On-tissue Direct Monitoring of Global Hydrogen/Deuterium Exchange by MALDI Mass Spectrometry: Tissue Deuterium Exchange Mass Spectrometry (TDXMS)*

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Hydrogen/deuterium exchange mass spectrometric (H/DXMS) methods for protein structural analysis are conventionally performed in solution. We present Tissue Deuterium Exchange Mass Spectrometry (TDXMS), a method to directly monitor deuterium uptake on tissue, as a means to better approximate the deuterium exchange behavior of proteins in their native microenvironment. Using this method, a difference in deuterium uptake behavior was observed when the same proteins were monitored in solution and on tissue. The higher maximum deuterium uptake at equilibrium for all proteins analyzed in solution suggests a more open conformation in the absence of interacting partners normally observed on tissue. We also demonstrate a difference in the deuterium uptake behavior of a few proteins across different morphological regions of the same tissue section. Modifications of the total number of hydrogens exchanged, as well as the kinetics of exchange, were both observed. These results provide information on the implication of protein interactions with partners as well as on the conformational changes related to these interactions, and illustrate the importance of examining protein deuterium exchange behavior in the presence of its specific microenvironment directly at the level of tissues. Molecular & Cellular Proteomics 15: 10.1074/mcp.O116.059832, 3321–3330, 2016.

Hydrogen/Deuterium Exchange Mass Spectrometry is a robust technique that can be used to study structural changes of proteins. It has been demonstrated, for example, in the study of protein folding dynamics (1), and protein-protein (such as antigen-antibody interactions (2), allosteric binding (3), aggregation (4), etc.) and protein-ligand interactions in solution (5). This can be done by analyzing global changes observed by subjecting the protein of interest to deuterium exchange and analyzing the amount of deuterium incorporation directly (6). It can also be used to determine the specific regions that are affected by conformational change, by digesting the protein using pepsin, and analyzing the subsequent peptides generated after doing a rapid chromatographic separation ensuring that the extent of back exchange will be minimal (6).

Several methods exist for the study of protein conformation by deuterium exchange in addition to mass spectrometry, such as Fourier Transform Infrared Spectroscopy (FTIR), Circular Dichroism (CD), and Nuclear Magnetic Resonance (NMR). Methods such as FTIR (7, 8) and CD (8) are normally restricted to global conformational change studies. Far ultraviolet (UV) CD can be used to study secondary structural elements whereas near UV CD can be used to study tertiary structure focusing mainly on aromatic amino acid residues. Positional specificity down to the amino acid level can be achieved using NMR; it is limited however to the analysis of low mass proteins typically below 30 kDa (9), for example; beyond this the complexity of the analysis of 2D NMR correlation increases. Protein structure is mainly studied by x-ray crystallography, and like NMR, it provides unequivocal information down to single amino acid positional specificity. A limitation of this technique though is the need to crystallize the protein, thus, not only restricting the analysis to its stable, crystal conformation, but also requiring that the protein to be analyzed is in its purest form (10). In addition, it is difficult to study proteins with partial amorphous structures using this technique because they are difficult to crystallize. Compared with the last two techniques mentioned, H/DXMS currently offers only limited positional specificity defined by the extent which the proteolytic enzyme used cleaves the protein, but it can be advantageous in other respects. For example, it only requires picomoles of protein and does not require the protein to be of high purity because of the high sensitivity offered by recent advances in the MS instrumentation (11). Also, the analysis has been demonstrated for large macromolecular complexes having masses above 180 kDa, such as the brome mosaic virus (12).
Despite its successful application, deuterium exchange experiments are still restricted to studies in solution. Indeed, there has been a gradual shift toward performing protein dynamics studies when interacting with their stable ligands and other small or polymeric biomolecules in an attempt to mimic the behavior of these proteins in vivo. By taking the protein dynamics in vitro to be identical in vivo, however, these studies suffer the risk of underestimating the extent of interactions that might be present in the protein microenvironment which varies depending on its localization (13). Recent evidence shows that when deuterium exchange is performed in vivo, the thermodynamic stability of the protein studied increased in response to a hyperosmotic change in its environment, compared when the protein is analyzed in vitro (14). In this work, the protein was overexpressed in E. coli cells, which were then exposed to deuterated media to enable the incorporation of deuterium inside the cells via passive diffusion. The idea of studying proteins in vivo using deuterium exchange has been described as early as 1930 (15), but until recently only the aforementioned publication has described the application of MS to study this. The major drawback of the aforementioned study was its limited application to single cell organisms where the protein has been overexpressed. Overexpression of the protein of interest allowed its detection and enabled deuterium exchange experiments to be performed on it. This means though that the protein is not anymore in its native environment where it may have different interacting partners which may not be reflected anymore in the case of the single cell organism. Furthermore, in order to express the protein on the host cell, modifications such as truncation of several amino acid sequences might be necessary. If we want to study protein dynamics in its native state, a wise approach would be to examine them in the tissues where they are originally expressed. This is however difficult to do in practice, because of limitations imposed by the deuterium exchange method itself.

