Fast Reversed-Phase Liquid Chromatography to Reduce Back Exchange and Increase Throughput in H/D Exchange Monitored by FT-ICR Mass Spectrometry

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In solution-phase hydrogen/deuterium exchange (HDX), it is essential to minimize the back-exchange level of H for D after the exchange has been quenched, to accurately assign protein conformation and protein–protein or protein–ligand interactions. Reversed-phase HPLC is conducted at low pH and low temperature to desalt and separate proteolytic fragments. However, back exchange averages roughly 30% because of the long exposure to H₂O in the mobile phase. In this report, we first show that there is no significant backbone amide hydrogen back exchange during quench and digestion; backbone exchange occurs primarily during subsequent liquid chromatography separation. We then show that a rapid reversed-phase separation reduces back exchange for HDX by at least 25%, resulting from the dramatically reduced retention time of the peptide fragments on the column. The influence of retention time on back exchange was also evaluated. The rapid separation coupled with high-resolution FT-ICR MS at 14.5 T provides high amino acid sequence coverage, high sample throughput, and high reproducibility and reliability. (J Am Soc Mass Spectrom 2009, 20, 520–524) © 2009 Published by Elsevier Inc. on behalf of American Society for Mass Spectrometry

Solution-phase hydrogen/deuterium exchange (HDX) coupled with mass spectrometry is a non-perturbative method to study protein conformational changes in protein–ligand and protein–protein interactions [1, 2]. The exchange reaction rate minimizes at pH 2.0–3.0 and at a temperature of about 0 °C. To minimize back exchange of amide backbone –ND to –NH, the procedures after quench are performed under quench conditions. However, back exchange still averages nearly 30% [3], depending on various factors, e.g., peptide sequence [4]. With matrix-assisted laser desorption/ionization, the back-exchange level is even higher [5].

In addition to low pH and low temperature, the H₂O concentration should be kept low and exposure to H₂O should be minimized during and after quench. Thus, elution of peptide fragments should occur as fast as possible. Our standard reversed-phase liquid chromatography (RPLC) C₅ column

elutes peptide fragments in about 4.5 min. Supercritical fluid chromatography (SFC) reduces back exchange because its mobile phase is mainly CO₂ instead of H₂O [6]. Dramatic reduction of back exchange has been achieved with SFC separation. However, short column life and reliability of the experiment still require improvement.

Here we present results with a high flow rate reversed-phase ProZapTM C₁₈ column to reduce back exchange, while also increasing sequence coverage and total fragment number. Compared to a conventional C₅ column, the ProZap C₁₈ column has a wider inside diameter, shorter length, smaller particle size, and larger pore size, enabling higher flow rate and lower back pressure. The retention time is thereby reduced from 4.5 min (JupiterTM C₅ column) to 1.5 min, for a 3-fold reduction in H₂O exposure time and concomitant reduction in back exchange for the fragment peptides. Besides reduction in back exchange, the ProZap column also provides much improved elution efficiency for increased fragment number and amino acid sequence coverage. The fast elution of many peptide fragments results in increased mass spectral complexity, which greatly benefits from a high-resolution (high peak ca-

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capacity) 14.5 T Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer [7].

Experimental

Materials

Myoglobin from equine heart, protease type XIII from *Aspergillus saitoi*, deuterium oxide, formic acid, and luteinizing hormone releasing hormone (LHRH) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Hydrogen/Deuterium Exchange

The entire HDX experiment was automated with a LEAP robot (HTS PAL, Leap Technologies, Carrboro, NC, USA) [8]. A 5 μ L stock protein solution (80 μ M for myoglobin) was mixed with 45 μ L 50 mM sodium phosphate buffer in D₂O (pH meter reading = 7.8) to initiate each H/D exchange period at about 0 °C. For the 56 h exchange experiments (at 25 °C), LHRH (5 μ M) was incubated in 99.5% D₂O; myoglobin (8 μ M) in 95% deuterated buffer; and KIT (a receptor tyrosine kinase at 8 μ M) in 92% D₂O buffer. Each H/D-exchanged sample is then subjected to simultaneous quench and proteolysis [9].

On-line LC/SFC ESI FT-ICR MS

A Jasco HPLC/SFC instrument (Jasco, Easton, MD, USA) was interfaced to the LEAP robot. For liquid chromatography (LC), the protein digest was injected from a 30 μ L loop to either a C₅ column (Jupiter™, Phenomenex, Torrance, CA, USA; 1 $\times$ 50 mm, 5 μ m particle size, 300 Å pore size) or a ProZap™ C₁₈ column (Grace Davidson, Deerfield, IL, USA; HR, 1.5 μ m, 500 Å, 2.1 $\times$ 10 mm). The gradient is 2% B to 95% B in 1.5 min (short) or 15 min (long) (A: acetonitrile/H₂O/formic acid, 5/94.5/0.5; B: acetonitrile/H₂O/formic acid, 95/4.5/0.5), at 50 μ L/min (C₅ column) or 0.3 mL/min (C₁₈ column).

