Published in final edited form as:

Anal Chem. 2010 February 1; 82(3): 942. doi:10.1021/ac9021874.

Controlling hydrogen scrambling in multiply charged protein ions during collisional activation: Implications for top-down HDX MS utilizing collisional activation in the gas phase

Rinat R. Abzalimov and Igor A. Kaltashov *

Department of Chemistry, University of Massachusetts-Amherst

Abstract

Hydrogen exchange in solution combined with ion fragmentation in the gas phase followed by MS detection emerged in recent years as a powerful tool to study higher order protein structure and dynamics. However, certain type of ion chemistry in the gas phase, namely internal rearrangement of labile hydrogen atoms (the so-called hydrogen scrambling), is often cited as a factor limiting the utility of this experimental technique. Although several studies have been carried out to elucidate the roles played by various factors in the occurrence and the extent of hydrogen scrambling, there is still no consensus as to what experimental protocol should be followed in order to avoid or minimize it. In this study we employ fragmentation of mass-selected subpopulations of protein ions to assess the extent of internal proton mobility prior to dissociation. A unique advantage of tandem MS is that not only does it provide a means to map deuterium content of protein ions whose overall levels of isotope incorporation can be precisely defined by controlling mass selection window, but also correlates this spatial isotope distributions with such global characteristic as the protein ion charge state. Hydrogen scrambling does not occur when the charge state of the precursor protein ions selected for fragmentation is high. Fragment ions derived from both N- and C-terminal parts of the protein are equally unaffected by scrambling. However, spatial distribution of deuterium atoms obtained by fragmenting low charge-density protein ions is consistent with a very high degree of scrambling prior to the dissociation events. The extent of hydrogen scrambling is also high when multi-stage fragmentation is used to probe deuterium incorporation locally. Taken together, the experimental results provide a coherent picture of intra-molecular processes occurring prior to the dissociation event and provide guidance for the design of experiments whose outcome is unaffected by hydrogen scrambling.

Keywords

Protein conformations; protein ions, multiply charged; hydrogen exchange (HDX); mass spectrometry; ion fragmentation; tandem mass spectrometry, MS/MS; collision-activated dissociation (CAD); hydrogen scrambling; gas-phase structure

INTRODUCTION

Characterization of protein conformation and dynamics using a combination of hydrogen/deuterium exchange in solution (HDX) and mass spectrometry (MS) has become a powerful tool in biophysics and structural biology.¹⁻³ Among many advantages offered by MS as a

*Address correspondence to: Igor A. Kaltashov, Department of Chemistry, University of Massachusetts-Amherst, 710 North Pleasant Street, Lederle Graduate Research Tower #701, Amherst, MA 01003, tel. (413) 545-1460, fax (413) 545-4490, kaltashov@chem.umass.edu.

method of monitoring the progress of HDX in solution, are the speed of analysis and very modest requirements for protein sample quantities. Furthermore, MS does not suffer from the rather unforgiving limitations on the molecular weight of protein species, which restrict the usage of other techniques, such as NMR. Finally, a very unique and attractive advantage offered by HDX MS is its ability to observe and characterize under certain conditions distinct conformational states, which can be populated either transiently⁴ or at equilibrium.⁵

Patterns of deuterium distribution along the polypeptide backbone can be obtained by fragmenting the protein molecule either in solution under the slow-exchange conditions⁶ or in the gas phase prior to MS detection.⁷ The methodology employing enzymatic fragmentation in solution remains the most popular choice in HDX MS studies, as it can be implemented using a variety of (often inexpensive) platforms^{8–10} and in some favorable cases allows the deuteration patterns to be mapped down to the single amide level.¹¹ Alternatively, site-specific information on deuterium incorporation can be obtained using the so-called “top-down” approach to HDX MS, where the enzymatic step is substituted with the fragmentation of intact protein ions in the gas phase.¹² This approach offers several advantages, including a possibility to avoid back-exchange by excluding protein manipulation under the slow exchange conditions from the experimental scheme. It has been applied to characterize dynamics and structural features of proteins in their native¹³ and non-native^{14, 15} states, as well as to elucidate the role of protein dynamics in ligand binding.¹⁶ One unique feature of such experiments that is only beginning to be exploited is their ability to correlate isotope distribution patterns with specific protein conformations using mass selection prior to ion activation as a means to obtain fragment ions originating from a particular conformer with a specific level of deuterium incorporation.¹²

A major factor limiting the utility of the top-down HDX MS is a concern over the possibility of hydrogen scrambling accompanying fragmentation of protein ions in the gas phase. Indeed, several reports have been published suggesting that proton mobility in the gas phase does influence the outcome of HDX CAD MS measurements for short peptides^{17–19} and in some cases for proteins.^{13, 20} Recently, several groups reported that the specter of hydrogen scrambling may be alleviated by employing non-ergodic fragmentation processes, where ion-electron interactions (such as electron capture dissociation, ECD, or electron transfer dissociation, ETD) are used to activate small peptides^{21, 22} and proteins.^{12, 23, 24} Although these reports are very encouraging, it is very unusual for ECD and ETD to provide complete sequence coverage, especially for larger polypeptides. Even though the fragmentation patterns produced by CAD are typically less extensive, they are often complementary to ECD and ETD. Therefore, inclusion of both approaches to protein ion activation in the arsenal of top-down HDX MS is likely to result in significant enhancement of the spatial resolution with which protein conformation and dynamics can be probed.

