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Measuring the Hydrogen/Deuterium Exchange of Proteins at High Spatial Resolution by Mass Spectrometry: Overcoming Gas-Phase Hydrogen/Deuterium Scrambling

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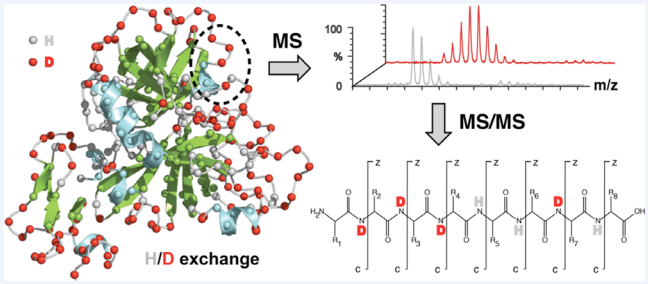
CONSPECTUS: Proteins are dynamic molecules that exhibit conformational flexibility to function properly. Well-known examples of this are allosteric regulation of protein activity and ligand-induced conformational changes in protein receptors. Detailed knowledge of the conformational properties of proteins is therefore pertinent to both basic and applied research, including drug development, since the majority of drugs target protein receptors and a growing number of drugs introduced to the market are therapeutic peptides or proteins. X-ray crystallography provides a static picture at atomic resolution of the lowest-energy structure of the native ensemble. There is a growing need for sensitive analytical tools to explore all of the significant molecular structures in the conformational landscape of proteins. Hydrogen/deuterium exchange monitored by mass spectrometry (HDX-MS) has recently emerged as a powerful method for characterizing protein conformational dynamics. The basis of this method is the fact that backbone amides in stable hydrogen-bonded structures (e.g., α-helices and β-sheets) are protected against exchange with the aqueous solvent. All protein structures are dynamic, however, and eventually all of the protecting hydrogen bonds will transiently break as the protein—according to thermodynamic principles—cycles through partially unfolded states that correspond to excited free energy levels. As a result, all of the backbone amides will eventually become temporarily solvent-exposed and exchange-competent over time. Consequently, a folded protein in D_2O will gradually incorporate deuterium into its backbone amides, and the kinetics of the process can be readily monitored by mass spectrometry. The deuterium uptake kinetics for the intact protein (global exchange kinetics) represents the sum of the exchange kinetics for the individual backbone amides. Local exchange kinetics is typically achieved by using pepsin digestion under quench conditions (i.e., under cold acidic conditions where the amide hydrogen exchange rate is slowed by many orders of magnitude). The ability to localize the individual deuterated residues (the spatial resolution) is determined by the size (typically ~7−15 residues) and the number of peptic peptides. These peptides provide a relatively coarse-grained picture of the protein dynamics. A fundamental understanding of the relationship between protein function/dysfunction and conformational dynamics requires in many cases higher resolution and ultimately single-residue resolution.



In this Account, we summarize our efforts to achieve single-residue deuterium levels in proteins by electron-based or laser-induced gas-phase fragmentation methods. A crucial analytical requirement for this approach is that the pattern of deuterium labeling from solution is retained in the gas-phase fragment ions. It is therefore essential to control and minimize any occurrence of gas-phase randomization of the solution deuterium label (H/D scrambling) during the MS experiment. For this purpose, we have developed model peptide probes to accurately measure the onset and extent of H/D scrambling. Our analytical procedures to control the occurrence of H/D scrambling are detailed along with the physical parameters that induce it during MS analysis. In light of the growing use of gas-phase dissociation experiments to measure the HDX of proteins in order to obtain a detailed characterization and understanding of the dynamic conformations and interactions of proteins at the molecular level, we discuss the perspectives and challenges of future high-resolution HDX-MS methodology.

1. INTRODUCTION

Protein function and dysfunction are intimately connected to the dynamic properties of the protein structure. For example, many cellular processes are triggered by ligand-induced changes in the conformational dynamics of receptors.[1] Hydrogen/deuterium exchange (HDX), which is typically monitored by either NMR spectroscopy or mass spectrometry (MS), has become an important method for characterizing protein conformational dynamics. Until recently, the major advantage of HDX-NMR was that this

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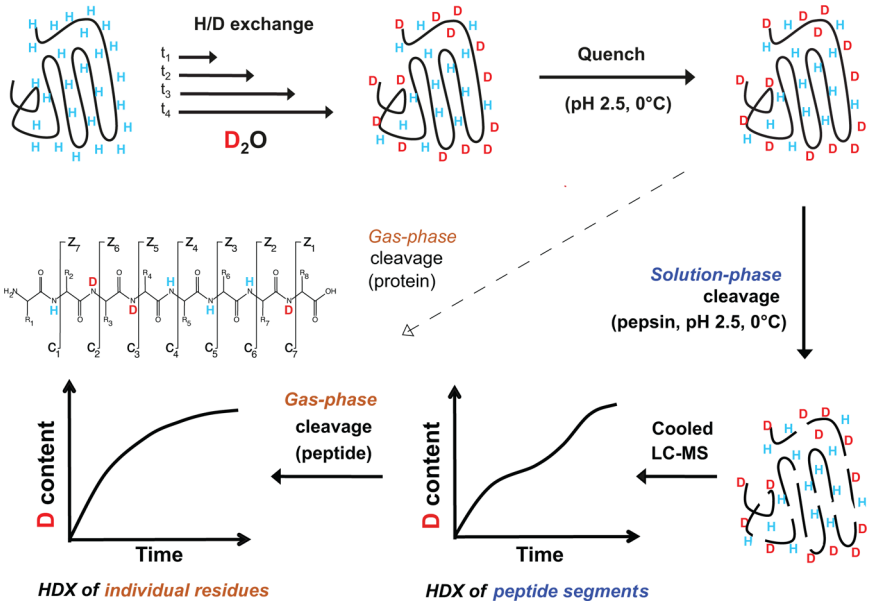

