RESEARCH ARTICLE

Polar Aprotic Modifiers for Chromatographic Separation and Back-Exchange Reduction for Protein Hydrogen/Deuterium Exchange Monitored by Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstract

Hydrogen/deuterium exchange monitored by mass spectrometry is an important non-perturbing tool to study protein structure and protein–protein interactions. However, water in the reversed-phase liquid chromatography mobile phase leads to back-exchange of D for H during chromatographic separation of proteolytic peptides following H/D exchange, resulting in incorrect identification of fast-exchanging hydrogens as unexchanged hydrogens. Previously, fast high-performance liquid chromatography (HPLC) and supercritical fluid chromatography have been shown to decrease back-exchange. Here, we show that replacement of up to 40% of the water in the LC mobile phase by the modifiers, dimethylformamide (DMF) and *N*-methylpyrrolidone (NMP) (i.e., polar organic modifiers that lack rapid exchanging hydrogens), significantly reduces back-exchange. On-line LC micro-ESI FT-ICR MS resolves overlapped proteolytic peptide isotopic distributions, allowing for quantitative determination of the extent of back-exchange. The DMF modified solvent composition also improves chromatographic separation while reducing back-exchange relative to conventional solvent.

Key words: FT-ICR, FTMS, Myoglobin, Luteinizing hormone releasing hormone (LHRH)

Introduction

Hydrogen/deuterium exchange [1–6] coupled with mass spectrometry has been widely used in structural biology to study protein structure and dynamics [7, 8], including folding and unfolding of proteins [9, 10], protein/

protein interactions [11, 12], peptide/membrane complex interactions [13], conformational changes induced by ligand binding [14], and binding of small inhibitors [15]. The exchange rate of an amide hydrogen reflects solvent accessibility, which is in turn governed by secondary and tertiary structure. Protons involved in hydrogen bonding in an alpha-helix or beta-sheet exchange much slower than protons in unstructured regions or at the surface of a protein. The effect of primary structure including various amino acid side chains, pH, and temperature has been systematically studied by Englander and co-workers [16–18]. Mass spectrometry is appropriate if the system is inaccessible by

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X-ray crystallography or NMR due to inability to crystallize and/or insolubility and/or too high molecular weight.

Previously, H/D exchange (HDX) monitored by mass spectrometry has been successfully applied to probe structural features of intact proteins including peptide binding [19] and conformational changes [20]. Enzymatic digestion of labeled protein into peptides (first shown by Rosa and Richards) [21] as well as a fragment separation method [22], followed by mass spectrometric analysis [23, 24] significantly enhances sensitivity and spatial resolution. The enzymatic digestion is typically carried out with pepsin [23] at low temperature (0.1 °C) and low pH (pH ~2.3) [3] to minimize the loss of incorporated deuterium. Recently, we have confirmed the advantages of protease type XIII [25] for enhanced digestion efficiency, more efficient electrospray ionization, and higher sequence resolution [26]. Hence, all digestions were performed with protease XIII.

Previously, we have shown reduced back-exchange with a shorter sub-2-micron stationary phase (ProZap C₁₈ resin, Grace/Vydac) and fast chromatography (1.5 min gradient) [27]. This methodology resulted in an average back-exchange of 22% relative to conventional reversed-phase chromatography (RPLC) with a C₅ column that averaged 30% back-exchange [12]. High-resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) analysis [28] greatly aids in the interpretation of the complex spectra due to the fast chromatography. Supercritical chromatography (SFC) also dramatically reduces back-exchange in HDX experiments [29], but short column life has limited its use. Back-exchange associated with matrix-assisted laser desorption ionization is high due to the additional sample preparation step to introduce the matrix [30, 31]. The extent of back-exchange depends on peptide sequence [18, 32], duration of exposure to solvents with exchangeable hydrogens [27], temperature, pH [3, 16], and choice of chromatographic medium [33].