A major obstacle to implementing this strategy is the lingering uncertainty *vis-à-vis* reliability of HDX CAD MS measurements due to the possibility of hydrogen scrambling. Clearly, wider utilization of HDX CAD MS in biophysics and structural biology is contingent upon identifying the factors leading to hydrogen scrambling and establishing conditions where HDX CAD MS measurements can be carried out in a scrambling-free regime. Although the initial steps in finding such conditions were made several years ago,¹³ there is still no consensus among the HDX MS practitioners how this scrambling-free regime can be achieved. The purpose of this work was to evaluate the influence of several experimental parameters on the occurrence of hydrogen scrambling in the gas phase upon collisional activation of protein ions in the collision cell of a hybrid quadrupole-TOF mass spectrometer, which arguably represents one of the most popular platforms utilized for HDX MS work.

While the previous report on using this configuration in top-down HDX MS work was rather disappointing,²⁰ we found conditions that allow these experiments to be carried out in a scrambling-free regime. Specifically, we have found a surprisingly strong dependence of scrambling on the charge state of the precursor ion. The proton mobility upon collisional activation of high charge-density precursor ions appears to be greatly suppressed, as suggested by the measurements of deuterium content of both *b*- and *y*-fragment ions. At the same time, massive scrambling is observed within the fragments derived from low charge-density protein ions, which usually represent compact structures in solution. Extensive scrambling also accompanies multi-stage fragmentation, effectively ruling out the possibility to use MSⁿ as a means to further enhance spatial resolution in HDX CAD MS measurements. The results of this study allow us to formulate recommendations as to how and when top-down HDX MS experiments with collisional activation can be reliably used to probe protein higher order structure and conformational dynamics in solution.

EXPERIMENTAL

Materials

Variants of cellular retinoic acid binding protein I (CRABP I) were expressed in *E. coli* using a procedure described in detail elsewhere²⁵ from plasmids generously provided by Prof. Lila M. Gierasch (UMass-Amherst, Department of Biochemistry and Molecular Biology). Deuterium oxide (D₂O) and d-acetic acid (CD₃CO₂D) were purchased from Cambridge Isotopes, Inc. (Andover, MA). All other chemicals were of analytical grade or higher.

Methods

HDX reactions were carried out by first deuterating the proteins via several cycles of dissolving in D₂O/CD₃CO₂D followed by overnight incubation and lyophilization. The stock solutions of completely deuterated proteins were made in D₂O/10 mM CH₃CO₂NH₄ several hours prior to HDX measurements and were kept at +4°C. The exchange reactions were initiated by diluting the stock solution 1:25 (v:v) in the exchange solvent (H₂O/10 mM CH₃CO₂NH₄), whose pH was adjusted to a desired level using acetic acid. The protein solution was placed in a syringe and continuously infused in the ESI source of a hybrid quadrupole/time-of-flight mass spectrometer (QStar-XL, ABI/Sciex, Toronto, Canada). The progress of HDX reactions was monitored in MS1 regime by observing the mass shift of the intact protein ions.

Fragmentation of protein ions was carried out in the RF-only quadrupole using argon as collision (6 mTorr) gas following mass-selection of the precursor ion in the preceding quadrupole filter. Typically, fragmentation of protein ions was achieved by using collision energy in the 650–900 eV range. Fragmentation of the entire ionic population in the ESI interface region without mass-selection was achieved by raising the value of DP (declustering potential) to 200V. Mass-selection of some of these fragments and their collisional activation in the RF-only quadrupole (*vide supra*) was used to produce the second generation of fragment ions, a process referred to as MS³ in the subsequent sections, although it would be more appropriate to call it pseudo-MS³ (no mass selection prior to the first fragmentation step).

RESULTS AND DISCUSSION

Hydrogen scrambling and the precursor ion charge state

The extent of internal proton mobility in protein ions prior to their dissociation can be determined by either using intrinsic reporters of hydrogen scrambling (such as flexible loops lacking well-defined structure under native conditions¹³ or engineered unstructured segments, such as His-tags²⁶) or by comparing the patterns of backbone protection generated by top-down HDX MS to those obtained by either NMR or bottom-up HDX MS.²⁴ While the direct

comparison of top-down HDX MS and NMR data provides the most rigorous way to assess the validity of the former approach, it works best when the sequence coverage during protein ion dissociation is close to 100%, so that for every amide detectable by NMR, a corresponding piece of data can be obtained from HDX MS. While it works in some cases, particularly for smaller proteins,^{23, 24} top-down fragmentation of protein ions by CAD is notorious for leaving multiple gaps in the sequence and generating fragmentation patterns where cleavage of all (or even most) amide bonds is very rare. In fact, all published examples of successful utilization of CAD in top-down HDX MS measurements focus on obtaining segment-specific (rather than residue-specific) backbone protection patterns.^{13, 14, 16} Bottom-up HDX MS measurements also produce information on backbone protection with limited spatial resolution (*i.e.*, at the peptide level), however, direct comparison of these protection patterns with those derived from top-down HDX MS measurements is even less straightforward due to the obvious incongruence of proteolytic fragmentation in solution and dissociation in the gas phase.

