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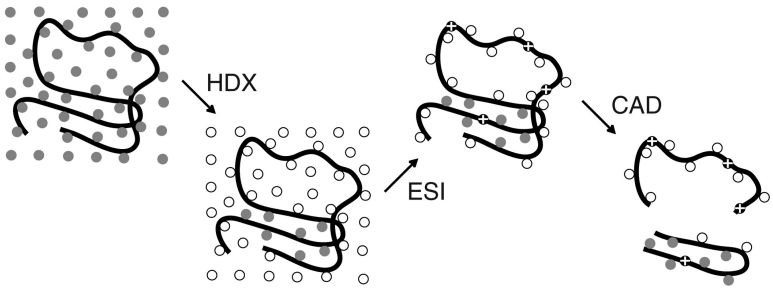

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Is There Hydrogen Scrambling in the Gas Phase? Energetic and Structural Determinants of Proton Mobility within Protein Ions

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Abstract: The extent of internal hydrogen exchange (scrambling) within multiply charged solvent-free protein ions was investigated using a small model protein. The site-specific backbone amide protection data were obtained using protein ion fragmentation in the gas phase and compared with the available NMR data. Only minimal scrambling was detected when relatively high-energy collisional activation was used to fragment intact protein ions, while low-energy fragmentation resulted in more significant but not random internal exchange. Increased conformational flexibility of protein ions in the gas phase did not have any effect on the extent of hydrogen scrambling under the conditions of higher-energy collisional activation but resulted in totally random redistribution of labile hydrogen atoms when the protein ion fragmentation was induced by multiple low-energy collisions.

Introduction

Hydrogen−deuterium exchange (HDX) of proteins in solution has been an indispensable tool in probing protein conformation and dynamics in the past several decades.[1] While the majority of HDX studies employ high-resolution NMR as a means to monitor the exchange kinetics, mass spectrometry (MS) is currently enjoying a dramatic surge in popularity in this field as well.[2−5] It offers several important advantages over NMR, namely faster time scale, tolerance to high-spin ligands and cofactors, and ability to monitor the exchange in a conformer-specific fashion, as well as much more forgiving molecular weight limitations. On the other hand, HDX MS rarely achieves the degree of structural detail that is easily accessible to HDX NMR. One of potential remedies to this problem was suggested almost a decade ago, namely utilization of tandem capabilities of modern mass spectrometry (MS/MS) in the course of HDX MS measurements.[6] Recent dramatic expansion of the molecular weight range of proteins, for which nearly complete sequence coverage is possible by methods of MS/MS,[7] may potentially pave the way for HDX MS/MS to become a leading technique in the studies of protein conformations and dynamics. HDX MS/MS of entire proteins has been used in recent years by several research groups to study protein dynamics,[8−10] as well as binding site topology in protein complexes[11] and polypeptide aggregates.[12,13] However, the validity of data obtained in such experiments is often (and rightly so) questioned based on the possibility of hydrogen scrambling in the gas phase (prior to protein ion dissociation).[14] Johnson and co-workers were perhaps the first to identify this problem.[15] Subsequently, McLafferty reported extensive hydrogen scrambling when fragmenting gaseous cytochrome *c* ions in the cell of a Fourier transform ion cyclotron resonance mass spectrometer (FT ICR MS).[16]

The significance of this problem extends far beyond the question of analytical utility of HDX MS/MS methodology. Indeed, the extent of proton mobility within peptide and protein ions plays an important role in a variety of processes, most notably dissociation.[17] Finally, understanding the factors governing internal mobility of protons within large proteins unaffected by solvent may have significant consequences for our understanding of charge transfer alongside proteins in lipophilic environments, particularly proton pumps.[18]

Recently, this journal led a discussion on the occurrence, causes, and consequences of hydrogen scrambling. Following the initial McLafferty report,[16] Smith[19] and Deinzer[20] evaluated

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scrambling within small peptic fragments subjected to collision-activated dissociation (CAD) in the trapping cell of a quadrupole ion trap instrument. In both cases, the "correctness" of the amide protection deduced from the HDX MS/MS measurements was judged against the available HDX NMR data. No detectable scrambling was observed within the *b*-type (N-terminal) fragment ions, while the deuterium content of the *y*-type (C-terminal) fragment ions often showed significant departures from the protection levels assessed by NMR.[19,20] We have examined the scrambling issue within larger protein ions (>18 kDa) by monitoring the deuterium content of an intrinsically unstructured His-tag.[10] Protein ion dissociation was achieved by using CAD in the ESI interface region (the so-called nozzle-skimmer dissociation) of an FT ICR mass spectrometer. We argued that the absence of scrambling in the gas phase would render the fragment ions derived from the His-tag region essentially label-free (due to the lack of any protection of this segment in solution). On the other hand, even limited scrambling was expected to introduce some labels into that segment prior to protein ion fragmentation. No evidence of the latter had been found, leading us to a conclusion that scrambling did not occur or else was very limited.[10] Finally, Heck and co-workers have examined proton mobility within shorter model transmembrane peptides and reported detectable scrambling whose extent could be linked to the nature of the charge carrier (i.e., protonated and alkali metal-cationized peptide ions behaved differently).[21]

A lack of consensus in these and other[22] studies led us to a suggestion that there may be several factors that govern proton mobility in peptide and protein ions.[14] Outcome of any particular experiment would then depend on a combination of such factors. Two factors have been suggested as prime candidates for being major determinants of scrambling, namely collision energy and flexibility of the polypeptide chain in the gas phase.[14] Higher collision energy favors direct cleavages, while slow heating methods[23] often lead to extensive internal rearrangements (which could include scrambling) prior to dissociation. Chain flexibility in the gas phase should also be important, as it is likely to enhance proton mobility by allowing two distant segments of the protein to make random contacts. Such transient association in the gas phase seems to be a prerequisite for transferring a labile hydrogen atom from one segment to another, since the two exchanging sites must come into direct contact with one another (akin to a bimolecular H/D exchange[24]). Lack of such flexibility due to either (partial) retention of the solution structure in the gas phase[25] or the "cementing" role of metal cations[26] would limit the extent of scrambling.

The goal of the present work is to evaluate the influence of both collision energy and gas phase conformational flexibility on the proton mobility within multiply charged protein ions.

