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Supercritical Fluid Chromatography Reduction of Hydrogen/Deuterium Back Exchange in Solution-Phase Hydrogen/Deuterium Exchange with Mass Spectrometric Analysis

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The single biggest problem with solution-phase H/D exchange as a mass spectrometric probe of surface exposure in a protein (or protein complex) is back-exchange of H for D after the initial H/D exchange has been quenched. Back-exchange results in loss of pertinent data and also greatly hampers data analysis. Previously, very fast, cold (0−4 °C) HPLC was performed to help reduce back-exchange, but calculated back-exchange still averages ~30%. In this report, supercritical fluid chromatography replaces HPLC as the desalting/separation technique prior to mass analysis, providing a dramatic reduction in back-exchange compared to the fast, cold HPLC methods.

Determination of protein−protein interaction is best accomplished by X-ray crystal diffraction and NMR[1] because both methods provide the highest spatial resolution at the sites of interaction. On the downside, both methods require large (milligram) quantities of protein. Often crystals are difficult or impossible to obtain. NMR requires high sample concentration (limited by solubility), and analysis is difficult as molecular weight increases (~50 000 Da and above). Other techniques rely on chemical or photoinduced reactions to reveal functional groups that are exposed to the solvent. The sites of solvent accessibility are most often identified by mass spectrometry to determine the site(s) of modification.[2−5] These methods can suffer from limited availability of specific solvent-available residues.[4] Additionally, covalent cross-linking has the potential to alter conformation.

Hydroxyl radical reactions with alkyl C−H bonds are fast (10^8 M^{-1} s^{-1}, i.e., within a factor of 100 of diffusion-limited), and thus, the OH radical tends to react mainly with surface-exposed residues, providing a good footprint of the solvent exposed surface of the protein(s). The OH radicals may be generated chemically from N_2O solution,[6] with a Fenton reagent,[7] from UV irradiation of peroxide solution,[8] or by synchrotron-generated X-rays.[9] The great advantage is that the modification is covalent and thus irreversible. On the other hand, each such modification can potentially change the conformation of the protein, so that one is never sure that the inferred properties are those of the native protein. Finally, pulse−chase experiments monitored by quantitative mass spectrometry follow the binding of ^{15}N-labeled protein to a complex chased with ^{14}N-labeled protein at specific intervals.[10] These experiments are excellent for determination of the binding of whole proteins in a multiprotein complex but lack the resolution of intraprotein (peptide) binding.

Exchange of labile hydrogen for deuterium (HDX) as a probe of protein surface accessibility[11,12] does not change the conformation of the protein. The experiment is initiated by dilution of the protein solution into a biological buffer made with D_2O. Solvent-accessible hydrogens exchange with deuterium. The exchange is quenched by dropping the pH to ~2.3 and lowering the temperature to 0−4 °C. The greatest disadvantage is that back-exchange during subsequent analysis results in the loss of data. Some back-exchange is advantageous—side chains (e.g., arginine) typically exchange much faster than backbone amides. This "good" back-exchange usually eliminates the side-chain contributions before MS analysis, leaving only direct information about the backbone, but further back-exchange greatly hampers HDX data analysis by smoothing exchanged populations as recently described by Hotchko et al.[13] Back-exchange begins during the peptic digest of the H/D-exchanged protein and continues during

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the reversed-phase separation. To limit back-exchange, a very fast elution gradient (at 0−4 °C) during desalting/separation limits the period that the sample is in contact with the aqueous mobile phase. Reversed-phase chromatographic resolution is thereby reduced (increasing the complexity of the mass spectra). In this laboratory, the spectral complexity is dealt with by use of high-resolution Fourier transform ion cyclotron resonance (FT-ICR) mass analysis. Even with the rapid, cold HPLC separations, the back-exchange can easily reach ~30%, resulting in "lost" information.

