Optimizing Electron Transfer Dissociation Conditions for Hydrogen/Deuterium Exchange Mass Spectrometry and Its Application to the Study of Protein Conformation

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ABSTRACT

Purpose: A HDX workflow utilizing electron transfer dissociation (ETD) was developed to pinpoint protein conformation with near single amino acid residue level resolution.

Methods: A model peptide and protein were used to develop the designed workflow. Both an MS full scan and an ETD MS^2 scan were conducted in a single experiment. The MS full spectra were used to probe region of significant change in deuterium incorporation. The ETD MS^2 spectra were then used to pinpoint deuterium incorporation at the single amino acid level for the specific peptides that showed significant deuterium change.

Results: 100% sequence coverage was obtained for the bottom-up ETD MS^2 experiment for cytochrome C. More than 160 peptides were identified and 127 peptides were used for deuterium uptake measurement. Single amino acid resolution was obtained for many peptides. Around 85% sequence coverage from 46 fragments was obtained for the cytochrome C HDX-ETD top-down experiment. Near single amino acid deuterium incorporation results were obtained from the consecutive N and C terminal fragments.

INTRODUCTION

Proteins possess primary, secondary, ternary and quaternary structures. Hydrogen/deuterium exchange mass spectrometry (HDX) has emerged as a powerful tool to study protein conformation, conformation dynamics and protein-protein interactions. 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 by CID and related techniques is perturbed by "hydrogen scrambling". Electron transfer dissociation (ETD) is known in the literature as a potential technique that would yield very low levels of hydrogen scrambling and therefore allows for localization of incorporated deuterium with single residue resolution[1]. A model peptide and protein were used to evaluate this technique as to its feasibility at single residue resolution.

MATERIALS AND METHODS

Sample Preparation

A model peptide, HHHHHHIIKIIK, was prepared in both H_2O and D_2O at 100 µmol/L and kept at 4 ºC over 24 hours allowing complete deuterium exchange. The solution was diluted 50-fold with cold H_2O buffer (50% MeOH, 0.5 M acetic acid, pH 2.5) and infused into the ESI source. Source and capillary temperatures were evaluated to measure hydrogen scrambling and deuterium back exchange.

Cytochrome C was studied by both top-down and bottom-up HDX-ETD experiments on a fully automated HDX workflow station incorporating a Thermo Scientific™ Orbitrap Fusion™ mass spectrometer, Figure 1. Cytochrome C was diluted (1 to 9 ratio) with D_2O/HEPES pH 7.4 labeling buffer, and incubated for multiple time points. The labeled protein samples were then quenched with 2M guanadine, 100mM citric acid, pH 2.2 quenching buffer and subjected to online pepsin digest prior to injection into a trapping column and elution to a Thermo Scientific™ Hypersil GOLD™ C18 reversed phase column. Top-down method was performed with a C8 column.

Experimental Method Conditions

Mass Spectrometry

Parameters \ Workflow	Bottom-up	Top-down
MS Full Resolution	60K	120K
MS AGC	2E5	2E5
MS Mass Range	300-1000	200-2000
RF Lens	40	40
Sheath	20	20
Source Temperature ºC	50	50
Capillary Temperature ºC	220	220
Isolation	3 or 5	5
MS2 Resolution	60k	120k
MS2 AGC	1E5	2E5
MS2 Mass Range	200-2000	
MS2 Microscan	2	5
Reagent Target	4E5	4E5

LC Method

Bottom-up

Retention [min]	Flow [µl/min]	%B
4.9	45.00	3.0
5.0	45.00	10.0
17.0	45.00	30.0
19.0	45.00	90.0
20.0	45.00	3.0

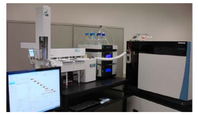
Top-down

Retention [min]	Flow [µl/min]	%B
0.5	45.00	30.0
2.0	45.00	70.0
2.1	45.00	90.0
3.0	45.00	90.0
3.1	45.00	30.0

Data Analysis

Peptide identification, mapping, and PTM analysis were performed with Thermo Scientific™ BioPharma Finder™ 2.0 software. Both top-down and bottom-up HDX experimental data were analyzed with HDExaminer™ 2.2 beta software (Sierra Analytics).