In this study, we applied the deuterium exchange approach to study proteins on tissue to determine if the deuterium uptake is affected in the tissue by comparison to the solution and if localization on the tissue matrix has also significant effects. Direct MS measurements on tissue are advantageous in that the analysis is rapid, information can be obtained on hundreds of molecular signatures at the same time, and direct measurements of proteins/peptides are performed on tissue. In this report, we show that the effect of the tissue environment has varying effects on the deuterium uptake depending on the examined protein.

MATERIALS AND METHODS

Reagents—HPLC grade water, trifluoroacetic acid (TFA), acetonitrile (ACN) and ammonium acetate (NH4OAc) were obtained from BioCell BV (Dieuze, France). Sinapinic acid (SA, puriss) and deuterated water (D2O) enriched up to 99.9% with deuterium were purchased from Sigma-Aldrich (St. Quentin-Fallavier, France). Bovine ubiquitin and chick lysozyme C protein standards were purchased from Promega (Charbonnières-les-Bains, France) and were used without further purification.

Sample Preparation

H/D Exchange On Standards in Solution—The deuterium exchange procedure used to analyze protein standards was performed as follows. Lysozyme C and Ubiquitin standards were prepared in 100 μl of 50 mM NH4OAc in H2O (pH 7.6) at a concentration of 1.5 mg/ml each. The mixture was allowed to equilibrate for at least an hour before working solutions were obtained. To realize deuterium exchange, 3 μl working solution was diluted in 30 μl 50 mM NH4OAc prepared in D2O. 1 μl of the solution was taken for every time point and diluted to 10 μl with a quenching buffer (2% TFA in H2O, pH = 2.2). The solution was immediately frozen in liquid nitrogen until analysis. To examine the amount of deuterium uptake, the frozen samples were partly melted and 0.5 μl was spotted on a MALDI target. To this, 0.5 μl ice-cold matrix solution composed of 20 mg/ml SA suspended in 500 μl 2% TFA in H2O + 500 μl ACN/0.1% TFA in H2O (7:3, v/v) (quenching matrix solution) was added. The spots were dried for at least 5 min under vacuum in a cold room (4 °C) and analyzed immediately.

H/D Exchange On Tissue Extracts in Solution—A 12-μm coronal section of fresh frozen rat brain was dispersed in 10 μl 50 mM NH4OAc in H2O by vortex mixing after placing the tissue section in a micro-tube. The mixture was then centrifuged for 10 min at 10,000 × g and the supernatant was collected and dried in a speedVac. The dried extracts were reconstituted with 10 μl 50 mM NH4OAc in H2O and allowed to equilibrate for at least an hour before solutions were taken for deuterium uptake experiments. Three microliters of the extract was diluted in 30 μl 50 mM NH4OAc made up in D2O to achieve exchange. A 0.5 μl sample was taken for each time point and spotted on a MALDI target, to which 0.5 μl of the quenching matrix solution was added. The spots were dried under vacuum for at least 5 min and analyzed immediately.

