J. Am. Soc. Mass Spectrom. (2017) 28:971–977
DOI: 10.1007/s13361-017-1612-4

RESEARCH ARTICLE

Regio-Selective Intramolecular Hydrogen/Deuterium Exchange in Gas-Phase Electron Transfer Dissociation

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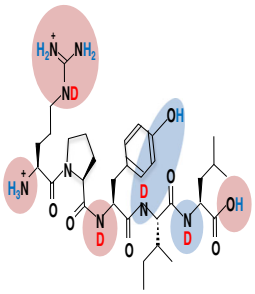

Abstract. Protein backbone amide hydrogen/deuterium exchange mass spectrometry (HDX-MS) typically utilizes enzymatic digestion after the exchange reaction and before MS analysis to improve data resolution. Gas-phase fragmentation of a peptic fragment prior to MS analysis is a promising technique to further increase the resolution. The biggest technical challenge for this method is elimination of intramolecular hydrogen/deuterium exchange (scrambling) in the gas phase. The scrambling obscures the location of deuterium. Jørgensen's group pioneered a method to minimize the scrambling in gas-phase electron capture/transfer dissociation. Despite active investigation, the mechanism of hydrogen scrambling is not well-understood. The difficulty stems from the fact that the degree of hydrogen scrambling depends on instruments, various parameters of mass analysis, and peptide analyzed. In most hydrogen scrambling investigations, the hydrogen scrambling is measured by the percentage of scrambling in a whole molecule. This paper demonstrates that the degree of intramolecular hydrogen/deuterium exchange depends on the nature of exchangeable hydrogen sites. The deuterium on Tyr amide of neurotensin (9–13), Arg-Pro-Tyr-Ile-Leu, migrated significantly faster than that on Ile or Leu amides, indicating the loss of deuterium from the original sites is not mere randomization of hydrogen and deuterium but more site-specific phenomena. This more precise approach may help understand the mechanism of intramolecular hydrogen exchange and provide higher confidence for the parameter optimization to eliminate intramolecular hydrogen/deuterium exchange during gas-phase fragmentation.

Keywords: Electron transfer dissociation, Electrospray ionization, Hydrogen/deuterium exchange, Intramolecular reaction, Mass spectrometry, Neurotensin, Hydrogen scrambling

Received: 23 October 2016/Revised: 29 December 2016/Accepted: 17 January 2017/Published Online: 13 February 2017

Introduction

While protein backbone amide hydrogen/deuterium exchange coupled with proteolysis, liquid chromatography, and mass spectrometry (HDX-MS) has become a popular analytical tool for protein characterization [1–4], one of the biggest technological challenges is to obtain single amide resolution data [5]. The ultimate goal of the HDX-MS technology is to determine the exchange rates of all backbone amide hydrogen at single amide resolution [6, 7]. Currently, the size of peptic fragments generated by proteolysis control the resolution in a typical HDX-MS study. Four different approaches potentially further improve the resolution of HDX-MS data; (1) sub-localization of deuterium by subtracting the deuterium incorporations in two analogous peptic fragments utilizing non-specificity of acid proteases [6, 8–10], (2) development of new software that can utilize the isotope envelope shape of an peptide instead of the centroid value [7, 11], (3) development of new proteases to cut the analyte protein at different sites [5, 12–15], and (4) sub-localization of deuterium within a peptide by gas-phase fragmentation [16–23].

Sub-localization of deuterium by gas-phase fragmentation is the most active area of investigation among the four approaches. From the 1990s, various groups explored this possibility using collision induced dissociation (CID) for sub-localization of deuterium [24–29]. However, the current consensus is that sub-localization of deuterium by CID may not be reliable, at least for protonated peptides, because it induces

Electronic supplementary material The online version of this article (doi:10.1007/s13361-017-1612-4) contains supplementary material, which is available to authorized users.

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intramolecular exchange (scrambling) among the exchangeable hydrogens and deuteriums [16, 19, 30–35].

More recently, electron capture dissociation (ECD) and electron transfer dissociation (ETD) showed better potential for sub-localization of deuterium within a peptide. Jørgensen's group developed synthetic peptides for the detection of scrambling in gas-phase fragmentation [16] and paved the way for the practical application of this method [17–19]. However, the optimization of ECD/ETD parameters may be challenging because the mechanism of scrambling is not clear and the degree of scrambling depends on various parameters. Nonetheless, several groups demonstrated the utility of ECD/ETD for deuterium sub-localization in HDX-MS studies [20, 21, 36–40].