Under these circumstances, reliance on intrinsic reporters of hydrogen scrambling remains the most viable strategy that can be used to detect the occurrence of hydrogen scrambling in the gas phase prior to protein ion fragmentation. In this work, we use both N- and C-terminal His-tags as reporters of hydrogen scrambling in CRABP I constructs (Figure 1). The initial purpose of introducing each His-tag in the protein sequence was facilitation of protein purification; however, presence of the 21 amino acid residues long unstructured segment in the N-terminus of the protein (*e.g.*, HT_N-CRABP, Figure 1A) provides an easy and convenient way to detect the occurrence of hydrogen scrambling. While the protein itself is folded into a β -barrel conformation, which remains stable across a wide pH range, the His-tag portion of the protein is entirely unstructured and its backbone should lack any protection in solution. HT_N-CRABP has already been used to detect occurrence of scrambling in top-down HDX MS measurements carried out with FT ICR MS (CAD in the ESI interface region,^{26, 27} ECD inside the ICR cell, 12 and ETD in the external hexapole²⁸).

In the past, we used CAD in the ESI interface region (which is frequently referred to as “nozzle-skimmer fragmentation” or “cone fragmentation”) to induce dissociation of protein ions without prior mass selection to maximize the abundance of fragment ions. Mass selection of protein ions at a single charge state as precursor ions obviously reduces the overall abundance of the fragment ions; however, a large number of abundant fragments can still be observed in MS/MS experiments. The charge state envelope of protein ions under the experimental conditions used in this work covers a wide range (from +9 to +24), but the bimodal character of this distribution results in very low abundance of two charge states in the middle of the envelope (+12 and +13). As a result, we were unable to obtain MS/MS data of adequate quality using these two protein ions as precursors. CAD of most other protein ions, including low charge density ones (+9, +10 and +11) generated abundant fragments. An example of MS/MS experiment where protein ions at a single charge state were used as precursors is shown in Figure 2.

Although fragmentation patterns observed in CAD mass spectra depend on the charge state of the precursor ion, there are a few ions that can be detected in most MS/MS experiments, regardless of the extent of multiple charging of protein ions undergoing CAD. We were particularly interested in “recurring” fragment ions that are derived from the His-tag segment of the protein. While short fragments (*e.g.*, b_{10}^{+}) are detected in nearly all MS/MS experiments, we were interested in using larger fragments derived from the His-tag as indicators of hydrogen scrambling. A larger number of labile hydrogen atoms at both backbone amide and side chain groups should make the detection of hydrogen scrambling (if it does occur) within a longer unprotected protein segment easier. For example, the b_{19} fragments observed in a large number of CAD spectra contain 38 labile hydrogen atoms (not counting charges), or 12% of the total number of such atoms within the entire protein. Calculations of the intrinsic exchange rate for

amide hydrogen atoms within this protein segment using the kinetic data assembled by the Englander group²⁹ indicate that only 1.45 deuterium atoms should be retained within this segment following 5 min of exchange in solution. However, selection of a highly protected precursor ion at low charge state (inset A in Figure 2) gives rise to an abundant b_{19}^{2+} fragment ion, whose isotopic distribution clearly suggests much more significant deuterium content compared to the estimates that are based on the kinetics of the intrinsic exchange alone (inset B in Figure 2). Indeed, the difference between the average mass of this fragment ion and the one representing the end-point of the exchange reaction (gray trace in (inset B in Figure 2) is 11.3.

A more accurate assessment of deuterium content of the b_{19}^{2+} ion can be made using a deconvolution algorithm developed in our laboratory, which removes contribution of natural isotopes.³⁰ This procedure, when applied to b_{19}^{2+} fragment derived from a highly protected population of M^{+9} ions clearly signals the occurrence of hydrogen scrambling (Figure 3A). Not only is the deuterium content of this ion significantly higher than that of the b_{19}^{2+} fragment derived from the M^{+9} ions representing the end-point of the exchange reaction, but it actually correlates with the amount of the deuterium atoms expected to be present within this segment following a totally random redistribution of all labile hydrogen atoms across the protein ion prior to its dissociation.