Figure 1. HDX-MS/MS workflow using gas-phase backbone cleavage to determine deuterium uptake for individual residues. HDX, quenching, and solution-phase cleavage with proteases working at low pH is analogous to the classical bottom-up workflow. In addition, the HDX kinetics of individual residues is determined by ETD or ECD of the proteolytic peptides by LC−MS (bottom-up approach). Alternatively, the intact protein can be directly fragmented, typically in direct infusion experiments on high-resolution instruments (top-down approach).

technique can provide exchange rates of individual backbone amides,[2] while HDX-MS requires much lower sample quantities, can cope with larger (>40 kDa) proteins and more complex sample mixtures, and provides the ability to resolve coexisting populations of a given protein if they exchange differently.[3,4] As outlined above, the traditional HDX-MS approach based on proteolytic digestion yields a coarse-grained picture of protein structural dynamics.[5,6] However, high spatial resolution is required in order to pinpoint key individual residues that, for example, mediate the allosteric function of a protein or the intermolecular interactions with other proteins or ligands. Another example arises when comparing members of the same protein family. The latter proteins have similarity in their sequences and similar 3D structures, yet their dynamical properties can differ vastly. Since differences in protein sequence typically change the pepsin cleavage pattern significantly, a direct comparison of the HDX kinetics for members of the same protein family is extremely difficult at the peptide-resolution level. Increased spatial resolution is required in order to address this limitation. This can be achieved either by fragmentation of the peptic peptides or proteins in the gas phase using mass spectrometry[7] or by further digestion in solution with another protease.[8,9] Our recent developments in using gas-phase dissociation as an experimental tool to increase the spatial resolution in bottom-up and top-down HDX-MS experiments (i.e., HDX-MS/MS) are outlined in Figure 1. We have over the last years developed sensitive model peptide systems for detecting hydrogen/deuterium (H/D) scrambling and made use of such systems to demonstrate that prompt fragmentation techniques including electron-capture dissociation (ECD), electron-transfer dissociation (ETD), and matrix-assisted laser desorption/ionization in-source decay (MALDI ISD) dissociate peptide or protein ions without inducing H/D scrambling.

2. DEVELOPMENT OF MODEL PEPTIDES FOR QUANTITATING GAS-PHASE H/D SCRAMBLING

H/D scrambling in the gaseous peptide or protein ion is the main issue that has to be addressed in order to utilize gas-phase dissociation to increase the spatial resolution in HDX-MS experiments. H/D scrambling is the result of multiple reversible proton/deuteron transfers among the labile sites in the peptide (i.e., all N-, S-, and O-linked hydrogen/deuterium atoms). Extensive H/D scrambling causes full positional randomization among these sites. Consequently, if scrambling is prevalent, it will erase the solution labeling pattern, and the deuterium distribution derived from gas-phase fragment ions will not correctly reflect the labeling in solution. It is therefore of pivotal importance to keep the scrambling at negligible levels in HDX-MS/MS experiments.

To detect and quantitate the occurrence of H/D scrambling during an MS experiment, it is critical that one uses a polypeptide model system with well-characterized and selective deuterium labeling in solution. The labeling pattern should be as different as possible from the random distribution of deuterium across all labile sites, which is the state obtained after complete H/D scrambling (Figure 2).

The level of H/D scrambling that such a model system has undergone before or upon peptide fragmentation can then be quantitated by comparison of the measured H/D distribution to the known solution labeling pattern (0% scrambling) and the calculable fully random distribution (100% scrambling). Measurement of the deuterium levels in fragment ions in an MS/MS experiment will be associated with an inherent experimental error, typically with a standard deviation in the range of 0.01−0.2 D, depending on sample preparation, model system, instrument type, and the abundance of the fragment ion. Thus, the greater the difference in deuterium contents of fragment ions between the limiting cases of 0% and 100% scrambling, the more sensitive and accurate the model system will be for the purpose of quantifying H/D scrambling. On the basis of these considerations,

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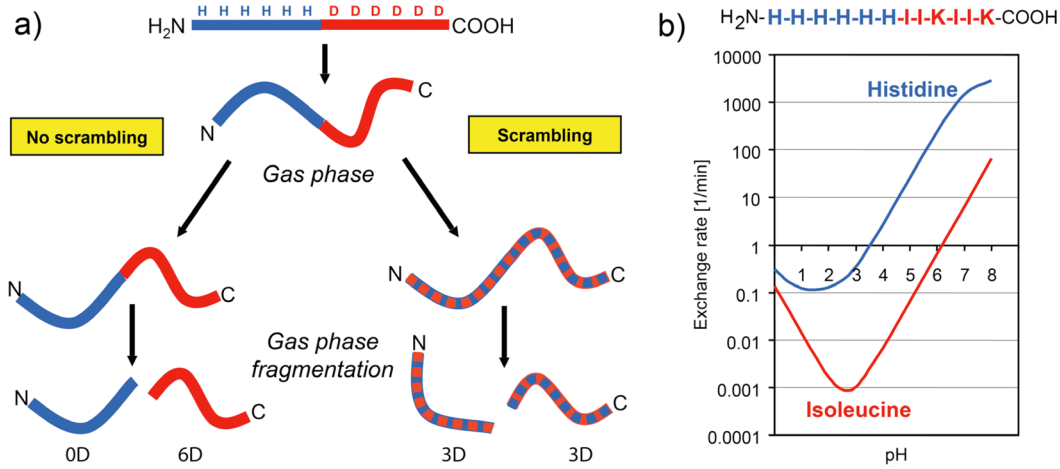

Figure 2. Principle of measuring H/D scrambling during gas-phase fragmentation using selectively labeled model peptides. (a) For sensitive detection of scrambling, the deuterium labeling pattern in solution must be known and should be as different as possible from the fully random distribution over all labile sites obtained upon complete gas-phase scrambling. By measuring the deuterium content of product ions after gas-phase fragmentation, one can detect and quantitate the level of H/D scrambling. (b) Selective deuterium labeling in solution can be achieved by exploiting primary structure effects on the intrinsic amide exchange rate (His vs Ile side chains).

