Hydrogen Exchange-Mass Spectrometry

OPTIMIZATION OF DIGESTION CONDITIONS*

Lintao Wang, Hai Pan, and David L. Smith‡

The direct linkage between folded structures of proteins and their function has increased the need for high resolution structures. In addition, there is a need for analytical methods for detecting and locating changes in the folded structures of proteins under a wide variety of conditions. The rates at which hydrogens located at peptide amide linkages undergo isotopic exchange have become the basis for an important method for detecting such structural changes. When detected by mass spectrometry, hydrogen exchange can be used to study dilute solutions of large proteins and protein complexes with very high sensitivity. To locate structural changes, labeled proteins are often digested with acid proteases to form peptides whose hydrogen/deuterium levels are determined by mass spectrometry. This approach is successful only when the protein can be digested rapidly under conditions where isotope exchange is slow. This study describes how columns packed with immobilized pepsin can be used to reduce the digestion time and to provide an effective means for separating the pepsin from the isotopically labeled fragments. These columns are part of an on-line system that facilitates both rapid digestion of low concentrations of protein and concentration of the peptides. Molecular & Cellular Proteomics 1:132–138, 2002.

Proteomes may be studied at different levels of complexity and structural detail. One may be interested only in identifying which proteins are present, or one may wish to follow quantitatively changes in the levels of specific proteins. Proteome studies requiring more detailed structural information may focus on the type and abundance of post-translational modifications. Given the direct relationship between the structures of folded proteins and their functions, there is also an increasing need to know the structures of folded proteins. High resolution three-dimensional structures of folded proteins are most often determined by x-ray diffraction or NMR. As a result, studies of the folded structures of proteins under native or non-native conditions most often rely on lower resolution methods, such as fluorescence, circular dichroism, and ultracentrifugation.

The rates at which hydrogen located at peptide amide linkages undergo isotopic exchange has proven to be another important method for studying the three-dimensional structures of proteins under a variety of conditions (1–3). All peptide amide linkages except proline have an amide hydrogen that may exchange with other labile hydrogens in solution, most often water. Studies performed 40 years ago showed that the isotope exchange rates of these hydrogens are very sensitive to the structure and dynamics of a protein (3, 4). Although many useful applications of this approach where protium was replaced with tritium have been reported, NMR detection of hydrogen/deuterium exchange has proven more useful, because it can be used to determine the exchange rates at specific peptide amide linkages. The ability to detect subtle structural changes in different forms of the same protein using amide hydrogen exchange (HX) has been demonstrated using oxidized and reduced cytochrome c (5–7).

Approximately 10 years ago, it was demonstrated that amide hydrogen exchange in polypeptides could also be detected by mass spectrometry (MS) (8, 9). Since these initial demonstration experiments, HX MS has been used to study structural changes in proteins (10–13), protein folding (14–18), and protein aggregation/oligomerization (19–22). Today, NMR and MS appear to be highly complementary for detecting amide hydrogen exchange. The relative advantages of the two approaches for detecting HX have been discussed (23–25). NMR continues to be the preferred method for determining isotope exchange rates at specific amide linkages. That is, NMR gives higher spatial resolution than MS.

Approximately 20 years ago, Rosa and Richards (26) showed that proteins labeled with tritium could be digested with pepsin under conditions that minimized hydrogen exchange. Englander et al. (27, 28) have substantially refined this approach. Following general principals laid down in these early studies, Zhang and Smith (29) showed that peptide digestion was compatible with HX MS. Key to all of these approaches is the use of low pH and temperature to minimize hydrogen exchange during digestion and high concentrations

* The abbreviations used are: HX, hydrogen exchange; ESIMS, electrospray ionization mass spectrometry; HPLC, high performance liquid chromatography; MS, mass spectrometry.

From the Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588-0304
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of pepsin to minimize digestion time. Although many applications of this approach have been reported, high concentrations of pepsin can create problems. The present study focuses on an on-line sample-handling system that uses a column packed with immobilized pepsin to fragment proteins and a trapping column to concentrate the peptic fragments prior to analysis by HPLC ESIMS.

