performed generating electrons from a heated hollow cathode (1.5 A), with a 10 V pulse to inject the electrons into the ICR-cell for ~0.2 s prior to detection (ECD bias 1.5 V); 3072 scans of 512 k 16-bit data points were acquired over a mass range of *m/z* 368.2–3000 *m/z* on the vertical axis and *m/z* 147.5–3000 *m/z* on the horizontal axis; total time of acquisition was ~30 min. 2D MS data were processed with SPIKE, using *urQRd* with rank 10. All spectra were internally calibrated using known fragment peaks with a quadratic calibration function, and manually interpreted and assigned as reported in previous studies [14, 16].

Results

The results of the 2D ECD MS analysis of tryptic-digested calmodulin are showed in Figure 1. Figure 1a shows the resulting 2D mass spectrum, with the extraction of the autocorrelation line (1b) and a horizontal fragment ion scan (1c).

The ECD 2D mass spectrum in Figure 1a shows a clear autocorrelation line, and different horizontal lines of signal

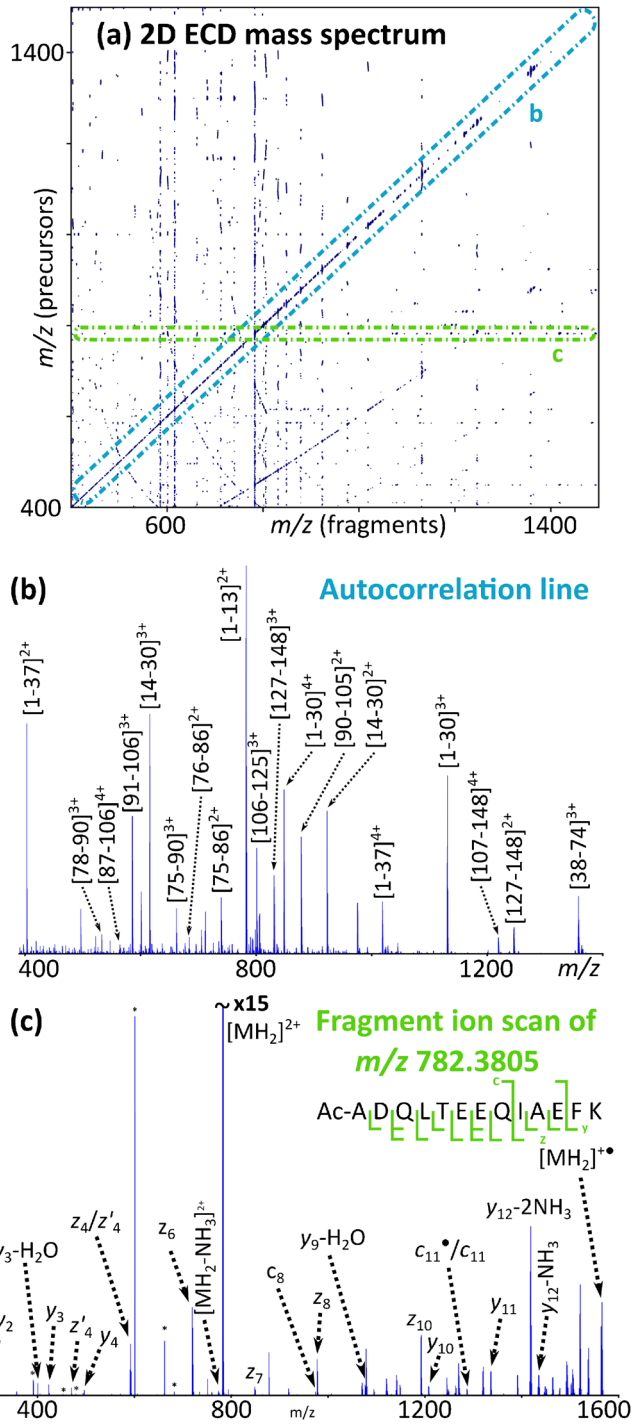

Figure 1. Bottom-up 2D ECD MS analysis of calmodulin. **(a)** ECD 2D mass spectrum of tryptic digested CaM, with highlight on the autocorrelation line and a precursor ion scan. The vertical lines represent the residual scintillation noise, heavily reduced by urQRd. **(b)** Extraction of the autocorrelation line, with the assigned tryptic peptides. **(c)** Fragment ion scan of the peptide with *m/z* 782.3805, with its sequence and relative cleavage coverage

corresponding to the fragmentation of each precursor. Vertical "noise streaks" are also clearly visible, representing the residual scintillation noise [24], together with curved lines departing

from the bottom left part of the spectrum, constituting harmonic signals. The characteristics of the spectrum are reported in Table S1 in the Supporting Information.

