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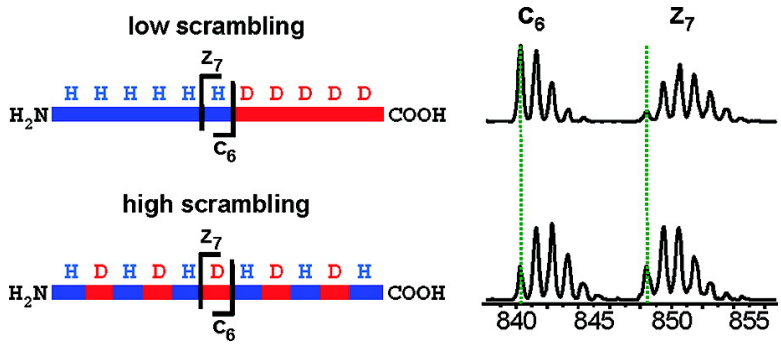

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Electron Transfer Dissociation Facilitates the Measurement of Deuterium Incorporation into Selectively Labeled Peptides with Single Residue Resolution

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Abstract: Mass spectrometry is routinely applied to measure the incorporation of deuterium into proteins and peptides. The exchange of labile, heteroatom-bound hydrogens is mainly used to probe the structural dynamics of proteins in solution, e.g., by hydrogen-exchange mass spectrometry, but also to study the gas-phase structure and fragmentation mechanisms of polypeptide ions. Despite considerable effort in recent years, there is no widely established mass spectrometric method to localize the incorporated deuterium to single amino acid residues, and typically, only the overall deuterium content of peptides or proteins is obtained. The main reason for this is that CID and related techniques induce intramolecular migration of hydrogens ("hydrogen scrambling") upon vibrational excitation of the even-electron precursor ion, thus randomizing the positional distribution of the incorporated deuterium atoms before fragmentation. In contrast, decomposition of radical gas-phase peptide cations upon electron capture dissociation was recently demonstrated to proceed with a very low level of amide hydrogen scrambling. Employing model peptides developed to enable sensitive detection of hydrogen scrambling, we show in the present study that electron transfer dissociation in a 3D-quadrupole ion trap retains the site-specific solution-phase deuterium incorporation pattern and allows for localization of incorporated deuterium with single residue resolution. Furthermore, we exploit this finding to monitor how collisional activation induces proton mobility in a gaseous peptide ion at various levels of vibrational excitation.

Introduction

Hydrogen isotope exchange is a renowned tool to study the structure of molecules that possess labile, heteroatom-bound hydrogens.[1] The determination of the solution-phase deuterium incorporation into proteins is of particular interest in structural biology. The exchange rates of the backbone amide groups in a folded protein can span a range of several orders of magnitude and reflect the protein structure and structural dynamics. The hydrogen exchange of proteins is mainly measured by NMR and mass spectrometry (MS). Whereas MS is generally considered to be more sensitive and able to deal with very large protein assemblies and more complex sample mixtures, NMR has the advantage that it readily yields site-specific exchange rates for some, though typically not all, backbone amide groups.[2−5]

The most established hydrogen exchange mass spectrometry (HX-MS) approach involves incubation of the protein of interest in deuterated buffer (i.e., an "exchange-in" experiment). After defined time intervals, aliquots are harvested and the amide hydrogen exchange reaction is quenched by cooling (0 °C) and acidification (pH ∼2.5). The mass spectrometric analysis at this stage provides the overall deuterium content of the whole protein (i.e., a "global exchange analysis"). To localize the incorporated deuterium to different segments of the protein, it is enzymatically cleaved by pepsin under quench conditions. The resulting peptic peptides are then typically analyzed by reversed-phase chromatography coupled online to an electrospray ionization (ESI) mass spectrometer (i.e., a "local exchange analysis").[3−6] The obtainable resolution of this approach is limited by the size and degree of overlap of the peptic peptides. In most cases, only a few site-specific hydrogen exchange rates at individual amino acid residues can be determined in this manner.[4,5]

When performing hydrogen exchange in the gas phase, MS can also be applied to probe the gas-phase structure of peptide and protein ions.[7,8] Furthermore, deuterium labels are used to trace proton transfer and proton mobility upon gas-phase fragmentation of polypeptide ions.[9−13] Clearly, an MS technique that would allow for localization of labile deuterons to single

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amino acid residues would make all these approaches significantly more powerful.