The present goal is to further reduce back-exchange during the HDX experiment by replacement of H₂O in the aqueous mobile phase with polar aprotic modifiers [dimethylformamide (DMF) or *N*-methylpyrrolidone (NMP)] during the HPLC step. The mobile phase modifiers, DMF and NMP, cannot contribute to back-exchange due to the lack of rapidly exchangeable hydrogens in their structures. Minimizing the concentration of protic solvent with these aprotic modifiers should thus significantly reduce back-exchange due to reduced exposure to H₂O. Organic solvents have been historically shown to decrease the amide hydrogen exchange rate of proteins, most likely due to peptide bond solvation effects and decrease in acid dissociation constant of water, K_w (due to lower [OH⁻] concentration) [22, 34] to reduce tritium losses in HPLC fragment separation [22]. Englander and co-workers demonstrated progressive shift in pH_{min} (H/D exchange rate) to higher pH with increase

in concentration of co-solvents such as methanol, acetonitrile, tetrahydrofuran (THF), and dioxane, by use of a spectrometric method based on acetamide [22]. In this report, our goal is to replace protic water in reversed-phase aqueous buffer with a significantly polar aprotic modifier (THF and dioxane are not suitable in buffer A here due to higher elution strength, i.e., poor retention of peptides) to enable acceptable salt separation, peptide retention, and back-exchange reduction with electrospray ionization (ESI) [35] detected by FT-ICR MS. DMF/DMSO have been shown to increase the solubility of hydrophobic compounds and are used in electrospray ionization (ESI) [35] of peptides and sugars [36]. We have recently demonstrated the use of these modifiers for enhanced charging and improved chromatographic separation of intact proteins with higher sensitivity [37]. DMF and dimethylacetamide (DMAA) has been used to significantly improve recovery of tryptic peptides and yield higher sequence coverage in in-gel digestion protocols for protein identification. The increase in protocol efficiency is attributed to the higher solubility of peptides in those solvents [38, 39]. In addition to their MS compatibility, the solvents tested in this paper, DMF and NMP, were chosen for their ability to solubilize H/D exchange buffer, peptides, and proteins. We also compare various RPLC columns with respect to speed and chromatographic separation for complex protein mixtures.

Experimental

Materials

Dimethylformamide, *N*-methylpyrrolidone, equine heart myoglobin, protease type XIII from *Aspergillus saitoi*, formic acid, deuterium oxide, and luteinizing hormone releasing hormone (LHRH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade water and acetonitrile were purchased from J.T. Baker (Philipsburg, NJ, USA). Sodium phosphate (monobasic and dibasic) was purchased from Fisher Biotech (Fairlawn, New Jersey, USA).

Hydrogen/Deuterium Exchange and Digestion

The entire HDX experiment was automated with a LEAP robot (HTS PAL; Leap Technologies, Carrboro, NC, USA) [40]. A blank control, in which the protein or peptide sample under study was diluted in H₂O buffer instead of D₂O, provides a reference point for deuterium incorporation analysis. Stock solution concentrations were: 80 μM myoglobin, ~40 μM FKBP, and 100 μM LHRH. Stock myoglobin solution (5 μL) was diluted 1:10 into 50 mM sodium phosphate buffer (monobasic and dibasic) in D₂O (pH meter reading=7.8). HDX incubation periods were 1, 8, 30 min, and 2 h. All

HDX incubations were performed in triplicate. Each HDX reaction was simultaneously digested and quenched with an equal volume of protease XIII [25, 26] in 2% formic acid for 2 min, and a final pH of ~ 2.5 . The digested sample was then injected onto a reversed-phase C₁₈ HPLC column to desalt and separate peptides. The entire HDX process was performed at 0.4 °C controlled by a Huber recirculating water bath (Peter Huber, Offenburg, Germany).

As a separate control that did not require proteolysis, 100 μ M LHRH was diluted 1:10 into sodium phosphate buffer in D₂O for 5 min to initiate H/D exchange. The reaction was then immediately quenched by adding 2% formic acid and decreasing the temperature to ~ 0.4 °C. The sample was injected onto a Jupiter C₁₈ column for desalting with a 1.5 min gradient (2% B–95% B) as described below. The average mass of LHRH for each experiment was calculated, and back-exchange was determined as explained elsewhere [27].