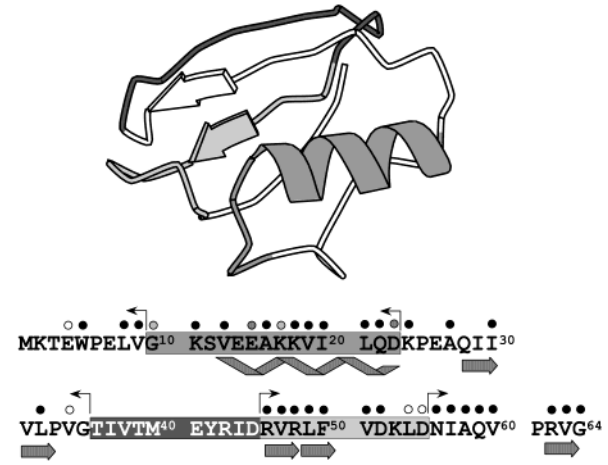

Figure 1. Tertiary (top) and primary (bottom) structures of native CI2. Symbols underneath the protein sequence indicate the secondary structural elements of CI2. Circles on top of the sequence indicate residues whose amide hydrogen atoms remain protected long enough to be detected by HDX NMR at pH 5.3. Black circles indicate residues whose protection can be confidently measured by HDX NMR at pH 6.8 (gray circles mark residues whose apparent lack of protection at pH 6.8 may be caused by signal interference, as discussed in the text). Protein segments whose dynamics are discussed in the text are highlighted with various shades of gray in both diagrams.

Barley chymotrypsin inhibitor 2 (CI2) was chosen as a model protein, since its backbone amide HDX kinetics has been thoroughly characterized by NMR.[27] CI2 is one of a relatively few proteins that lack both kinetic and equilibrium intermediate states (except for some rather exotic conditions[28]) and conform to a classic two-state folding/unfolding scheme. Furthermore, CI2 possesses a rather long flexible internal loop (residues 31−45), which is totally unprotected on the time scale of an NMR experiment,[27] flanked by stable regions (Figure 1). This renders CI2 an attractive model, which can be used to reliably verify the presence and/or extent of scrambling in the gas phase (with the deuterium content of the loop being a "scrambling gauge"). A variety of ion fragmentation techniques that can be implemented with FT ICR MS allow HDX CAD MS experiments to be carried out at both relatively high (*nozzle-skimmer* or *cone* CAD) and very low (SORI CAD) collision energy. The results of the present work clearly indicate that the collision energy is a major determinant of hydrogen scrambling, with low energy collisions favoring more extensive internal proton exchange. However, flexibility reduction of the protein ions in the gas phase (e.g., due to residual higher order structure) can significantly decrease the proton mobility even under the conditions of low-energy collisional activation. On the other hand, a combination of low energy collisions and significant disruption of the protein higher order structure in the gas phase inevitably lead to a totally random (statistical) redistribution of labile isotopes within the protein, a situation we term *perfect scrambling*.

Experimental Section

Materials. CI2 was a generous gift from Dr. Sophie E. Jackson (Cambridge University Department of Chemistry, Cambridge, U.K.). Deuterium oxide (^2H_2O) and $^2H_4$-acetic acid ($C^2H_3CO_2{}^2H$) were

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purchased from Cambridge Isotopes, Inc. (Andover, MA). All other chemicals were of analytical grade or higher.

Methods: HDX CAD MS. Initial deuteration of CI2 was performed by incubating the denatured protein in ^2H_2O at pD ~2 (unadjusted for the isotope effect) and 45 °C for 1 h followed by lyophilization. This procedure was repeated three times to ensure complete replacement of all labile hydrogen atoms with deuterium (^{2}H-CI2). The pD of the final stock solution of ^{2}H-CI2 in ^2H_2O was adjusted to 6.8 using $^2H_4$-acetic acid. Completeness of protein deuteration was verified by ESI MS. HDX reactions were initiated by diluting an aliquot of the 350 μM solution of ^{2}H-CI2 in 10mM $CH_3CO_2^-NH_4^+$ (pH 6.8) at a 1:50 (v/v) ratio. Data acquisition has been carried out in two different modes, which are referred to as an *on-line* and *off-line* HDX MS. The on-line monitoring of the HDX reactions was carried out by continuously infusing the protein solution into the ESI source of a mass spectrometer using a syringe pump. The off-line experiments were performed by quenching the HDX reactions prior to measuring the extent of the exchange. HDX quenching was achieved by rapid acidification (to pH 2.5) and subsequent freezing of the protein solution, the conditions known to minimize the intrinsic exchange rate of unprotected amide hydrogen atoms.[29] Partial protein denaturation in the "quenched" solution (i.e., under the slow exchange conditions[29]) was achieved by adding alcohol, 60 vol %, to the frozen acidified CI2 sample while thawing on ice. The protein solution temperature was maintained near 0 °C during infusion into the ESI source in these experiments.

All mass spectra were acquired with an APEX-III FT ICR mass spectrometer (Bruker Daltonics, Inc., Billerica, MA) equipped with a 4.7 T magnet and a standard ESI source. The samples were introduced into the ESI source in a continuous infusion regime at flow rates of 80−125 μL/min. Nozzle-skimmer dissociation of protein ions was initiated by increasing the capillary exit and skimmer voltages and setting the hexapole accumulation time to 2 s. Each spectrum was recorded by averaging eight scans, resulting in a total acquisition time of 30 s. SORI CAD experiments were carried out on isolated CI2 ions (+5 charge state, *m*/*z* 1468). Each SORI fragmentation spectrum was recorded by averaging 32 scans. Each fragmentation spectrum contained at least 15 abundant fragment ions, seven of which (b_7^+, b_{11}^+, b_{20}^{2+}, b_{23}^{2+}, y_9^+, y_{12}^+, and y_{19}^{2+}) were consistently detected in both nozzle-skimmer CAD and SORI CAD experiments. The conditions of ion activation were selected such that the presence of the internal fragment ions in the spectra was minimal (presence of abundant internal fragment ions in the CAD spectra was used as an indicator of facile production of the second generation of fragment ions).

The deuterium content of each fragment ion was calculated based on the difference between the centroid of its isotopic cluster and that of a fragment derived from unlabeled protein (Figure 2).

