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Probe the protein conformation using top-down hydrogen exchange mass spectrometry at higher resolution with electron transfer dissociation

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Abstract

Purpose: Study protein, protein/complex conformations by hydrogen/deuterium exchange mass spectrometry (HDX-MS) using secondary fragmentation technique, electron transfer dissociation (ETD) to improve the resolution from peptide level to single amino acid resolution.

Methods: A model peptide, referred as P1 peptide, was used to confirm the proper ETD experimental condition for minimum deuterium scrambling and level of back exchange. Apo- and holo forms of calmodulin were then used to study by both top-down and bottom-up HDX-ETD experiments using a fully automatic HDX workflow station, TRAJAN CHRONECT system, Thermo Scientific™ Vanquish Flex/Binary pump N LC system with a Thermo Scientific™ Orbitrap ™ Ascend™ Structural Biology Edition mass spectrometer.

A full scan (MS) and an ETD MS^2 scan were both conducted at the same time for bottom-up experiments. The MS full spectra were used to probe region of significant change in deuterium incorporation, and the ETD MS2 spectra were then used to pinpoint deuterium incorporation at the single amino acid level for the specific peptides that showed significant deuterium change.

Results: Optimal instrument parameters were tuned on the Orbitrap Ascend Structural Biology Edition mass spectrometer and were used for following studying. Near 100% sequence coverage was obtained by bottom-up ETD MS2 experiments for apo- and holo- calmodulin, while to-down ETD gave 88% sequence coverage. Overall, the apo calmodulin showed more deuterium incorporation than the holo- calmodulin, which is consistent with previous results. Notably, nearly single amino acid deuterium incorporation results were observed from consecutive N and C terminal fragments combining top-down and bottom-up experiment.

Introduction

HDX has emerged as a powerful tool to study protein conformation in native states. When HDX is analyzed at the peptide or protein level, only the overall deuterium uptake of peptides or proteins is obtained. Determination of deuterium incorporation at single amino acid resolution requires a secondary fragmentation which may induce “hydrogen scrambling” issue. ETD, as a nonergodic, fragmentation technique that could yield a low level of hydrogen scrambling and therefore allows single residue localization of incorporated deuterium.

A model peptide P1, HHHHHHIIKIIK, was used to evaluate this technique to obtain single residue resolution. Calcium binding protein, calmodulin, was studied with top-down and bottom-up approaches to compare the protein conformation for its apo-and holo-forms.

Materials and methods

Scrambling optimization

A model peptide, HHHHHHIIKIIK, was prepared in both H2O and D2O at 10 µmol/L and kept at 4 ºC over 24 hours allowing complete deuterium exchange. 10 µL of the solution was injected to a C18 trap column for 2 min trapping allowing full back-exchange at N-term and then the peptide was eluted with a 4 min short gradient elution. The effect of source, capillary temperatures, and voltages at different ion transfer stages on hydrogen scrambling and deuterium back exchange were further investigated individually.

Calmodulin ETD HDX workflow

Apo- and holo- calmodulin samples were diluted at a 1:1 ratio with labeling D2O buffer and incubated for various time points, 30 sec, 90 sec, 900 sec and 3600 sec. In the bottom-up ETD workflow, the samples were quenched and digested before being loaded onto a Thermo Scientific™ Hypersil GOLD™ C18 analytical column, 1mm x 5cm ID. For bottom-up experiments, simultaneous full scan (MS) and ETD MS2 scan were conducted. The MS full spectra were utilized to identify regions of significant change in deuterium incorporation, while the ETD MS2 spectra were subsequently employed to pinpoint deuterium incorporation at the individual amino acid level for specific peptides exhibiting notable deuterium changes.

Conversely, for the top-down ETD workflow, the denatured samples were desalted using the C18 trap and directly eluted to the Orbitrap Ascend Structural Biology Edition mass spectrometer.

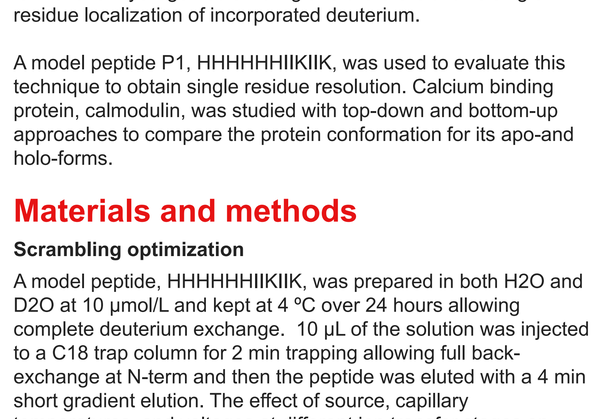

Figure 1. Scheme of bottom-up ETD experiment workflow.

