SAIDE: A Semi-Automated Interface for Hydrogen/Deuterium Exchange Mass Spectrometry

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Abstract

Deuterium/hydrogen exchange in combination with mass spectrometry (DH MS) is a sensitive technique for detection of changes in protein conformation and dynamics. Since temperature, pH and timing control are the key elements for reliable and efficient measurement of hydrogen/deuterium content in proteins and peptides, we have developed a small, semiautomatic interface for deuterium exchange that interfaces the HPLC pumps with a mass spectrometer. This interface is relatively inexpensive to build, and provides efficient temperature and timing control in all stages of enzyme digestion, HPLC separation and mass analysis of the resulting peptides. We have tested this system with a series of standard tryptic peptides reconstituted in a solvent containing increasing concentration of deuterium. Our results demonstrate the use of this interface results in minimal loss of deuterium due to back exchange during HPLC desalting and separation. For peptides reconstituted in a buffer containing 100% deuterium, and assuming that all amide linkages have exchanged hydrogen with deuterium, the maximum loss of deuterium content is only 17% of the label, indicating the loss of only one deuterium molecule per peptide.

Introduction

Dynamics of proteins in solution are of great interest because knowledge of types, locations, and rates of movements lead to a better understanding of how proteins carry out their functions *in vivo*. Deuterium/hydrogen exchange in combination with mass spectrometry (DH MS) is a sensitive technique for detection of changes in protein conformation and dynamics. This technology has several advantages over more conventional methods, such as crystallography and multidimensional NMR; among them are the possibility to study native proteins in solution, the requirement for reduced protein concentrations and the potential to discriminate multiple coexisting conformations. In addition, DH MS has no upper limit to the size of protein or protein complex that can be analyzed. DH MS approaches rely on the fact that hydrogen atoms on the amide linkages of the protein backbone can exchange with solvent deuterium at rates that reflect the dynamics of particular regions of the protein. The associated relative mass changes can be measured with a mass spectrometer.

The theory and methodology used to study protein conformation and dynamics has been described in several recent reviews [1-7]. The first experimental setup to measure rates of deuterium/hydrogen exchange by mass spectrometry and to map them to specific regions of the polypeptide sequence was described by Smith [8]. The procedure is based on the greatly reduced rate of exchange at low pH and temperature and on the activity of pepsin at pH 2.3. Thus, the typical exchange experiment is performed as follows: 1) Isotopic exchange is initiated by incubating the protein sample in a deuterated buffer at the desired pH and temperature. 2) At different time periods an aliquot is removed and deuterium/hydrogen exchange is quenched by reducing the pH of the solution to pD 2.4 and the temperature is lowered to 0^{o}C or below. 3) The global exchange rate is measured on the intact protein following fast desalting on a reverse phase HPLC column. Alternatively, to obtain detailed structural information, the protein is fragmented into small peptides by hydrolysis with pepsin

and the deuterium content in these peptides is analyzed by reverse phase HPLC connected on-line to an electrospray mass spectrometer. DH MS has been utilized successfully in the study of protein structure and dynamics [3], including protein folding [9], protein dynamics during catalysis and allosteric regulation [10-12]. In addition, DH MS has been used to map substrate binding sites on the primary structure of the protein [13].

In order to minimize loss of deuterium, mass measurement must be taken quickly, usually within the first few minutes following quenching. However, there is no standard equipment to interface the different stages of analysis described above. Initially, temperature control during the quench and mass measurement stages was provided by an ice bath, and control of pH by a manual dilution of the protein sample with a highly concentrated acidic buffer (for example 200 mM ammonium phosphate, pH 2.3) [8, 14-19]. This is still the most common experimental procedure. To reduce back exchange and increase in sensitivity, the use of capillary columns and fast flow chromatography have been included in many instrumental configurations [16]. Also, multiple proteases have been used to enhance peptide coverage and, therefore, sequence resolution [2, 20].