Similar behavior is exhibited by all b_{19}^{2+} fragments derived from the low charge density protein ions, *i.e.* +9, +10, and +11, as shown in Figure 4 (the low charge density protein ions are defined here as the ionic species derived from the part of a bimodal charge state distribution, which represents native or near-native protein conformers in solution, see Supplementary Material for more detail). However, a markedly different retention of deuterium is observed in these same fragment ions derived from high charge-density precursor ions (+14 through +17). Although the deuterium content of the precursor ions is the same as in the case of low charge-density precursors, the amount of deuterium retained within the b_{19}^{3+} fragments is dramatically lower and in fact matches the estimates that are made based on the kinetics of the intrinsic exchange alone (Figure 4).

Signal interference did not allow us to observe distinct isotopic distributions for b_{19}^{3+} fragments derived from protein ions at charge states higher than +17. In these cases, b_{23}^{4+} fragments were used as surrogate reporters of hydrogen scrambling. These fragments encompass the entire His-tag segment (residues 1–21 of HT_N-CRABP), as well as two residues of the wild type sequence which are unstructured in solution. An estimate of the deuterium content of this segment based on the intrinsic exchange kinetics²⁹ predicts that 1.6 deuterium atoms should be retained following 5 min of exchange in solution, the same level of retention as that expected for b_{19}^{3+} . The deuterium content of b_{23}^{4+} fragments derived from the highly protected protein ions at charge states +18, +19, and +20 matches this residual level of deuterium retention within the experimental error (Figure 4).

Unfortunately, the low abundance of protein ions at charge states +12 and +13 prevented us from acquiring MS/MS spectra whose quality would be sufficient for the top-down HDX MS measurements. Nevertheless, it is quite clear that the dependence of the deuterium content of large fragment ions derived from the unstructured N-terminal segments of the protein follows a sigmoidal curve. One wing of this curve represents a totally random redistribution of labile hydrogen atoms with the protein ions prior to their fragmentation (charge states +9, +10, and +11), while another is consistent with the absence of hydrogen scrambling during protein ion activation (charge states +14 through +20).

While the strong dependence of the extent of hydrogen scrambling on the protein ion charge density may seem surprising, it must be noted that this phenomenon is likely to be related to

the influence of Coulombic interactions on both kinetic parameters of ion dissociation and the mechanistic aspects of ion fragmentation (dynamic events occurring during collisional activation of protein ions). According to the “charges on a string” model,³¹ the precursor ion charge increase is translated into higher dissociation rates as a result of greater electrostatic repulsion within the protein ions. Furthermore, low charge density protein ions (which represent native or native-like conformations in solution and usually retain compactness in the gas-phase) have been shown to undergo large-scale unfolding and re-folding transitions upon collision activation.^{32, 33} These transitions frequently involve large-scale charge (proton) redistribution, which is likely to facilitate hydrogen scrambling. In contrast, increased electrostatic repulsion within protein ions at higher charge density tends to constrain a polymer chain to more extended form during collision activation, thus limiting the occurrence of charge redistribution processes.

Both N- and C-terminal fragment ions generated from the high charge-density precursor ions indicate absence of hydrogen scrambling

In the experiments presented in the preceding section, the occurrence of hydrogen scrambling was determined by monitoring the deuterium content of *b*-ions derived from the unstructured N-terminal segment (His-tag) of HT_N-CRABP. However, earlier work with short peptide ions suggested that *b*-ions may be less prone to scrambling compared to *y*-ions.^{34, 35} In order to evaluate the extent of hydrogen scrambling among *y*-ions, we used another CRABP variant, HT_C-CRABP, in which an eight-residue His-tag is attached to the C-terminus of the protein (Figure 1B).

Since the His-tag region of HT_C-CRABP is very short, the *y*-ions derived from this segment do not incorporate a significant number of labile hydrogen atoms, and, therefore, are not very useful as indicators of hydrogen scrambling. However, the fragmentation pattern within the “wild type” portion of the HT_C-CRABP sequence bears significant resemblance to that of HT_N-CRABP. This allows the C-terminal His-tag to be used as hydrogen scrambling indicator by comparing the levels of retained deuterium within fragment ions derived from the cleavage of the same peptide bond in HT_C-CRABP and HT_N-CRABP. For example, the *y*₁₀ fragment derived from HT_N-CRABP ions may be compared with the *y*₁₈ fragment derived from HT_C-CRABP ions. The latter incorporates eight additional amino acid residues (the C-terminal His-tag), which obviously increases both the number of backbone amides (17 vs. 9) and the total number of labile hydrogen atoms (44 vs. 26). Should hydrogen scrambling occur, the level of retained deuterium atoms within the HT_C-CRABP-derived *y*₁₈ would be significantly higher compared to that of the HT_N-CRABP-derived *y*₁₀ (provided the deuteration levels of the two precursor ions are equal). However, if the internal redistribution of labile hydrogen atoms during protein ion activation does not occur, the amount of deuterium label retained within these two fragment ions should be identical.