Comparison of the HDX CAD MS and the HDX NMR Data. Information on the protection of individual backbone amide hydrogen atoms within CI2 was deduced from the reported ^{1}H/^{2}H exchange rates (measured with heteronuclear single quantum coherence spectroscopy, ^{1}H−^{15}N HSQC NMR[27]). These data were used to construct a cumulative *NMR protection curve* for CI2 (Figure 3, bold solid lines) in the following fashion. Each backbone amide was assigned protection of either "0" or "1" depending on whether its protection could be detected on a time scale of an HSQC NMR experiment (exchange rates exceeding ca. 0.1 min^{-1} could not be measured by HSQC NMR,[27] and the corresponding amide hydrogen atoms were considered as lacking protection). All proline residues were also assigned zero protection. The cumulative curve was then assembled by iterative summation of such numbers beginning from the N-terminus of the protein. Therefore, a horizontal line connecting residues $n-1$ and n would be indicative of no amide protection at position n (detectable by NMR), while a line with a slope equal to one would indicate that the nth amide hydrogen atom is protected. The last point on the curve (64th in case of CI2) would be indicative of the overall backbone protection. *Totally randomized protection* curves, representing purely probabilistic distributions of deuterium atoms within a peptide, were constructed based on the overall protection measured protection (P^{total}) assuming no bias with respect to their distribution among all available sites. In other words, it was assumed that any given labile deuterium atom from a total pool of P^{total} could be found with equal probability on any backbone amide, with the exception of proline residues, side chain, as well as protein termini. It follows then that the distribution of deuterium atoms within the protein in such a case, i.e., a *perfect scrambling curve* (thin solid lines in Figure 3), can be calculated simply as

$$P_k = \frac{\sum_{i=1}^{k}(N_b^i + N_s^i + N_t^i)}{\sum_{j=1}^{64}(N_b^j + N_s^j + N_t^j)} \times P^{\text{total}}$$

where N_b^i, N_s^i, and N_t^i represent the number of sites within the ith residue occupied by labile hydrogen atoms at backbone amide, side chain, and protein terminal sites, respectively. Here P_k is the expected number of deuterium atoms located within the first k residues of the protein under the perfect scrambling scenario. These calculations are similar to the statistical state model used by Heck to represent a limiting case of extensive hydrogen scrambling.[21]

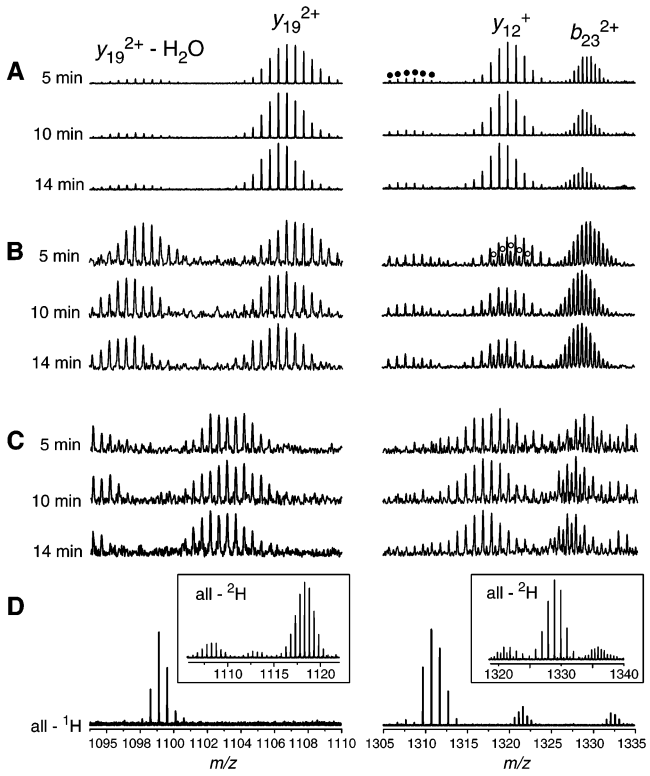

Figure 2. Evolution of deuterium content of several fragment ions as revealed by on-line (A, B) and off-line (C) HDX CAD MS measurements. Fragments are generated by collisional activation of protein ions in the ESI interface (A) and SORI CAD in the ICR cell (B, C). Isotopic clusters of unlabeled and fully labeled (deuterated) fragment ions are shown in panel (D). Isotopic peaks marked with filled circles correspond to a singly charged internal fragment, most likely [Gly^7→Lys^{18}]. Isotopic peaks marked with open circles correspond to a $(b_{23} - H_2O)^{2+}$ fragment ion.

Results and Discussion

Hydrogen Scrambling under the Rapid Collisional Activation Conditions. Analysis of the deuterium content of CI2

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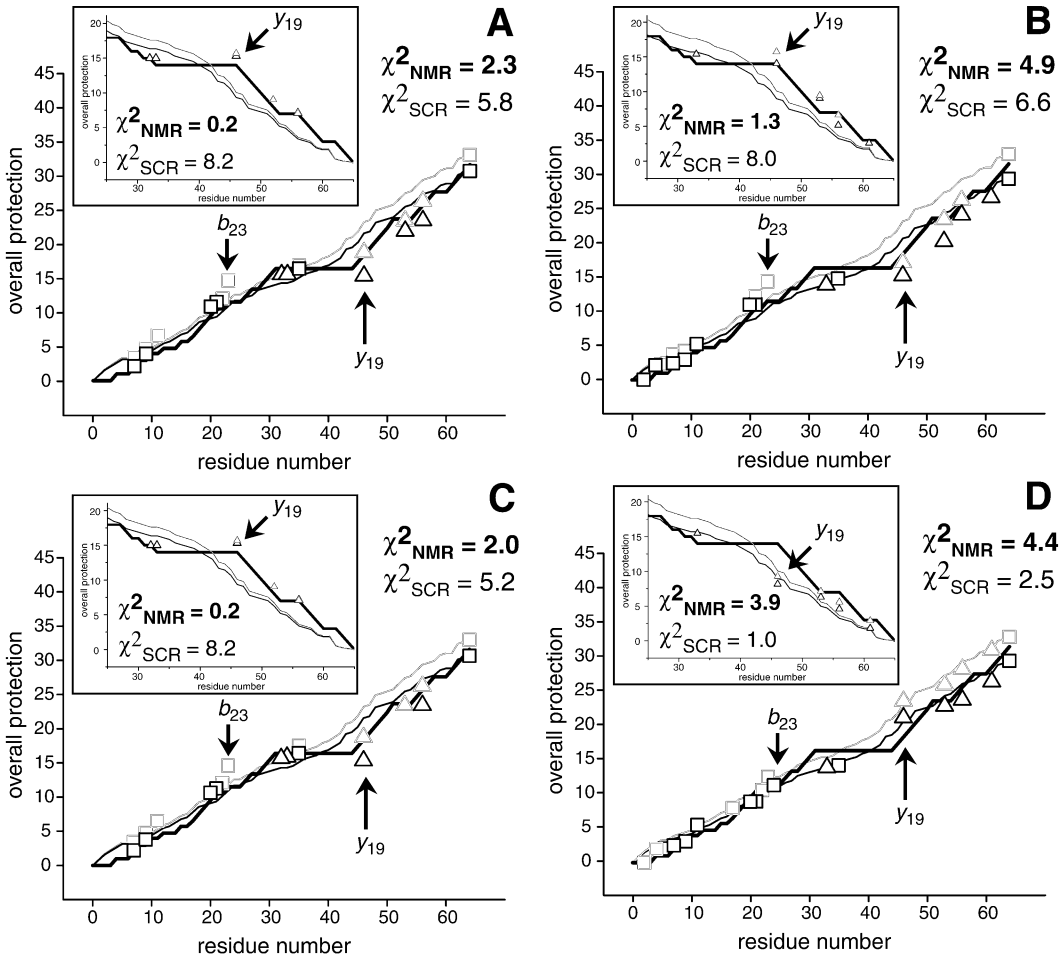