On-Tissue H/D Exchanges—On-tissue deuterium exchange analysis was done as follows. Serial 12-μm coronal rat brain sections were obtained using a cryostat and mounted on ITO-covered glass slides, at one section per slide. The sections were dried under vacuum for 15 min. A 10 μl sample of 50 mM NH4OAc was then deposited throughout the entire section, and each slide was placed inside a Petri dish. The Petri dish contained paper towels wetted with 50 mM NH4OAc solution to prevent drying of the deposited liquid. The sections were allowed to equilibrate for at least 1 h before use, after which the excess liquid was withdrawn and replaced with 15 μl of 50 mM NH4OAc made up in D2O to achieve deuterium exchange. The slides were then dried under vacuum for at least 5 min and analyzed immediately. A control section was prepared following the same procedure but with H2O replacing D2O in all solutions. In experiments monitoring the deuterium exchange of the proteins across different regions, sagittal sections of the rat brain were used. The same protocol was applied except that only 1 μl of the exchange solution and quenching matrix solution were used per region, allowing for local H/D exchanges to occur and reducing the drying time to not more than 2 min.

MALDI TOF Mass Spectrometry—Mass spectrometric analysis was performed using a MALDI UltraFlex II time-of-flight (TOF) instrument equipped with a Smartbeam laser (355 nm emission wavelength) having a maximum repetition rate of 200 Hz and controlled by a FlexControl 3.0 (Build 158) software from Bruker Daltonics, GmbH (Bremen, Germany). The full MS spectra were acquired in positive
linear mode with delayed extraction within the 3000–20,000 \( m/z \) range by accumulating 5000 laser shots at a repetition rate of 200 Hz. The laser energy was fixed to be just slightly above the threshold energy for ion detection. Medium deflection of the low mass ions below \( m/z \) 3000 was used to enhance the detection of proteins. The MALDI target was always calibrated prior to analysis using Protein Calibration Standard 1 (Bruker Daltonics, GmbH), which contains a mixture of six proteins covering a mass range of \( m/z \) 5500–17,000. Mass values expressed in the text correspond to the centroid of the \( m/z \) values of the \([M+H]^+\) ions. The MALDI-TOF raw data of the H/D exchanges have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD004630.

**Protein Identification by Top-Down Microflow LC-MS—**Proteins were extracted from a consecutive 12-\( \mu \)m rat brain tissue section. One microliter of the protein extract was suspended in either 10 or 30 \( \mu l \) 5.95 ACN/0.1% FA in \( H_2O \) and 4 \( \mu l \) of these samples were injected in a 200 \( \mu m \times 50 \) cm ProSwift RP-4H monolithic capillary column (Thermo Fisher Scientific, Bremen, Germany). Proteins were eluted using a linear gradient from 3% to 40% ACN for 60 min, then to 95% ACN for 10 min, followed by a wash out until 90 min, at a flow rate of 1.4 \( \mu l/min \). Spectra were acquired in data-dependent mode using an LTQ Orbitrap XL mass spectrometer and subjecting the top 2 most intense peaks for fragmentation. The full MS scans were acquired at 100,000 resolution for \( m/z \) 400 and acquiring 2 FTMS microscans at a maximum ion injection time of 1000 ms. On the other hand, the MS/MS scans were acquired at 60,000 resolution for \( m/z \) 400 and acquiring four microscans at a maximum injection of 500 ms. High-energy collision dissociation (HCD) activation was used with the activation time set at 1s. The isolation width was set at \( \pm 15 \) ppm and the default charge state at +10. The normalized collision energy (NCE) was fixed at 30.0 V. Dynamic exclusion was enabled and the exclusion mass width was set to \( \pm 9 \) ppm by mass. The repeat count was set to 1 and repeat duration to 240 s. The exclusion list size was set to 500 and the exclusion duration to 180 s. +1, +2 and +3 charge states were rejected.