The goal of this work is to reduce or eliminate back-exchange during the HDX experiment by replacement of HPLC with supercritical fluid chromatography (SFC) as the desalting/separation technique. The mobile phase for SFC separation is predominately CO_2, which cannot contribute to hydrogen back-exchange. SFC has been most commonly used as a rapid separation technique for hydrophobic to somewhat polar molecules and thus was not used for routine analysis of hydrophilic compounds such as peptides. SFC was demonstrated in the separation of hydrophobic peptides by Smith[14] and later Stringham.[15] The first SFC analysis of peptides with continuous flow ESI MS detection was reported by Greig et al. at Pfizer in 2001[16] and published by Bolaños et al. in 2004.[17] Recently, Zheng et al. at Procter and Gamble demonstrated the separation of up to 40-mer polypeptides by SFC with MS detection.[18]

EXPERIMENTAL SECTION

All preliminary SFC HDX data was acquired in Greig's laboratory at Pfizer Inc., La Jolla Laboratories. Automated HDX of myoglobin and injection of sample was performed by a LEAP (Carboro, NC) robot programmed with interlaced software developed at NHMFL (unpublished). Supercritical fluid chromatography was performed with a Jasco system (Easton, MD) consisting of a PU-1580 CO_2 delivery pump, a PU-1580 Intelligent HPLC pump (for modifier delivery), and a BD-158-81 back-pressure regulator. The SFC effluent was monitored with a Waters Micromass (Milford, MA) LCT ESI-TOF mass spectrometer equipped with a Z-spray ESI source. The HDX sample was injected (10 μL at either 4 or 40 pM/μL) onto a 4.6 mm × 50 mm Waters Atlantis HILIC 5-μm silica column. Final SFC gradients were performed from 80 to 40% CO_2 in 4 min at a flow rate of 3.5 mL/min. The mobile-phase modifier was 40% acetonitrile, 40% methanol, 19% H_2O, and 1% formic acid. All SFC was performed at ~1 °C. Data from automated HDX of myoglobin with SFC separation and ESI-TOF analysis were compared to automated HDX of myoglobin with fast, cold HPLC separation and analyzed with a 9.4-T FT-ICR MS at the NHMFL under conditions as previously described.[19]

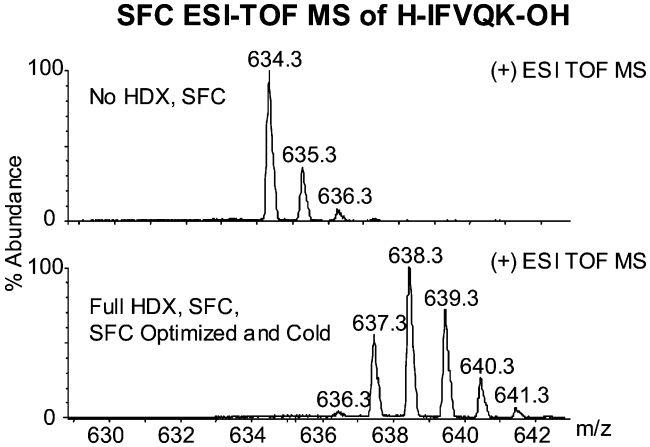

Figure 1. ESI-TOF mass spectra following SFC of the pentapeptide, IFVQK. (Top) 10-μL injection at 4 pM/μL undeuterated peptide to demonstrate that the peptide was retained on the SFC column. (Bottom) Fully deuterated sample (loaded at the same concentration) that had been stored in deuterated buffer for several days. SFC conditions: gradient elution of 80 to 40% CO_2 in 4 min at a flow rate of 3.5 mL/min. The mobile-phase modifier was 40% acetonitrile, 40% methanol, 19% H_2O, and 1% formic acid. Modifier, injector, and SFC column were all cooled to ~1 °C. Back-exchange is only 7.5% based on the theoretical maximally exchanged mass for this pentapeptide with four exchangeable amide hydrogens.