Figure 3. CI2 protection patterns derived from HDX CAD MS based on deuterium content of *b*- (□) and *y*-ions (△) following 10 min (gray symbols) and 14 min (black symbols) of exchange. Protein ions were generated on-line under near-native conditions in solution (A, B) and under denaturing slow exchange conditions (C, D). Ion fragmentation was induced by collisional activation in the ESI interface region (A, C) and SORI CAD in the ICR cell (B, D). The *NMR protection curve* is shown as a bold solid line in each panel. The two *total scrambling curves* (calculated based on the overall protein protection following 10 and 14 min of exchange in solution) are shown as thin lines (10 min, gray; 14 min, black). Calculations of χ^2 were based on the data sets collected following 14 min of exchange in solution, when the overall protein protection level is closest to that detected in the NMR experiments. The insets show the comparison of the *NMR protection curve*, the two *total scrambling curves*, and the HDX CAD MS data deduced from the *y*-fragment ions (protection is counted from the C-terminus of the protein).

fragment ions using nozzle-skimmer CAD was carried out at several time intervals following initiation of HDX reactions. The overall protein protection measured at the earliest time point was significantly higher than that measured by NMR. Thus, the surviving molecular ion peaks of CI2 contained 35 deuterium atoms following a two-minute exposure of ^{2}H-CI2 to a protiated buffer (Figure 4D), while only 30 backbone amides remain protected on the time scale of an HDX NMR experiment (≥ 10 min, vide supra) under similar conditions.[27] The superior data acquisition rate of MS allows it to "visualize" the exchange processes that are too fast to be detected by NMR. To have a valid comparison between the MS and NMR data, we have selected two data sets acquired following 10 and 14 min of ^{2}H-CI2 exposure to a protiated buffer. The overall protein protection levels measured by MS at these time points "bracket" the overall backbone protection measured by NMR.

The protection levels of CI2 segments of varying length were deduced from the deuterium content of the corresponding fragment ions. Since a number of both *b*- and *y*-type ions were prominent in the nozzle-skimmer CAD spectra of CI2, it was possible to achieve a fairly uniform sequence coverage (Figure 3A). Since the major goal of the present work was to evaluate the extent of hydrogen scrambling during the ion activation process prior to the fragmentation event, a special effort was made to avoid more extensive fragmentation, which could give rise to a second generation of fragment ions (see the Experimental Section). Scrambling that may occur between the two consecutive fragmentation events would undoubtedly complicate the data analysis.

To make a comparison between the HDX CAD MS and the HDX NMR data more transparent, measured deuterium contents of both *b*- and *y*-type ions were used to calculate the protection of corresponding N-terminal segments of CI2. In the case of *y*-ions, this was done by subtracting their deuterium content from that of the surviving molecular ions (Figure 3A). The inset in Figure 3A shows the protection of the C-terminal segments of CI2 corresponding to every *y*-fragment ion generated in these experiments. The solid lines in the inset correspond to the cumulative protection of the C-terminal segments calculated based on the NMR protection data (bold solid line) and the statistical distribution of deuterium atoms among all available sites, i.e., *perfect scrambling curves* calculated for HDX MS data following 10 min (gray thin line) and 14 min of the exchange (black thin line).