On-Line LC ESI FT-ICR MS and Data Analysis

A Jasco HPLC/SFC instrument (Jasco, Easton, MD, USA) was interfaced to a LEAP robot (HTS PAL; Leap Technologies, Carrboro, NC, USA) to desalt and separate the HDX peptides after proteolysis. Three LC platforms were compared: Jupiter C₁₈ column (Phenomenex, Torrance, CA, USA; 1 $\times$ 50 mm, 5 μ m particle size, 300 Å pore size), HALO C₁₈ column (MAC-MOD Analytical Inc., PA, USA; 2.1 $\times$ 50 mm, 2.7 μ m, 90 Å pore size), and ProZap C₁₈ column (Grace Davidson, Deerfield, IL, USA; 2.1 $\times$ 10 mm, HR, 1.5 μ m, 500 Å pore size). The HPLC gradient (including the loading period for salt separation), data acquisition period, and flow rate with each of various columns is shown in Supplementary Figure S1. Briefly, the gradient was 2% B to 95% B in 1.5 min [27] (A: H₂O:DMF:formic acid, 60:40:0.5 vol/vol/vol; B: acetonitrile:DMF:formic acid, 98:2:0.5 vol/vol/vol) at 50 μ L/min with a Jupiter C₁₈ column. NMP was also tested in the same proportion as DMF. The results were compared to conventional HPLC solvent (A: H₂O:acetonitrile:formic acid, 98:2:0.5 vol/vol/vol; B: H₂O:acetonitrile:formic acid, 2:98:0.5 vol/vol/vol). A post-column splitter reduced the eluent flow to ~ 400 –500 nL/min for efficient microelectrospray ionization [41].

The LC eluent was microelectrosprayed into a custom-built 14.5 T FT-ICR mass spectrometer [42] equipped with front-end modified hybrid linear quadrupole ion trap (LTQ-FT; Thermo Fisher Corp., Bremen, Germany) [43, 44]. It is imperative to optimize front-end parameters for non-volatile modifiers. Higher capillary temperature (275 °C) and tube lens voltage (190 V) yielded enhanced desolvation and better ion mobility and transmission. Data was collected with Xcalibur 2.0 software (Thermo Fisher). FT-ICR mass spectra were acquired at high mass resolving power ($m/\Delta m_{50\%}=200,000$ at m/z 400, in

which $\Delta m_{50\%}$ denotes mass spectral peak full width at half-maximum peak height). The total data acquisition period for each sample varied from 5 min (ProZap, 300 μ L/min) to 10 min (Jupiter, 50 μ L/min), depending on the chromatographic column and the flow rate (Figure S1). External ion accumulation [45] was performed in the front-end linear ion trap with a target ion population of three million charges for each FT-ICR acquisition. LTQ-accumulated ions were transferred (~ 1 ms) through three octopole ion guides (2.2 MHz, 250 V_{*p-p*}) to a capacitively coupled [46] open cylindrical ICR cell (55 mm i.d.) for analysis. The robust automatic gain control [43] for transferring the same number of ions for each FT-ICR MS scan coupled with the high magnetic field [47] provides excellent external calibration mass accuracy (typically better than 500 ppb rms).

Data was analyzed with custom software [48] permitting reliable peptide isotopic distribution and charge state identification. The software also accurately computes the number of deuteriums incorporated into each proteolytic peptide segment for each H/D exchange period, relative to the blank control (no D₂O).

Back-Exchange Calculations

Fragments common to conventional and modified mobile phases for each chromatographic column are identified. Relative back-exchange for a common peptide is then calculated for each D₂O incubation period separately (1, 8, 30 min, and 2 h) for modifiers relative to conventional protic solvent (higher back-exchange). Then, we calculate average back-exchange (higher deuterium level) for that specific peptide for all incubation periods and finally compute the global relative average back-exchange percentage for all common peptides, taking into account retention time differences due to altered chromatographic selectivity with less polar modifiers relative to water [49–51].