FIGURE 1. HDX work station coupled online to the Orbitrap Fusion mass spectrometer

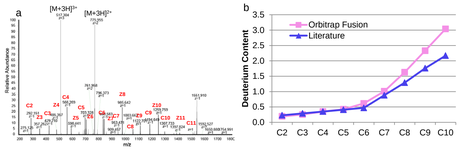


RESULTS

ETD experimental conditions

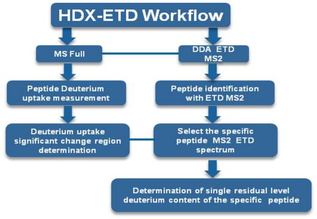
The synthetic peptide P1, with the sequence HHHHHHIIKIIK, was used to evaluate the ETD experimental condition[1]. ESI source needle temperatures of 30, 50, 100 ºC, ion transfer tube temperatures of 220, 270 and 300 ºC, and different isolation widths were evaluated to minimize back exchange and deuterium scrambling. In general, lower needle and transfer capillary temperatures reduced the back exchange, but did not have much impact on deuterium scrambling. The isolation width also did not have a significant effect on deuterium scrambling (data not shown). Spectra with a complete series of c- and z- fragment ions are shown in Figure 2a. Minimal deuterium scrambling described in the literature[1,2] for the fully deuterated P1 peptide 3+ precursor (N-terminal should contains hydrogen up to C6 while C-terminal should retain deuterium) was observed under typical bottom-up HDX experimental conditions, as shown in Figure 2b. The ESI source temperature of 50 ºC, ion transfer capillary temperature of 220 ºC were chosen to obtain sufficient sensitivity and low deuterium back exchange.

Figure 2. a) Model peptide P1 ETD spectrum with near complete c-, z- fragment ions. b) Fragment deuterium content measurement

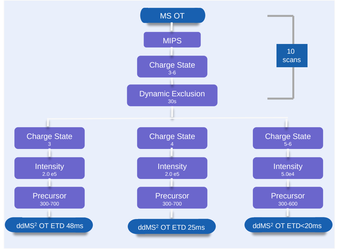


The HDX-ETD bottom–up experiment was conducted with MS full scan followed with data dependant ETD MS^2 (Figure 3 top panel). ETD MS^2 spectra of the undeuterated sample were used for peptide identification. The MS full scans were used to measure the deuterium content of the identified peptides at different time points and conditions. Once the significantly different deuterium uptake region was observed, the MS^2 ETD spectra of that region would be investigated to get the higher resolution (near single amino acid) to pinpoint the exact position.

Figure 3. Top panel: Bottom-up HDX-ETD workflow scheme. Bottom panel: HDX- ETD experimental method set up with the Orbitrap Fusion MS



To get the best ETD fragmentation efficiency, monoisotopic precursor selection (MIPS) was used. Only charge states of 3 and above precursors were set to trigger ETD MS^2. Values of intensity, precursor range, AGC, isolation width, injection time, and ETD reaction time were optimized and used for the different charge state precursor as shown in Figure 3, bottom panel.



Cytochrome C bottom-up HDX-ETD

Cytochrome C bottom-up experiments with 5 labeling time points were performed using the optimized HDX-ETD experimental conditions. Around 160 peptides were identified and 100% sequence coverage was obtained. 127 peptides were used for the deuterium content measurement for the two labeling conditions. Based on the MS full scan data, similar deuterium uptake profiles were observed for the two labeling conditions as shown in Figure 4 a). The residual plot of the two conditions showed that there was more deuterium uptake from the HEPES buffer labeling condition for all the detected peptides. The differences ranged from 0.2 to 4 (Figure 4 b).

Figure 4. a) Cytochrome C deuterium uptake mirror plot. The X axis represents the number of identified peptides. The Y axis is the number of deuterium uptake of each identified peptide. b) Cytochrome C deuterium uptake residual plot of the two labeling conditions

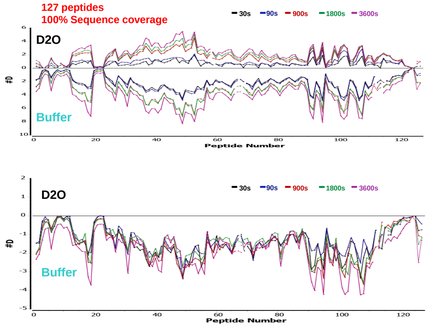
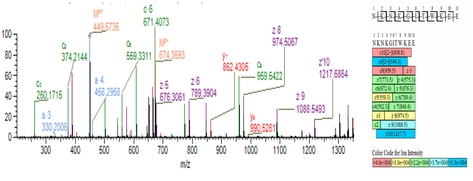


Figure 5. ETD MS2 spectrum of NKNKGITWKEE peptide



After the deuterium content significant change area had been identified from the residual plot, the specific ETD MS^2 spectra of the peptides in that region were investigated. Figure 5 presents the examples of the peptide from 52-62. The spectrum contains almost complete c- and z- fragment ions. The selected peptide ETD MS^2 data of the two labeling conditions were processed with HDExaminer software. The deuterium uptake for the peptide was calculated and the peptide fragments were obtained as shown in Figure 6a. Consistent deuterium uptake measurements were obtained from charge state 3 and 4 precursors (Figure 6b and c).