The results of such an experiment are shown in Figure 5, where the top panel shows the isotopic distribution of the *y*₁₀²⁺ fragment derived from HT_N-CRABP following 6 min HDX (the deuteration level of the precursor protein ion at charge state +16 is 95 ± 4), and the bottom panel shows the isotopic distribution of a *y*₁₈³⁺ fragment derived from HT_C-CRABP ions having a very similar deuteration level. The *m/z* scales in Figure 5 are adjusted in such a way that the comparison of the number of deuterium atoms retained by each fragment is straightforward. However, since the two fragment ions in question differ in size, their isotopic distributions have different contributions from the natural isotopes, as well as the residual deuterium present in the exchange solvent. These contributions can be removed from the experimentally obtained isotopic distributions by carrying out the deconvolution procedure,³⁰ as was done in the preceding section for the *b*-ions. The results of deconvolution are presented in insets in Figure 5.

The HT_N-CRABP segment represented by the y_{10}^{2+} fragment is expected to be highly protected (since it represents one of the most stable elements of CRABP structure³⁶), retaining 8.5 deuterium atoms under the experimental conditions in the absence of hydrogen scrambling (the experimentally measured level of deuterium retention is 8.3). At the same time, the HT_C-CRABP segment represented by y_{18}^{3+} is expected to retain only 1.2 more deuterium atoms in the absence of hydrogen scrambling (due to the finite rate of exchange of fully unprotected amide hydrogen atoms in the His-tag segment). The actual difference in deuteration levels of the two fragment ions is 1.1 ± 0.5 .

This comparison clearly demonstrates the absence of any apparent protection within the C-terminal His-tag segment of HT_C-CRABP, providing strong evidence that no internal transfer of labile hydrogen atoms occurs during protein ion activation in the gas phase at the C-terminal segment of the polypeptide chain. Therefore, formation of y -fragments upon dissociation of high charge-density protein ions occurs in the scrambling-free regime.

Hydrogen scrambling in multi-stage fragmentation schemes

Inadequate sequence coverage of protein ions by CAD is one of the factors limiting the utility of this fragmentation technique in top-down HDX MS experiments, as it inevitably leads to less-than-desirable spatial resolution.¹² Sequence coverage in top-down sequencing of protein ions can often be enhanced by employing multi-stage fragmentation schemes (the so-called MSⁿ experiments), where large fragments produced upon initial dissociation of a protein ion can be isolated and subjected to further collisional activation. A similar approach may be used to enhance the spatial resolution in top-down HDX MS studies, provided hydrogen scrambling can be avoided in such experiments.

In order to evaluate the extent of hydrogen scrambling in top-down HDX MS measurements utilizing multi-stage CAD, HT_N-CRABP was collisionally activated in the ESI interface region (a process, which is frequently referred to as nozzle-skimmer, or cone fragmentation), and several first-generation fragment ions were mass-selected and subjected to further collisional activation in the RF-only quadrupole of a hybrid Qq-TOF mass spectrometer. In the absence of collisional activation in the ESI interface region, the charge state distribution of HT_N-CRABP ions is bimodal, although the high charge-density protein ions (charge states $> +12$) make up the largest fraction of the total ionic signal.

Dissociation of these ions in the ESI interface region gives rise to over 20 highly abundant fragment ions, both b - and y -type. Since the reporter of hydrogen scrambling (the His-tag) was located at the N-terminus of the protein, we examined behavior of b -ions only. Despite the significant retention of deuterium label within the intact protein ions subjected to collisional activation in the ESI region (see the inset in Figure 6A), the deuterium content of short b -ions derived from the His-tag region (*e.g.*, b_{10}^{2+} in Figure 6B) is consistent with slow intrinsic exchange within a fully unprotected chain. This is in agreement with the results of our earlier studies, where no scrambling was observed following protein ion dissociation in the ESI source.^{26, 27} We also note that the high charge density ions appear to be the major contributors to the fragmentation processes induced by in-source collisional activation, as the fragmentation patterns observed in these experiments closely match MS/MS patterns obtained by fragmenting ions at charge states higher than +15 (data not shown). This observation is hardly surprising, given the fact that the kinetic energy of protein ions is proportional to their charges, and under identical conditions the ions with higher charge density experience greater activation upon collisions. The disproportionately high contribution of high charge density protein ions to in-course fragmentation may also be an important factor contributing to the absence of scrambling in these experiments.

Significant deuterium label is retained within larger *b*-ions, which contain both the His-tag and a relatively long portion of the wild type sequence (*e.g.*, b_{38}^{4+} in Figure 6C), consistent with the presence of protected segments beyond the His-tag. Mass-selection of this fragment followed by collisional activation in the RF-only quadrupole gives rise to over a dozen of abundant fragment ions. However, the deuterium content of these second-generation fragment ions indicates significant redistribution of the labile isotope label prior to dissociation. For example, deuterium content of the second-generation b_{10}^{2+} fragment ion (Figure 6D) is very significant, 29.5% of the total deuterium label of the precursor ions. This number is much higher compared to the deuterium content of b_{10} ions generated by in-source fragmentation (Figure 6B), but is consistent with total random redistribution of deuterium label within the b_{38}^{4+} ion prior to its dissociation (b_{10}^{2+} incorporates 29.3% of all labile hydrogen atoms contained within the b_{38}^{4+} ion). Similar behavior is displayed by other second-generation CAD fragments, clearly suggesting that their formation is accompanied by massive hydrogen scrambling, and effectively ruling out the possibility to use multi-stage CAD as a means to increase spatial resolution in top-down HDX MS measurements.