The measurement of the deuterium content of fragment ions generated by collision-induced dissociation (CID) of peptide or protein ions seems to be a straightforward approach to increase the resolution of HX-MS, and this strategy has been applied several times.[14−20] However, multiple studies have revealed extensive intramolecular gas-phase migration of exchangeable hydrogens (i.e., "hydrogen scrambling") within peptide and protein ions upon CID, thus questioning the general applicability of this approach.[8−12,19−25] In contrast, electron capture dissociation (ECD) of gas-phase peptide cations was recently demonstrated to proceed with a very low level of amide hydrogen scrambling.[26]

In ECD, multiply protonated peptides or proteins are reacted with near-thermal electrons to yield hydrogen abundant radical cations that can undergo decomposition to c- and z-type fragment ions.[27] The mechanism of the peptide backbone $N-C_\alpha$ bond cleavage upon electron capture is not fully understood. Different mechanisms are currently proposed, which, however, have in common that the reactive intermediate state is either an aminoketyl radical or an amide anion radical.[27−37] A very important, yet unanswered question is whether the backbone $N-C_\alpha$ bond cleavage is an ergodic or a nonergodic process.[27−32,38] A nonergodic mechanism, as originally proposed by Zubarev et al., can nicely explain the lack of hydrogen scrambling upon ECD without having to consider whether the threshold energy for proton mobilization is actually reached with this technique.[27] However, recent ab initio calculations have shown that the energy barrier for the $N-C_\alpha$ bond cleavage in the aminoketyl radical is so low that dissociation can be expected to occur even after intramolecular vibrational energy redistribution (IVR) of the recombination energy.[30] In the case of an ergodic mechanism, the observed lack of hydrogen scrambling indicates that either the internal energy after IVR is too low to enable hydrogen scrambling or the $N-C_\alpha$ bond cleavage is kinetically favored compared to hydrogen scrambling.

Electron transfer dissociation (ETD) is a recently developed fragmentation technique that also yields c- and z-type fragment ions.[39] This method utilizes the transfer of an electron from a radical molecular anion to multiply protonated peptides or proteins to produce radical cations. The subsequent peptide backbone $N-C_\alpha$ bond cleavage is assumed to proceed by the same mechanisms as discussed for ECD.[35] The main advantage of ETD is that ion/ion reactions can be performed in a variety of standard instrument types that use radio frequency (RF) oscillating electric fields to trap ions.[39−41] Efficient ECD requires static magnetic and electric fields and is nearly exclusively implemented in the very expensive Fourier transform ion cyclotron resonance mass spectrometers (FT-ICR-MS).[29] No systematic comparison between ETD and ECD has been performed so far, and there are significant differences between these two techniques. The major difference is that the recombination energy upon electron transfer is significantly lower compared to the recombination energy upon capture of a free thermal electron.[36,42] In addition, ETD is typically performed at pressures several orders of magnitudes higher than those used for ECD in an FT-ICR-MS. This facilitates efficient collisional cooling throughout the whole ion/ion reaction time.[43] The internal energies of the hydrogen abundant radical ions produced by either electron transfer or electron capture are thus significantly different, which might affect the branching ratios of different gas-phase reaction processes, such as backbone cleavage, proton/hydrogen migration, and dissociation of noncovalently associated fragment ions. Considering the current level of understanding of the underlying mechanism, it is thus not possible a priori to predict whether ETD in an ion trap yields fragment ions whose deuteration levels reflect the solution-phase deuterium incorporation pattern. In the present study, we have utilized peptides with unique selective labeling to demonstrate that ETD proceeds with a very low level of hydrogen scrambling

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provided that collisional activation prior to the ETD event is carefully minimized.