In this paper, the optimization of ETD parameters is first described using previously characterized commercially available neurotensin (9–13), RPYIL (Figure 1 for the structure), to minimize scrambling [35]. It was then found that the intramolecular hydrogen exchange occurs in site-specific manner, unlike previously assumed complete randomization among all exchangeable sites. This type of more site-specific approach and results may shed the light on the mechanistic understanding of intramolecular hydrogen exchange in gas phase and help optimize the MS/MS parameters to minimize the scrambling.

Materials and Methods

Materials

Neurotensin (9–13), RPYIL, and (Lys^{15})-amyloid β-protein (15–21), KKLVFFA, was purchased from Bachem (King of Prussia, PA, USA). Deuterium oxide and all other reagents were obtained from Sigma (St. Louis, MO, USA).

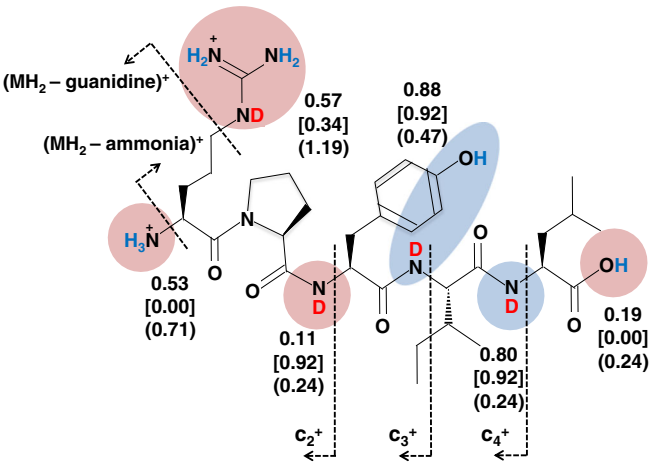

Figure 1. Deuterium distribution of fully deuterated neurotensin (9–13), with isolation width 32. Desalting and elution steps washed away all deuteriums except four positions (red). Deuterium incorporation at each segment was calculated from the deuterium incorporation in parent and daughter ions listed in Table 1. Top number, observed deuterium incorporation; middle number, calculated deuterium incorporation with 0% scrambling; and bottom number, calculated deuterium incorporation with 100% scrambling. Pink indicates fast exchanging sites and light blue indicates slow exchanging sites

Mass Analysis of a Peptide

To a 20 µL solution of 0.1 mg/mL non-deuterated (prepared in 100% water) or fully deuterated (prepared in 98% deuterium oxide for neurotensin peptide and prepared in 90% deuterium oxide for amyloid peptide) peptide was added 30 µL of a chilled 0.8 M GuHCl, 0.8% formic acid solution to practically stop the amide hydrogen exchange reactions. The mixture was loaded onto a reverse phase column chilled at 0 °C and washed with a chilled 0.05% aqueous TFA solution at 200 µL/min for 1 min to remove most of deuteriums from N- and C-terminal as well as the side chains. The peptide was then eluted by a chilled 95% acetonitrile, 5% water, 0.0025% TFA at 10 µL/min. The elution was analyzed by LTQ Orbitrap XL (Thermo Fisher Scientific, San Jose, CA, USA) with a capillary temperature of 200 °C. Typical ETD parameters are: parent mass, 331 for +2 neurotensin peptide and 426 for +2 amyloid peptide; isolation width, 32 (these make the mass range of 315–347 and 410–442); normalized collision energy, 35%; activation Q, 0.250; and activation time 150 ms.

Results

Prior to ETD investigation, fully deuterated neurotensin (9–13) parent ion was found to carry a higher than expected amount of deuterium. The fully deuterated neurotensin (9–13) parent ion retained 3.08 deuterium atoms per molecule, while prepared in 98% D_2O (Figure 2b and Table 1). The generally accepted assumption of HDX-MS technology is that only backbone amides can retain deuterium after downstream process (desalting and elution steps). Therefore, the maximum amount of deuterium attached to the fully deuterated peptide is 2.94 (=3 amides × 0.98; Figure 1 for the structure) in the current study. Counting inevitable deuterium loss during desalting and elution steps, the deuterium incorporation should be even lower.