Data analysis

Peptide identification was performed with Thermo Scientific™ BioPharma Finder™ 5.2 software (BPF). Both top-down and bottom-up HDX experimental data were analyzed with HDExaminer software 3.4.1 (Trajan).

Results

ETD experimental condition optimizations

The synthetic peptide P1,HHHHHHIIKIIK, was used to evaluate ETD experimental condition. 10 µL of 10 µMol fully deuterated P1 was injected into a C18 trap column. Multiple MS parameters were evaluated and optimized to reducing scrambling to a negligible level while minimizing the impact on sensitivity on a high-resolution OT

MS full and ETD MS2 spectra were collected during the same injection (**Figure 2**). Global parameters for ion source conditions and several voltages in mass spectrometer ion transmission showing in **Figure 3**, were further investigated and tuned for low level of scrambling.

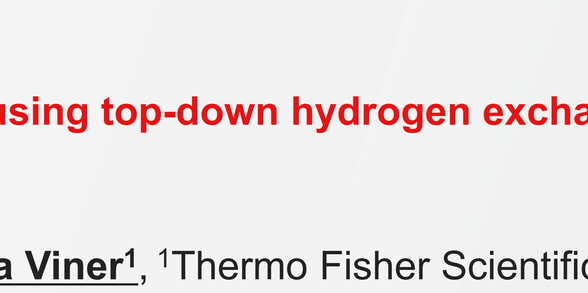

Figure 2. Model peptide P1 MS full scan and ETD spectrum of +3 precursor, *m/z* 518.9800.

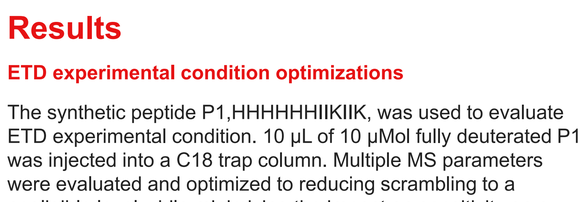

Figure 3. Architecture of Orbitrap Ascend Structural Biology Edition mass spectrometer.

The low deuterium incorporation between c2 and c6 is indication of a low level of deuterium scrambling. The deuterium content for the c fragments of peptide P1 is comparable to what is reported in the literature, as showing in **Figure 4a** for different OT tribrids MS. Additionally, the scrambling level was also evaluated using the ammonia loss as a reporter ion of hydrogen scrambling, as showing in **Figure 4b**.

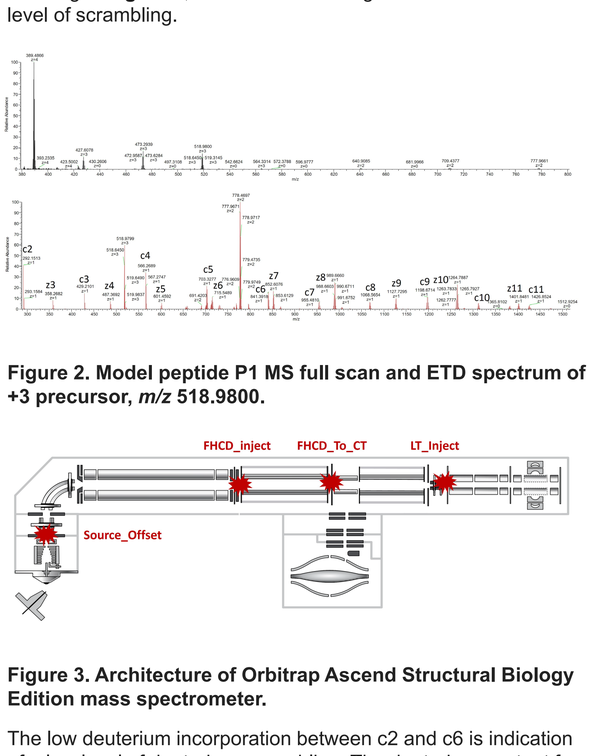

Figure 4. Measurements of P1 ETD Fragment deuterium content on OT Tribrid Mass Spectrometers.

Minimal fragment deuterium scrambling measurement was obtained with optimized instruments and source operation conditions. Recommended settings for tuning OT Ascend are summarized in **Table 1**.