Experimental Section

Mass Spectrometry. Model peptides with the sequences HH-HHHHIIKIIK (P1), HHHHHHIITIIT (P2), HHHHHHIIIIII (P3), HHHHHHPPPPPPIIKIIK (P4), KKDDDDDDIIKIIK (P5), and RRDDDDDDIITIITR (P6) were obtained from Genscript Corp. (Piscataway, NJ). The deuterium labeling and direct infusion procedure are described in detail elsewhere.[26,44] Briefly, regioselective labeling of peptides P1 and P2 was achieved by a 50-fold dilution of the fully deuterated peptide (100 μM in 99.9% D_2O) into precooled methanol/0.5 M acetic acid (pH 2.5) 1:1 (v/v) followed by short mixing and immediate freezing either in liquid nitrogen or on dry ice. The samples were thawed immediately before measurement and directly infused into the ESI source through a short peek tubing (~13 cm length, 0.004" ID) at a flow rate of 300 μL h^{-1} with a syringe pump (kdScientific, Holliston, MA). The syringe was cooled with dry ice throughout the measurement. All mass spectra were obtained on a 3D quadrupole ion trap instrument equipped with an orthogonal ESI source and a separate chemical ionization source to produce the fluoranthene radical anions required for ETD (Agilent 6340 Ion Trap, Agilent Technologies, Santa Clara, CA). A detailed list of relevant instrumental settings is provided as Supporting Information.

Data Analysis. The mass spectra were processed with the DataAnalysis software (Agilent Technologies), and mass lists were exported to Excel (Microsoft, Redmond, WA). Average masses of the obtained fragment ions and charge reduced species were calculated as the intensity-weighted average of the masses of the isotopes. The mass increase of fragment ions obtained from labeled peptides (Δm_{exp}) was calculated relative to corresponding fragment ion masses of the nonlabeled peptide, which were recorded on every measurement day to compensate for fluctuations in the calibration. For each individual mass spectrum and fragment ion, theoretical mass differences for the limiting cases of 0% [eq 1] and 100% scrambling [eq 2] were calculated

$$\Delta m_{0\%} = (n_{txf} - n_{sxf}) \times \Delta m_{equ} + n_{sxf} / n_{sxp} \times [\Delta m_{precursor} - (n_{txp} - n_{sxp}) \times \Delta m_{equ}] \quad (1)$$

$$\Delta m_{100\%} = n_{txf} / n_{txp} \times \Delta m_{precursor} \quad (2)$$

where n_{txp} and n_{txf} is the total number of exchangeable (i.e., N- and O-bound) hydrogens in the precursor and given fragment ion, respectively, n_{sxp} and n_{sxf} is the number of slow exchanging hydrogens in the precursor and given fragment ion, respectively, Δm_{equ} is the mass increase per exchangeable hydrogen after equilibration of the peptide in the spray solvent (experimentally determined to be 0.029 ± 0.001 Da), and $\Delta m_{precursor}$ is the experimentally determined mass difference between the labeled and the nonlabeled precursor ion. $\Delta m_{precursor}$ was determined from the highly abundant charge-reduced molecular ions that are formed by proton transfer from the peptide cation to the ETD reagent anion and by nonproductive electron transfer (i.e., electron transfer that does not result in peptide dissociation).

The level of scrambling (F_{scr}) was calculated according to eq 3.

$$F_{scr} = (\Delta m_{exp} - \Delta m_{0\%}) / (\Delta m_{100\%} - \Delta m_{0\%}) \times 100 \quad (3)$$

Each reported scrambling level (F_{scr}) represents the average ± the standard deviation of at least 10 values, i.e., five values per measurement obtained from the ions c_2' to c_6' and at least two (typically three) independent measurements.

Results

Measuring Gas-Phase Hydrogen Scrambling. In the present study, we investigate whether ETD in a 3D-quadrupole ion trap (QIT) allows for gas-phase fragmentation of peptides while retaining the site-specific solution-phase deuterium incorporation pattern. To enable sensitive detection of hydrogen scrambling, we have employed our recently developed model peptide with the sequence HHHHHHIIKIIK (P1).[44] When the fully deuterated peptide is diluted into cooled acidic ^1H_2O solution, only the amides in the C-terminal half retain their deuterium, while those in the N-terminal half undergo very rapid back-exchange. This selective labeling pattern will be distorted upon the occurrence of gas-phase hydrogen scrambling in the mass spectrometer.