Four positions of neurotensin (9–13) retained a significant amount of deuterium in the fully deuterated peptide (Figure 1 and Table 1). To understand the higher than expected deuterium recovery and find the distribution of deuterium in the fully deuterated peptide, the isotope envelope of fully deuterated peptide was deconvoluted (Figure 3; Table S1 in Supplemental Information). The isotope envelope was best fit by the four positions deuterated at 92%, 92%, 92%, and 34%, respectively. Additional deuterium incorporation at the fifth position did not improve the fit. The first three positions must be backbone amide hydrogen positions because the downstream process was carried out at pH 2.3, which is optimal to minimize the back (deuterium to hydrogen) exchange reaction of backbone amide hydrogens [41, 42].

The fourth deuterated position is the NHδ of arginine (Figure 1). The presence of the fourth position may be a little surprising, since it is usually assumed that all side-chain deuteriums are lost rather quickly during the

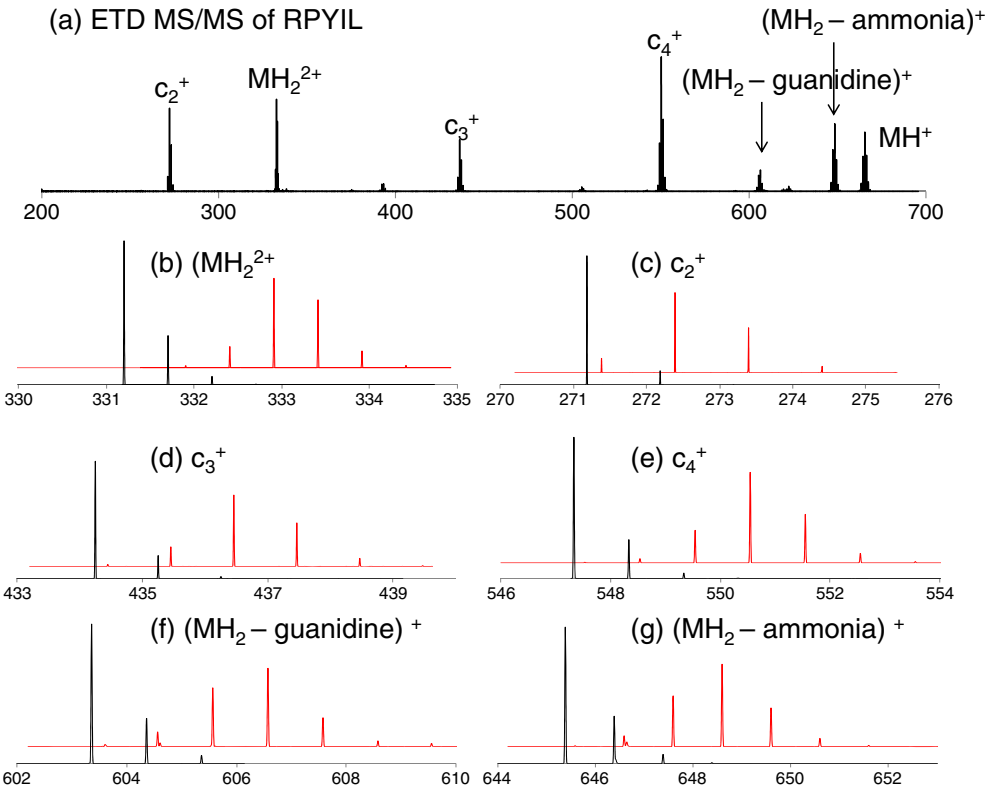

Figure 2. ETD MS/MS spectra of neurotensin (9–13). (**a**) ETD MS/MS full spectrum of non-deuterated neurotensin (9–13). (**b**)–(**g**) Non-deuterated (black) and fully deuterated (red) isotope envelopes of each daughter ion. Fully deuterated spectra are shifted to the right and up for clarity

downstream process. However, the current experimental condition not involving digestion and liquid chromatography steps probably led to better deuterium retention than the usual bottom-up HDX-MS analysis. Calculation using the study of Bai et al. [41] showed that the hydrogen exchange rate of NHδ of arginine is only five times faster than that of oligo-alanine and 25 to 60 times faster than those of three amide hydrogens in neurotensin (9–13) at pH 2.3 (Table S2 in Supplemental Information). The simulation predicts approximately 94% to 96% deuterium retention in the three amide positions, when the NHδ position retained 34% of deuterium, reasonably consistent with the deuterium distribution observed (Figure S1 in Supplemental Information).