Wide application of DH MS continues to be hindered by the lack of instrumentation to handle sample exchange. To the best of our knowledge, there is only one fully automatic system capable of performing automatic measurements in the millisecond to 24 hr time range [4]. This system was built around a series of individual components: 1) two auto samplers equipped with Peltier-cooled sample stacks, 2) a mechanical micro syringe and 3) a three valve unit build into a custom built thermal chamber. These components were in addition to a standard HPLC and high resolution mass spectrometer. This is a complex system that requires the assembly of different parts into a unit that works as a single instrument, and the entire system is under computer control by dedicated software. This instrument has been used successfully to study protein-ligand interactions [4] and protein dynamics [2]. Alternative approaches by quench flow methods have been used to investigate fast protein folding reactions [21]. In this approach, the protein sample was mixed rapidly with the protein-free solvent to initiate exchange. At various time periods after the initiation of exchange, the reaction was quenched by an additional mixing at low pH and temperature. Samples were then flash frozen and stored at -80^{o}C until they could be analyzed by mass spectrometry. The main advantage of this automated system is that quench flow instruments are robust and standard pieces of equipment. However, thawing of the samples for peptic digestion and mass spectrometry analysis could be problematic; considerable loss of deuterium content is likely if not performed under strict conditions and, therefore, reproducibility can be compromised. These automatic setups are complex and expensive. Here we describe a simple but efficient Semi-Automated Interface for Deuterium Exchange (SAIDE). This interface allows strict temperature and timing control during protein digestion, HPLC peptide desalting and separation and direct injection for mass analysis. This system is not complex as compared to those described above. However, on the basis of our preliminary studies our system is as effective and can be applied to most DH MS experimental procedures without modification.

Materials and methods

Reagents.

Cytosolic aspartate aminotransferase (cAAT) was purified as previously described [22]. Protein concentration was estimated from the absorbance of the pyridoxal-phosphate bound to its active site, using the molar absorption coefficient of 8500 M^{-1} cm^{-1} and a M = 46,399. TCPK treated trypsin was from Worthington. D_2O was from ACROS Organics. All other reagents were of the highest purity available.

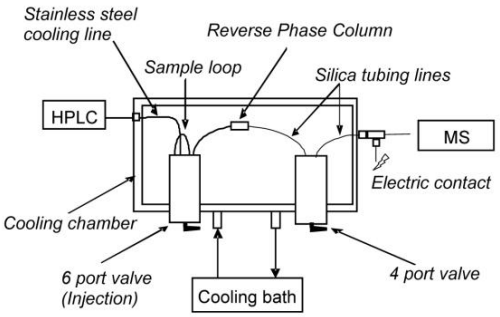

Figure 1. Schematic representation of SAIDE, a semiautomatic interface for DH MS. *The scheme shows the components contained inside of a thermostatic box. The temperature probe is located on the sample loop and is set to -2 ºC. The system contains the electric contact of the electrospray source. The entire assembly in installed on the front end of a LTQ FT mass spectrometer.*

Trypsin digestion of cAAT.

A stock solution of cAAT at 1 mg/ml in 2mM Tris HCl, pH 7.5 was denatured in 6 M guanidine hydrochloride (Gnd HCl). The sample was sequentially reduced and alkylated and finally diluted with 50 mM ammonium bicarbonate to 0.6 M Gnd HCl and incubated with trypsin, at 1:100 trypsin:cAAT subunit molar ratio, overnight at room temperature. The resulting peptides were desalted on a reverse phase guard column (Zorbax C18SB Wide pore guard Column, MicroTech Scientific, 1 cm x 0.32 mm), and were eluted using 200 µl 0.01 % TFA in 60% acetonitrile, and 10 µl aliquots were evaporated to dryness and stored at -20ºC.

Mass spectrometry.