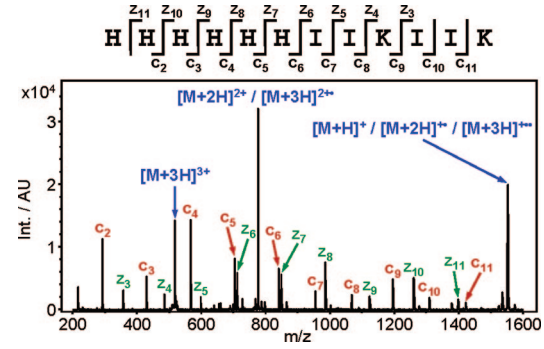

Figure 1. ETD MS/MS of the $[M+3H]^{3+}$ ion of fully protonated peptide P1 (HHHHHHIIKIIK) yields an extensive series of c and z fragment ions. Settings optimized for low scrambling ("Cap. Exit" 100 V; "Oct. One DC" 6 V) have been used to record this mass spectrum.

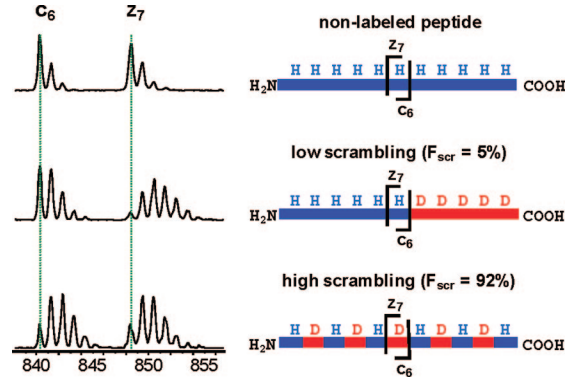

Figure 2. Hydrogen scrambling in the selectively labeled model peptide P1 leads to migration of deuterons from the C-terminal half to the N-terminal half. This is readily detected by examining the isotopic pattern of ETD fragment ions. The extensive level of scrambling in the lowest spectrum was induced by collisional activation in the ion trap with an RF amplitude of 0.8 V before performing ETD.

ETD of the triply protonated peptide P1 yields an extensive series of c and z fragment ions that serve as scrambling probes (Figure 1). In the absence of scrambling, fragment ions that contain only fast exchanging backbone amides (e.g., c_6') contain only a very low amount of deuterium (Figure 2). Accordingly, the majority of deuterium is localized in fragment ions that contain all of the slow exchanging backbone amides (e.g., $z_7^{\bullet}$). Upon mobilization of the backbone amide hydrogens, deuterons are transferred from the C-terminal to the N-terminal half of the peptide. This hydrogen scrambling results in a mass increase of c-type fragment ions and a concomitant mass decrease of z-type fragment ions (Figure 2).

For each fragment ion, the level of scrambling (F_{scr}) was quantitatively determined by comparing its deuterium content with the theoretical values for the two limiting cases of no (F_{scr} = 0%) and complete (F_{scr} = 100%) scrambling. In the 100% scrambling model, the deuterium is equally distributed over all

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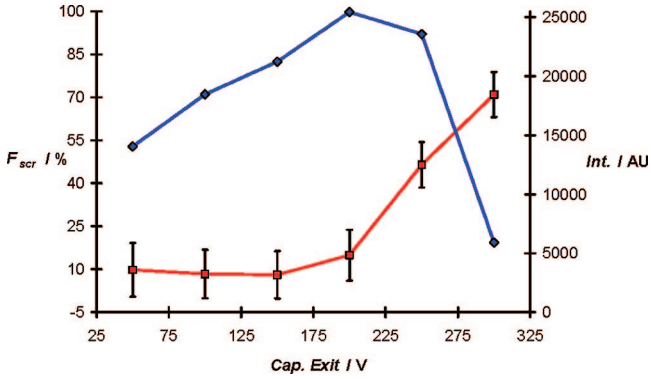

Figure 3. Effect of varying an ion source declustering potential ("Cap. Exit") on the level of scrambling (F_{scr}) as measured by ETD MS/MS (red line). The ion transmission was measured by the intensity of the charge-reduced peptide ion (blue line, Int.).

exchangeable sites (i.e., N- and O-bound hydrogens). In our 0% scrambling model, the majority of the incorporated deuterium is equally distributed over the five backbone amides in the C-terminal half of the peptide (i.e., due to the lack of independent reference data, all slow exchanging backbone amides are assumed to exchange with the same rate).[45] All results shown in the next paragraph were obtained using the $[M + 3H]^{3+}$ ion of peptide P1 as a precursor ion for ETD.