ETD fragmented +2 charge state of neurotensin (9–13) very well (Figure 2). The high quality daughter ions generated, such as c_2^+, c_3^+, c_4^+, $(MH_2 - ammonia)^+$, and $(MH_2 - guanidine)^+$, enable investigating the intramolecular hydrogen/deuterium exchange in a fully deuterated peptide. Using these daughter ions, isolation width and capillary temperature were optimized to minimize scrambling.

The isolation width prior to ETD fragmentation had significant effects on the extent of hydrogen/deuterium scrambling of fully deuterated neurotensin (9–13) (Figure 4a). Isolation width

Table 1. Deuterium Incorporation in Parent and Daughter Ions of Fully Deuterated Neurotensin (9–13) after ETD with Isolation Width 32

	C_2^{+1}	C_3^{+1}	C_4^{+1}	$(MH_2\text{-ammonia})^{+1}$	$(MH_2\text{-guanidine})^{+1}$	MH_2^{+2}
(a) D observed	1.21 ± 0.01	2.09 ± 0.02	2.89 ± 0.03	2.55 ± 0.02	2.51 ± 0.02	3.08 ± 0.02
(b) D calculated with 0% scrambling	1.25	2.17	3.08	3.08	2.75	3.08
(c) D calculated with 100% scrambling	2.13	2.61	2.85	2.37	1.90	3.08
(d) Scrambling percentage	<0%	<0%	81%	75%	28%	-
(e) D calculated with 29% scrambling	1.51	2.29	3.01	2.88	2.50	3.08
(f) D calculated with two rates	1.13	2.04	2.96	2.71	2.46	3.08

The numbers in (b) were calculated using the values obtained in Figure 3. The numbers in (c) were calculated assuming complete randomization. The scrambling percentage in (d) was calculated using the values in (a)–(c) of the same column. This means that complete randomization of hydrogen and deuterium (no regioselectivity) is assumed. The numbers in (e) were calculated assuming 29% of all exchangeable positions were randomized. This value gives the best results with the randomization model. The numbers in (f) were calculated assuming all hydrogen and deuterium atoms on amino, guanidine, Tyr-NH, and C-terminal carboxyl groups scrambled 100% and Ile-NH, Leu-NH, and phenol-OH scrambled 0% (see Figure 1).

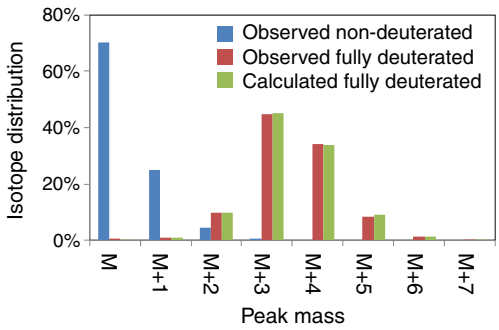

Figure 3. Isotope distribution of observed non-deuterated, observed fully deuterated, and calculated fully deuterated neurotensin (9–13). The isotope distribution of calculated fully deuterated peptide was obtained using observed non-deuterated isotope distribution and deuterium incorporation at four positions (92%, 92%, 92%, and 34%). See Supplemental Information for isotope distribution calculation of fully deuterated peptide

greater than 10 clearly suppresses intramolecular hydrogen exchange. When isolation width was 6, the deuterium incorporation in each daughter ion was close to the value expected for 100% scrambling, presumably due to sideband excitation [17]. On the other hand, when isolation width was 32, the deuterium incorporation in c_2^+ and c_3^+ appeared with no/little scrambling. The extent of scrambling kept decreasing as the isolation width increased. The greater the isolation width, the lower the deuterium incorporation in c_2^+ and c_3^+ and the higher the deuterium incorporation in $(MH_2 - \text{ammonia})^+$ and $(MH_2 - \text{guanidine})^+$.