Implications for devising reliable top-down HDX MS strategies

The results of the work presented in the preceding sections strongly suggests that the design of experimental schemes in top-down HDX MS measurements is of paramount importance as far as occurrence of hydrogen scrambling during collisional activation of protein ions. While some previous work carried out with shorter polypeptides suggested that scrambling may be a pervasive problem when top-down HDX MS experiments are executed with popular quadrupole-TOF analyzers,^{20, 37} we have been able to find conditions when the extent of internal rearrangement of labile hydrogen and deuterium atoms is minimal. The extent of multiple charging plays a particularly prominent role in dictating the magnitude of hydrogen scrambling, with the high charge-density ions (representing the unfolded protein conformers in solution) showing virtually no scrambling in either *b*- or *y*-ions, while the low charge-density ions (compact structures in solution) have their deuterium label randomized during the collisional activation process.

The apparent importance of the extent of multiple charging for the internal rearrangement of labile hydrogen atoms during collisional activation may explain the apparent occurrence of hydrogen scrambling in several published studies. To the best of our knowledge, nearly all of the studies that reported either partial or complete scrambling have been carried out with relatively short peptides, whose mass was generally below 3 kDa and the charge did not exceed +3.^{17–20, 34, 35} The only report of scrambling within larger polypeptide ions (7.5 kDa ubiquitin³⁷) was based upon experiments where non-uniform labeling was observed across the polypeptide sequence, and the measurement outcomes were apparently affected by partial H/D exchange of protein ions in the gas phase (ESI interface region), a phenomenon reported previously by Katta and Chait.³⁸ The results of the present work provide unequivocal evidence that no scrambling occurs upon collisional activation of high charge-density protein ions whose mass approaches 20 kDa. Importantly, absence of scrambling is verified in both N- and C-terminal fragments ions, whereas the results of earlier work on short singly charged peptide ions suggested that *y*-ions were more likely to be affected by scrambling.^{34, 35}

Our initial motivation in working with precursor protein ions at a single charge state was the desire to design an effective top-down HDX MS strategy that would allow us to carry our measurements in a conformer-specific fashion. Deuterium content of protein ions in HDX MS reflects conformations sampled by the protein prior to its desorption from solution to the gas phase; if the exchange follows the so-called EX2 regime,⁵ different conformations can be visualized in HDX MS based on the differences in the deuterium content. However, sampling of a less structured state by a protein molecule in solution under these conditions would erase

all memory of the more structured conformations sampled previously. Therefore, only the low charge-density protein ions (representing native conformations in solutions³⁹) would typically have a chance to retain memory of all conformations sampled by a protein molecule prior to its transfer to the gas phase, while the higher charge states (representing less structured protein species) would suffer “isotopic amnesia” under exchange conditions favoring EX2 regime. Since the results of the present study suggest that collisional activation of low charge-density protein ions inevitably leads to extensive hydrogen scrambling, HDX MS/MS cannot be used directly to probe protein conformation by mass selecting ionic populations with specific deuterium content prior to CAD.

Nevertheless, this task (conformer-specific HDX MS/MS) can still be accomplished by introducing a quench step in solution prior to MS measurements. Since acidification of the protein solution (to pH 2.5–3.0) is a mandatory step in the HDX quench procedure, most protein molecules will unfold under these circumstances and the ESI mass spectrum would be populated mostly by high charge-density protein ions. At the same time, dramatic deceleration of the exchange kinetics under these conditions will allow the convoluted isotopic distributions to be mostly preserved and, therefore, will enable the conformer-specific HDX MS/MS measurements whose outcome would be unaffected by hydrogen scrambling. As an added benefit, high charge-density protein ions typically give rise to more extensive fragmentation, as collisional energy is directly proportional to the ionic charge.

Unfortunately, hydrogen scrambling cannot be kept at bay when multi-stage protein ion fragmentation is carried out in an effort to increase spatial resolution in probing the backbone protection patterns. Randomization of the deuterium label is seen very clearly in larger fragment ions that are collisionally activated to produce smaller fragments. While this effectively rules out the possibility to use multi-stage CAD as a means to obtain high resolution HDX data, HDX MS/MS/MS might still be feasible if the first stage of fragmentation is carried out using non-ergodic methods (such as ECD or ETD). Feasibility of using these techniques to probe conformation of proteins exceeding 15 kDa was recently demonstrated,^{12, 24} and if the ECD and/or ETD fragment remain cold, a second-stage of dissociation employing CAD may provide an opportunity to enhance the sequence coverage and, therefore, spatial resolution in top-down HDX MS experiments. We are currently beginning to explore these HDX MSⁿ schemes that are based on ECD/CAD and ETD/CAD combinations.