The tryptic cAAT peptides were analyzed by on-line HPLC mass spectrometry, using SAIDE as the injection interface, as described in Results. Mass analysis was done on a LTQ FT mass spectrometer (ThermoFinnigan) under automatic control to perform MS followed by tandem mass scans of the four most intense ions, using an exclusion list of 2 min. Data in the m/z range 600–2000 was collected. Tandem mass spectra were analyzed using the Sequest algorithm [23] included in the Xcalibur version software package (ThermoFinnigan). Sequest was set up to search a protein database containing a cAAT FASTA formatted data file and assuming full tryptic digestion, using a fragment ion mass tolerance of 20 ppm Da and a parent ion tolerance of 0.30 Da. Peptide identifications were accepted if they could be established at Xcorr score of at least 1.5, 2.0 or 2.5 for peptides with 1, 2 or 3 charges, respectively and a Delta Correlation score larger than 0.08.

Measurement of deuterium content.

Peptide masses were calculated from the centroid of the isotopic envelope using in-house developed software and were verified manually using the MagTran software. The extent of deuterium content was determined from the mass shift of labeled peptides relative to unlabeled peptides. The percentage of deuterium label was calculated as $D(\%) = 100*(M_D-M_H)/(n-2)$; were M_D and M_H are the neutral masses of the labeled and unlabeled peptide, respectively, n is the total number of amino acid residues of the peptide and 2 is a factor described by Englander [24]

Results and discussion
SAIDE interface.

Central to D/H exchange studies is that exchange is temperature and pH sensitive. Therefore, exchange reactions are performed at near neutral pH at controlled temperature. Then, the exchange is quenched using low pH and low temperature. However, quenching slows, but does not eliminate further exchange and/or back-exchange. For this reason, analysis of quenched samples must be performed quickly (within 30 minutes) and quenching conditions (low temperature/low pH) must be maintained during this analysis. Most instrumental designs used in D/H studies immerse solvents, columns and HPLC injection valves in an ice bath. Since temperature, pH and timing control are the key elements for reliable and efficient measurement of deuterium content in protein and peptides, we have developed a small, semiautomatic system that is easy to interface with a mass spectrometer and provides efficient temperature and timing control in all stages of HPLC separation and mass analysis.

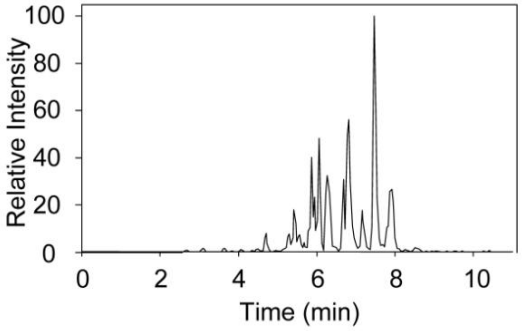

Figure 2. HPLC trace of tryptic peptides. *Base peak chromatogram showing the rapid elution of the peptide mixture obtained after trypsin digestion of cAAT. Most peptides elute in less than 10 minutes.*

The designed interface, SAIDE, is used to perform timed protein digestion followed by automatic peptide desalting and fast HPLC peptide separation under controlled conditions of pH and temperature. A schematic of the interface is shown on Figure 1. This interface is installed between the HPLC and the mass spectrometer. It consists of a temperature controlled chamber that holds two valves and a reverse phase column. Valve 1 is a six port valve (VALCO Cheminert) with an additional front-end port for sample introduction. This valve is equipped with an electric contact closure that triggers simultaneously the start of the gradient formation on the HPLC and the acquisition of data on the mass spectrometer. Port 2 of this valve is connected to the in-line gradient formed by the HPLC, ports 1 and 4 hold a 20 µl loop, port 3 is connected to the reverse column and ports 5 and 6 are waste lines. The outlet of the reverse phase column is connected to a 4 way valve (VALCO Cheminert), which acts as a switch valve to direct the eluent from the column to either the mass spectrometer or to the waste. The temperature of the box is connected to a cooling bath (Polyscience). For the procedures described in this report, the temperature of the thermostatic box was measured on the outside of the sample loop and was adjusted to -2ºC. The entire assembly was built by Mecour (Groveland, MA) according to our specifications. A thermo sensor (Digisense) was added to measure the temperature at the sample loop. The cooling line for this interface is connected to a 96 well cooling sample tray. To minimize changes in temperature during transfer of the sample from the reaction vial to the interface, an independent cooling line is used to maintain a 25 µl micro syringe (Hamilton) at the same temperature as the cooling box.