**EXPERIMENTAL PROCEDURES**

Pepsin was immobilized on POROS-20AL support by a linkage consisting of a secondary amine formed by reducing a Schiff’s base between the support and the pepsin. Freshly purchased pepsin (200 mg) was dissolved in 2 ml of pH 4.4 buffer (50 mM sodium citrate) at 0 °C. The solution was loaded on a pre-equilibrated G-25 gel filtration column (pD-10; Amersham Biosciences, Inc.) and eluted with 4 ml of the same buffer. The coupling reaction was performed at 0 °C in several steps in a 25-ml beaker. First, 0.66 ml of ALD coupling solution (stabilized 1.0 M sodium cyanoborohydride; Sterogene Inc.) was added to the 4-ml pepsin solution, followed by slowly adding 2.3 ml of 1.5 M Na2SO4. Then, 1.4 g of POROS-20AL powder (PerSeptive Biosystems) was added to form a homogeneous suspension. Finally, 4.6 ml of 1.5 M Na2SO4 was added drop by drop in a time span of about 2 h. The suspension was then transferred into a centrifuge tube where the reaction continued at 4 °C for 15–18 h. Throughout this time, the tube was turned end over end to ensure gentle mixing. To quench the reaction, 1 ml of 0.1 M ethanolamine was added. This solution was incubated for 5 more hours. Free and immobilized pepsin were separated by washing the support in a sintered glass funnel with 50 ml of pH 4.4 citrate buffer and 50 ml of pH 4.4 citrate buffer. The clean immobilized pepsin was resuspended in 50 ml pH 4.4 citrate buffer (volume ratio 50:50) and stored at 4 °C.

The immobilized pepsin was packed in clean stainless steel columns, typically 2-mm inner diameter × 50-mm length (Alttech). These columns were assembled such that one end had a frit and a normal cap. The other end had no frit but had a packing cap modified to have an opened-up hole inside. PEEK tubing (0.030-inch inner diameter, 180-cm length) was filled with 400 μl of an immobilized pepsin slurry (50% v/v in pH 4.4 citric buffer) and connected inline with the frit-free end of the empty column. An HPLC pump was used to flow 0.1% formic acid through the line, driving the slurry into the column. The flow rate started from 1 ml/min and was gradually increased until the back pressure reached 2500 p.s.i., the maximum pressure recommended for POROS packing. The flow continued for 10–15 min to obtain compact packing and then decreased slowly to 0 p.s.i. The modified packing cap was replaced with a normal, fritted cap, and the column was washed with 0.1% formic acid at 9 ml/min for 5 min before use.

**RESULTS**

A general procedure commonly used to study the hydrogen exchange properties of proteins is illustrated in Fig. 1. Protic solutions of proteins are diluted 20-fold with deuterated solutions to initiate isotope exchange. After various labeling times, this solution is acidified and cooled to quench isotope exchange. The deuterium level in labeled proteins may be determined by analyzing the intact protein by directly coupled HPLC ESIMS. These results provide a global view of the hydrogen exchange properties of a protein averaged over all peptide amide linkages. Alternatively, the hydrogen exchange properties of proteins in specific regions of the polypeptide backbone may be determined by similar analyses of peptic fragments of the labeled protein.

This general approach to HX MS measurements is most successful when the amount of deuterium lost during analysis is relatively small. Studies using completely labeled proteins show that experimental conditions limiting total deuterium loss to ~10% can be found for analyses of intact proteins (21, 30). This small amount of loss was attributed to exchange occurring during HPLC (typically 2–5 min) and possibly electrospray ionization. When labeled proteins are digested prior to analysis by HPLC ESIMS, the deuterium losses are larger, because some exchange occurs during the 4–5 min typically used for digestion. In addition, the deuterium losses measured for different peptides may span a wide range (e.g. 10–50%), because hydrogen exchange depends on the amino acid sequence (11, 22, 31–34). Controlling these losses is particularly important for studies where the hydrogen exchange properties of a protein have changed little. For example, a minor structural change may change the deuterium level in a peptide, as measured by its molecular mass, by less than 1 Da.