For top-down analysis, the data were analyzed with ProSightPIC 3.0 (Thermo Fisher Scientific) and Proteome Discoverer 2.1 (Thermo Fisher Scientific) utilizing the ProSightPD 1.0 node. Spectra were then searched using a three-tiered search tree. The first search was an Absolute Mass search with MS1 tolerance of 100 Da, MS2 tolerance of 10 ppm, against a *Rattus norvegicus* UniProt database containing 35,953 canonical and isoform sequences accessed on July 8, 2016, or the ProSightPD 1.0 database available at their website (ftp://prosight-pc.northwestern.edu/). The second search was a ProSight Biomarker search with MS1 tolerance of 10 ppm, MS2 tolerance of 10 ppm, against the same databases. Finally, a second Absolute Mass search was performed with MS1 tolerance of 1000 Da, MS2 tolerance of 10 ppm, using Delta M mode, against the same databases. Protein assignments can be found in supplemental Data S1. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the same data set identifier (PXD004630).

The "*.xml" Search files for 20160719_TopDown2 correspond to searches done using the UniProt database, whereas those for 20160719_topDown correspond to searches performed using the ProSightPD database.

**RESULTS**

Hydrogen/Deuterium (H/D)\(^1\) Exchange of Standard Proteins—Optimization of the deuterium exchange protocol in

\(^1\) The abbreviations used are: H/D, hydrogen/deuterium; TFA, trifluoroacetic acid; H/DXMS, hydrogen/deuterium exchange mass spectrometry; FTIR, Fourier Transform infrared Spectroscopy; CD, Circular Dichroism; NMR, Nuclear Magnetic Resonance; \( NH_4OAc \), ammonium acetate; \( D_2O \), deuterium oxide; SA, sinapinic acid; ACN, acetonitrile; MALDI, matrix-assisted laser desorption/ionization.
protein containing 144 labile hydrogens, 72 in the amide backbone and 72 in the amino acid side chains (19). The tightly folded protein contains an α helix at residues Ile23-Glu34, and two β strands adjacent to each other at residues Met1-Thr7 and Thr12-Glu16 comprising a mixed β sheet. The α helix backbone amide hydrogens are virtually nonexchangeable because of their strong interaction with adjacent carbonyl oxygens, whereas those of the β strands are slow exchanging (20). These structures, together with uncorrected back exchange (21), limit the maximum deuterium incorporation observed to 53%. A similar and even more pronounced effect is observed in the deuterium uptake of lysozyme C; which is also a globular protein containing a well-protected core stabilized by four disulfide bridges. This region is comprised of 30 well-protected backbone amide hydrogens that do not exchange even after 14 days (17, 18).

**On Tissue H/D Exchange (TDXMS)**—The deuterium exchange protocol was then adapted to examine the deuterium uptake of proteins on tissue sections. For on-tissue experiments, the choice, order and combination of D2O, quench acid, and matrix are crucial parameters to be able to observe deuterium exchange directly on tissue, while avoiding protein denaturation and minimizing back exchange processes. These parameters thus need to be carefully studied and optimized. Fig. 2 presents the designed strategy to perform and measure the deuterium uptake of proteins on tissue sections. To prevent denaturation of the proteins on tissue, the sections were immediately incubated in NH4OAc buffer for at least 1h after sectioning and drying under vacuum. After incubation of the tissue sections in NH4OAc, the buffer was aspirated and the sections were transferred to a medium saturated with the deuterated buffer prior to addition of the exchange solution onto the tissue section. The deuterated buffer in the medium prevents drying of the exchange solution and the possible gas phase exchange that occurs between the exchange solution and the environment. When the Petri dish was sealed with parafilm, it was observed that the exchange solution can be maintained for extended periods (6 days in these experiments) without drying. This workflow is designed to monitor the proteins that remain within their local environment during the H/D exchange, avoiding de facto, the contribution of the proteins undergoing diffusion or extraction (here the lower mass soluble proteins) during the conditioning or exchanging step. Indeed, the exchange solution is removed just before adding the matrix solution that is used to quench the reaction. Nonetheless, to check this aspect we analyzed the NH4OAc buffer used for on-tissue conditioning and compared it with that of the tissue after removing the conditioning solution (supplemental Fig. S2). The MALDI-TOF MS spectrum of the conditioning medium reveals much fewer signals than the on-tissue one. The observed signals are common with the tissue and correspond to the highly abundant proteins that are of the lowest mass and highest solubility. Some of these show very high intensities compared with the tissue because of the lower complexity of the studied system. This demonstrates that only a small portion of a few abundant and soluble proteins diffuse into the buffer solution and that the largest majority of the proteins remain within their local environment.