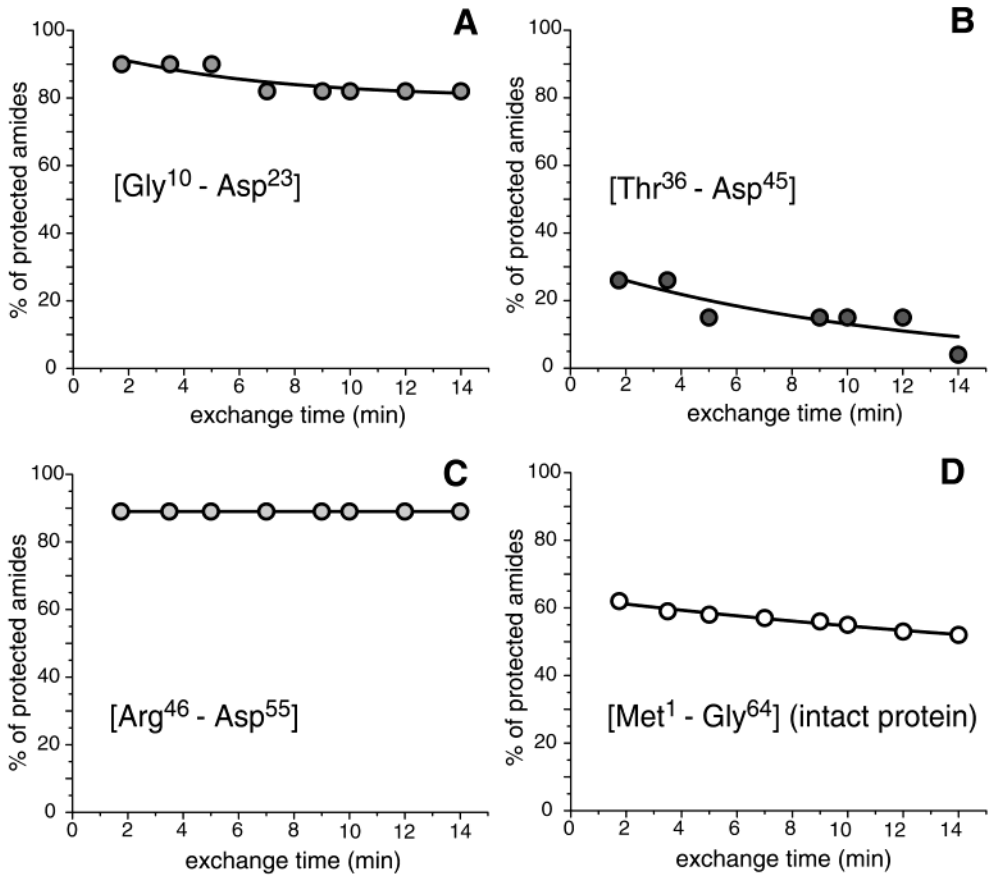

Figure 4. Evolution of amide protection within CI2 segments corresponding to the helical region [Gly^{10}→Asp^{23}] (A), flexible loop [Thr36→Asp^{45}] (B), and the β-strand-β-turn segment [Arg^{46}→Asp^{55}] (C) calculated based on the deuterium contents of the b_9/b_{23}, b_{35}/y_{19}, and y_9/y_{19} fragment ion pairs measured by HDX CAD MS (nozzle-skimmer fragmentation) and the total amide protection of CI2 measured as the deuterium content of the surviving intact protein ions (D).

Clearly, collisional activation carried out in the ESI interface results in minimal deviation of the HDX CAD MS data points from the NMR protection curve. In quantitative terms, such deviation is only $\chi^2 = 2.25$ (at 14 min), while the correlation between the HDX CAD MS data and the *perfect scrambling curve* is rather poor ($\chi^2 = 5.81$ at 14 min). Still, there are two data points that show noticeable deviation from the NMR protection curve, namely the b_{23}^{2+} and y_{19}^{2+} fragment ions. We note that the corresponding fragmentation processes are likely to be charge-directed, as they both result from cleavage of peptide bonds separating basic and acidic residues (Asp^{23}/Lys^{24} pair for b_{23}^{2+} and Asp^{45}/Arg^{46} for y_{19}^{2+}). Peptide bond dissociation in such regions often involves proton transfer between the adjacent residues.[30] Such processes have been implicated before in local internal hydrogen exchange leading to limited "apparent" scrambling in HDX CAD MS experiments.[20] Therefore, it would be reasonable to assume that the observed local and rather limited discrepancy between the HDX MS/MS and HDX NMR data can be explained in terms of proton transfer occurring during, but not prior to, the dissociation event. Another explanation that should not be discounted is the possibility of HDX MS experiments "picking up" protection of residues that are missed by NMR. For example, the Asp^{23} residue, which is a terminus in the b_{23} fragment, apparently exchanges too fast on the NMR time scale at pH 6.8, although this residue is involved in the hydrogen-bonding network in the native CI2 (and its protection can be detected at pH 5.7).[27] As stated by the authors of the original work, the cross-peak of this residue "could not be measured accurately because of ... intrinsically small coupling constant" and interference from other peaks.[27]

The anomalous behavior of b_{23}^{2+} and y_{19}^{2+} fragment ions emphasizes the fact that it is unrealistic to expect that HDX CAD MS experiments can provide reliable information on protection of each individual amide even if the requisite "ladder" fragment ions can be generated. The goal, therefore, should be shifted toward evaluating suitability of HDX CAD MS measurements for establishing *protection patterns* within the protein. Instead of localizing each deuterium atom at the highest spatial resolution, i.e., at a single amino acid residue level, such protection patterns will characterize protein dynamics at somewhat lower resolution by looking at the deuterium content of various protein segments that group several residues together. If the residue grouping is based upon the protein structure, it would allow dynamics of various structural elements to be characterized with high confidence, since the total deuterium content of a segment should not be influenced significantly by *local* scrambling events. Indeed, the measured deuterium content of each segment would only be affected by proton mobility within its terminal residues, unless a significant long-distance proton transfer occurs as well.

Three CI2 regions representing different structural elements of the protein were chosen to evaluate the correlation between

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the local structure/stability and the evolution of the deuterium content of the corresponding protein segments. These segments are the α-helical region of the protein [Gly^{10}→Asp^{23}], the flexible loop [Thr^{36}→Asp^{45}], and the β-strand [Arg^{46}→Asp^{55}] (Figure 1). The latter segment is the most stable, while the former is known to exhibit some flexibility at the N-terminal part of the helix,[27] which is also exposed to solvent (Figure 1). On the other hand, the flexible loop region lacks any backbone protection as detected by NMR, although its N-terminal part is somewhat stabilized due to the presence of a hydrogen bond acceptor[27] (Thr^{36}) and hydrophobic clustering between residues Ile^{29} and Ile^{37} even in the denatured state of the protein.[31]

The time-evolution of the deuterium content of these three segments as measured by HDX CAD MS is presented in Figure 4. The kinetic data clearly indicate a high degree of backbone protection within the helical segment [Gly^{10}→Asp^{23}]. Gradual decrease of deuterium content of this segment over time is probably due to the increased flexibility and solvent exposure within the N-terminal part of the helix (vide supra). Protection within the [Arg^{46}→Asp^{55}] segment is also very high (Figure 4C). Unlike the helical region of the protein, this β-strand appears to be much less dynamic, as its deuterium content remains constant, with 9 out of 10 amides retained during the entire time interval on the time scale examined. On the other hand, the [Thr^{36}→Asp^{45}] segment shows some retention of deuterium atoms early on but loses them after 10 min of exchange (Figure 4B), consistent with the NMR measurements.[27] Small initial protection of this segment on the time scale not accessible to HDX NMR may reflect reduced backbone flexibility in the N-terminal part of the loop due to hydrophobic clustering (vide supra). The latter two segments represent neighboring regions of CI2. Comparison of the evolution of the deuterium content in these two segments (Figure 4B,C) indicates that not only their protection levels are different (closely matching the NMR data) but also the local exchange kinetics is very different, in agreement with the dynamic parameters of the corresponding structural elements. These results provide clear indication that intramolecular proton exchange between these two segments in the gas phase does not occur or else it is minimal, since no sign of deuterium content averaging between the two segments has been detected despite their close proximity.