we have developed a range of simple model peptides with unique regioselective deuterium labeling for sensitive detection of the occurrence of H/D scrambling. These peptides can be prepared in a manner that ensures that deuterium is retained only at backbone amides of the C-terminal half of the peptide prior to MS analysis (Figure 2). This was achieved by decreasing the exchange rates of amides of the C-terminal half either by higher-order structure effects in our first-generation model peptides[10,11] or by primary structure effects in the model peptide series of the second generation.[12−14]

In detail, the first-generation model peptide is a construct of an urokinase-type plasminogen activator receptor (uPAR)-binding sequence in the C-terminal half and a nonbinding sequence in the N-terminal half.[11] In the high-affinity complex between the peptide and the receptor, the C-terminal half of the peptide adopts a stable α-helical structure, while the nonbinding N-terminal half is solvent exposed. When D-to-H isotopic exchange is initiated by diluting a fully deuterated peptide−uPAR complex into protiated buffer, the stable α-helix strongly protects the amides in the C-terminal half against exchange. The unprotected N-terminal half, on the other hand, rapidly exchanges with ^1H_2O buffer, and after a few seconds of incubation at neutral pH, only the amides in the C-terminal half are deuterated. Isotopic exchange is quenched by acidification (pH 2.5), which also dissociates the uPAR−peptide complex and makes the selectively labeled peptide available for mass spectrometry experiments. The drawbacks of this peptide model system are that the sample preparation protocol is not trivial[10,11] and that recombinant uPAR is rather expensive (~$2200/mg).

In contrast, the primary analytical advantage of the second-generation peptides, which rely exclusively on primary structure effects to achieve regioselective labeling, is that these can be infused continuously into the ion source in an electrospray ionization (ESI)-compatible solvent while retaining their selective labeling. The first such model peptide (P1) consisted of the sequence HHHHHHIIKIIK[12] and exploited the fact that the presence of an Ile side chain adjacent to an amide hydrogen reduces the intrinsic exchange rate of that amide by over 2 orders of magnitude relative to a His residue in the same position (Figure 2b). Thus, this peptide has a slow-exchanging C-terminal half and a faster-exchanging N-terminal half. Additionally, two lysines were added to facilitate higher charge states of the peptide upon ESI and aid solubility. Prolonged selective labeling of this peptide was achieved by the use of an ESI solvent of low pH containing 50% methanol, which in addition to the pH effect also enabled supercooling of the spray solution to subzero temperatures (below −20 °C) on dry ice to further reduce the solution HDX rates. This concept of supercooling in mixed aqueous/organic solvents to reduce back-exchange (i.e., unintended loss of deuterium) was later used in top-down and bottom-up HDX-MS applications.[15,16] On the basis of an earlier systematic study by Bai et al.,[17] the intrinsic exchange rates for amides in unstructured peptides can be predicted. Interestingly, we found that while the slow exchange of amides in the C-terminal half was in good accordance with expected primary structure effects, the amides of the His-containing N-terminal half exchanged considerably faster than predicted under the conditions used for direct infusion.[12,13] Thus, P1 could be continually infused for up to 60 min as a selectively labeled species, enabling the monitoring of H/D scrambling in real time during MS/MS analysis while tuning the instrument parameters. Building on the sequence of model peptide P1, we designed a family of peptides to investigate the effect of primary structure on the scrambling process itself.[14] To test the influence of the Lys in P1, this residue was substituted by Thr (P2) or Ile (P3). P2 and P3 showed more uniform labeling of the slow-exchanging C-terminal peptide half than P1, and P3 in particular retained more deuterium. Peptide P4 contained a segment of six Pro residues between the fast- and slow-exchanging halves of P1 (i.e., HHHHHHPPPPPPIIKIIK). In two other peptides, the fast-exchanging six-His repeat was replaced by fast-exchanging Asp-rich sequences (P5, KKDDDDDDIIKIIK; P6, RRDDDDDDIITIITR). Overall, all six peptides performed similarly in terms of regioselective labeling and detection of H/D scrambling.

For the use of selectively labeled model peptides, it is important to ensure that the labeling is fully controlled and free of artifacts. For instance, the carbon-bound hydrogen at position C-2 in the imidazole ring of the histidine side chain will exchange with deuterium upon incubation for prolonged periods of time at elevated temperatures and neutral pH.[18,19] Such undesired side-chain labeling of His residues in model peptide P1 would pretend the occurrence of H/D scrambling as a result of the presence of

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deuterium in the N-terminal half and should therefore be prevented by avoiding the above-mentioned labeling conditions.

Our second-generation model peptides have the advantage that the level of scrambling can be judged and even quantified in a relatively easy and fast way. They are, however, not fully representative of all peptides generated in a bottom-up HDX-MS/MS workflow or of intact proteins analyzed by top-down HDX-MS/MS approaches. For the comprehensive validation of such studies, proteins with well-studied solution-phase HDX behavior (i.e., with high-quality HDX-NMR reference data) are needed. Ideally, such proteins should be commercially available, easy to handle and label, and rather small in order to avoid making the data interpretation too time-consuming. Examples include β2-microglobulin, ubiquitin, and CRABP-1, as described later in this Account.