RESULTS AND DISCUSSION

Initially, we tested a pentapeptide (IFVQK, the size of the smallest peptic fragments normally produced by peptic digestion of a protein), for retention and sensitivity on the SFC ESI-TOF system. This peptide has four exchangeable amide hydrogens (exchangeable amide hydrogens = (A) − 1 − (P), in which A is the number of amino acids, A − 1 is the number of peptide bonds, and P is the number of prolines). Figure 1 (upper panel) shows the ESI-TOF spectrum of normal-abundance IFVQK peptide as it eluted from the SFC column. The peptide (monoisotopic $[M + H]^+$ appears at m/z 634.3) was retained and eluted from the SFC column. The calculated number-average molecular weight of the peptide was m/z 634.8. If all amide hydrogens were replaced by deuteriums, the theoretical average molecular weight would be m/z 638.8 (back-exchange calculations used this theoretical value). Before optimization of the SFC conditions for reduction of back-exchange, the HDX exchanged pentapeptide eluted with an average mass of m/z 636.2 (data not shown), corresponding to ~65% back-exchange. Figure 1 (lower panel) shows an ESI-TOF spectrum of HDX SFC of the same peptide eluting under the optimized mobile-phase conditions stated above with cooling of the SFC modifiers, injector, and column (~1 °C). The peptide eluted from the column in ~30 s, compared to ~2 min for a typical HPLC run. The number-average molecular weight from the experimental isotopic distribution was m/z 638.5, corresponding to ~7.5% back-exchange for this small peptide with a maximum of only four exchangeable amide hydrogens!

After confirming that peptides were retained on the SFC column and that back-exchange was greatly reduced, we per-

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Table 1. Mass Analysis Following Solution-Phase HDX of Myoglobin[a]

	SFC-ESI TOF			HPLC-ESI FT-ICR 9.4 T		
time	av mass	std dev	D uptake	av mass	std dev	D uptake
	Myoglobin Fragment: KASEDLKKHGTVVL (aa 56−69)					
0 ctrl	1526.32	0.02	0.0	1526.01	0.12	0.0
30s	1526.63	0.66	2.3	1526.55	0.08	0.5
60s	1529.36	0.36	3.0	1526.40	0.08	0.4
120s	1529.36	1.57	3.0	1526.33	0.14	0.3
240s	1530.70	1.44	4.4	1526.42	0.08	0.4
480s	1530.56	0.41	4.2	1526.47	0.14	0.5
900s	1529.97	0.19	3.7	1526.91	0.22	0.9
1800s	1530.54	0.15	4.2	1527.23	0.18	1.2
3600s	1530.87	0.08	4.6	1527.53	0.14	1.5
	Myoglobin Fragment: FRNDIAAKYKELGFQG (aa 138−153)					
0 ctrl	1859.14	0.02	0.0	1858.00	0.14	0.0
30s	1864.45	0.89	5.3	1860.17	0.33	2.2
60s	1865.85	0.93	6.7	1860.43	0.14	2.4
120s	1865.85	1.12	6.7	1860.08	0.30	2.1
240s	1867.02	0.37	7.9	1860.56	0.18	2.6
480s	1866.70	0.52	7.6	1860.29	0.67	2.3
900s	1866.27	0.25	7.1	1861.07	0.55	3.1
1800s	1866.50	0.22	7.4	1861.74	0.61	3.7
3600s	1867.01	0.38	7.9	1862.38	0.30	4.4
	Myoglobin Fragment: AIIHVLHSKHPGDFGADAQAMTKA (aa 110−134)					
0 ctrl	2576.36	0.02	0.0	2575.27	0.09	0.0
30s	2581.73	0.04	5.4	2578.33	0.03	3.1
60s	2582.84	0.39	6.5	2578.28	0.02	3.0
120s	2582.84	1.67	6.5	2578.52	0.03	3.3
240s	2584.03	0.47	7.7	2578.53	0.10	3.3
480s	2583.70	0.65	7.3	2578.69	0.15	3.4
900s	2583.20	0.58	6.8	2578.89	0.14	3.6
1800s	2583.56	0.07	7.2	2579.20	0.09	3.9
3600s	2583.95	0.01	7.6	2579.39	0.03	4.1