The retention of the deuterium atoms within the [Gly^{10}→Asp^{23}] segment of the protein ion in the gas phase is also very remarkable, since it contains three lysine residues, plus one more lysine residue is adjacent to this segment in the CI2 sequence (Figure 1). Lysine residues (or, more generally, protonated amine groups) when found in high proportion dramatically increase the rates of gas-phase H/D exchange for the peptide and protein ions.[32,33] The increased susceptibility of lysine residues to hydrogen exchange is most likely caused by the highly efficient formation of proton-bridged intermediates, which usually account for the relatively high reactivity of peptide ions with deuterating agents.[32] Previous mechanistic studies of the gas-phase H/D exchange reactions identified several mechanisms by which a proton (or a deutron) can be transferred from a peptide ion to a small molecule.[24,34] Among the several mechanisms identified, two are of particular interest within the context of this work. The *relay mechanism* appears to be favored when the disparity between the proton affinities of the reagents prevents direct H/D exchange. When the proton affinities of the reagents are comparable, the *tautomer mechanism* becomes highly favored, as it involves extensive solvation of the products by the tautomerized peptide.[24] It is plausible that the intramolecular H/D exchange follows the mechanism that resembles the *tautomer mechanism* of the intermolecular exchange (Figure 5). Although this proposed mechanism of hydrogen scrambling involves both deuterium atom *and* charge transfer, there will be no net charge gain or loss, since the transfer occurs intramolecularly. Internal solvation of a single amide group by two lysine residues (as depicted in Figure 5) does not appear to be entropically costly. Nevertheless, formation of such an internal complex also requires significant flexibility of the polypeptide chain (e.g., to enable the swinging motion in Figure 5). Furthermore, the elevated internal energy of the polypeptide ion acquired during rapid collisional heating in the ESI interface may destabilize this internal complex and prevent efficient internal exchange, analogous to the suppression of the intermolecular H/D exchange at elevated internal energies of peptide ions.[35] The influence of these two parameters (polypeptide ion internal energy and flexibility in the gas phase) on the extent of hydrogen scrambling is considered below.

Hydrogen Scrambling under the Slower Collisional Heating Conditions. The extent of hydrogen scrambling in the gas phase under the slower heating conditions was evaluated using SORI (sustained off-resonance irradiation) CAD as a means of producing CI2 fragment ions in the gas phase. The fragmentation efficiency of SORI CAD compared to the nozzle-skimmer CAD is significantly lower, hence, a noticeable deterioration of the signal-to-noise ratio for the observed fragment ions (Figure 2B). Poor signal-to-noise ratios required longer acquisition times for the SORI CAD spectra and precluded carrying out a kinetic analysis similar to that shown in Figure 4. Instead, evaluation of the deuterium content of the fragment ions produced by SORI CAD was carried out only on a time scale similar to that used in HDX NMR experiments. Analysis of the HDX SORI CAD data (Figure 3B) reveals somewhat higher intramolecular proton mobility, as compared to the experiments that use faster collisional heating (Figure 3A). There are several noticeable deviations of the HDX SORI CAD data points from the "NMR protection curve," some of which cannot be linked to the presence of basic and acidic residues at or near the cleavage site(s). Specifically, deuterium content of the fragment ion derived from the cleavages in the flexible loop region (b_{35}) indicates that some internal redistribution of the isotopic label occurs prior to protein ion fragmentation. It is noted, however, that such redistribution is not consistent with total randomization of the ^2H label within the polypeptide. A reasonable qualitative agreement is seen between the NMR protection curve and the measured deuterium levels within the smaller C-terminal fragment ions (y_{19} and below). In quantitative terms, the correlation between the HDX SORI CAD and HDX NMR data is relatively poor ($\chi^2 = 4.9$) but even less consistent with a perfect scrambling scenario ($\chi^2 = 6.6$).

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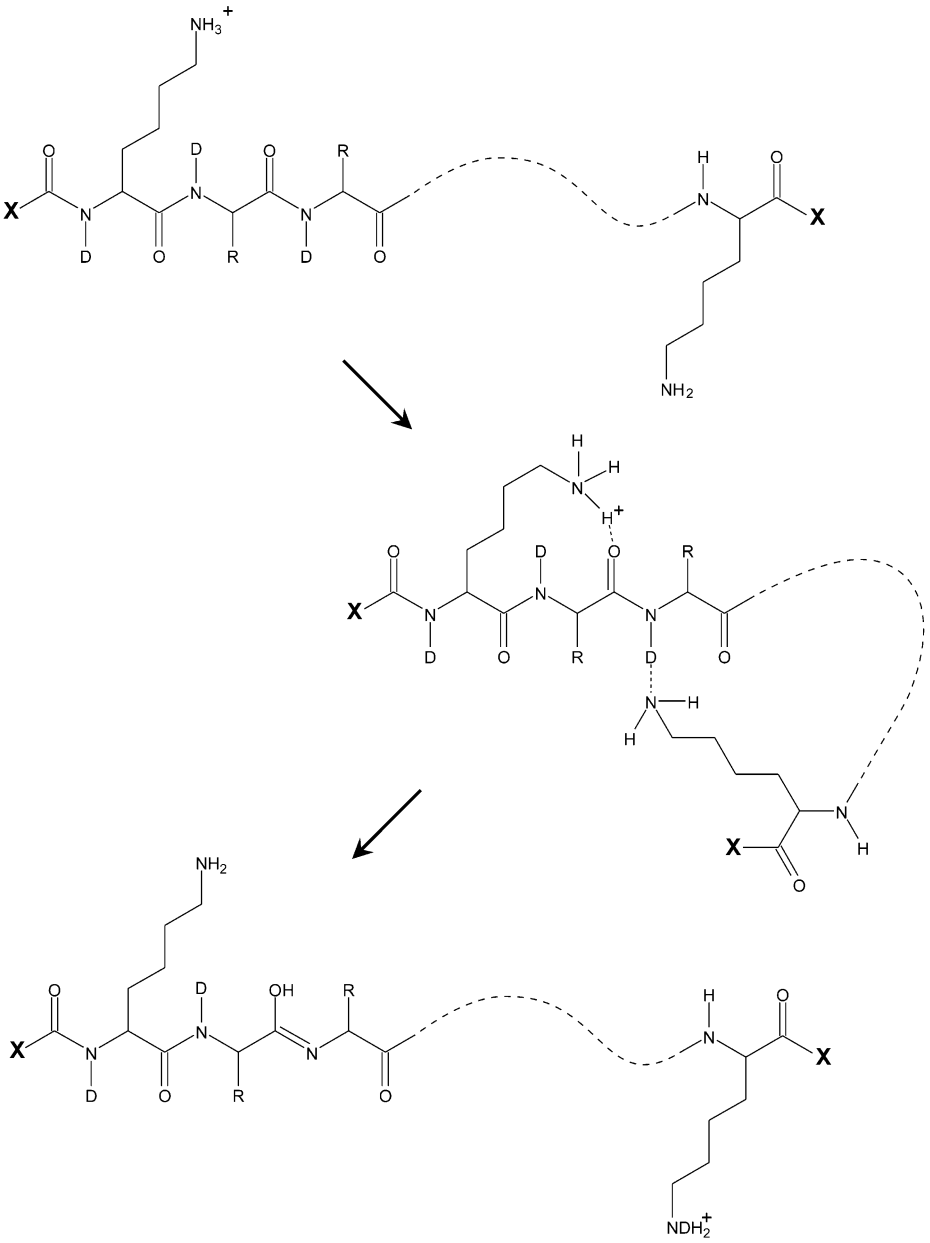