Optimization of reverse phase chromatography.

The first step in the analysis of DH MS experiments involves the fast separation of peptides at low temperature. Thus, it was desirable to determine conditions for optimal separation of a peptide mixture at the lowest operational temperature of the SAIDE. Three parameters had to be measured or optimized: 1) the minimum operative temperature of the cooling chamber, 2) the chromatographic gradient and 3) the degree of back exchange of deuterium present in labeled peptides. With that purpose, a set of tryptic peptides derived from cAAT was prepared as indicated in Materials and Methods and analyzed under various chromatographic gradient profiles and temperatures. An independent probe was used to check the effectiveness of the cooling chamber and temperature readings were taken inside the chamber (air), the beginning and end of the cooling line as well as at the sample loop of the injection valve. As expected, a temperature gradient was observed between the air and the sample loop, but it was only 1ºC. The temperature gradient on the sample loop and cooling line was also minimal, with a difference of only 0.2ºC. Thus, the cooling line is able to effectively decrease the temperature of the solvents. This is particularly important since all of the chromatographic equipment and solvent reservoirs were at room temperature. Subsequently only the temperature at the sample loop was monitored. The minimum operational temperature of the thermostatic box was determined to be -2ºC at the sample loop, and this was used for all experiments involving deuterium measurements. Under this temperature condition the solvent in the capillary solvent lines will not freeze provided that there is at least a flow of 5 µl/min.

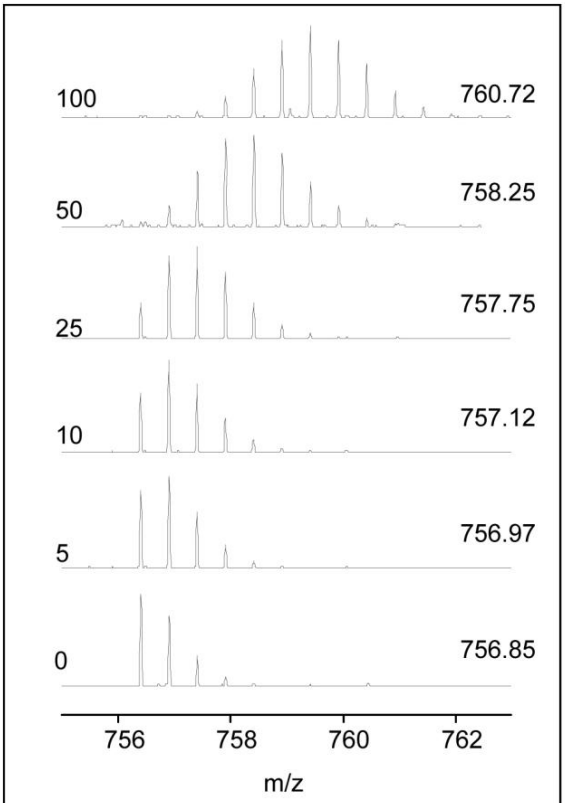

Figure. 3. Isotopic envelopes of a doubly charged peptide-ion reconstituted in different content of D2O. *The figure shows the isotopic envelopes for the doubly charged ion of the peptide TDDSQPWVLPVVR dissolved in deuteron-ammonium phosphate containing different amount of D2O.The content in D2O is indicated at the lower left corner of each trace. For each isotopic envelope, its average mass is given on the upper right corner.*

Next, to optimize a chromatographic profile the test mixture was analyzed utilizing several chromatographic gradients; changes include the flow of the mobile phase, and the slope and shape of the gradient. For all these experiments, the temperature of the cooling chamber was maintained at -2ºC. A trace of a typical optimized gradient is shown on Figure 2. Peptides were loaded on the loop and following a 2 min. desalting step at 200 µl/min of 0.05% TFA, were eluted using a 5 min 10% to 80% gradient of acetonitrile in 0.05% TFA at a flow rate of either 25 or 100 µl/min. Most peptides eluted within 15 minutes from injection, yet the chromatographic trace maintains the high degree of chromatographic resolution required for the detection of a large number of peptides. The resulting protein coverage was 98%, and peptides lengths ranged from 8 to 23 residues.