More recently, we introduced a universal test for H/D scrambling in actual peptic peptides subjected to ETD during a bottom-up HDX-MS/MS-type workflow.[20] Advantageously, this test is completely independent of the protein under study and exploits the loss of ammonia from the N-terminal amino group, which is a common feature in ETD spectra of peptides. As the N-terminal amino group of peptic peptides cannot retain deuterium because of fast back-exchange in solution, the loss of ammonia from deuterium-labeled peptides resulting from a HDX-MS experiment should not deplete the deuterium content of the peptide unless scrambling has occurred.[20] Thus, by a simple comparison of the deuterium contents of the intact peptide and its deammoniated counterpart, one can verify the absence of H/D scrambling in fragment ions of the individual peptic peptides by inspection of the actual ETD spectra used to obtain site-specific HDX information. This universal test for the occurrence of H/D scrambling in bottom-up HDX-MS/MS experiments is not as sensitive as measurements using c/z-type fragment ions of the model peptides designed for this purpose. However, depending on the deuterium content of the peptide, the masses of the deammoniated peptide ions in the non- and fully scrambled cases typically differ by about 1 Da. The approach is thus highly suitable to universally verify the absence of scrambling in the actual peptides analyzed during any given HDX-ETD/ECD experiment instead of relying on a model system as a single benchmark for the propensity of a multiplicity of peptide sequences and ion species to undergo H/D scrambling. The practical utility of this built-in test for scrambling has been further demonstrated in more recent HDX-MS studies employing ETD.[21,22]

3. ION ACTIVATION METHODS AND SCRAMBLING

3.1. Collision-Induced Dissociation

Collision-induced dissociation (CID) is the most common method to fragment peptides and proteins in commercial mass spectrometers, for instance to identify proteins in proteomic studies. The use of CID to fragment deuterated peptides and proteins in the attempt to obtain information on site-specific HDX in solution is based on the assumption that the level of scrambling of backbone amide hydrogens is negligible upon collisional activation. However, this assumption is in general not true, especially in the case of protonated peptides and proteins.

The "scrambling controversy" was initiated by Deng et al.[23] and Kim et al.,[24] who reported that b-type fragment ions from CID of protonated peptides derived from pepsin digestion of deuterated proteins yielded site-specific deuterium levels that correlated qualitatively with amide hydrogen exchange rates determined by NMR spectroscopy. Their findings suggested that the level of H/D scrambling for the formation of b-type fragment ions was negligible and that CID could be used to increase the spatial resolution in HDX-MS experiments. Paradoxically, the reported level of H/D scrambling for y-type fragment ions was extensive. Such a difference between b- and y-type fragment ions requires a priori that they originate from different precursor ion populations. That is, b-type fragment ions should be formed from a nonscrambled population of precursor ions while y-type fragment ions should originate from a scrambled population. Such a scenario is difficult to reconcile with the fact that fragmentation of multiply protonated peptide ions typically yields several complementary b- and y-type fragment ion pairs, where each pair originates from the same peptide molecule by a single covalent bond cleavage. While an exhaustive review is beyond the scope of the present Account, several earlier as well as later studies have demonstrated extensive H/D scrambling upon collisional activation of protonated peptides and proteins for both b and y ions.[10−12,25−30] All of these observations of prevalent H/D scrambling are well in line with the current theoretical framework for understanding CID-type fragmentation of protonated peptides, which is based on the "mobile proton" model.[31,32] The first step in the charge-directed fragmentation mechanism is an endothermic intramolecular proton transfer facilitated by internal energy uptake through energetic collisions, causing the proton to move from a basic site (e.g., an amino group) to a less basic backbone amide (Figure 3). Protonation of the amide

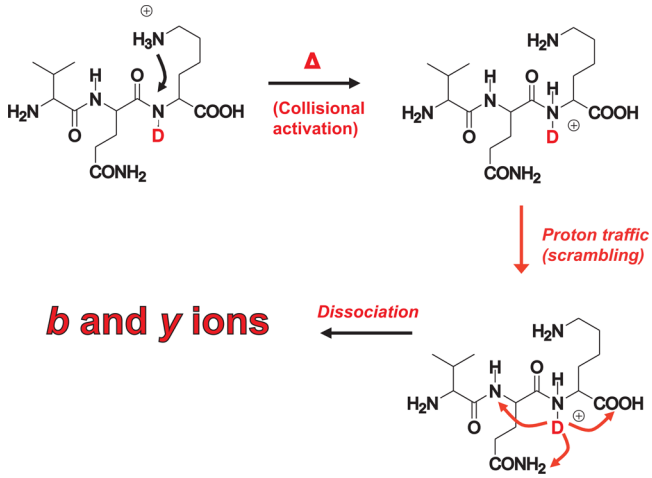

Figure 3. Proton mobility in peptides during CID. In low-energy CID, activation of the precursor ion occurs through multiple energetic collisions on a relatively slow (>μs) time scale. This induces extensive proton trafficking across all of the available protonation sites and thus randomization (scrambling) of all of the heteroatom-bound hydrogens in the peptide.

nitrogen weakens the peptide bond, and subsequent cleavage occurs via the formation of a cyclic structure of the N-terminal fragment (e.g., an oxazolone or diketopiperazine derivative).[31] Since the protonation of the backbone amide should be reversible in nature, this mechanism also nicely explains the displacement and migration of the deuterium labels on amide sites. Consequently, the energy landscape for peptide fragmentation predicts the occurrence of scrambling through multiple reversible proton transfers among backbone amide nitrogens and other protonation sites prior to peptide bond cleavage. Experimentally, we have measured the level of H/D scrambling among all exchangeable hydrogens to be ~100% for CID of protonated peptides utilizing selectively deuterium-labeled

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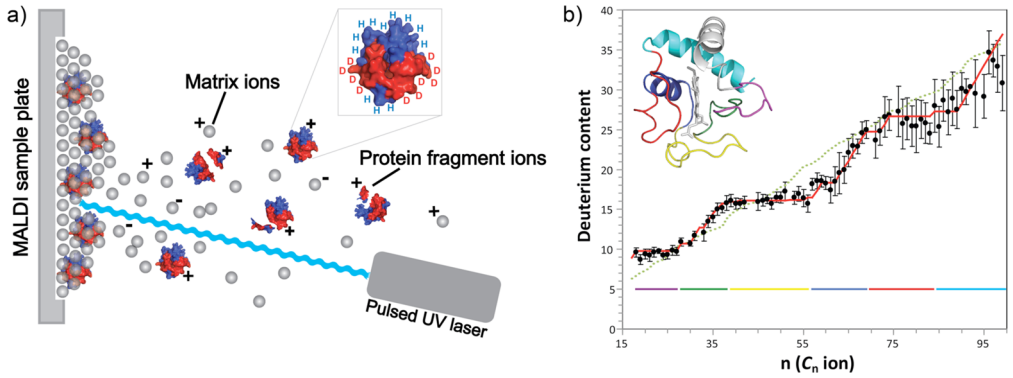