In order to demonstrate the importance of the tissue environment on the protein conformation, we need to compare the H/D exchange kinetics for the same proteins both in solution and in the tissue context. Extraction of intact proteins from a consecutive tissue section using the NH4OAc buffer allowed us to obtain proteins whose rate of deuterium uptake can be monitored both in solution and on the tissue section. Results show that signals can be detected in linear positive mode from an extract obtained from an entire tissue section (Fig. 3A, top). Likewise, the full MS spectrum acquired from the tissue section incubated in NH4OAc buffer also showed abundant signals (Fig. 3A, bottom), even when no lipid removal steps were incorporated into the protocol to enhance the detection of intact proteins (22). More importantly, several of the signals from the full MS spectrum of the extracted proteins in solution can be matched with those observed from the direct tissue analysis, as shown in Fig. 3 and supplemental...
Data S1 allowing for the comparison of the deuterium uptake in solution versus in the tissue.

In order to identify the proteins common to the tissue extracts and in situ on the tissue, proteins were extracted from a consecutive tissue section of same thickness and submitted to Top-Down analysis. Extracted intact proteins were analyzed in LC-MS by separation on a monolithic column and fragmented using the HCD cell of the LTQ-orbitrap instrument before identification by database interrogation. Protein assignments are given in supplemental Data S1. An example of the monitoring of deuterium uptake in both conditions is shown in Fig. 3B for Ubiquitin (m/z 8563). Because of experimental limitations such as the time interval between the analyses of two data points, the first data point was monitored only after 10 min of exchange. Nonetheless, results indicate that the deuterium uptake can still be monitored using this approach and H/D exchange could monitored both in solution and on tissue sections up to 43 h. Fig. 4 compares the deuterium uptake of several proteins in solution and on tissue. In the four examples plotted, it can be observed that overall the maximum amount of deuterium uptake for proteins in solution is much higher than when observed on tissue. If the tissue environment does not affect the conformation and interaction of proteins, we should expect that the deuterium uptake behavior would be similar. Moreover, the deuterium uptake behavior of the proteins matched under both conditions is not uniform. For example, the deuterium incorporated by Thymosin beta-4 (m/z 4960) in solution is already high during the initial stages of the exchange compared with that on tissue, possibly indicating that in solution it attains a more open conformation than in the latter (Fig. 4A). Meanwhile, for the Ubiquitin-40s ribosomal protein S27a fragment (m/z 8452), the deuterium uptake profile over the time course of exchange is similar both in solution and on tissue, suggesting a similar rate of deuterium uptake in both conditions, although the amount of deuterium incorporated in solution is much higher all throughout (Fig. 4B). On the other hand, for Ubiquitin (m/z 8563) and Cytoplasmic dynein light chain 2 (m/z 10258), the rate of deuterium uptake varies under both conditions, with the proteins attaining maximum incorporation and equilibrium much faster in solution than on tissue (Fig. 4C and 4D). These differences could be attributed to the change in the type of interactions which the proteins are exposed to. In solution, proteins are mainly interacting with -OH coming from water, which stabilizes the conformers formed during local and/or global opening events that occur as the protein

FIG. 2. Tissue deuterium exchange MS (TDXMS) workflow. For tissue exchange, the tissue must first be dried and preincubated in NH₄OAc buffer so that the proteins are in their native form prior to performing the H/D exchange. H/D exchange is performed in a humid chamber in order to avoid drying of the exchange solution and back to exchange. Exchange is then stopped by addition of the matrix solution on the tissue. After matrix crystallization (SA), the tissue section is directly analyzed using MALDI-TOF MS. To examine the uptake across different regions on the same tissue section, the procedure was modified by reducing to 1 µl the volume of the equilibration and exchange buffers and quenching matrix instead.
changes from one state to another (23). In order for deuterium exchange to occur, these intramolecular hydrogen bonds need to be broken and reformed after exchange, in accordance with the Linderstrom-Lang equation (24). Specifically, during these equilibrium fluctuations, the intrinsic rate of amide hydrogen exchange overwhelms the rate of reforming of these intramolecular hydrogen bonds, so that the rate is in fact dictated by the rate of bond opening (assuming that the exchange is governed by the EX1 regime, which is majority of the cases). In the solid state, as demonstrated by Topp and co-workers (25), the protein loses some of this free movement (and consequently the rate of bond opening) not only because of decrease in the water content but also because of the added interaction with the solid matrix. The situation would be