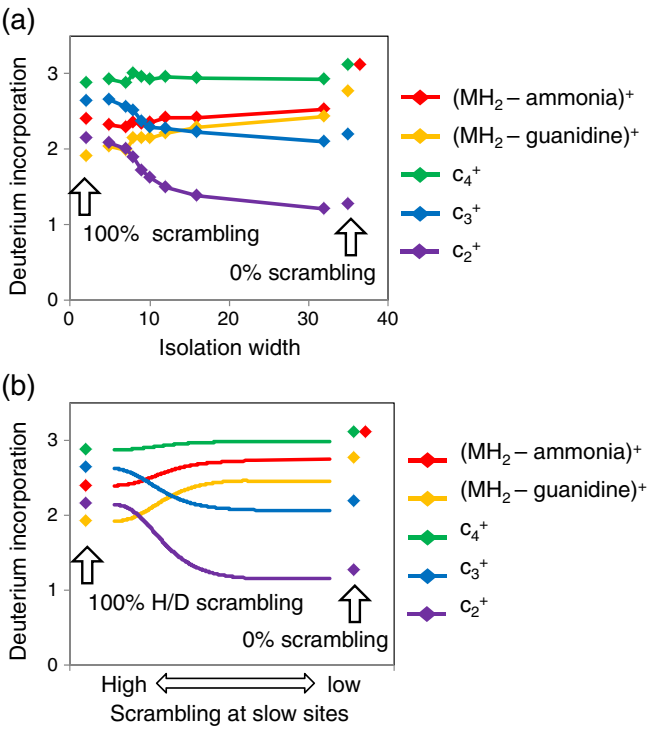

Figure 4. Deuterium incorporations in fully deuterated neurotensin (9–13) daughter ions. The deuterium incorporation with 0% scrambling and 100% scrambling for each ion were calculated with assuming the following deuterium incorporation in the parent ion; Tyr-NH, 92%; Ile-NH, 92%, Leu-NH, 92%, and Arg-NHδ, 34%. (**a**) Observed deuterium incorporation in various daughter ions with increasing isolation widths. (**b**) Simulated deuterium incorporation in various daughter ions. While all exchangeable sites on amino, guanidine, Tyr-NH, and C-terminal carboxyl groups are always assumed 100% scrambling, the scrambling percentage at Ile-NH, Leu-NH, and phenol-OH decreases from left to right

When isolation width was 32, simple randomization of hydrogen and deuterium among all the exchangeable positions cannot explain the deuterium incorporation observed in the daughter ions of neurotensin (9–13) (Table 1). On one hand, the deuterium incorporation in both c_4^+ and $(MH_2 - \text{ammonia})^+$ ions suggests a very high degree of scrambling. While both ions should have the same amount of deuterium as the parent ion in the absence of scrambling, their deuterium incorporations were 0.19 and 0.54 lower than that of parent ion, respectively (Table 1a and b). The decreased deuterium incorporation in the two ions indicates that some deuterium migrated from three amides and/or Arg-NHδ to C-terminal carboxylate group in the case of c_4^+, and N-terminal amino group in the case of $(MH_2 - \text{ammonia})^+$. On the other hand, the low deuterium incorporation in c_2^+ and c_3^+ ions suggests minimum scrambling. Randomization of deuterium should lead to higher deuterium incorporation at amino and guanidine groups and, thus, higher deuterium incorporation in c_2^+ and c_3^+ ions [35]. Table 1d shows the scrambling percentage calculated for each daughter ion using the simple randomization model. If the intramolecular hydrogen/deuterium exchange reactions occur through the simple randomization among all exchangeable hydrogen and deuterium, the scrambling percentages of all daughter ions should be the same. However, observed scrambling percentages of these daughter ions vary widely. Table 1e shows the deuterium incorporation in each daughter ion when 29% of all exchangeable positions were randomized. This value minimizes the sum of the squares of deviation between observed and calculated deuterium incorporations in all daughter ions with the simple randomization model. This best fit value for the simple randomization model cannot simulate the deuterium incorporation of the daughter ions, indicating that the model does not describe the phenomena well.