Figure 4. MALDI ISD enables in-source fragmentation of proteins without H/D scrambling. (a) Hydrogen radical transfer reactions from the matrix to the analyte upon laser irradiation induce prompt cleavage of N−C_α bonds, yielding c- and z-type fragment ions that retain the labeling pattern from solution. (b) MALDI-ISD analysis of the HDX of cytochrome *c* reveals accurate information on the conformation and dynamics of the protein in solution. Distinct regions for which site-specific HDX information was obtained are colored onto a structural representation of cytochrome *c* and indicated by a line in matching colors for corresponding c ions. Adapted from ref 42. Copyright 2011 American Chemical Society.

peptides described earlier in this Account.[10−12,20] Interestingly, we have found that H/D scrambling is also prevalent in negative ion mode for CID of deprotonated peptides.[33] In this case, a "mobile deprotonation site" causes extensive internal rearrangement of deuterons initially located on the backbone amides. With the present knowledge of the prevalence of scrambling in protonated peptides and proteins, there is now a consensus that CID should *not* be used as an experimental tool to obtain information on the local deuterium uptake in solution.

3.2. MALDI In-Source Decay

MALDI ISD is used as a top-down fragmentation approach to obtain sequence information, typically for purified proteins.[34,35] Fragmentation of proteins by MALDI ISD occurs when the laser fluence is somewhat above the threshold for production of protonated analyte ions (Figure 4a). Mechanistic studies suggest that backbone cleavage by MALDI ISD is triggered by a hydrogen radical transfer from an electronically excited matrix molecule to a carbonyl oxygen in the backbone.[35−37] Recent results suggest that this step occurs very early in the MALDI process, namely, in the matrix crystal during dissipation of the energy from the laser pulse and before desorption of the ions.[38] Subsequently, but still on a nanosecond time scale after the laser pulse, the N−C_α bond undergoes radical-induced cleavage, yielding c- and z-type fragment ions. Interestingly, phosphorylated residues in protonated peptides retain their phosphate group after backbone cleavage by MALDI ISD,[39] suggesting either that the fragmentation process is nonergodic or that the dissociation threshold for the radical-induced backbone cleavage is substantially lower than the threshold for the loss of the modification. For both scenarios, we speculated that the proton mobility in the precursor ion was likely to be low. This prompted us to investigate the occurrence of H/D scrambling in MALDI ISD of selectively labeled model peptides. The deuterium content of the gaseous c ions closely mimicked the known solution deuteration pattern of the model peptides.[40] Consequently, the level of scrambling in MALDI ISD of the peptides was low. However, it should be noted that the gas-phase data indicated the occurrence of an intraresidue H/D migration between the backbone amide of a lysine residue and its side-chain ε-amino group, but there were no indications of any inter-residue H/D migration, so the effect on the overall solution labeling pattern was negligible. The MALDI ISD process using 2,5-dihydroxybenzoic acid (DHB) caused an unexpected deuterium loss for the z ions, as their deuterium content was ∼20% lower than the value expected on the basis of the D content of the precursor ion. This loss occurs presumably through those ion−molecule reactions in the dense MALDI plume that are also responsible for the formation of the abundant even-electron z′ ions via hydrogen radical transfer to $z^\bullet$ ions.[41] Consequently, the c ions are the preferred fragment ions for obtaining site-specific deuterium levels by MALDI ISD.

As a proof of concept, we used MALDI ISD to probe the solution structural dynamics of equine cytochrome *c* by native-state D-to-H exchange (Figure 4b).[42] For small proteins like cytochrome *c* (104 residues), MALDI ISD yields a comprehensive series of c ions that allows for a detailed picture of the backbone dynamics. The deuterium content of the c-ion series matched closely the known solution dynamics of cytochrome *c*, with low deuterium levels for highly dynamic regions such as an Ω loop at positions 40−57 and much higher retention of deuterium for very stable structures such as the adjacent 60s α-helix (Figure 4b). These measurements were conducted in linear time-of-flight (TOF) mode, where the higher laser fluence used to induce fragmentation decreases the mass accuracy, in particular for the larger fragment ions.[42] As a result, the deuterium uptake profile of the N-terminal region of cytochrome *c* was more accurately determined than that of the C-terminal region. A higher mass accuracy for MALDI ISD fragments is achievable by using a reflectron[43] or instruments that otherwise compensate for the kinetic energy dispersion of the MALDI ISD fragment ions.

From the rather limited number of studies on this topic,[40,42,44] one can conclude that MALDI ISD is not prone to hydrogen scrambling and seems to provide valid data on local hydrogen exchange kinetics. This technique is thus an alternative to ECD and ETD, particularly for the top-down HDX-MS/MS approach, although the difficulties of achieving high-quality data [i.e., good mass accuracy and sufficient signal-to-noise (S/N) ratio for a larger number of fragment ions] are somewhat limiting in practice.

3.3. Electron-Capture Dissociation and Electron-Transfer Dissociation

In electron-based dissociation (ExD), multiply positively charged ions are reacted with low-energy electrons, which triggers cleavage of covalent bonds. The difference between ECD and ETD is the source of the electrons. For ECD, which is nearly

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exclusively performed on Fourier transform ion cyclotron resonance (FTICR) instruments, the cations are reacted with free, near-thermal electrons,[45,46] while for ETD, which is implemented on the more widespread ion trap and Q-TOF instruments, the electron is transferred from a radical molecular anion to the positively charged analyte ion.[47] If applied to multiply protonated peptides or proteins, hydrogen-abundant radical cations are formed that undergo decomposition to c- and z-type fragment ions (Figure 5).