The autocorrelation line is extracted in Figure 1b. It shows all the species present in the mixture under analysis, derived from the digestion of CaM with trypsin. A total of 19 peaks could be correctly assigned to peptides generated by the tryptic digestion, based on their *m/z* with an average mass accuracy of 0.71±2.86 ppm (mean ± standard deviation, SD) (Table S.1 in the Supporting Information). The peaks on the spectrum are present with a range of charges between 2+ and 5+, over a single-scan dynamic range of ~190.

For each precursor, a horizontal ion scan has been extracted, generating a mass spectrum showing the fragmentation pattern of the chosen species. An example is reported in Figure 1c for the ion at *m/z* 782.3805, corresponding to $ADQLTEEQIAEFK^{2+}$, labeled on the autocorrelation line as $[1–13]^{2+}$. The fragment ion scan in Figure 1c shows the fragmentation pattern of the peptide, exhibiting a range of *c/z* ions and *y* ions. Peaks corresponding to experimental noise are marked with a * symbol. The spectrum shows cleavages for all the bonds in the peptide.

The horizontal scans extracted for each precursor have been analyzed (Tables S.3 to S.8 in the Supporting Information), reporting in total 69/148 cleavages for the entire protein, for a resulting cleavage coverage of ~47%.

Discussion

The analysis of a mixture of peptides generated by tryptic digestion of calmodulin has been performed using 2D MS with ECD as fragmentation technique. The 2D ECD MS analysis of tryptic-digested CaM generated a single 2D mass spectrum containing all the available tandem mass spectrometry information of the entire mixture.

In 2D MS, analysis of the autocorrelation line constitutes the first means of exploration of the sample, providing information of what constitutes the mixture before the simultaneous fragmentation of the precursors. The extraction of the autocorrelation line shows a range of peptides generated by the tryptic digest of calmodulin. The assigned peptides cover almost all of the protein, with a gap between amino acids in positions 31 to 34 (Table S.2 in the Supporting Information).

Analysis of the horizontal ion scans of the fragmenting analytes allows improving the exploration of the coverage obtained with 2D IRMPD MS studies on calmodulin. The use of different fragmentation techniques is known to bring complementary information to the structural analysis of proteins, above all when the techniques generate different kinds of fragment ions, such as IRMPD and ECD. The 2D ECD mass spectrum and the 2D IRMPD mass spectrum of the tryptic digest of calmodulin yielded good cleavage coverage for different tryptic peptides, thus resulting in higher cleavage coverage. As a result, combining fast 2D ECD and 2D IRMPD analysis can be a powerful tool for bottom-up proteomics. In fact, although similarities can be observed in the cleaved bonds for the two techniques, the 2D MS experiment performed with ECD enhances the cleavage coverage of the whole protein by fragmenting extensively ions that showed only multiple water losses with 2D IRMPD MS. However, as for the IRMPD study, not all the precursors showed significant fragmentation because using a set fragmentation parameter combination is not ideal for all charge states.

By summing the information obtained with this work and the bottom-up and top-down 2D MS analysis of calmodulin, the cleavage coverage of the protein has risen to ~68%.

Conclusions

The 2D IRMPD MS analysis of CaM resulted in a ~40% cleavage coverage of the protein under analysis. In this work, the cleavage coverage of CaM has been improved of more than 20% by performing a single additional 30-min BUP ECD 2D experiment, for a total of 68%.

Acknowledgments

The authors thank EPSRC (grants J003022/1 and N021630/1) and Bruker Daltonics, UK, for funding, and Andrew Soulby, Hayley Simon, Alice Lynch, Cookson Chiu, Meng Li, Sam Bisby, and Tomos Morgan for helpful conversations.

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