Results and Discussion

Solubility Comparisons for New Mobile Phase Modifiers

Solubility and retention of solute on the stationary phase media are two of the most basic requirements for HPLC. We therefore began by determining precipitation for sodium phosphate buffer (monobasic and dibasic) and LHRH (10 μ M) as a function of DMF and NMP concentration. Table 1 shows structure, viscosity, and polarity of these modifiers. At 40% (vol/vol) the modifiers were compatible with the HDX buffer and peptides; higher concentration resulted in precipitation of salts and peptides at 0 °C. Therefore, 40% of the water could be replaced by DMF or NMP in the HPLC separation step. DMSO is a common NMR solvent because it can

Table 1. Structure, Polarity, and Viscosity of Polar Aprotic Solvents Relative to Water and Acetonitrile

Solvent	Polarity	Viscosity(cP) @ 25°C
Acetonitrile	5.8	0.34
N,N-Dimethylformamide	6.4	0.80
Dimethyl Acetamide	6.5	1.95
N-methylpyrrolidone	6.7	1.65
Dimethyl Sulfoxide	7.2	1.99
Water	10.2	0.89

solubilize both hydrophobic and hydrophilic compounds. However, DMSO and DMAA are not suitable for chromatography due to their high viscosity, higher back pressure during HPLC separation, and reduction in electrospray ionization (ESI) efficiency at 0 °C.

LHRH Retention on a Reversed-Phase LC Column with DMF-Modifier (UV Detection)

To confirm that peptides are retained by the reversed-phase column, 20 µL of LHRH (50 µM) was injected onto a Jupiter C₁₈ column (Shimadzu HPLC system) at room temperature, with UV detection at 214 nm with a 10 min gradient (2% B–95% B) at a flow rate of 50 µL/min. LHRH was retained for ~6.5 min with conventional solvent (10 min gradient, 2%–95% organic), ~1.5 min with conventional solvent (isocratic flow at 70% acetonitrile), and ~3 min with DMF-modified solvent (10 min gradient, data not shown). Hence, LHRH is clearly retained on the chromatographic column with DMF-modifier.

Back-Exchange Tested with LHRH

Preliminary back-exchange studies were conducted with LHRH, pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-CONH₂, a decapeptide. The number of potentially exchangeable amide hydrogens for LHRH is nine (minus one for proline and plus one for the pyroGlu N-terminus), for total of nine backbone amide hydrogens and maximum of 19 potentially exchangeable hydrogens (including side chains). The deuterium incorporation for 5 min exchange period serves to establish the relative back-exchange level between different solvent systems. 100 µM LHRH (10 µL) was incubated in 90 µL D₂O buffer (1:10 dilution) for 5 min followed by 1:1 quenching with 2% formic acid (in H₂O, no protease present, sham digestion) for 2 min. A 30 µL aliquot was injected onto a Jupiter C₁₈ HPLC column for desalting under identical conditions with conventional, DMF- and

NMP-modified solvents at 50 µL/min. The average mass of LHRH (M_{avg}) was computed from

$$M_{\text{avg}} = \sum(M_i I_i) / \sum I_i \quad (1)$$

in which M_i is the mass of the i^{th} peak in the isotopic distribution and I_i is its relative abundance. NMP produced the highest back pressure (~12 MPa, 1740 psi) followed by DMF (~9 MPa, 1305 psi) and conventional solvents (~5 MPa, 725 psi) at 98% A and 2% B mobile phase composition. LHRH exhibited shorter retention time with modifiers (~5.2 min with DMF and ~5.4 min with NMP) relative to conventional solvents (~6.2 min). The spectrum with the highest signal-to-noise ratio was used to calculate the average mass of LHRH. The average number of incorporated deuteriums is simply the increase in LHRH average mass in Da. The DMF-modified solvent system exhibited the highest D level and hence, the least back-exchange relative to conventional and NMP-modified systems (Table 2).