Deuterium recovery on tryptic peptides.

To determine the effectiveness of the SAIDE interface in the retention of deuterium incorporated into these peptides, six aliquots of cAAT tryptic peptides were subjected to three cycles of lyophilization/solubilization in deuterium ammonium phosphate, pH 7.5, containing 0, 5, 10, 25, 50 and 100% deuterium oxide. Each aliquot was analyzed in duplicate and the deuterium content was determined as described in Materials and Methods. A set of 43 well characterized semi tryptic peptides was selected (Table 1). The length and retention time of these peptides ranged from 8 to 23 amino acid residues and 10 to 15 min, respectively. Thus, providing a wide range of peptide length and retention times to measure deuterium back-exchange during their chromatographic separation. Figure 3 shows the isotopic envelopes for the doubly charged ion of the peptide TDDSQPWVLPVVR obtained at different D_2O content in the reconstitution buffer. The recovery of deuterium label for each peptide was measured as described in Materials and Methods. The deuterium content for each experimental condition was averaged among all peptides independently of its length or retention time. The results are shown in Figure 4, which shows the average recovery of deuterium label as a function of the content on deuterated water in the reconstitution step. There is a clear linear correlation between the amount of deuterium present in the sample and the amount of deuterium label recovered on the peptides, with a correlation score of 0.998. For peptides reconstituted in a buffer containing 100% deuterium oxide we recovered 83% of the label in the resulting peptide ions (assuming that all amide linkages have exchanged hydrogen with deuterium), this is equivalent or better than the deuterium recovery obtained by other methods [16] including more complex systems [4]. More specifically, this represents a loss of only 17% of the label incorporated in peptides, or about 1 deuterium atom from a 100% labeled decapeptide.

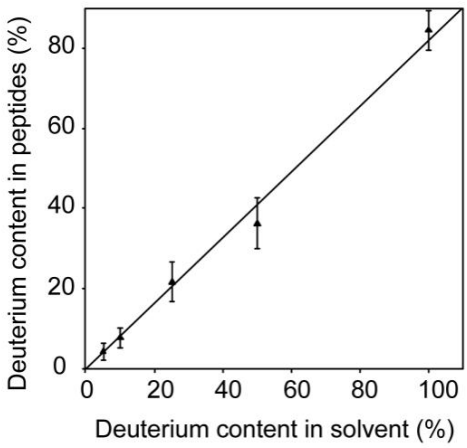

Figure.4. Retention of Deuterium in tryptic peptides reconstituted in D2O. *A set of peptides obtained by trypsin treatment of cAAT were lyophilized and reconstituted in buffer containing increasing amounts of deuterium oxide (5, 10, 25, 50, 75 and 100%). Fourteen well characterized peptides by mass spectrometry were selected and the deuterium content measured as indicated in experimental procedures. The graph shows the linear plot fit of triplicate experiments. The experimental data were fitted to a single linear equation with a correlation score is 0.998.*

Conclusions

The use of SAIDE, a semiautomatic interface device, has been described and its efficiency for the elucidation of deuterium content in proteins has been demonstrated. This interface provides efficient temperature and time control for most basic DH MS experimental procedures. Reduction of D/H back- exchange has been achieved not only by the operational low temperature, but also by the fact that this small box is located immediately in front of the mass spectrometer. This design reduces the length of the solvent lines and quickly delivers the peptide mixture directly onto the ESI source. Back-exchange is kept to a minimum as demonstrated by the detection of deuterium content in peptides. The simplicity of the SAIDE design will make it easy to interface with other instruments, such as an autosampler or stopped or quench flow instrument, for a variety of workflows, including the use of nano-columns operating at low flow rates. Currently, our SAIDE is being retrofitted with automated valves to convert it to a fully automatic instrument, thus the digestion time in the loop, desalting, elution of peptides and data acquisition on the mass spectrometer will be events automatically controlled by a computer started by the injection of a sample into the loop.

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