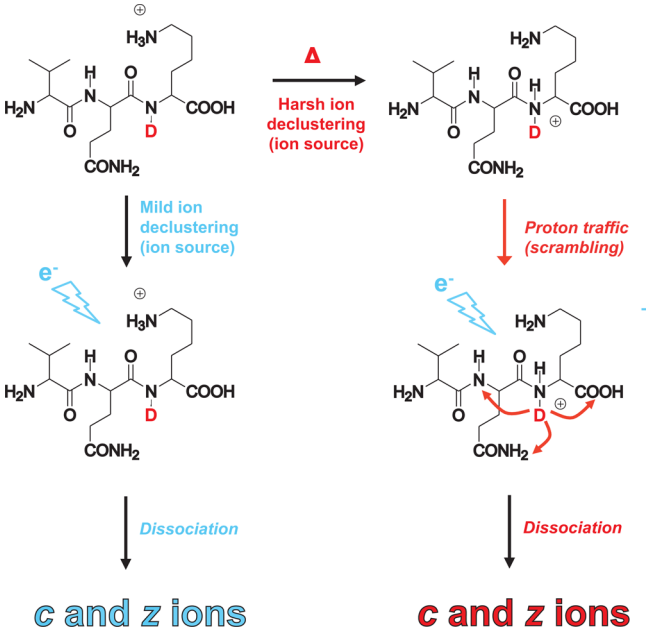

Figure 5. ExD facilitates peptide fragmentation without scrambling, provided that the precursor ion is not excessively heated. In ECD and ETD, receiving an electron leads to prompt fragmentation on a picosecond time scale without intramolecular proton traffic. However, proton traffic can be induced prior to receiving the electron if harsh ion declustering or ion transport conditions are used in the higher-pressure regions of the front end of the mass spectrometer.

The exact mechanism of the peptide backbone N−Cα bond cleavage upon ExD is still a matter of some debate, but it is evident that these fragmentation techniques are gentle enough to facilitate peptide backbone fragmentation while retaining post-translational modifications that are very labile under CID conditions.[46,48] The idea that these fast, electron-based dissociation techniques might overcome the H/D scrambling problem associated with slow heating techniques such as CID was already conceived in the very early days of their development. Along with the first top-down ECD spectra of multiply protonated proteins published in 1998, Zubarev et al.[45] briefly reported data from gas-phase HDX experiments with equine cytochrome *c* indicating that ECD proceeds faster than H/D scrambling. Despite this encouraging result, only two studies further investigating ECD as a tool to localize labile deuterium in peptide or protein ions appeared in the next 10 years.[49,50] However, the results obtained from these early studies were not conclusive with respect to the occurrence of H/D scrambling.

Employing our second-generation peptide probes, we were able to clearly demonstrate that both ECD[51] and ETD[14] generate fragment ions from multiply protonated peptides without distorting their specific labeling from solution. Therefore, localization of solution-phase deuterium labels to single residues was achieved for large parts of the model peptides. Although we did not observe the induction of H/D scrambling by ExD itself, we realized that careful optimization of the ion source and ion transfer parameters is required in order to avoid H/D scrambling by excessive vibrational excitation of the peptide ions *before* the MS/MS step (Figure 5).[51] We found that the extent of ion activation upon desolvation and transfer depends on the ion source design, and consequently, the instrument parameters that control the internal energy uptake in this stage must be individually optimized for each instrument type to find the best compromise between the lowest possible H/D scrambling and still acceptable ion transmission efficiency (Figure 6). Our model peptides are ideally suited for this parameter optimization step during method development, as they provide particularly high sensitivity in the detection of H/D scrambling and can be stably applied over longer time intervals (up to 1 h) via direct infusion. The parameter set obtained with model peptide P1 was also found to give negligible scrambling levels for all of the other second-generation peptide probes (P2−P6), indicating the generality of our finding that ETD proceeds without significant hydrogen scrambling.[14]

Our ECD results on model peptide P1,[51] published in 2008, were confirmed and extended to the top-down case in the same year by Pan et al.,[52] who provided convincing data that localization of the solution-phase deuterium labels by ECD is also feasible for small, intact proteins. For that, they compared the results obtained for the model protein ubiquitin to HDX-NMR reference data and found very good agreement. In a proof-of-principle study in 2009, we employed β2-microglobulin, a 99-residue amyloidogenic protein, to demonstrate the feasibility of a bottom-up HDX-MS/MS workflow on an ETD-capable ion trap instrument to achieve single-residue resolution (Figure 7).[7].

Also in 2009, Abzalimov et al.[53] reported top-down ETD experiments with a His-tagged model protein that strongly indicated the absence of H/D scrambling. These proof-of-principle studies, which established ExD as an experimental tool to approach the desired single-residue resolution in HDX-MS experiments, have laid the groundwork for a number of recent studies that have demonstrated the ability of such "high-resolution" HDX-MS/MS experiments to provide detailed insights into the dynamic conformation and interactions of both native and non-native protein states in solution.[15,21,22,54−63]

4. FUTURE DEVELOPMENTS IN BOTTOM-UP AND TOP-DOWN HDX-MS/MS METHODOLOGY

The main analytical challenge of identifying methods and conditions for preventing the occurrence of H/D scrambling during gas-phase ion fragmentation has been overcome, greatly aided by the use of well-defined model peptides and proteins as described in this Account. Slow-heating fragmentation techniques, such as CID, have been shown to give rise to extensive H/D scrambling in model peptides and proteins,[11,12,26,27,30,64] which can be fully reconciled within the framework of the mobile proton model for CID of protonated peptides. Prompt fragmentation methods such as MALDI ISD, ETD, and ECD, on the other hand, provide an attractive option for increasing the spatial resolution of the HDX-MS experiment. However, the implementation of these techniques into the HDX-MS workflow still presents significant challenges.