The sources of uncertainty in quantifying significant changes in deuterium levels may be divided into two categories, sample preparation and mass measurement. For strong signals with minimal interference from other ions, modern mass spectrometers can determine the molecular masses of polypeptides with an uncertainty of 10–100 ppm. This limitation translates to an uncertainty of 0.01–0.1 Da for a peptide of molecular mass 1000 Da and an uncertainty of 0.5–5 Da for a protein of molecular mass 50,000 Da. Factors associated with sample preparation usually contribute more to the variation in deuterium levels detected. For this reason, pH, time, and temperature must be controlled carefully at every step of labeling and analysis.

For analyses of peptides, the need to control pH and temperature during digestion is minimized when the digestion
time is short. Two approaches used to digest labeled proteins under hydrogen exchange quench conditions are illustrated in Fig. 2. In the more commonly used procedure (Fig. 2A), labeled proteins are digested in solution using a pepsin:substrate ratio of 1:1. After 5 min of digestion, the sample is injected onto a reversed phase column and analyzed by ESI-MS. Although this approach has been used extensively, it is not ideal, because significant deuterium is lost during the 5-min digestion time, and because the large quantity of pepsin required for rapid digestion may plug the HPLC column or interfere with the ESI process. Plugging of HPLC columns, either by pepsin or undigested substrate, is particularly common when using high performance columns packed with small pore materials. In addition, we have found that pepsin may adhere irreversibly to the HPLC column. This pepsin may retain activity and fragment proteins injected some time later.

An on-line approach that may be used to rapidly digest labeled proteins and concentrate the peptic fragments prior to their separation and analysis by HPLC ESIMS is illustrated in Fig. 2B. A similar approach using an immobilized pepsin column has been reported previously (34). Labeled protein, in a volume as large as 10 ml, is rapidly loaded into the sample loop of the injection valve (left in Fig. 2B). Solvent (H2O, 0.1–1% formic acid) from pump C is used to carry the sample though the pepsin column to the peptide trap located on the switch valve (right in Fig. 2B). Following a short desalting period, the switch valve is set so that solvent from pumps A and B elutes peptides from the peptide trap, enabling their separation and analysis by HPLC ESIMS. The digestion time is determined by the volume of the pepsin column and the flow rate of pump C. Although the pepsin column has been used either at ambient temperature or at 0 °C, all other parts of the system are submerged in an ice bath to minimize deuterium loss.

Peptic digestion of cytochrome c in solution and in a column packed with immobilized pepsin is illustrated in Fig. 3. The peak shaded black indicates the elution of intact cytochrome c whereas other peaks indicate elution of peptic fragments. Analysis of the peak areas indicates that ~40% of the cytochrome c remained intact when digestion was performed in solution. However, only 5% of the cytochrome c remained intact when digestion was performed using a column packed with immobilized pepsin. These results show that the immobilized pepsin column used under these conditions increased the extent of digestion 8-fold. Furthermore, the effective digestion time was reduced 15-fold. Chromatograms in panels B and C illustrate the reproducibility of peptic digestion using the immobilized pepsin column. Although pepsin cleavage sites cannot be predicted accurately in advance, digestion with immobilized pepsin is highly reproducible.

Studies performed using model peptides indicate that the rate of hydrogen exchange will increase 10-fold as the temperature is increased from 0 to 20 °C (32). However, the digestion time, and hence the time for deuterium loss, was...
in 1.5 M GdHCl. Although high concentrations of denaturants, and no signal for intact protein was detected.

The effect of temperature of the immobilized pepsin column on the extent of digestion is illustrated in Fig. 4. These experiments were performed using a 170-μl pepsin column (2 mm × 50 mm) and a flow rate from pump C of 200 μl/min. For these conditions, the effective digestion time was 50 s. Results obtained for digestion at 20 and 0 °C (Fig. 4, A and B) show that digestion of cytochrome c is far more efficient at the higher temperature. However, essentially all of the cytochrome c was digested at 0 °C for 50 s when the sample was digested in 1.5 M GdHCl. Although high concentrations of denaturants, such as GdHCl or urea, are generally incompatible with ESIMS, these reagents can be used with the on-line sample system illustrated in Fig. 2B, because it uses two separate steps of purification. Most of the denaturant exits through the waste port inside the column, the actual temperature there may be less than 20 °C.