CONCLUSIONS

There is still no consensus regarding the experimental protocol that should be followed in order to significantly minimize or indeed completely eliminate hydrogen scrambling in top-down HDX CAD experiments. In this work we employed fragmentation of mass-selected subpopulations of protein ions to assess the extent of internal proton mobility prior to dissociation. Hydrogen scrambling does not occur when the charge state of the precursor protein ions selected for fragmentation is high (e.g., for protein ions representing non-native conformations). Fragment ions derived from both N- and C-terminal parts of the protein are equally unaffected by scrambling. However, spatial distribution of deuterium atoms obtained by fragmenting low charge-density protein ions (which represent highly compact species in solution) is consistent with a very high degree of scrambling prior to the dissociation events. The extent of hydrogen scrambling is also high when multi-stage fragmentation is used to probe deuterium incorporation locally, suggesting that pre-fragmentation history of precursor ions is an important determinant of scrambling. Taken together, the experimental results provide a coherent picture of intra-molecular processes occurring prior to the dissociation event and provide guidance for the design of top-down HDX MS/MS experiments whose outcome is unaffected by hydrogen scrambling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by a grant R01 GM061666 from the National Institutes of Health. The authors wish to thank Prof. Lila M. Gierasch (UMass-Amherst, Department of Biochemistry and Molecular Biology) for providing plasmids of CRABP I constructs and Dr. Virginie Sjoelund for help with protein expression and purification.

REFERENCES

1. Engen JR, Smith DL. *Anal. Chem* 2001;73:256A–265A.
2. Hoofnagle AN, Resing KA, Ahn NG. *Annu. Rev. Biophys. Biomol. Struct* 2003;32:1–25. [PubMed: 12598366]
3. Konermann L, Tong X, Pan Y. *J. Mass Spectrom* 2008;43:1021–1036. [PubMed: 18523973]
4. Konermann L, Simmons DA. *Mass Spectrom. Rev* 2003;22:1–26. [PubMed: 12768602]
5. Eyles SJ, Kaltashov IA. *Methods* 2004;34:88–99. [PubMed: 15283918]
6. Wang L, Pan H, Smith DL. *Mol. Cell. Proteomics* 2002;1:132–138. [PubMed: 12096131]
7. Kaltashov IA, Eyles SJ. *J. Mass Spectrom* 2002;37:557–565. [PubMed: 12112737]
8. Mandell JG, Falick AM, Komives EA. *Anal. Chem* 1998;70:3987–3995. [PubMed: 9784743]
9. Woods VL Jr, Hamuro Y. *J. Cell. Biochem* 2001;37S:89–98.
10. Wu Y, Engen JR, Hobbins WB. *J. Am. Soc. Mass Spectrom* 2006;17:163–167. [PubMed: 16406808]
11. Del Mar C, Greenbaum EA, Mayne L, Englander SW, Woods VL Jr. *Proc. Natl. Acad. Sci. U. S. A* 2005;102:15477–15482. [PubMed: 16223878]
12. Kaltashov IA, Bobst CE, Abzalimov RR. *Anal. Chem* 2009;81:7892–7899. [PubMed: 19694441]
13. Hoerner JK, Xiao H, Dobo A, Kaltashov IA. *J. Am. Chem. Soc* 2004;126:7709–7717. [PubMed: 15198619]
14. Hoerner JK, Xiao H, Kaltashov IA. *Biochemistry* 2005;44:11286–11294. [PubMed: 16101313]
15. Kaltashov IA. *Int. J. Mass Spectrom* 2005;240:249–259.
16. Xiao H, Kaltashov IA. *J. Am. Soc. Mass Spectrom* 2005;16:869–879. [PubMed: 15907702]
17. Hamuro Y, Tomasso JC, Coales SJ. *Anal. Chem* 2008;80:6785–6790. [PubMed: 18666782]
18. Jørgensen TJ, Gardsvoll H, Ploug M, Roepstorff P. *J. Am. Chem. Soc* 2005;127:2785–2793. [PubMed: 15725037]
19. Demmers JA, Rijkers DT, Haverkamp J, Killian JA, Heck AJ. *J. Am. Chem. Soc* 2002;124:11191–11198. [PubMed: 12224967]
20. Ferguson PL, Pan J, Wilson DJ, Dempsey B, Lajoie G, Shilton B, Konermann L. *Anal. Chem* 2007;79:153–160. [PubMed: 17194133]
21. Rand KD, Adams CM, Zubarev RA, Jørgensen TJD. *J. Am. Chem. Soc* 2008;130:1341–1349. [PubMed: 18171065]
22. Zehl M, Rand KD, Jensen ON, Jørgensen TJ. *J. Am. Chem. Soc* 2008;130:17453–17459. [PubMed: 19035774]
23. Pan J, Han J, Borchers CH, Konermann L. *J. Am. Chem. Soc* 2008;130:11574–11575. [PubMed: 18686958]
24. Pan J, Han J, Borchers CH, Konermann L. *J. Am. Chem. Soc* 2009;131:12801–12808. [PubMed: 19670873]
25. Sjoelund V, Kaltashov IA. *Biochemistry* 2007;46:13382–13390. [PubMed: 17958379]
26. Eyles SJ, Speir JP, Kruppa GH, Gierasch LM, Kaltashov IA. *J. Am. Chem. Soc* 2000;122:495–500.
27. Eyles SJ, Dresch T, Gierasch LM, Kaltashov IA. *J. Mass Spectrom* 1999;34:1289–1295. [PubMed: 10587623]
28. Abzalimov RR, Kaplan DA, Easterling ML, Kaltashov IA. *J. Am. Soc. Mass Spectrom* 2009;20:1514–1517. [PubMed: 19467606]