Let us first consider sensitivity. To determine accurate site-specific deuteration levels in HDX-MS/MS experiments, high-quality spectra with good fragment ion statistics (i.e., high S/N ratio) are required. The intensities of the peptides (i.e., precursor

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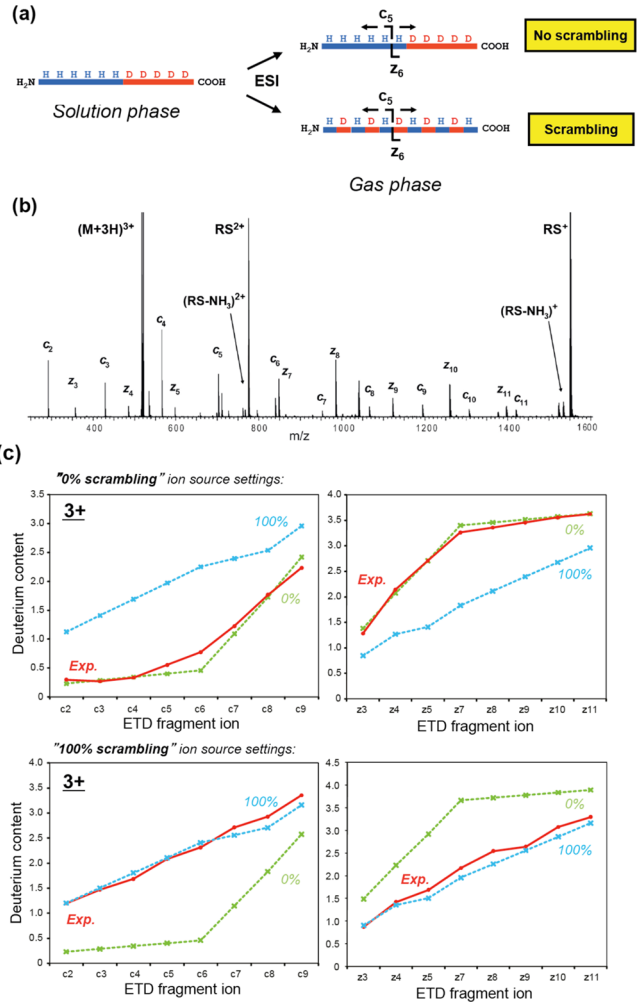

Figure 6. Principle of measuring H/D scrambling by ExD of selectively labeled model peptides. (a) For sensitive detection of scrambling, the labeling pattern in solution must be known and should be as different as possible from the fully random distribution over all labile sites obtained upon complete gas-phase scrambling. (b) During ExD analysis of the multiply protonated peptide ions (shown is the ETD spectrum of triply protonated peptide P1 obtained on a high-resolution Q-TOF instrument), the labeling pattern remains unaffected and is reflected in the deuterium content of the c- and z-type fragment ions. (c) Since the solution-phase labeling pattern of the model peptides is known, the theoretical deuterium content in the limiting case of no (0%) scrambling can be easily calculated for each individual fragment ion, as can the values for full (100%) scrambling. By comparison of the measured deuterium content of the ExD fragment ions with these two theoretical values, the level of scrambling that the peptide ion has undergone before ExD can be accurately quantified. Adapted with permission from ref 57. Copyright 2011 American Society for Mass Spectrometry.

ions) from a peptic digest differ vastly as a result of differences in their ESI efficiencies and the fact that the peptides are not present in stoichiometrically equal amounts because pepsin has broad specificity. Furthermore, deuteration in high-percentage D_2O decreases the S/N ratio by dispersing the signal intensity over a broader isotopic distribution. Therefore, it can be challenging to obtain an HDX-MS/MS spectrum of sufficient quality in a single chromatographic run for a given peptic peptide. One possibility to increase the sensitivity of HDX-MS/MS experiments is to improve the typically rather low fragmentation efficiency of ETD and ECD, in particular for ions with high m/z values.[22] Recently developed strategies to overcome this challenge, such as pre-, concomitant, or postactivation of the peptide/protein ions to disrupt gas-phase secondary or tertiary structures and to dissociate non-covalently bound c/z-fragment ion pairs,[22,65] should be further tested for their applicability in top-down and bottom-up HDX-MS/MS workflows, with particular emphasis on the occurrence of H/D scrambling. Additionally, novel MS/MS fragmentation methods should also be explored for the same purpose.

While the in-source decay and desorption/ionization of ions occur nearly simultaneously in MALDI ISD, leaving no time for H/D scrambling to take place before the fragmentation, ETD and ECD are fully decoupled from the generation of ions in time and space. This means that H/D scrambling may be induced by excessive vibrational excitation of the ions before the dissociation event. Since the current instruments are typically not designed to keep the ions at particularly low internal energies along the whole pathway through the ion optics, the sensitivity can be significantly compromised upon tuning of the ion source and ion transfer parameters for low H/D scrambling. The data obtained to date indicate that declustering of the ions through collisions with neutral gas molecules in the intermediate-pressure region is the most critical step. The development of novel ion sources that enable efficient declustering and ion transmission while maintaining minimal H/D scrambling would be highly desirable for HDX-MS/MS experiments. The design and adaptation of new MS instruments with a focus on the requirements of HDX-MS/MS might become similarly important in the evolution of this technique as it was in the case of, for example, native mass spectrometry.[66]