The deuterium incorporation of parent (MH_2^{2+}) and daughter ions can provide the distribution of deuterium at each segment of the fully deuterated neurotensin (9–13) with isolation width 32 (Figure 1). Various evidences suggest most of the intramolecular hydrogen exchange reactions occur prior to the dissociation reactions [17, 39, 43]. If so, it is possible to calculate the deuterium incorporation at each exchangeable site prior to the dissociation reaction. The difference between the deuterium incorporations in parent and in c_4^+ gave the deuterium incorporation at the C-terminal carboxyl group. Similarly, the deuterium incorporations in $(MH_2 - \text{ammonia})^+$ and $(MH_2 - \text{guanidine})^+$ in comparison with the parent ion yielded the deuterium incorporations at the N-terminal amino and

guanidine groups, respectively. The difference between the deuterium incorporation in c_4^+ and that in c_3^+ gave the deuterium incorporation at the Leu-NH group. Similarly, the comparison between the deuterium incorporations of c_3^+ and c_2^+ yielded the total deuterium incorporation at Ile-NH and phenol-OH groups. Finally, the deuterium incorporations in previously calculated amino and guanidine groups and c_2^+ can provide the deuterium incorporation at Tyr-NH group.

The deuterium distribution at each segment of neurotensin (9–13) infers the degree of intramolecular hydrogen exchange at the site with isolation width 32 (Figure 1). For example, high deuterium incorporation at Ile-NH and Leu-NH indicates small loss of deuterium from these sites and, thus, low degree of intramolecular hydrogen exchange involving the two sites. Moderate deuterium incorporation at N-terminal amino and C-terminal carboxyl groups indicates deuterium migration from other sites and high degree of intramolecular hydrogen exchange involving the two sites. Low deuterium incorporation at Tyr-NH group indicates high degree of intramolecular hydrogen exchange involving this site.

A simple two-exchange rate model is proposed to better describe the deuterium distribution of fully deuterated neurotensin (9–13) when isolation width is 32 (Figure 4; Table 1f). In this model, it is assumed that all hydrogen and deuterium atoms on amino, guanidine, Tyr-NH, and C-terminal carboxyl groups exchange very fast (100% scrambling) and that those on Ile-NH, Leu-NH, and phenol-OH groups do not exchange at all (0% scrambling). This rather simple model can explain the deuterium incorporation in all daughter ions very well (Table 1f). Moreover, increasing the exchange rates (or scrambling percentage) at Ile-NH, Leu-NH, and phenol-OH groups describes the deuterium incorporation changes with isolation width very well (Figure 4b).

The capillary temperature had no effect on the extent of hydrogen/deuterium scrambling of the +2 charge state of fully deuterated neurotensin (9–13) between 50 °C and 200 °C. Prior to the detection of regio-selective intramolecular hydrogen exchange, the effects of capillary temperature on scrambling were tested. This result may be a little surprising because both Rand et al. and Landgraf et al. reported that lower capillary temperature suppresses scrambling in their systems [17, 39]. While these results may contradict the current result, the discrepancy may be due to the difference in ion source configurations and/or the nature of model peptides used.

Another commercially available peptide, amyloid peptide (KKLVFFA), showed no evidence of scrambling under the same ETD conditions (Table 2). The two exchange rate model proposed above hypothesizes regio-selective intramolecular hydrogen exchange reactions. This means that the exchange reaction rate depends on the nature of exchangeable sites involved and that the sequence of a peptide should have influence on the degree of intramolecular hydrogen exchange reaction. To check the effects of peptide sequence, the degree of scrambling in fully deuterated amyloid peptide was also investigated with isolation width 32 and capillary temperature 200 °C (Table 2). While ETD fragmented amyloid peptide very well and generated c_2^+ through c_6^+ and $(MH_2 - \text{ammonia})^+$ ions (Figure S2 in Supplemental Information), the deuterium incorporation in c_2^+ and c_3^+ were not reliable due to the presence of respective c-1 ions (Figure S3 in Supplemental Information). Nonetheless, the deuterium incorporation in all four reliable daughter ions, c_4^+, c_5^+, c_6^+, and $(MH_2 - \text{ammonia})^+$, suggested negligible scrambling (Table 2d).