Higher Deuterium Level with DMF or NMP on Jupiter C₁₈

We aim to minimize back-exchange by modifying only the chromatographic conditions because most back-exchange occurs post-digestion/quench, during the HPLC separation step [27]. Given that LHRH showed higher deuterium level (due in part to earlier elution) by replacement of 40% of protic H₂O by aprotic DMF/NMP modifiers, we proceeded to deuterate myoglobin and separate protease XIII fragments on a Jupiter C₁₈ column with modified and unmodified solvents. A longer gradient yields more efficient chromatographic separation, but at the cost of significantly higher back-exchange due to longer retention [12, 27]. Consequently, in order to minimize back-exchange, we perform fast chromatographic separation (1.5 min short gradient), and employ ultrahigh resolution FT-ICR MS to address the resultant spectral complexity, thereby achieving significantly higher deuterium level [27] due to less exposure to water. Here, salts reproducibly elute from ~1.5 to 2.8 min followed by peptides at 95% buffer B (only 5% aqueous phase to minimize back-exchange) [27]. Table 3 shows the myoglobin digest results after separation with conventional, DMF-, and NMP-modified solvents with a 1.5 min gradient (1 min loading period). With DMF, more and longer fragments (on average) were observed than for the other two solvent systems. Surprisingly, the protease XIII fragments were retained longer with modifiers than with conventional solvent [37] at low temperature, presumably due to higher back pressure caused by the high viscosity of these modifiers as well as inter- and intra-molecular hydrogen bonding. Nevertheless, the deuterium level was higher for the fragments eluted with modifiers due to less exposure to H₂O. Enhanced chromatographic separation between salts and smaller hydrophilic peptides was achieved by slight

Table 2. Effect of Modified Solvents on LHRH Back-exchange After 5 min Incubation in D₂O Followed by Elution from a Jupiter C₁₈ Column at 50 μ L/min

Solvent system	LHRH number-average mass (Da) ^a	No. of incorporated deuteriums	% Decrease in back-exchange relative to conventional HPLC solvent
Conventional	1186.616	4.47	—
DMF-modified	1187.522	5.38	20.36
NMP-modified	1187.093	4.95	10.73

^aLHRH number-average mass without exchange=1182.141 Da.

modification of loading condition gradient [i.e., washing for 1 min (Table 3) rather than 0.5 min (Supplementary Table S1)] with 98% solvent A with a subsequent increase in fragment number, higher sequence coverage, and decrease in average peptide length.

Figure 1 shows the charge state distribution for myoglobin digest protease XIII fragments. Fewer protease XIII fragments were observed with NMP for all charge states greater than 2+. Singly and doubly charged peptide fragments were more abundant than those of higher charge states.

Deuterium level with modifiers was compared with that for conventional solvents for each of the HDX incubation periods for individual fragments, and average higher deuterium level was computed for all identical fragments. Average deuterium level for four identical representative myoglobin digest peptide fragments was significantly higher (~22% and ~11%) with DMF (Figure 2) and NMP-modified solvents (Supplemental Information, Figure S2). Fragments retained longer in the presence of modifiers ([1–11] and [94–106]) also show higher deuterium level. We discounted the use of NMP for further high speed separation due to fewer fragments, lower deuterium level, higher back pressure, and longer retention time relative to DMF modifier. Interestingly, only 26 common fragments were found with conventional solvent (99 total fragments) and DMF-modified solvent (109 total fragments) (see below).

Higher Deuterium Level with HALO C₁₈ than Jupiter C₁₈

We then tested two HALO C₁₈ columns (30 and 50 mm length). HALO is a fast separation resin because of its smaller particle size (2.7 μ m and its porous shell [0.5 μ m]) that reduces solute diffusion path and minimizes axial dispersion. The claimed result is a higher number of theoretical plates and better separation at higher flow rate. **Table 3.** Results of Protease XIII Digestion of Myoglobin, for Three Different Solvent Systems (1 min Loading Period)

Solvent system	No. of fragments	Sequence coverage	Average length	Elution time for peptide fragments (min)
Conventional	99	100%	12	3.3–6.6
DMF-modified	109	94%	17	3.9–7.2
NMP-modified	77	97%	15	4.7–7.8

The shorter HALO C₁₈ column gave poor separation with 40% DMF and was not pursued.