Next, let us consider size. To date, the majority of HDX-MS/MS studies have been conducted on relatively small proteins in the size range that is also covered by HDX-NMR. However, we expect that HDX-MS/MS will increasingly be used to provide data at single-residue resolution for larger and more complex proteins as well. The top-down approach has recently been shown to have several advantages, such as the ability to disentangle the HDX and dynamics of coexisting conformers by selecting individual conformers (i.e., narrow parts of the whole isotopic distribution) for gas-phase fragmentation,[55,62] and it will certainly yield very interesting biological data on peptides and small proteins. However, despite the continuous technical improvements, top-down HDX-MS/MS measurements on larger proteins (>50 kDa) will most likely be compromised by the size effect, where the proportion of inter-residue cleavages decreases with protein size. This translates into limited spatial resolution, especially for the region in the middle of the protein. Furthermore, covalent protein modifications, including cysteine bonds and glycosylations, pose a particular challenge to top-down analysis. The bottom-up HDX-MS/MS approach is applicable to such large and complex protein systems. Perhaps most realistically in a targeted approach as shown recently,[22] where only a few peptides are selected for ExD in order to extract detailed information about the HDX of protein regions of particular interest, while resolving the HDX of the rest of the protein to the level of peptide segments. The main challenge of this strategy is to obtain LC−ExD−MS spectra of sufficiently high quality during the limited acquisition time and under the constraints on the LC separation due to the necessity to limit back-exchange. An interesting alternative, namely, skipping the LC separation in the bottom-up approach and instead relying on the high resolving power of an FTICR-MS instrument, has recently been demonstrated.[63] In this approach, the peptide mixture is directly infused into the MS, which allows longer acquisition times

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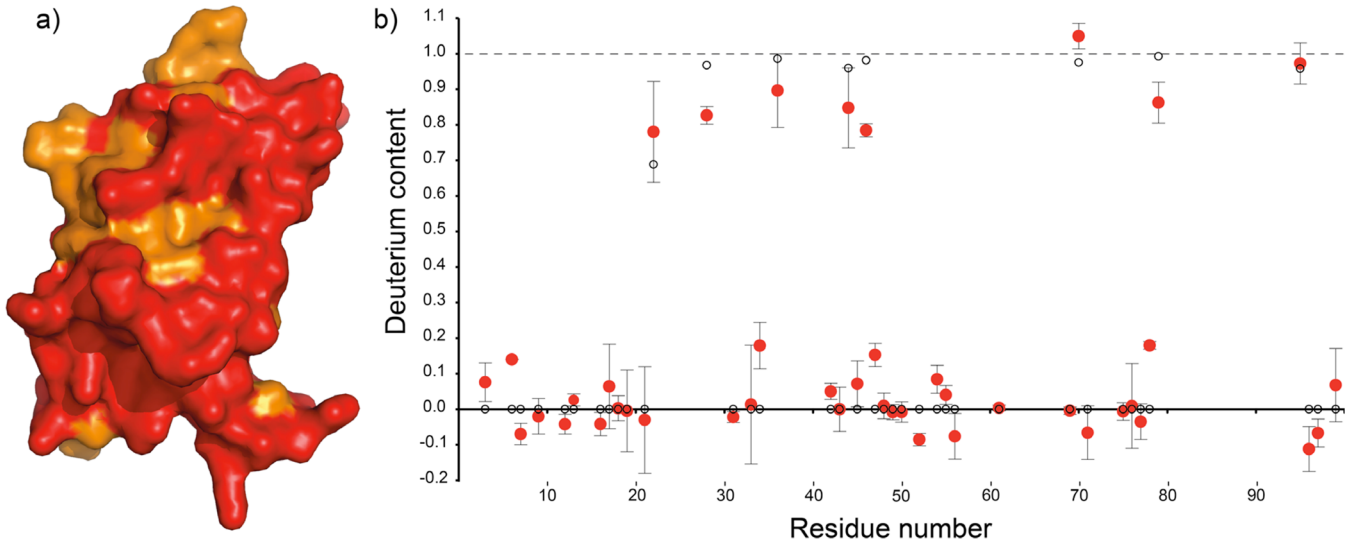

Figure 7. Analysis of HDX of β2-microglobulin at single-residue resolution by a bottom-up HDX-MS/MS workflow coupled to ETD. (a) ETD allowed analysis of site-specific HDX of 60 amides (red color) and analysis of the remaining 38 amides at a resolution of 2−4 residues (orange). (b) In agreement with NMR data, only a few highly protected backbone amides were found to efficiently retain deuterium after 40 min exchange-out, which represents a labeling pattern that is highly different from the scrambled case and thus provides clear evidence for the validity of the bottom-up HDX-MS/MS approach. Adapted from ref 7. Copyright 2009 American Chemical Society.

and results in lower levels of back-exchange but precludes the use of nonvolatile buffer systems and reagents. Furthermore, the problems of peak overlap and peak suppression will certainly increase significantly with increasing protein size, particularly if proteases with broad specificity such as pepsin are employed.

In whatever way prompt gas-phase fragmentation is implemented in the HDX-MS workflow, we are confident that HDX-MS/MS is an invaluable extension of this biophysical method. Although the resolution of the conventional HDX-MS setup has also been increased significantly by combining the use of multiple proteases with advanced computational methods,[9] MS peak overlap due to the complexity of the resulting peptide mixture can be a significant challenge, and typical back-exchange levels (>20%) can be problematic for such computational approaches that rely on information extracted from the shape of the isotopic envelope. MS/MS is not similarly sensitive to back-exchange and, at least theoretically, can access every single backbone amide without introducing additional MS peak overlap (as fragment ions are generated and isolated in the gas phase for each peptide ion). Most importantly, only bottom-up HDX-MS/MS allows the targeted high-resolution analysis of protein regions of interest from large and complex protein samples. This unique feature, together with future developments in the above-mentioned areas, will certainly further drive the applicability of bottom-up and top-down HDX-MS/MS approaches to reveal high-resolution conformational dynamics of proteins of ever larger size and complexity.

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Notes

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Martin Zehl studied Chemistry at the University of Vienna and received his Ph.D. in 2005. After a short postdoctoral stay at the Vienna University of Technology, he joined the Department of Biochemistry and Molecular Biology of the University of Southern Denmark in 2006 as a postdoctoral fellow. In 2008, he returned to the University of Vienna, where he is currently a Senior Scientist at the Department of Pharmacognosy and the Department of Pharmaceutical Chemistry. His research focuses on the development and application of mass spectrometry methods for the characterization of natural products, proteins, and biomolecular interactions.

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