![Fig. 3. A, MALDI-TOF MS spectra of a rat brain extract (Top) versus the direct analysis of the tissue section (Bottom). B, Zoomed MALDI-TOF MS spectra showing the progression of deuterium uptake for the peak at m/z 8563.8 ± 0.1 Da (Ubiquitin) in solution (left) versus on tissue (right) during an extended period of exchange. The exchange was quenched by adding SA matrix acidified with 2% TFA to pH 2.2. The summary of aligned peaks can be found in supplemental Data S1.](image-url)
similar as when the protein is in the solid state, except that this time the proteins will be interacting with components present on the tissue section. Thus, the extent by which the deuterium uptake will be affected will also depend on the nature of the interaction present, and if the interaction is stable or transient.

Spatially Resolved TDXMS—To support this finding, we examined the deuterium uptake of proteins detected across different regions of the rat brain. For this purpose, we used sagittal sections of the brain because distinct regions are easily recognizable from these types of sections. Fig. 5 shows examples of the deuterium uptake behavior of several proteins across three different regions: cerebellum (R1), hippocampus (R2), and midbrain (R3). For m/z 8572 (Fig. 5A) and other ions (mitochondrial ATP synthase epsilon subunit (m/z 5638), Purkinje cell protein 4 (m/z 6653), m/z 8934, and m/z 9986, supplemental Fig. S3), no discernible differences can be observed in the deuterium uptake behavior across the three different regions. However, for m/z 8128 (Fig. 5B), the deuterium uptake and the maximum number of deuterium incorporated once equilibrium is reached are enhanced in the midbrain region (R3). The change in deuterium uptake behavior across different regions is further shown in Fig. 5C and 5D by plotting the natural logarithm of the number of accumulated deuterium, D, against the time of incubation, t, and fitting to equation (1):

\[
D = N - \sum_{i=1}^{N} \exp^{-k_i t}
\]  

(Eq. 1)

where \( N \) is the number of amide linkages and \( k_i \) is the pseudo-first order rate constant of exchange for the given backbone amide linkage (26, 27). It can be observed from these plots that two populations of hydrogens undergo deuterium exchange at different rates designated as “fast” (i.e. exchange occurring in the first minutes of exchange corresponding to rapidly exchanging hydrogens exposed at the protein surface) and “slow” (i.e. exchange occurring after 30 min or more of exchange corresponding to protected hydrogens because of closed conformation and H-bonding interactions) (28, 29). For m/z 8572, the fitted curve of the slow exchanging population across the three different regions show very little deviation from each other. This suggests that for this protein, the conformation does not fluctuate across the different regions. However, for m/z 8128, the deviation is greatly enhanced, especially for the R3 curves. This difference compared with its deuterium uptake

![Graphs showing deuterium uptake plots of proteins in solution (red diamonds) versus on tissue (black squares) for (A) m/z 4960 (Thymosin beta-4), (B) m/z 8452 (Ubiquitin -40S Ribosomal Protein S27a), (C) m/z 8563 (Ubiquitin), and (D) m/z 10258 (Dynein Light Chain 2 Cytoplasmic acetylated in the N-terminal part), respectively. Error bars indicate standard deviation obtained from two measurements.](image)
behavior in the two other regions suggests that this protein has a less restricted conformation with fewer interacting partners (hence higher maximum deuterium incorporation at equilibrium for the slow exchanging population) in the midbrain.