Avoiding Hydrogen Scrambling before ETD. The analysis of the deuterium content of the fragment ions revealed that the level of scrambling varies strongly with the extent of collisional activation prior to ETD. The electrospray process yields highly solvated peptide ions that must be desolvated before they enter the mass analyzer. This desolvation is achieved in the intermediate pressure region of the ion source by collisions with neutral gas molecules. The energy of these collisions depends on the kinetic energy of the peptide ions, which in turn is controlled by potential differences (ΔV) between the ion optic elements in the ion source. The magnitude of these "declustering potentials" determines not only the efficiency of the desolvation but also the internal energy of the peptide ions.[46] Accordingly, the first declustering potential ("Cap. Exit", i.e. the potential difference between the transfer capillary and the first skimmer) was found to be critical for the onset of scrambling (Figure 3). A dramatic increase in the level of scrambling was observed above 200 V. Similarly, a DC voltage above 9 V applied to the first octopole ("Oct. One DC"), which is situated in the second vacuum stage, also induces a considerable level of scrambling (Figure S1A in the Supporting Information). In addition, a moderate increase in scrambling due to sideband activation was observed when applying a very narrow precursor ion isolation width (Figure S1B in the Supporting Information). All other tested instrumental parameters were found to have no or only minor influence on the level of scrambling when varied across a meaningful range and were thus optimized for maximum ion transmission.

Since the above-mentioned effects are additive, nearly 100% scrambling was observed with "harsh" ion source settings ("Cap. Exit" 250 V; "Oct. One DC" 15 V). Under optimized conditions ("Cap. Exit" 50 V; "Oct. One DC" 6 V), the deuterium content of small c fragment ions resembled closely the 0% model

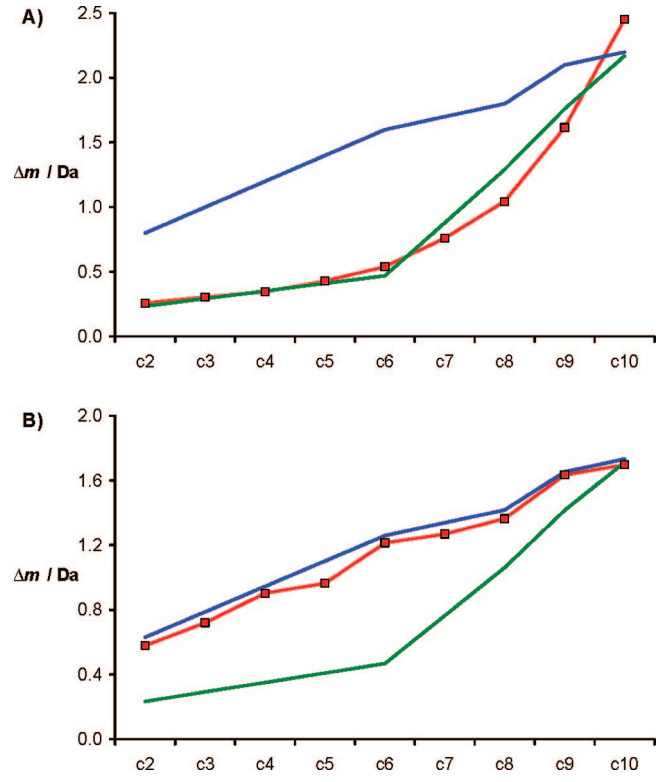

Figure 4. Deuterium content of the fragment ions $c_2'-c_{10}'$ obtained by ETD of selectively labeled peptide P1 (red line) at (A) gentle and (B) harsh declustering conditions in the electrospray ion source. The theoretical deuterium contents for 0% (green line) and 100% scrambling (blue line) are also shown. Nearly complete scrambling occurs upon collisional activation at harsh conditions (B) whereas scrambling is reduced to a negligible level at gentle conditions (A). The precursor ion contained (A) 2.7 and (B) 2.1 deuterons.

