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Protein species-specific characterization of conformational change induced by multisite phosphorylation

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ABSTRACT

Phosphorylation is a central mechanism for regulating the structure and function of proteins in the cell, but accurate characterization of a specific protein phospho-species is challenging due to the difficulty of separating it from other species, as well as the limitations of the traditional structural methods. By using selective top-down ETD, we were able to identify six specific phospho-species of calmodulin (CaM). Phosphorylation of CaM at four sites by CK2 was found to follow a sequential order, with Ser81 as the first, Thr79 as the second, and Ser101 or Thr117 as the third. By combining top-down ETD with hydrogen/deuterium exchange, the impact of phosphorylation on CaM's structure was elucidated in a species-specific manner. A negligible structural effect was observed for mono-phosphorylation at Ser81, or di-phosphorylation at Ser81-Thr79, or tri-phosphorylation at Ser81-Thr79-Ser101 or Ser81-Thr79-Thr117. However, it was found that a significant phosphorylation-induced conformational change in CaM was caused by simultaneous phosphorylation at Ser101 and Thr117. The dramatically increased deuterium incorporation for residues between 102 and 119 strongly suggests that the structure of this region has been greatly changed.

1. Introduction

Post-translational modification (PTM) of proteins plays crucial roles in the regulation of their cellular functions, and has been one of the main focuses of modern proteomic research [1,2]. As one of the most common and important PTMs that occur within the cell, phosphorylation typically leads to changes in a protein's conformation, activity, localization, and interactions with other biomolecules [3,4]. With kinases and phosphatases accounting for 2–4% of eukaryotic proteomes, it has been estimated that about one-third of eukaryotic proteins are phosphorylated [5]. Phosphorylation not only modulates protein interaction by increasing their negative charge on the surface, but also through changing the protein's structure, as in the case of many kinases, receptors, ion channels, and protein degradation processes [6–9]. Due to the central regulatory role that phosphorylation plays in signal transduction within a living cell, it is of key interest to develop better strategies for structural characterization of phosphorylated proteins. However, the reversible, dynamic nature, and low stoichiometry of phosphorylation, and the low relative abundance of many phospho-species make structural characterization of these proteins extremely challenging. In particular, for a protein with multiple phosphorylation sites, it is almost impossible to purify one specific phospho-species out of a complex solution-phase mixture [5].

Compared to the tremendous advancements in PTM identification and quantitation [10–12], higher-order structural characterization of post-translationally modified protein species [13–17] – which is crucial to a better understanding of their functions – has far lagged behind. X-ray crystallography and NMR spectroscopy are two classical methods used for high-resolution structural analysis of proteins [18]. However, due to critical requirements for sample amount and purity, application of these two methods is largely limited to abundant or recombinant proteins which are devoid of naturally-occurring PTMs. Hydrogen/deuterium exchange mass spectrometry (HDX-MS) has become a powerful tool for characterizing protein structure and dynamics [19–21]. Two workflows are usually employed in this method, one is "bottom-up", the other is "top-down". In the traditional bottom-up approach, the protein is subjected to rapid pepsin digestion after D_2O labeling, followed by analysis of the deuteration status of these individual protein segments using LC-MS at ~0 °C [19,20]. In the more recent top-down HDX-MS approach, the deuteration pattern of a labeled protein is determined by the direct fragmentation of intact proteins by electron capture dissociation (ECD) [22–26] or electron transfer dissociation (ETD) [27–31] in the gas-phase.

Although each of the two HDX-MS approaches has its own advantages, the capability of analyzing a protein conformer/isoform selectively

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using gas-phase precursor ion isolation [25,32,33] makes the top-down approach an attractive choice for the characterization of PTM species. If a protein is digested into small peptides (as in the bottom-up approach), the deuteration information on each peptide will be a mixture of all the PTM species originally present. In addition, because top-down MS with ETD/ECD preserves labile phosphoryl groups during the fragmentation process, it serves as an ideal approach for characterizing phosphoproteins [7].

CaM is a highly-conserved calcium sensing protein in eukaryotic cells and plays crucial roles in a wide variety of intracellular Ca^{2+}-signaling pathways [34]. It is involved in the activation of over 300 target proteins including kinases, phosphatases, ion channels, and pumps [34,35]. Phosphorylation of CaM occurs extensively both in vitro and in vivo, and CK2 has been determined as one of the major enzymes responsible for this reaction [36,37]. Four phosphorylation sites, namely Ser81, Thr79, Ser101, and Thr117, have been reported for CK2 [36–38]. In theory, with four phosphorylation sites, there are 16 (2^4) possible phosphorylated protein species. However, the trypsin digestion-based bottom-up MS approach used in that work [37] could only identify the sites of phosphorylation, but not the actual phosporylated protein species, because the combination of phosphorylation sites on each original protein species no longer existed after the protein is digested into small singly-phosphorylated peptides. We also note that there is no X-ray crystallographic or NMR structural data available for the phosphorylated CaM species. In this work, we used top-down ETD to identify the true phospho-species of calmodolin (CaM) and selectively characterized their structures by top-down HDX-MS.