29. Bai Y, Milne JS, Mayne L, Englander SW. *Proteins* 1993;17:75–86. [PubMed: 8234246]
30. Abzalimov RR, Kaltashov IA. *J. Am. Soc. Mass Spectrom* 2006;17:1543–1451. [PubMed: 16934998]
31. Rockwood AL, Busman M, Smith RD. *Int. J. Mass Spectrom. Ion Proc* 1991;111:103–129.
32. Shelimov KB, Clemmer DE, Hudgins RR, Jarrold MF. *J. Am. Chem. Soc* 1997;119:2240–2248.
33. Clemmer DE, Jarrold MF. *J. Mass Spectrom* 1997;32:577–592.
34. Deng YZ, Pan H, Smith DL. *J. Am. Chem. Soc* 1999;121:1966–1967.
35. Kim MY, Maier CS, Reed DJ, Deinzer ML. *J. Am. Chem. Soc* 2001;123:9860–9866. [PubMed: 11583550]
36. Krishnan VV, Sukumar M, Gierasch LM, Cosman M. *Biochemistry* 2000;39:9119–9129. [PubMed: 10924105]
37. Ferguson PL, Konermann L. *Anal. Chem* 2008;80:4078–4086. [PubMed: 18459737]
38. Katta V, Chait BT. *J. Am. Chem. Soc* 1993;115:6317–6321.
39. Kaltashov IA, Abzalimov RR. *J. Am. Soc. Mass Spectrom* 2008;19:1239–1246. [PubMed: 18602274]

Figure 1.

Amino acid sequences of HT_N-CRABP (**A**) and HT_C-CRABP (**B**) showing location of elements of the secondary structure and the fragment ions discussed in this work.

Figure 2.

Mass spectrum of fragment ions produced by collisional activation of narrow subpopulation of HT_N -CRABP ions (charge state +9) with a high level of retained deuterium. The subpopulation of the protein ions mass-selected for consecutive fragmentation is shown in the left upper inset with a black trace. Distribution of the deuterium content of all protein ions at this charge state following 5 min. HDX in solution is shown in the same inset with a dark gray trace; the end-point of the exchange reaction is shown with a light gray trace. The upper right inset shows a zoomed view of the fragment ion mass spectrum containing isotopic distributions of two representative fragments (b_{19}^{2+} and y_{20}^{2+}) derived from the deuterium-rich precursor ions. Isotopic distributions of the same fragments at the end-point of the exchange reaction are shown with a light gray trace.

Figure 3. Distributions of retained deuterium atoms within the b_{19} fragments derived from deuterium-rich populations of intact HT_N -CRABP ions at charge states +9 (**A**) and +15 (**B**) following 5 min HDX in solution.

Figure 4.

The average number of deuterium atoms retained within b_{19} fragments derived from deuterium-rich populations of intact HT_N -CRABP ions (following 5 min HDX in solution) plotted as a function of the precursor ion charge state. No satisfactory data could be obtained for precursor ions +12 and +13 due to their low abundance. Signal interference prevented using b_{19} fragments derived from precursor ions at charge states +18 and higher, in which cases the plot shows the deuterium content of b_{23}^{4+} fragments (gray-shaded data points on the graph). Solid horizontal lines show the calculated average number of deuterium atoms corresponding to completely random placement of labile hydrogen and deuterium atoms within the protein ion prior to its dissociation (top) and the absence of hydrogen scrambling (bottom).

Figure 5.

Isotopic distributions of y_{10}^{2+} fragments derived from HT_N -CRABP (**A**) and y_{18}^{3+} derived from HT_C -CRABP (**B**). In both cases the precursors ions were selected at charge state +16 and had high deuterium content (95 ± 4). The gray traces in each panel show the isotopic distributions of these same fragment ions at the end-point of the exchange reactions. Insets on the right-hand side of each graph show the distributions of retained deuterium atoms for each fragment obtained by deconvolution.

Figure 6.

The entire population of HT_N-CRABP ions following 5 min HDX in solution (A) was collisionally activated in the ESI interface region, producing abundant b_{10}^{2+} (B) and b_{38}^{4+} (C) fragments. The latter was mass-selected and collisionally activated in the RF-only quadrupole of the Qq-TOF MS, yielding a second-generation b_{10}^{2+} fragment (D). Isotopic distributions of all fragment ions are shown in respective panels as gray traces.