Figure 6a Sequence 52-62 peptide, NKNKGITWKEE fragments prediction by HDExaminer software. 6b 3+ precursor deuterium uptake plots 6c 4+ precursor deuterium uptake plots

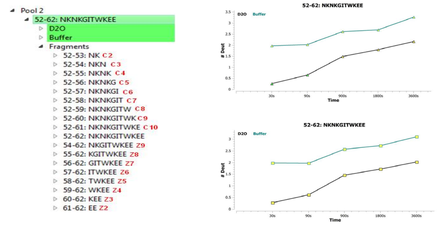


Figure 7. NKNKGITWKEE peptide fragment deuterium uptake plots Left panel: 900 second time point Right panel: 3600 second time point

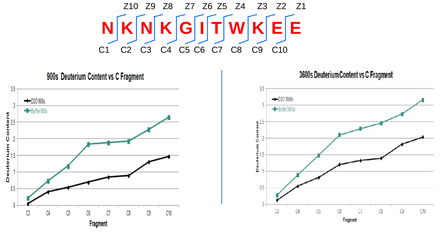
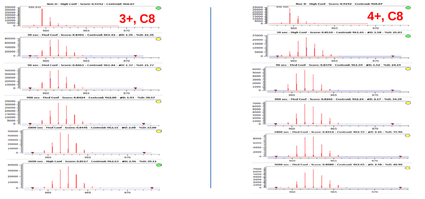
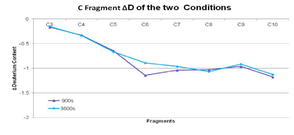


Figure 8. HEPES buffer labeled NKNKGITWKEE peptide C8 fragment isotopic cluster distribution Left panel: 3+ Right panel: 4+



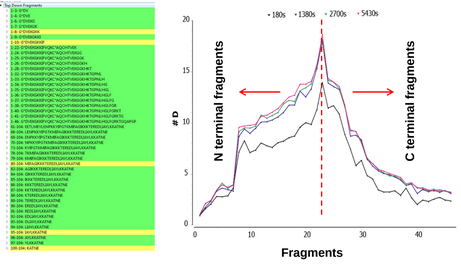
The fragment deuterium content was also calculated by HDExaminer. Figure 7 shows example fragment deuterium content plots for NKNKGITWKEE peptide. More deuterium was incorporated with increasing labeling time; similar deuterium uptake profiles for fragment C3 to C10 were observed for different labeling times. N and C terminal fragments incorporated more deuterium when compared to the middle fragments, C6 to C8. The sum of the deuterium content of the counter part C and Z fragment (C3, Z7 etc.) were consistent with the deuterium content of the peptide (data not shown). Furthermore, both charge 3 and charge 4 precursors generated very close fragment deuterium content values. Figure 8 was the C8 fragment isotopic cluster distribution as an example. Similar C8 deuterium content and isotopic cluster distribution were generated from 3+ and 4+ precursors.

Figure 9. C fragment deuterium content differences of the two labeling conditions



By comparing the deuterium uptake levels of C or Z fragments at the two conditions, discrepancies in deuterium uptake behavior at single amino acid level were obtained to pinpoint the conformation differences. Figure 9 was the C fragment deuterium difference plots (D_2O labeling as control) of peptide NKNKGITWKEE. There was more deuterium uptake for all the fragments, C3-C10, under the HEPES buffer labeling condition. Furthermore, the difference slope was shallower for the 3600s measurements. The differences were increased from C3 to C6, and remain flat after C6 for 900s.

Figure 10. Cytochrome C top-down with ETD Left panel: Identified fragments by HDExaminer Right panel: Deuterium uptake plot of the identified fragments.



Cytochrome C top-down with ETD

Cytochrom C targeted ETD top-down experiment was performed. The data were processed by HDExaminer, 46 fragments were identified from the Cytochrome C targeted ETD top-down spectrum as shown in Figure 10 left panel. Near complete fragments were identified for C terminal and N terminal except the heme modification area, C14-C18 . Figure 10 right panel was the fragment deuterium uptake plots.

CONCLUSIONS

- The HDX-ETD experimental conditions were identified and optimized.
- HDX-ETD bottom-up and top-down workflows were developed and successfully applied to study cytochrome C conformation.
- Single amino acid resolution was obtained for many number of peptides for the bottom-up approach. Near single amino acid resolution was obtained for the N, C terminal top-down approach.
- Both bottom-up and top-down HDX-ETD data were successfully processed by HDExaminer.

REFERENCES

1. K. Rand, C. M. Adams, R. A. Zubarev, T. J.D. Jorgensen, JACS, 2008, 01/03
2. D. Weis et al, Hydrogen Exchange Mass Spectrometry of Proteins