SFC was conducted with a 4.6 $\times$ 50 mm Atlantis™ HILIC 5- μ m silica column (Waters Corporation, Milford, MA, USA), with a 4-min gradient from 80 to 40% CO₂ at 3.5 mL/min. The mobile phase modifier was acetonitrile/methanol/H₂O/formic acid, 40/40/19/1. The ionized eluent was directed to a custom-built hybrid LTQ (Thermo Electron Corp., San Jose, CA, USA) 14.5 T FT-ICR mass spectrometer. Mass spectra were collected from 400 < m/z < 2000 at high mass resolving power ($m/\Delta m_{50\%}$ = 200,000 at m/z 400). High magnetic field [10] provided excellent external calibration mass accuracy (typically better than 500 ppb rms).

Data Analysis

Data were collected with Xcalibur software (ThermoFisher, San Jose, CA, USA) and analyzed by an in-house analysis package [11], permitting reliable identification

Figure 1. Elution profiles for myoglobin fragment peptides through an SFC Waters HILIC column (top), HPLC ProZap™ C₁₈ column (middle), and HPLC Jupiter™ C₅ column (bottom), with respective retention times of approximately 0.5–1.2 min, 0.7–1.5 min, and 4.0–4.5 min.

and accurate assignment of the deuterium incorporation in each of the peptide fragments in the spectrum.

Results and Discussion

Reduced Retention Time

Figure 1 shows the total ion chromatogram (TIC) of the peptic fragments of myoglobin from HPLC Jupiter C₅, HPLC ProZap C₁₈, or SFC Waters HILIC separations. With the 1.5-min short gradient, the ProZap C₁₈ column eluted peptic fragments from about 0.7 to 1.5 min—three times faster than the Jupiter C₅ column (~4.0–4.5 min). The fast elution with the ProZap C₁₈ column results from its wide i.d., short length, and large pore size, permitting high flow rate (0.3 mL/min versus 50 μ L/min for the C₅ column) at low pressure (~10 MPa). The small particle size and large pore size permit ultrafast and high-resolution peptide separations. In addition, the loading period was reduced to 0.5 min (versus 2 min for the C₅ column). All of these factors contribute to faster elution of the peptides, less exposure to H₂O, and less back-exchange during LC separation for HDX MS analysis.

The fast separation also reduces the experiment duration and memory storage for more efficient data reduction and potentially higher throughput (more LC separation runs for the same experiment duration). Furthermore, the short retention time also allows more time for the column to fully re-equilibrate for improved reproducibility.

For all three separation methods with a short gradient, most of the fragment peptides eluted in a 30-s window. The complex mixture of co-eluted fragments is nevertheless resolved with our 14.5 T FT-ICR mass spectrometer, at a routine resolving power of $m/\Delta m_{50\%}$ = 200,000 at m/z 400 for online LC operation. Charge

states and monoisotopic m/z values are accurately resolved with an in-house analysis package [11].

Higher Elution Capacity

The ProZap C₁₈ column demonstrated a high capability to elute and separate many peptides, with shorter peptide average length (~17 aa) than the SFC HILIC column (~23 aa). The Jupiter C₅ column retained the shortest peptides (~15 aa). The peptides eluted from the SFC HILIC column are relatively large because small hydrophilic peptides are not well solubilized by supercritical CO₂. Because of charge competition in the electrospray ionization (ESI) process, larger fragments are ionized more efficiently. The ProZap C₁₈ column yielded the most overlapping fragments (77 versus 48 for the C₅ column and 25 for the SFC HILIC column) and the highest sequence coverage (98.7% versus 96.8% for the C₅ column and 93.5% for the SFC HILIC column), enabling amino acid sequence resolution of HDX rates to a few backbone amide hydrogens (sometimes even one or two).

Reduced Back Exchange

Figure 2 illustrates deuterium incorporation after 240 and 900 s exchange periods for each of four myoglobin fragments, following each of three different separations. Deuterium incorporation for the SFC HILIC is higher than that for ProZap C₁₈, which is higher than that for Jupiter C₅. The order of ability to reduce back exchange is thus the same.

Backbone Amide Hydrogen Back Exchange Occurs Primarily during LC Separation, Not during Quench and Proteolysis

The deuterium incorporation for fully exchanged LHRH was used to determine the absolute back-exchange level, by comparing direct infusion and reversed-phase separation. LHRH, pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-CONH₂, has 10 amino acids. The number of potentially exchangeable backbone amide hydrogens is thus 9 (minus one for proline and plus one for the pyroGlu N-terminus), for a total of 9 backbone amide hydrogens and a grand total of 19 potentially exchangeable hydrogens (including side chains). Deuteration and back-exchange level were determined in three ways. First, direct infusion micro-electrospray from the deuterated buffer was performed after addition of 100% formic acid (protic, final concentration in the solution is 0.5%). Second, fully exchanged LHRH solution was injected onto a ProZap C₁₈ column (1.5-min gradient). Third, fully exchanged LHRH was injected onto a Jupiter C₅ column (1.5-min gradient). Back exchange during a 2-min sham digestion procedure was simulated by adding 1% formic acid (in H₂O, no protease present) at a ratio of 1:1 (vol/vol) into the LHRH solution at 0 °C, analyzed by direct infusion.