(Figure 4). The deviations from the 0% model in the case of the large c ions ($c_7'-c_{10}'$), giving rise to formally negative values for F_{scr}, originate most probably from different intrinsic exchange rates of the five slow-exchanging amide hydrogens that we were not able to implement in our current model.[44] For that reason, all scrambling levels reported herein are calculated from the series of $c_2'-c_6'$ ions, which provided consistent and accurate scrambling levels (Figure S2 in the Supporting Information).

Alternatively, we could have also used the high mass z-type fragment ions that contain all five slow-exchanging amide hydrogens ($z_6^{\bullet}-z_{11}^{\bullet}$), but the use of some of these ions was hampered by peak overlapping (e.g., $z_6^{\bullet}$) or comparably low ion statistics (e.g., $z_9^{\bullet}$ and $z_{11}^{\bullet}$). However, the levels of scrambling obtained from the other $z^{\bullet}$ ions produced by ETD of our model peptides were consistent with the ones obtained from the c′ ions (Figure S3 in the Supporting Information).

The above-mentioned results, in agreement with previous data, clearly show that the selectively labeled peptide serves as reporter ion that records the history of the internal energy of the gaseous ion in the form of a distorted deuterium-incorporation pattern.[26] The c and z fragment ions preserve this information and act as sensitive probes for the level of scrambling. By virtue of this reporter system, we have minimized the degree of collisional activation in the ion source and reduced the extent of scrambling to a negligible level. Since the design of the electrospray source has a large impact on the internal energy buildup of the analyte ions, it is clear that each type of mass spectrometer must be individually optimized for

(45) The 0% scrambling model contains a correction term to account for the small amount of deuterium incorporated into fast-exchanging sites due to the residual D_2O in the spray solvent.

(46) Gabelica, V.; De Pauw, E. *Mass Spectrom. Rev.* **2005**, *24*, 566–587.

low scrambling conditions.[46] If the instrumental settings are left unchecked then scrambling is likely to prevail.

ETD-MS of Other Model Peptides. To test the generality of our findings, we have performed ETD-MS on a series of other selectively labeled model peptides (Figure S4 in the Supporting Information). In the case of peptides P2 and P3, the lysine residues of peptide P1 were replaced by threonine or isoleucine, respectively (HHHHHHIIXIIX, where X is K in P1, T in P2, and I in P3). These two peptides retained more deuterium than P1 and also showed a more uniform labeling of the slow-exchanging C-terminal peptide half. Accordingly, the deviations from the 0% model were less pronounced. In peptide P4 we have inserted a stretch of six prolines between the fast and slow exchanging part of peptide P1 (HHHHHHPPPPPPIIKIIK), and in peptide P5 we have replaced the fast-exchanging hexahistidine stretch by the also fast-exchanging dilysine-hexaaspartic acid stretch. In contrast to the His-Ile backbone amide in P1−P3, the Pro-Ile and Asp-Ile backbone amides retained their deuterium and the ETD data showed a good match to the corresponding 0% model with six slow exchanging amide bonds in the C-terminal half. Finally, peptide P6 (RRDDDDDDDIITIITR) was designed to test the effect of arginines as charge carriers. In addition to the deuterium retained on the seven slow-exchanging amides in the C-terminal peptide half, a low amount of deuterium was also found to be localized on the two N-terminal arginines, presumably due to incomplete back-exchange of the side-chain guanidinium group. Clearly, for all these model peptides, ETD-MS proceeds without significant hydrogen scrambling and allows the localization of the incorporated deuterium at a resolution of one or two amino acid residues.