Table I

| Peptidic fragments of cytochrome c | Deuterium recoverya | Solution digestionb | Column digestionc |
|-----------------------------------|---------------------|---------------------|-------------------|
| 1–10                              | 78                  | 80                  |
| 1–21                              | 77                  | 80                  |
| 22–36                             | 63                  | 74                  |
| 37–47                             | 76                  | 79                  |
| 47–64                             | 72                  | 79                  |
| 68–80                             | 71                  | 73                  |
| 83–94                             | 73                  | 80                  |
| 95–104                            | 73                  | 77                  |

a Determined from the number of deuteriums found in a peptide divided by the total number of peptide amide hydrogens in the peptide.
b Solution digestion was performed for 5 min at 0°C using an enzyme:substrate ratio of 1:1 (w/w). c Column digestion was performed for 20 s at approximately 20°C using immobilized pepsin.

immobilized pepsin, the cleavage sites were rather similar. This behavior was also evident when denaturants were used to accelerate digestion. In general, the changes in proteolytic fragmentation found using these different digestion conditions were similar to changes found when using different digestion times. That is, peptide length of the most abundant peptides decreases as the digestion time increases. Optimization of digestion conditions to give peptides derived from the entire length of a protein is an important step in most HX MS studies.

Cytochrome c is a good model protein for developing digestion procedures compatible with HX MS, because it is relatively difficult to digest. Most of the larger proteins that have been studied using these techniques in our laboratory digest more easily. For these proteins, the pepsin columns are typically positioned in the ice bath. Results for triosephosphate isomerase are presented in Fig. 5. This test was performed using a large volume (1 ml) and low concentration (150 fmol/μl), conditions typical of many HX MS studies. Digestion in solution for 5 min using a triosephosphate isomerase:pepsin ratio of 1:1 gave few peptides (Fig. 5A). However, digestion of the same sample solution for 20 s using immobilized pepsin located in the ice bath led to effectively complete digestion (Fig. 5B). Approximately 60 peptides were identified, and no signal for intact protein was detected.
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Fig. 5. Total ion chromatograms of peptic digests of triosephosphate isomerase. A, digestion performed in solution (digestion time 5 min). B, digestion performed using a column packed with immobilized pepsin. The digestion time of 20 s was calculated from the column volume (170 μl) and flow rate (500 μl/min). The temperature (0 °C), sample concentration (150 fmol/μl), and sample volume (1 ml) were the same for both digestion procedures.

DISCUSSION

Detection and precise location of changes in the folded structures of proteins are often important to our understanding of their function. The rates at which hydrogens located at peptide amide linkages undergo isotopic exchange when folded proteins are dissolved in D₂O often span a range of 10⁴. This wide range of exchange rates, combined with the sensitivity of exchange to intramolecular hydrogen bonding and solvent exposure, make hydrogen exchange very sensitive to many structural changes. Because every peptide amide linkage except proline has an amide hydrogen, structural changes along the entire polypeptide backbone may be detected by hydrogen exchange. Two-dimensional NMR has been particularly useful for detecting isotope exchange of these hydrogens, because exchange rates for all but the most rapidly exchanging amide hydrogens can usually be determined. That is, the exchange rates for individual hydrogens can be determined by NMR.

Although mass spectrometry offers several advantages for detecting amide hydrogen exchange, the spatial resolution is generally low. When intact proteins are analyzed, changes in molecular mass indicate changes in hydrogen exchange summed over all peptide amide linkages in the protein. Although this information may be important, it cannot be used by itself to determine the location of a structural change. However, a structural change can be located if the labeled protein is digested under conditions where little isotope exchange occurs. In this case, peptide molecular masses indicate changes in hydrogen exchange summed over all peptide amide linkages in the peptide. The actual gain in spatial resolution depends on the size of the peptides and is often in the range of 5–10 residues. Higher spatial resolution has been obtained by using multiple proteases (27), differences in overlapping fragments (29), and collision-induced dissociation (MS/MS) (10, 35, 36). The success of all of these approaches depends on being able to digest proteins under conditions where isotopic exchange is slow.