Figure 3 shows ESI FT-ICR mass spectra of LHRH with the direct infusion after 56-h deuterium exchange (Figure 3b) and after 2-min sham digestion (Figure 3c). Figure 3a resulted from direct infusion of LHRH in H₂O. In Figure 3b, LHRH incorporated close to 17 deuteriums (out of a possible 19), indicating that the LHRH backbone amide hydrogens were fully exchanged and two of the exchanged deuteriums on the

Figure 2. Deuterium uptake for myoglobin peptides after exchange for 240 and 900 s and separation with the JupiterTM C₅ and ProZapTM C₁₈ columns with 1.5-min short gradient and SFC Waters HILIC columns.

Figure 3. LHRH isotopic distribution and back exchange under various conditions. (a) LHRH natural abundance isotopic distribution from blank control (no deuterium exposure). (b) Fully exchanged LHRH (direct infusion after 56 h HDX). (c) Fully exchanged LHRH after sham digestion procedure (direct infusion after 2-min incubation in quench buffer). (d) Fully exchanged LHRH after ProZap™ C₁₈ LC separation. (e) Fully exchanged LHRH after Jupiter™ C₅ LC separation.

side chains were back exchanged to hydrogens (presumably arising from the 0.5% formic (protic) acid in the spray solution). In Figure 3c, close to 11 deuteriums were incorporated; thus nearly 8 deuteriums on the side chains back-exchanged and no backbone amide deuteriums were lost during the sham digestion period. The Arg D δ side-chain exchange rate is similar to that for a backbone amide hydrogen [12], but since 11 deuteriums were incorporated, clearly all of the 9 backbone amide hydrogens are retained, plus the D δ side chain of Arg and one additional side-chain hydrogen. Thus, there is no significant backbone amide hydrogen back exchange during digestion and the main back exchange occurs during the LC separation procedure.

Following the ProZap column separation, about 7 (78% of a possible 9, with 0.71% error) backbone amide deuteriums remained on LHRH (Figure 3d), compared to about 6 (69%, with 0.63% error) for the Jupiter C₅ column separation (Figure 3e). The rapid chromatography provided by the ProZap column reduced back exchange by roughly one third (i.e., 22% versus 31% for the Jupiter C₅ column).

For the Jupiter C₅ column, peptides eluted at 4.0–4.5 min (i.e., 95% organic eluent), whereas peptides eluted from ProZap C₁₈ at 0.7–1.5 min, i.e., highly aqueous eluent (14–64% organic). Back exchange is thus more extensive with Jupiter C₅ than with the ProZap C₁₈ column. These results imply that for longer retention, more aqueous mobile phase flows past the bound peptides and thus back exchange increases. Finally, the fast RPLC column is also compatible with normal LC pumps and pressure systems (operating at ~10 MPa maximum pressure).

To assess the need for fast elution of the peptides to reduce back exchange, we tested a longer (15 min)

gradient for both of the RPLC columns (see Supplemental Figure 1). Supplemental Figure 2 shows deuterium incorporation for myoglobin fragments with the two columns, for both short (1.5 min) and long (15 min) gradients. Supplemental Figure 3 illustrates the 56-hour deuterated myoglobin and KIT deuterium uptake for identical fragments following short-gradient separation with the two columns. Supplemental figures may be found in the electronic version of this article.

Conclusions

Because of its wide inside diameter, short length, small particle size, and large pore size, the reversed-phase ProZap C₁₈ column provides fast separation of digested peptides. Rapid elution of peptic fragments is essential to reduce back exchange and ensure accurate assignment of protein conformation. Based on fully exchanged LHRH data, we demonstrate that there is no significant backbone amide hydrogen back exchange during quench and digestion; back exchange occurs primarily during subsequent LC separation. The calculated absolute back exchange for the ProZap C₁₈ column was about 22% and for the Jupiter C₅ was roughly 31%. The reduction in back exchange is especially promising for the faster exchanging hydrogens, which are crucial to elucidate protein structure change upon ligand binding, posttranslational modifications, or mutagenesis. Rist and Mayer [13] demonstrated structural changes in enzymes in the subsecond to second time-scale by use of a quenched-flow apparatus, and low back exchange is necessary and/or advantageous for those types of analysis. The fast chromatography also provides high reproducibility and high elution capacity, and has great potential for higher throughput. The column yielded a large number of overlapped fragments with high sequence coverage, for improved amino acid sequence resolution for HDX conformational studies. It is also compatible with conventional RPLC, operating at normal pressure with standard solvent pumps, and has become our standard reversed-phase method for HDX analysis.

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