Global Parameters	Ion Transfer Tube Temp.	Vaporizer Temp.*	RF Lens (%)
Recommended Settings	220 ºC	50 ºC	25

Transmission Voltages	Source Offset	FHCD_ Inject	FHCD_To_ CT	LT_Inject
Recommended Settings	10-11	-3 to -4	4 to 5	-12

* Vaporizer Temp. is recommended based on flow rate at 40 µl/min. Need to be adjusted accordingly for different flow rate.

* Transmission efficiency will decrease along with changing the transmission voltages.

Table 1. Summarized recommending parameter settings.

Calmodulin and Ca^{2+} binding system

Calmodulin is a calcium binding protein. It is known that once calcium binds to the protein, the conformation has changed. C terminal folding orientation changes more than N terminal after binding.

There are four Ca binding sites at region 21-32, 57-68, 94-105, and 130-141, highlighted in yellow in **Figure 5**.

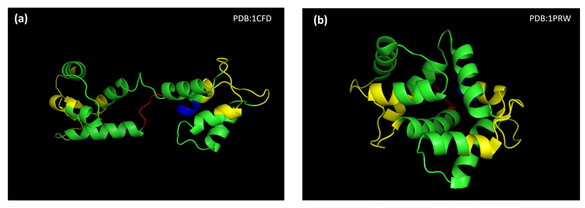

Figure 5. Crystal structure of Apo-, holo- calmodulin forms. Ca^{2+} binding sites are colored in yellow.

Bottom-up ETD MS2 experiment

Bottom-up ETD at four labeling time points experiments were conducted for the apo-, holo- calmodulin forms. 140 peptides were identified and used to do deuterium uptake measurements for both apo-, holo- calmodulin forms, giving 97% sequence coverage (shown in **Figure 6**). The apo calmodulin showed more deuterium incorporation than the holo- form, and there were more deuterium uptake on the C-terminal peptides than N-terminal peptides.

One representative peptide at the binding region was used to show how to use ETD data to obtain near single amino acid resolution, showing in **Figure 7**.

Figure 6. Sequence coverage for bottom-up ETD experiment

One representative peptide, residue 20-32, covering one of the binding regions was used to show how to use ETD data to obtain near single amino acid resolution (Fig.7 b). As showing in **Figure 7 (a)**, c2 to c12 and z3 to z12 except z5, were identified and uptake curves were showed for each one of the c ions in **Figure 7 (c)**.

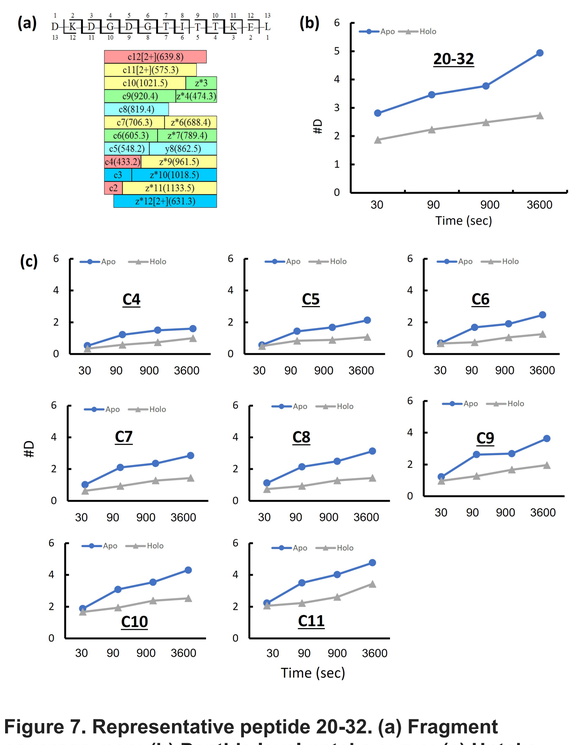

Figure 7. Representative peptide 20-32. (a) Fragment coverage map; (b) Peptide level uptake curve; (c) Uptake curve of c4 to c11 ions.

With the obtained fragment ion deuterium incorporation information, the fragment verses deuterium content could be plotted out as in **Figure 8**. For this particular peptide, deuterium incorporation was obtained at near single amino acid resolution by ETD fragment ions.