Testing the Mobile Proton Model of Collision-Induced Dissociation. In addition to being an obstacle for the localization of exchangeable deuterium, the proton mobility in peptide ions is of great interest for the understanding of the CID mechanism. According to the "mobile proton model", charge-directed cleavage of a peptide bond (yielding, e.g., b- and y-fragments) requires the migration of a proton from a site with higher gas-phase basicity to a backbone amide nitrogen.[47,48] This intramolecular proton-transfer reaction is facilitated by the vibrational excitation that results from collisional activation. However, the transfer of a proton to an amide nitrogen not only weakens the amide bond but also initiates an intramolecular interchange of the labile hydrogens (i.e., hydrogen scrambling). The occurrence of multiple reversible proton-transfer reactions involving all exchangeable sites is thus responsible for the complete positional randomization of exchangeable hydrogens in a "hot" precursor ion.[9−11,19,22] Consequently, the occurrence of hydrogen scrambling has been interpreted as a verification of the "mobile proton model".[9−11,48]

Previous systematic investigations of the phenomenon of hydrogen scrambling were based on measuring the deuterium content of CID-generated fragment ions, and naturally, information on the level of scrambling at activation energies lower than the dissociation energy was impossible to obtain. To fill this gap, we have utilized the multistage capability of the QIT to perform controlled RF-induced collisional activation of the $[M + 3H]^{3+}$ ion of P1 followed by analysis of the resulting deuterium distribution in this ion by ETD. In this experiment the precursor ion was transferred to the ion trap under "low scrambling conditions", isolated, subjected to collisional activation for 40 ms, isolated again, and finally fragmented by ETD. The level of scrambling as a function of the dipolar amplitude of the RF excitation field ("Frag. Ampl.") was compared to the abundance of the b_2 ion produced by CID under identical conditions (Figure 5). The results clearly show that the onset of scrambling is observed at much lower fragmentation amplitudes than the onset of peptide dissociation. Thus, the internal energy required for intramolecular hydrogen migration is significantly lower than the one required for the formation of the b_2 ion, which is one of the lowest-energy fragmentation channels in CID of our model peptide.

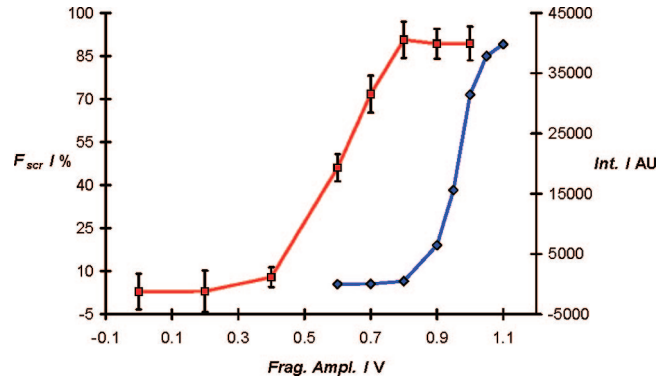

Figure 5. Mobilization of backbone amide hydrogens occurs at significantly lower internal energies than peptide fragmentation. Hydrogen scrambling (red line, F_{scr}) upon RF-induced collisional activation was monitored by ETD of the surviving intact precursor ion and compared to the intensity of the b_2 ion produced by CID under identical conditions (blue line, Int.).

Discussion

Proton Mobility in CID. The currently established frameworks to explain the unimolecular decomposition of protonated even-electron peptides upon CID are the "mobile proton" and the "pathways in competition" models.[47,48] According to these models, fragmentation of the peptide backbone proceeds via an endothermic intramolecular transfer of a proton to the backbone amide nitrogen. Protonation on the amide nitrogen causes a considerable weakening of the amide bond. A variety of peptide fragmentation pathways, jointly referred to as "charge-directed", are based on the mobility of the added protons to initiate backbone cleavage.

Hydrogen scrambling, which can be seen as an intramolecular gas-phase exchange of the labile hydrogens, is also explained as a direct effect of proton mobility. The considerable number of studies that have clearly proven the occurrence of hydrogen scrambling in ESI- and MALDI-generated peptide and protein ions upon CID[8−12,19−25] have therefore been interpreted as evidence for the validity of the "mobile proton" model.[48] The "mobile proton" model was also tested by theoretical quantum chemical techniques, which can, however, only be applied to relatively small model peptides. For the investigated systems (*N*-formylglycinamide, glycylglycine, *N*-formylglycylglycinamide, lysylglycine, and glycylglycylglycine), rather low energy barriers were found between the different protonation sites.[48−52] Consequently, these studies predict that the proton transfer between the protonation sites proceeds very fast even at internal energies well below the dissociation threshold energy.[48−52]