**DISCUSSION**

H/D exchanges can be adapted to fulfill the tissue context and H/D uptake kinetics can be monitored at the level of tissue. Tissue H/D Exchange (TDXMS) experiments demonstrate differences in the deuterium uptake of proteins in their native tissue context with respect to the solution. We further observed differences in the deuterium uptake behavior of certain proteins according to the region of the tissue where the protein is located. The deuterium uptake behavior is, thus, clearly dependent on the extent of the free movement of the protein influenced by the nature of its interacting partners. Different behaviors of deuterium uptake are observed when comparing solution and tissue. For certain proteins (e.g. Ubiq-uitin-40S ribosomal protein S27a at m/z 8452, Fig. 4B or Dynein light chain 2, cytoplasmic at m/z 10,258 Fig. 4D) the fast and slow kinetics are similar on tissue and in solution but the total number of deuterium exchanged is different (lower on tissue) leading to a simple shift of the plots. In other cases, there is a change in the kinetics of the fast exchange (e.g. Thymosin beta-4 at m/z 4960, Fig. 4A) or of the slow exchange (e.g. Myelin basic protein at m/z 8128, Fig. 5D). Fig. 6 provides a schematic representation of the different situations observed. The difference in deuterium uptake of proteins in solution and on tissue attributed to the difference in the number of H-bonded interactors can be observed in the plot (Fig. 6A) as a shift in the maximum number of deuterium that can be accumulated once equilibrium has been achieved. We can envision this difference as the amount of hydrogens protected by H-bonding induced by the presence of interacting partners (Fig. 6B and 6C, top). On the other hand, another variation in deuterium uptake behavior can also be observed in the presence of other interacting partners, which not only could enhance the extent of H-bonding protection, but also induce conformational change (Fig. 6A and 6C, bottom). In this case, the protein microenvironment becomes the major factor in

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**Fig. 5.** Deuterium uptake plots for (A) m/z 8572 and (B) m/z 8128 across 3 different regions of a rat brain tissue, denoted as R1 (cerebellum), R2 (hippocampus) and R3 (midbrain). C and D, show the kinetics of H/D exchange of the two proteins, respectively, with the natural logarithm of the amount of deuterium uptake ln (D) plotted as a function of time, following the pseudo-first order kinetics described in the text. The trace for each plot correspond to “slow” exchange data. For m/z 8572, R1: ln (D) = 0.0038t + 3.5 (r² = 0.91); R2: ln (D) = 0.0038t + 3.5 (r² = 0.90); and R3: ln (D) = 0.0026t + 3.6 (r² = 0.88). For m/z 8128, R1: ln (D) = 0.0033t + 3.2 (r² = 1.0); R2: ln (D) = 0.0033t + 3.2 (r² = 0.99); and R3: ln (D) = 0.0019t + 3.5 (r² = 0.94).
evaluating deuteration uptake evolution and consequently the protein structure. It should be pointed out that with this approach, only the deuteration uptake of abundant proteins was monitored as only they provide traceable signals discernible in MALDI.

To summarize, the study showed for the first time the possibility to perform H/D exchange directly at the tissue level. The current evidence thus provides an alternative way to look at protein structure as it occurs in response to variations in its microenvironment. The data showed differences in H/D exchange behavior for the same proteins in solution and on tissue and should be expected to provide a more approximate interpretation of protein structure as it occurs in situ. We also observed differences for the same proteins in different localizations of the tissue. The variations found are not only lying in the global amount of deuterium uptake across the different proteins, but also in the rates observed, which can be interpreted as both a change in the interacting partner environment as well as conformational change. In perspective, the next step will be to carry out local exchange analysis to determine the exact regions in the proteins affected by these interactions. To do so we will have to separate the components using short gradients and initially generate a database of tandem MS spectra from the undeuterated sample and use this to identify the proteins. This would be achieved by aligning the chromatographic traces and matching the undeuterated protein with the deuterated one using the retention times of their peptides. Another possible interesting perspective is the further development of a workflow for MALDI MS imaging of protein H/D exchanges across an entire tissue in order to provide understanding of the variation of conformation or interaction within different areas of a tissue.

* This research was supported by grants from Ministère de L'Education Nationale, de L'Enseignement Supérieur et de la Recherche (MENESR), Institut National de la Santé et de la Recherche Médicale (INSERM) and Université de Lille.

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The authors declare no competing financial interests in this work.

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