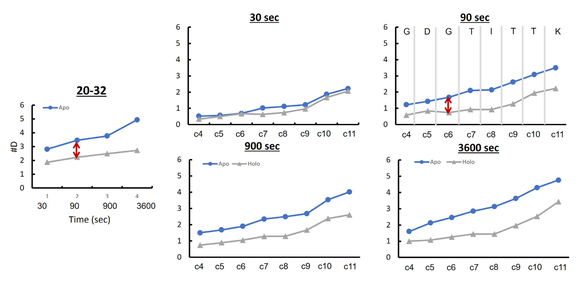

Figure 8. Fragments deuterium difference between holo- and apo- states.

Top-Down ETD experiment

Top-down workflow can aid in distinguishing variants. As showing in **Figure 9**, calmodulin with a N-terminus His-tag displayed a higher charge distribution compared to the variant without it. The His-tag introduces more charges to the N-terminus, resulting in increased coverage at the N-terminus but reduced coverage at the C-terminus.

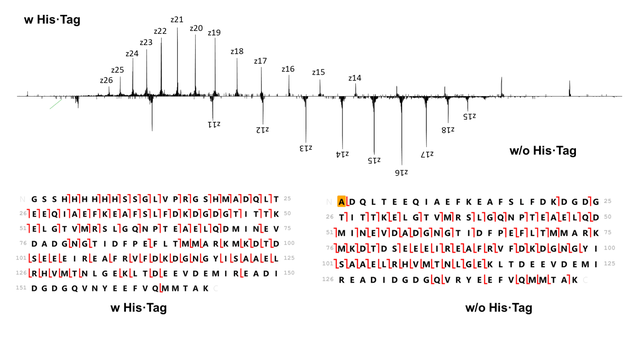

Figure 9. Top-down ETD MS full scan and sequence coverage map for two Calmodulin variants. Top panel: with His·Tag; Bottom-panel: without His·Tag.

Top-down MS full scan with targeted ETD MS2 was performed for apo-, holo- calmodulin (no His·Tag) at different labeling time points. The deuterium incorporation of the intact level was measured. The apo- calmodulin had more deuterium incorporation than the holo- form for all the measured time points. HDExaminer was used to calculate the deuterium incorporation for each fragment.

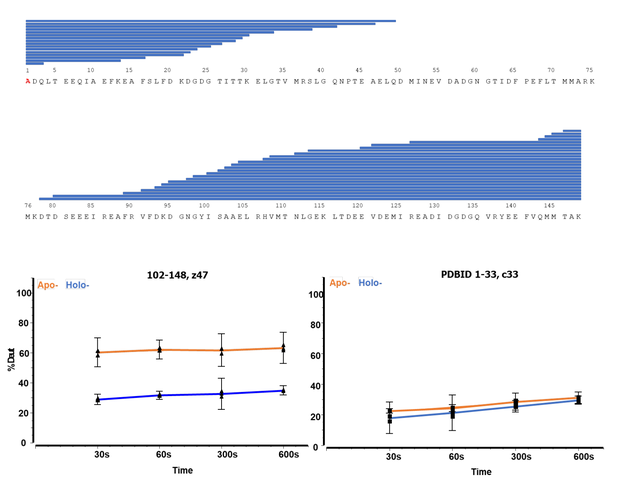

Figure 10. Sequence coverage for top-down ETD experiment and uptake curves of two representative fragments.

Conclusions

- The optimal parameters for low deuterium scrambling HDX-ETD on Orbitrap Ascend Structural Biology Edition mass spectrometer were tuned using P1 peptide as standard.
- HDX-ETD top-down and bottom-up experiments were successfully applied to a structural differential study for apo-, holo- calmodulin forms conformations. Consistent results were obtained from the two approaches and was matched with previous results published on literatures. Overall, the apo-calmodulin had more deuterium incorporation than the holo-calmodulin form and there were mode deuterium uptake on the C-terminal peptides than N-terminal peptides.
- Nearly single amino acid resolution was obtained for N-, and partial of C-terminals from the top-down experiment. Bottom-up ETD MS2 experiment data provided complementary information on nearly amino acid resolution to the top-down experiment.
- The top-down approach provides a possibility using HDX-MS as a high-throughput MS-based approach for protein-ligand binding study.

References

1. Kasper D. Rand and Thomas J. D. Jørgensen, Analytical Chemistry 2007 79 (22), 8686-8693, DOI: 10.1021/ac0710782
2. Kasper D. Rand, Martin Zehl, Ole N. Jensen, and Thomas J. D. Jørgensen, Analytical Chemistry 2010 82 (23), 9755-9762DOI:10.1021/ac101889b

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