Figure 5. Proposed mechanism of long-distance hydrogen scrambling within a positively charge protein ion.

The increased incidence of hydrogen scrambling in SORI CAD experiments most likely results from the slow collisional heating of the protein ion prior to dissociation.[23] The understanding of ion activation during the SORI process remains somewhat limited due to a very complicated sequence of events leading to ion dissociation. The precursor ion is continuously irradiated by an electric field oscillating at an off-resonance frequency. As a result, the ion undergoes alternate acceleration and deceleration cycles, during which numerous collisions with the collision gas molecules occur. In recent years, several groups attempted to characterize the energetics of ion activation by SORI. Williams showed that, under the typical SORI CAD experimental conditions, the effective temperatures of small peptide ions range between 200 and 400 °C,[36] similar to that achieved with BIRD (blackbody irradiative dissociation), the slowest ion activation technique. Futrell modeled internal energy distribution for small organic cations undergoing SORI CAD,[37] and Heeren used dissociation of small peptide ions under carefully controlled experimental conditions to define the energy scale of SORI CAD processes.[38] The average energy deposition rate for a pentapeptide ion was estimated to be in the range of 1−17 eV/s.[38] Even though the individual collisions in SORI CAD are in the eV range, the time intervals between the consecutive collisions are large enough to allow efficient radiative cooling,[39] hence, the significant decrease in the heating rate, especially for larger polypeptide ions. Slow elevation of the internal energy of protein ions during the SORI CAD process is likely to give rise to rearrangement reactions, such as hydrogen scrambling, within the protein ion prior to its dissociation.[14] On the other hand, rapid heating of the polypep-

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tide ions in nozzle-skimmer CAD results in a much faster transition to the ion internal energy range dominated by the direct cleavages, thus keeping hydrogen scrambling at a minimum. Even though the energy of the ion−neutral collisions in the ESI interface under the typical conditions of the nozzle-skimmer CAD are also in the eV range, the collisions occur much more frequently compared to SORI CAD due to significantly higher pressure, hence, a much lesser role of the radiative cooling. The energetics of nozzle-skimmer CAD is similar to ion fragmentation in triple quadrupole mass spectrometers (the so-called *eV multiple collision* regime,[23] with the typical ion activation times on the order of 10^{-4}−10^{-5} s[23]). On the other hand, the characteristic ion activation times in SORI CAD are several orders of magnitude above this range, typically 0.1−1 s.[23]

Influence of Protein Structure in the Gas Phase on the Extent of Hydrogen Scrambling. As noted above, the use of SORI CAD does not lead to a total random redistribution of the deuterium content of CI2 ions. In fact, some useful information on the protection of certain segments could be deduced from such experiments. It was hypothesized earlier that the extent of hydrogen scrambling can be limited by formation of a more or less rigid structure in the gas phase.[10,14] Lack of protein ion flexibility would hinder transient formation of random contacts between various protein sites. Such contacts can be important facilitators of proton mobility, as they lower the energy barriers for proton (or deutron) transitions. In fact, significant flexibility of the polypeptide ion backbone in the gas phase is required for the long-distance intramolecular proton transfer, which follows a mechanism similar to that presented in Figure 5.

Protonated polypeptides lacking stable native structures in solution usually display significant conformational freedom in the gas phase even at moderate temperatures.[40] At the same time, a large amount of experimental evidence has been accumulated in recent years, supporting the notion that protein ions in the gas phase largely retain their nativelike solution-phase structure, particularly if hydrogen bonding and other electrostatic interactions play a major role in stabilizing the latter.[25] Retention of solution conformation by polypeptide ions has been demonstrated by measuring kinetic energy release,[41] proton-transfer reactivity,[42] H/D exchange kinetics,[43] and ion mobility[44,45] of protein and peptide ions in the gas phase. In the experiments discussed in the previous section, both HDX and protein desorption were carried out under near-native conditions (pH 6.8). Furthermore, CI2 in its native state possesses a significant number of hydrogen bonds,[27] interactions that often survive transition from solution to the gas phase. Therefore, it is reasonable to assume that a significant proportion of these structural elements are preserved in the gas phase conformation of the protein. This would certainly decrease flexibility of the polypeptide chain in the gas phase, thereby decreasing the extent of scrambling. It is therefore conceivable that the limited character of hydrogen scrambling observed with SORI CAD is due to the partial retention of the native structure by the protein in the gas phase.