Supplemental Figure S3 presents a total ion chromatogram for myoglobin digest peptide fragments separated by conventional and 40% DMF-modified solvents with a longer HALO C₁₈ column at 150 μ L/min flow rate. Peptides elute gradually with improved separation over a wider window with DMF modified solvent composition (4.2 min) relative to conventional solvents (1.3 min). Moreover, more proteolytic fragments are observed with DMF modifier system. The Halo C₁₈ column coupled with the DMF-modified solvents yielded ~31% higher average deuterium level than conventional solvent for 34 common proteolytic fragments. The average deuterium level was ~20% higher for the HALO C₁₈ column relative to the Jupiter C₁₈ column with DMF due to higher flow rate (150 μ L versus 50 μ L) and concomitant reduced retention time.

Highest Deuterium Level with ProZap C₁₈ Column and DMF Modifier

The ProZap C₁₈ column, due to its wide i.d., short length, small particle size, and large pore size has demonstrated ultrafast peptide separations at 300 μ L/min [27]. The ProZap C₁₈ column with 40% DMF resulted in high back-pressure (>25 MPa) [52] and could not be used. Poor separation was noted even at a lower flow rate. However, 20% DMF in the mobile phase resulted in an acceptable separation at 300 μ L/min at a permissible back-pressure (~17 MPa, 2465 psi with DMF-modified solvent versus ~10 MPa, 1450 psi with conventional solvent) at 98% A and 2% B mobile phase composition. Figure 3 illustrates elution profiles for myoglobin digest peptide fragments with a ProZap C₁₈ column with conventional and DMF-modified solvents. Consistent with the HALO column, DMF modified solvent composition/polarity results in selective elution and improved separation (~2 min versus 30 s) of proteolytic fragments and subsequent higher fragment number (Table S2). Peptides elute gradually with the modified solvent system and some peptides exhibit shorter retention time relative to conventional solvents (Table S2), further minimizing exposure to H₂O.

Figure 4 shows deuterium level profiles for four representative myoglobin digest peptides for conventional and DMF-modified solvents. Fragments retained for similar/longer time in the presence of DMF also show significantly

Figure 1. Number (top) and percentage (bottom) charge state distributions of proteolytic peptides from myoglobin following H/D exchange, for conventional, DMF-modified, and NMP-modified HPLC solvents with a 1.5 min gradient. Note that DMF-modified solvent produces more fragments at charge states higher than 2+ than conventional or NMP-modified solvents. The higher charge states from the DMF-modified solvents also exhibit slightly longer fragments (Table 3)

higher deuterium incorporation (lower back-exchange) due to fewer exchangeable solvent hydrogens. Myoglobin frag-

ments show ~19% average higher deuterium level among 45 identical fragments with the DMF-modified solvent.

Figure 5 indicates deuterium levels for various myoglobin digest fragments after 30 min (top) and 2 h (bottom) exchange period, and subsequent separation with HALO (150 μ L/min) and ProZap (300 μ L/min) C₁₈ columns for conventional and DMF-modified solvents. DMF incorporation reduces back-exchange with both columns. Fragments separated with the ProZap column with 20% DMF show the highest deuterium level due to overall early elution (~2.5 min) relative to the HALO column (~6 min) due to faster flow rate and shorter column length.

Likewise, HDX of 40 μ M FKBP (human recombinant [C22A] FK506-binding protein, ~12 kDa) also exhibited ~18% higher average deuterium level with DMF-modified solvent (Supplementary Figure S4) among 38 identical fragments. Furthermore, consistent with our myoglobin results, we observe higher fragment number (118 versus 99) along with selective elution of FKBP fragments with DMF (0.7–2.3 min) relative to fast elution with conventional solvents (1.7–2.0 min). The elution profiles are different due to the overall polarity: i.e., rate of change of the solvent composition (DMF and H₂O), not just due to the added modifier.