Specific requirements for minimizing hydrogen exchange during digestion of labeled proteins are illustrated by the isotope exchange rate constants given in Table II. These rate constants, which were calculated for angiotensin at 0 °C and pH 2.5, are representative of the exchange rates of most unfolded polypeptides (32). These calculations show that exchange at peptide amide linkages is slowest when the pH is between 2 and 3. It follows that acid proteases, such as pepsin, are essential. These calculations also show that the half-life for exchange at most amide linkages under these conditions is in the range of 20 to 500 min. To have the maximum sensitivity for detecting structural changes, it is desirable to complete the digestion and HPLC MS analysis in a time frame that is shorter than the half-life for exchange. Note that the positive charge on the N terminus of the peptide increases the exchange rate at the adjacent amide linkage ~10-fold (32). Although it is difficult to find conditions where deuterium is retained at this position during analysis, such conditions can be found for most other amide linkages. Results of the present study show that pepsin immobilized on a porous support and packed in a column can be used to substantially decrease the time required to digest proteins under conditions where isotope exchange is minimized.

The efficiency of pepsin columns is particularly evident for hydrogen exchange studies where the protein concentration is low, often in the low nanomolar range. Previous studies have shown that labeled peptides can be detected readily when the substrate:pepsin ratio is as high as 1:1. However, increasing the pepsin concentration further overloads the small HPLC columns preferred for ESIMS and suppresses the ion signal during the spray process. The presence of any pepsin or undigested substrate is particularly detrimental when using capillary HPLC columns packed with high efficiency, small pore packing materials. The low concentration of the labeled protein, combined with the need to keep the substrate:pepsin ratio less than 1:1, limits the maximum con-

| Amide linkage | k (min⁻¹) | t₁/₂ (min) | pH minimum |
|---------------|-----------|------------|------------|
| Asp           | 0.75      | 0.92       | 1.3        |
| Arg           | 0.015     | 47         | 2.4        |
| Val           | 0.0021    | 336        | 3.1        |
| Tyr           | 0.0033    | 213        | 3.2        |
| Ile           | 0.0013    | 543        | 3.1        |
| His           | 0.015     | 47         | 2.4        |
| Pro           | 0.0030    | 229        | 3.3        |
| Phy           | 0.028     | 24         | 2.4        |
| Leu           | 0.0076    | 92         | 3.0        |
centration of the pepsin to the low nanomolar range. Long digestion times are inherent to use of these low concentrations of pepsin. However, immobilization of pepsin on PO-ROS-20AL leads to an effective pepsin concentration approaching the millimolar range and short digestion times, even when the substrate protein concentration is low.

Results of the present study show that the on-line sample handling system illustrated in Fig. 2B is effective for digesting labeled proteins and concentrating the peptides under conditions that minimize isotope exchange at peptide amide linkages. Volumes as large as 10 ml of dilute solutions of labeled proteins can be rapidly loaded in the loop of the injection valve and subsequently passed through the pepsin column. The digestion time is determined by the flow rate from pump C and the volume of the pepsin column. Peptidic fragments of the labeled proteins are concentrated into the small volume of the peptide trap on the switch valve. Additional washing with solvent from pump C is used to remove most of the salt or denaturants used to enhance digestion. This step also removes deuterium located in the side chains of the peptides, leaving deuterium only at the peptide amide linkages. The labeled peptides, which are eluted from the peptide trap by mobile phase from pumps A and B, are separated by the liquid chromatography column and analyzed by ESI-MS to determine the levels of deuterium at peptide amide linkages. The time required for the entire analysis is substantially less than the half-life for isotope exchange at most peptide amide linkages.

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‡ To whom correspondence should be addressed. Tel.: 402-472-2794; Fax: 402-472-9402; E-mail: dsmithe7@unl.edu.

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