2. Experimental

2.1. Materials

Human calmodulin (recombinant) was obtained from Enzo Life Sciences (Farmingdale, NY, USA), and deuterium oxide was from Cambridge Isotope Laboratories (Andover, MA, USA). The peptide enfuvirtide was bought from Thermo Scientific (Bremen, Germany). In vitro phosphorylation of CaM was carried out using kinase CK2 (New England Biolabs, Ipswich, MA) according to a procedure from the literature [38]. Briefly, CaM was incubated for 15 h at 37 °C and pH 7.5, under the following conditions: 50 mM Tris–HCl, 100 mM NaCl, 1 mM EGTA, 40 μg/mL kinase CK2, and 0.1 mg/mL CaM. To separate phosphorylated CaM (CaMp) from the kinase, the samples was injected onto a size-exclusion column (TSKgel G3000SWXL, TOSOH Bioscience, San Francisco, CA) operating at a flow rate of 0.4 mL/min with a mobile phase consisting of 20 mM sodium phosphate and 100 mM sodium chloride, at pH 6.7. The fraction containing CaMp was concentrated to approximately 0.1 mg/mL using a centrifugal membrane (1 K MWCO, Millipore, Billerica, MA).

2.2. Hydrogen/deuterium exchange

HDX was carried out by mixing CaMp (100 μM, pH 6.7) with D_2O buffer at a ratio of 1:4 (*v*/*v*). After incubation for 20 s, 10-μL aliquots were removed and quickly quenched by reducing the pH to 2.5 with 10 μL of phosphate buffer at pH 2.0. The samples without HDX were prepared similarly but without adding any D_2O. The samples were flash-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Liquid chromatography

LC-MS was conducted at −20 °C using a subzero setup described previously to reduce the back-exchange to a level of 2%, as determined using calmodulin [26]. Subzero temperature LC-MS was achieved by placing the column in a deep freezer (Twinbird Corporation, Tsubame city, Japan). The freezer is capable of controlling the temperature from +10 °C to −40 °C in 1.0 °C increments. 35% methanol was added to solvent A to avoid freezing of the solvents inside the column and solvent delivery lines, along with 0.1% formic acid. Solvent B was 100% acetonitrile with 0.1% formic acid. The sample injector (Rheodyne Model 7125, sample loop volume 20 μL) was embedded in an ice bath. A 15-min binary solvent gradient was used for protein elution, including a 2-min desalting time. Solvent A contained 35% methanol with 0.1% formic acid, while solvent B was 100% acetonitrile with 0.1% formic acid. The flow rate was 200 μL min^{-1}. The eluent was diverted to waste for the first 2 min to prevent salts from entering the instrument. Both the samples with and without HDX were thawed on ice and immediately injected and analyzed by LC-MS. The protein was eluted using a linear gradient containing 20% to 60% solvent B, and the flow rate was decreased to 100 μL min^{-1} for ETD.

2.4. Mass spectrometry

All MS data were acquired on a Thermo Scientific Orbitrap Fusion mass spectrometer equipped with ETD (Thermo Scientific, Bremen, Germany). Basic instrumental parameters for the Orbitrap were described previously [31] and are briefly given here as follows: spray voltage 3500 V (positive), transfer tube temperature 300 °C, vaporizer temperature 275 °C, sheath gas 25, auxiliary gas 10. The Orbitrap detection was calibrated to within <5 ppm error with Calmix (Thermo Scientific, Bremen, Germany). Detection of the intact proteins in the LC-MS experiments was performed in the Orbitrap mass analyzer over a m/z 500–2000 mass range in "standard pressure mode", with a resolving power of 60,000. In the ETD experiments, the reagent target value was 3×10^5, and the ETD reaction time was 9 ms. Online ETD experiments were done by selecting each species detected using an isolation window of 4 m/z units in the quadrupole, in a single HPLC run. Twenty scans were averaged for each ETD spectrum. The charge state selected for ETD was 16+. ETD fragment ions were detected in the Orbitrap using a scan range of 150–2000 m/z, with a resolution of 60,000.

2.5. Data analysis

The MS data were processed using Xcalibur software (version 3.0.63, Thermo Scientific). The ETD product ions were identified through ProteinProspector (http://prospector.ucsf.edu) as described previously [23,26]. The fragment tolerance was set as 10 ppm for this analysis. Only c and z ions were used for HDX analysis because they have been reported to be free of H/D scrambling [22,27]. The ETD cleavage maps in Fig. 2 were created using ProSight Lite (http://prosightlite.northwestern.edu), but the assignments were confirmed by comparing them to the match results from ProteinProspector. The centroid m/z values for the unlabeled ETD ions were obtained from ProteinProspector, and the centroid m/z values after HDX were determined using an in-house written excel spreadsheet (Microsoft, Redmond, WA). Only ETD fragment ions which had a signal-to-noise ratio (S/N) >5 were used for calculating the deuteration content. The number of deuterium atoms acquired by each protein segment, N_{exch}, was determined from analyses of ETD ion mass shifts before and after HDX. Because top-down ETD was performed after HPLC in the present study, the deuterium numbers obtained would only come from the exchanged amides, thus no exchanged hydrogens on the side chains and charge carriers need be taken into account. The details, including all of the equations, of calculating amino acid level deuteration information was described in a previous paper [26], and the error was on the order of ±0.1. The global HDX data and ETD data shown in the figures represent experiments made in triplicate. The error of the global HDX data was within ±0.5 Da.

3. Results and discussion

The LC-MS spectrum at charge state 16+ obtained on Orbitrap fusion is shown in Fig. 1A. The five peaks with zero to four phosphate

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