We previously showed that the ProZap C₁₈ column yielded the then-lowest back-exchange level of ~22% [27]. By further replacing H₂O with 20% aprotic DMF and ultrafast separation, we reduce average back-exchange by further ~19% (of 22%) to a final back-exchange level of

Figure 2. Deuterium level profiles for four selected proteolytic peptides from myoglobin. DMF-modified HPLC solvent resulted in ~22% higher average deuterium level than with conventional solvent. The retention time for each peptide fragment with DMF (shown above the curve) and conventional (shown below) clearly indicates higher deuterium level with aprotic modifier due not to early elution, but to partial removal of protic solvent. Error bars represent variation among triplicate experiments

Figure 3. Total ion chromatogram for myoglobin digest proteolytic fragments with a ProZap C₁₈ column for conventional and 20% DMF modifiers. The overall change in the polarity of solvent mixture results in improved chromatographic separation, more proteolytic fragments, and reduced back-exchange after DMF addition

~18%, the lowest yet achieved with HPLC ESI FT-ICR MS. Moreover, fast chromatography with the short ProZap column results in overall faster elution of peptides (fewer total mass spectral scans: memory storage) for more efficient data reduction and also allows more time for the column to fully re-equilibrate for better reproducibility relative to other longer columns.

Figure 5. Deuterium level for myoglobin peptides after exchange for 30 min (top) and 2 h (bottom) and separation with HALO C₁₈ and ProZap C₁₈ columns with a short (1.5 min) gradient; 20% DMF modifier with ProZap column yields the highest deuterium level and subsequent lowest back-exchange due to superfast elution at 300 μ L/min, short column length, and lack of exchangeable protons. Error bars represent variation among triplicate experiments

Modifiers Affect Chromatographic Separation, ESI, and Detection

Conventional and DMF-modified mobile phase with different columns yielded ~30%–40% identical fragments. The

Figure 4. Deuterium level profiles as for Figure 2, for four representative proteolytic peptides from myoglobin separated with a ProZap C₁₈ column and 20% DMF-modifier. Peptides retained for similar time show less back-exchange due to lack of exchangeable protons in the mobile phase with DMF. The final back-exchange level of ~18% is the lowest ever reported for H/D exchange monitored by HPLC ESI FT-ICR mass spectrometry

new fragments may be attributed to the reduced polarity of DMF/NMP relative to water, changing chromatographic separation and elution time. Peptides selectively elute from the column over a wider window depending on their affinity and partition characteristics with modifiers, versus small window (30 s) with conventional solvents. A given peptide may elute to yield only 1 or 2 mass spectral acquisitions with ultra-fast ProZap column and conventional solvent due to the steep elution gradient, but spread over ~4 to 5 acquisitions with DMF. Consequently, a wider elution window (gradual elution) considerably increases peptide detection for FT-ICR MS (1 acquisition/s), accounting for the higher proteolytic fragment number for DMF modified solvent system for both myoglobin and FKBP for all tested columns due to improved elution efficiency. Moreover, gradual elution is advantageous for ESI [35], due to fewer ionization interferences. Finally, physico-chemical properties of solvents reportedly affect charging during ESI under denaturing [53–56] and non-denaturing [57, 58] conditions. Indeed, we have recently characterized DMF/NMP and other modifiers for charge enhancement of intact proteins with improved sensitivity and chromatography [37]. Higher boiling point modifiers (DMF/NMP) may affect ionization by altering analyte distribution [59] in the ESI droplet, resulting in different peptide mapping. In addition, the previously reported enhanced solvating power of DMF for hydrophobic peptides [36, 38, 39] could result in different peptide maps. We are continuing to investigate the effects of modifiers on individual peptide fragment retention and back-exchange (sequence dependence); origin of longer retention time for some fragments [37]; improved LC separation; and applicability to higher molecular weight proteins.

Conclusion

We previously demonstrated that fast chromatographic separations could reduce total back-exchange to ~22% [27]. Replacing H₂O with polar aprotic modifiers further reduces back-exchange and improves chromatographic separation of peptide fragments applicable for HDX. Replacement of H₂O with 20% aprotic DMF in the chromatography solvent, coupled with fast chromatographic separation, reduces average back-exchange to ~18%, the lowest yet achieved in published HDX experiments. In addition, the overall polarity of the solvent system with aprotic modifiers increases the number of observable protease XIII proteolytic peptides (even with fast chromatography), for more detail about protein conformational changes upon ligand or protein binding, post-translational modifications, or mutagenesis, and mapping of contact surfaces in protein complexes.

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