To explore the relationship between the proton mobility and the protein ion flexibility in the gas phase, the protein structure must be disrupted prior to ESI MS analysis without affecting the outcome of the HDX measurements. This can be achieved in many cases by quenching the exchange reactions, i.e., by acidification of protein solution (to pH 2.5−3) and decrease in its temperature.[29] These conditions, however, would not work in the present case, as CI2 is extremely stable to acidic denaturation and only a small fraction of the protein unfolds at pH 3.[46] At the same time, a much more significant proportion of the CI2 molecules can be unfolded if solution acidification is accompanied by the addition of alcohol.[46] In this work, alcohol was added to an ice-cold quenched (acidic) CI2 solution just prior to the "off-line" HDX CAD MS measurements. Addition of even large quantities of methanol to the protein solution under these conditions did not alter the total deuterium content of the intact CI2 ions, as verified by ESI MS measurements.

Interestingly, the disruption of the protein higher order structure prior to its transition to the gas phase and fragmentation had virtually no effect on the backbone protection pattern obtained with the dissociation induced by rapid collisional heating, i.e., by nozzle-skimmer CAD. Both the protection pattern (Figure 3C) and the exchange kinetics of key internal segments (data not shown) were nearly identical to those observed without the quenching/protein denaturation step. The situation, however, is dramatically different when the local deuterium content of structurally compromised CI2 ions is assessed by SORI CAD. The protection pattern (Figure 3D) now matches the perfect scrambling curve, while showing only a weak correlation with the "NMR protection" curve.

Extensive hydrogen scrambling observed in this case, as well as the fact that the extent of scrambling was much less when SORI CAD, was used to fragment (partially) structured protein ions and provide clear experimental evidence that the protein ion gas phase structure plays an important role in governing proton mobility. Lack of chain flexibility hinders intramolecular proton transitions, thereby limiting scrambling even under conditions that typically favor internal rearrangements in the gas phase. On the other hand, gas-phase structure plays an insignificant role when higher collision energy favors direct cleavages of peptide bonds. As a result, chain flexibility of CI2 ions in the gas phase appears to be irrelevant as far as determining the extent of hydrogen scrambling when higher energy collisions are used to induce protein ion dissociation.

Energetics and Structure as Determinants of Scrambling in Other Studies. The present study appears to reconcile various seemingly different accounts on the occurrence and extent of hydrogen scrambling in the gas phase, as reported by several groups throughout the past several years.[8,10,14,16,19−21] A very significant level of scrambling reported by McLafferty[16] was likely caused by the slow activation rate of protein ions when ion fragmentation was carried out using SORI CAD. At the same time, the internal proton rearrangements were not totally random in McLafferty's work, consistent with the notion of a

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"residual" gas-phase structure of protein ions being an important limiting factor in the extent of hydrogen scrambling. Likewise, Heck's recent observation that short model peptide ions are less prone to scrambling when cationized with alkali metals, as opposed to simple protonation,[21] also seems to confirm that reduced flexibility of polypeptide ions in the gas phase is crucial for controlling the extent of scrambling under conditions that otherwise favor increased proton mobility, i.e., low collision energy. Indeed, measurements of ion mobility in the gas phase suggest that a common structural motif of small flexible chains, synthetic polymers and peptides alike, is charge solvation in the interior of the molecule by electron rich functional groups.[26] In the case of poly-Ala molecules,[21] the only electron-rich functional groups are backbone amides, whose flexibility should be greatly reduced once they form a solvation shell around large metal cations.

Smith[19] and Deinzer[20] observed deviations of site-specific protection data, at the level of individual amides, measured by HDX CAD MS from the results of the HDX NMR experiments. An explanation for such behavior invoked proton transfer in a charge-directed local rearrangement reaction leading to a peptide bond cleavage, a notion that agrees well with the anomalous behavior of two fragment ions of CI2 that are produced by the Asp-Arg and Asp-Lys amide bond cleavages. Although such processes are local and do not exert great influence over the deuterium content of segments spanning several residues,[10] they do seem to set a limit as to the spatial resolution that can be achieved in HDX CAD MS experiments.

The present work demonstrates that dynamic behavior of protein segments representing its structural elements can be adequately characterized using high-energy ion fragmentation of protein ions in the gas phase, while attempts to reach better resolution, down to the residue level, may sometimes result in artifacts. Despite such limitations, the "top-down" approach to HDX MS measurements[14] is useful for studies of protein dynamics. Since it does not require the quenching step to be a part of the experimental procedure, the method can be readily applied to study dynamics in the systems that aggregate and/or precipitate under the slow exchange conditions and, therefore, are not amenable to site-specific HDX MS measurements that utilize the traditional "bottom-up" approach. In a situation when both approaches can be used, the information provided by the two methods will not be redundant, since the peptic fragmentation pattern is different from the ion fragmentation pattern in the gas phase, leading to increased spatial resolution in mapping protein dynamics.

Conclusions

The results of the present work clearly indicate that the extent of hydrogen scrambling by internal long-distance proton exchange within multiply charged protein ions is controlled by both collision energy and the protein ion gas-phase structure. HDX MS measurements aided by the protein ion dissociation under rapid collisional heating conditions provide information on the backbone protection of protein segments, i.e., individual structural elements spanning several amino acid residues, that is in good agreement with the HDX NMR data. An increase in the protein ion conformational freedom in the gas phase, caused by protein denaturation prior to its desorption, does not lead to any noticeable increase in intramolecular hydrogen exchange as long as the rate of collisional activation remains high. In contrast, utilization of a slow heating technique to fragment protein ions results in detectable, but not totally random, scrambling when the protein higher order structure is partially maintained in the gas phase. The extent of scrambling increases dramatically upon increase in protein ion chain flexibility in the gas phase, resulting in totally random redistribution of labile hydrogen atoms within the protein. Thus, the rate of ion activation appears to be the major determinant of the extent of scrambling, although diminished flexibility of the polypeptide chain in the gas phase can limit the extent of scrambling even if it is favored energetically.

The results presented in this work provide a basis for further development of experimental HDX MS strategies and provide realistic limits as far as the spatial resolution that can be achieved by localizing labile deuterium atoms within polypeptides using the "top-down" HDX MS strategies. Our observation that conformational freedom is a prerequisite for efficient intramolecular proton transitions in the solvent-free environment may also hint at the importance of structural dynamics in charge-transfer processes occurring in aprotic nonaqueous environments, such as in a lipohilic membrane environment.

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