Discussion

For the optimization of instrument parameters to suppress intramolecular hydrogen exchange in the gas phase, usage of multiple probe peptides may be preferable. The current study suggests that the degree of intramolecular hydrogen exchange is site-dependent, and thus sequence-dependent. Therefore, different peptides may show different sensitivity toward various parameters. For example, neurotensin (9–13) showed regio-selective intramolecular hydrogen exchange with isolation width 32 and capillary temperature 200 °C, whereas amyloid peptide showed no scrambling under the identical conditions. Another example is the comparison between the study by Landgraf et al. and the current study. Landgraf et al. reported that both isolation width and capillary temperature affected the degree of scrambling in the hybrid peptide [39], whereas the current study showed that only isolation width influenced the degree of scrambling. It is possible that the hybrid peptide is more sensitive to capillary temperature than neurotensin (9–13). On the other hand, neurotensin (9–13) may be more sensitive to other parameters because the peptide still showed significant intramolecular hydrogen exchange with low capillary temperature and wide isolation width, which gave minimum scrambling for the hybrid peptide. While these differences may be due to the difference in instrument setting as the two studies were performed in different laboratories, the two laboratories used similar Thermo Orbitrap mass spectrometers.

It may be necessary to tune MS and MS/MS parameters for each peptide. So far, low capillary temperature, low capillary

Table 2. Deuterium Incorporation in Parent and Daughter Ions of Fully Deuterated (Lys^{15})-amyloid β–protein (15–21) after ETD with Isolation width 32

	C_4^{+1}	C_5^{+1}	C_6^{+1}	$(MH_2\text{-ammonia})^{+1}$	MH_2^{+2}
(a) D observed	2.73 ± 0.00	3.62 ± 0.01	4.39 ± 0.01	4.38 ± 0.00	4.41 ± 0.02
(b) D calculated with 0% scrambling	2.74	3.64	4.41	4.41	4.41
(c) D calculated with 100% scrambling	3.39	3.73	4.07	3.39	4.41
(d) Scrambling percentage	<0%	<0%	6%	3%	-

(b)–(d) were calculated in analogous manner as Table 1.

voltage, and wider isolation width appear to be important conditions to suppress intramolecular hydrogen exchange [17, 39]. However, these conditions are not preferable from other points of view. Low capillary temperature and low capillary voltage may lead to low signal-to-noise ratio due to poor desolvation of ions. A wider isolation width may result in a dirtier MS/MS spectrum by allowing contamination of other ions. In addition, all parameters contributing to intramolecular hydrogen exchange may not be known, because non-scrambling condition was not found for neurotensin (9–13).

Scrambling test with $(\text{parent} - \text{ammonia})^+$ is still valid with current regio-selective two exchange rate model. Rand et al. suggested testing the degree of scrambling by the loss of deuterium upon elimination of ammonia [18]. While no deuterium should be lost by the elimination of ammonia in the absence of scrambling, loss of ammonia may cause significant loss of deuterium in the presence of scrambling. The assumption of this test is that hydrogen and deuterium atoms on all exchangeable sites exchange in the same degree. Fortunately, this test is useful with the current two exchange rate model because the N-terminal amino group is one of the sites exchanging faster than the remaining sites. In fact, neurotensin (9–13) was considered heavily scrambled with isolation width between 6 and 32 (Figure 4a and Table 1d) by the test criteria. This test is valuable until some sites are found to exchange faster than N-terminal amino group.

Conclusion

Improving the data resolution is a critical technical challenge for HDX-MS methodology. While subtraction of proteolytic fragments and development of new proteases can yield data with higher resolution, there are limitations for both approaches. Gas-phase fragmentation can provide a new opportunity for high resolution HDX-MS data. However, prior to the application of ECD/ETD gas-phase fragmentation to HDX-MS, extra caution must be taken so that the MS/MS conditions cause no/little undesirable scrambling; especially considering that this field put quite an effort in the past on CID fragmentation, which is now regarded not to be reliable due to scrambling [19, 31].

The current study is the first step to investigate intramolecular hydrogen exchange in a more precise site-specific manner. When the issue of intramolecular hydrogen exchange was recognized and investigated first, it was implicitly assumed that all exchangeable hydrogens undergo positional randomization at the same rate in gas-phase (= scramble) [17, 35]. While this is useful as the first-order approximation, the assumption is not precise considering the difference in chemical nature and steric hindrance of exchangeable hydrogen sites as well as the conformation of a parent peptide ion in gas phase. The more detailed analysis like the current study should help understand the mechanism of intramolecular hydrogen exchange and optimize the MS/MS parameters to minimize the intramolecular hydrogen exchange in the gas phase.

Acknowledgements

The author thanks Stephen J. Coales, Sook Yen E, Jessica E. Lee, and Anita Ma for their technical support.

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