[a] Data from select peptic fragments of myoglobin with SFC or HPLC separation. Data represent average of three replicates for each H/D exchange period.

formed a fully automated HDX of a myoglobin digest (9 time points each performed in triplicate). After solution-phase HDX, the exchange was quenched with low temperature and formic acid (temperature ~0.1 °C, pH ~2.3) and the pepsin digest was initiated (2 min). The pepsin fragments of myoglobin were desalted and separated by SFC, resulting in 91.5% sequence coverage by SFC compared to 98.7% with HPLC. A total of 35 peptic fragments were observed in both the SFC and HPLC HDX experiments. The largest myoglobin peptic fragment observed for SFC (and HPLC) was the 34-residue, 70−103 fragment, TALGGILKKKGHHEAELKPLAQSHATKHKIPIKY (calculated neutral monoisotopic mass 3742.152 Da). The smallest myoglobin peptic fragment observed after SFC (and HPLC) was the 6-residue, 12−17 fragment, NVWGKV, (calculated neutral monoisotopic mass 701.386 Da).

Table 1 shows the actual deuterium uptake calculated from SFC-TOF MS analysis and optimized HPLC for HDX with 9.4-T FT-ICR mass analysis, for three myoglobin peptic fragments. Focusing on the 14-residue, 56−69 myoglobin peptic fragment, KASEDLKKHGTVVL, the SFC deuterium incorporation was much higher (from 2- to 4-fold higher) for all H/D exchange periods, due to the greatly decreased back-exchange. Note also the rapid incorporation of deuteriums for the shorter time periods. The 30-, 60-, 120-, and 240-s exchange periods exhibit back-exchange ranging from 78 (1−0.5/2.3) to 91% (1−0.4/4.4) for HPLC relative to SFC. Table 1 is representative of all of the myoglobin peptic fragments, which showed higher deuterium incorporation in the early time points with SFC compared to HPLC. Longer time points could not be fully interpreted with the SFC separation with ESI-TOF analysis due to isotopic overlap as deuterium exchange occurred. The 3 myoglobin peptic fragments in Table 1 are the 3 of the 35 myoglobin peptic fragments that could be followed out to the 3600-s exchange period by low-resolution ESI-TOF MS due to the overlap of isotope envelopes (data not shown).

CONCLUSIONS

Those preliminary data show that the sequence coverage for SFC (91.5%) was comparable to that for HPLC (98.7%). In these experiments, the peptide separation (chromatographic resolution) with SFC was not as good as HPLC, resulting in increased spectral complexity. However, the spectral complexity can be addressed by high-resolution FT-ICR MS in future experiments. We also expect to further optimize SFC chromatographic resolution, sequence coverage, and sample reproducibility. Comparison of HDX of myoglobin with low-resolution ESI-TOF MS to high-resolution FT-ICR MS demonstrates that much of the HDX information is lost in low-resolution MS due to the overlap of peptic fragment isotopic distributions.

Our preliminary SFC data demonstrate greatly reduced H/D back-exchange compared to HDX optimized HPLC conditions. The nonexchanging CO_2 mobile phase, fast flow rates, and short retention times of SFC all contribute to reduce the hydrogen/deuterium back-exchange. SFC shows increased deuterium incorporation for the shortest exchange periods, of the experiment, thereby recovering information lost in all previous conventional solution HDX experiments due to high back-exchange (up to ~90% back-exchange for the fastest exchanging hydrogens for HPLC relative to SFC separation—see Table 1). The increased deuterium incorporation observed at short exchange periods by SFC further substantiates the pursuit of improved coupling of quench/flow techniques to solution-phase HDX with SFC separation.[20] SFC separation shows great promise to significantly reduce back-exchange in solution-phase HDX with MS analysis.

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