Our experimental setup, involving controlled collisional activation of deuterated model peptides followed by detection and quantification of the extent of hydrogen scrambling, is, to

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the best of our knowledge, the first strategy shown to provide energy-resolved data on hydrogen scrambling. Considering the rather long ion activation times (40 ms) and that kinetic shift effects should be low under slow heating conditions,[53] our data show a large difference between the threshold energy for the onset of amide hydrogen scrambling and the fragmentation threshold energy. In the case of our model peptide, the entire precursor ion population has undergone complete hydrogen scrambling at collision energies required for backbone fragmentation to be observed.

Proton/Hydrogen Mobility in ETD and ECD. In the case of ETD and ECD, proton and hydrogen transfer reactions might occur before, during, and after the peptide backbone $N-C_\alpha$ bond cleavage. We have shown here, using a 3D QIT instrument, and in a previous study, using a hybrid linear ion trap/FT-ICR-MS,[26] that collisional activation upon desolvation in the intermediate pressure region of the ion source and upon precursor ion isolation must be carefully controlled to avoid hydrogen scrambling *before* ETD and ECD. In the case of the QIT instrument, settings could be found that cause very low levels of hydrogen scrambling while still providing the sensitivity and selectivity required for typical hydrogen exchange experiments. However, each type of mass spectrometer must be individually optimized for low scrambling, and depending on the instrument design, more or less sensitivity and selectivity might have to be sacrificed compared to "standard settings".

Intermolecular proton transfer reactions during ion/ion reactions are a potential source of hydrogen scrambling that is specific to ETD. Proton transfer from the peptide cation to the reagent anion occurs as a competitive process to electron transfer from the anion to the cation. To our knowledge, it has not yet been investigated whether intermolecular proton transfer reactions can trigger hydrogen scrambling in the charge-reduced peptide ions, but this scenario is not unlikely considering that proton transfer is usually more exothermic than electron transfer.[42,54] Similarly, hydrogen radical loss from peptide ions upon electron capture was suggested to result in sufficient vibrational excitation to yield b-type fragment ions via the "mobile proton" pathway.[55,56] However, since proton transfer reactions do not result in backbone cleavage,[42] they could only interfere with the localization of deuterium if a proton transfer event is followed by a dissociative electron transfer event. The probability for such a sequential process is certainly small for triply protonated peptide ions but might become relevant for higher charge states typically observed in larger peptide and protein ions.[34]

Our current and previous results clearly demonstrate that the $N-C_\alpha$ bond cleavage upon ETD and ECD proceeds without backbone amide hydrogen transfer reactions. This implies either that the $N-C_\alpha$ bond cleavage proceeds much faster than hydrogen scrambling (but not necessarily faster than IVR) or that the internal energy after IVR is below the threshold energy for hydrogen scrambling. Our results also match the fact that none of the currently proposed mechanisms for the radical-induced backbone $N-C_\alpha$ bond cleavage involves any hydrogen or proton transfer to the amide nitrogen before backbone cleavage. One mechanism assumes that the electron is initially attached to a protonation site (usually a basic side chain or the N-terminal amine). The $N-C_\alpha$ bond cleavage can then proceed via an intermediate aminoketyl radical that is formed by an intramolecular hydrogen atom transfer to a proximate amide carbonyl oxygen.[27−35] Alternatively, direct electron attachment to the amide carbonyl was suggested. The thus formed "superbasic" amide anion radical might exothermically abstract a proton from any protonated side chain to form a labile aminoketyl radical.[32] Clearly, the possibility of such proton or hydrogen transfer reactions must be considered when utilizing ETD or ECD in the analysis of peptides ions that are deuterated on the side chains. It should also be mentioned that other mechanisms have been suggested that do not necessarily involve any hydrogen or proton transfer to the cleavage site. These mechanisms assume Coulomb-stabilized direct electron attachment or through-bond electron transfer to an antibonding orbital of the amide bond followed by direct cleavage of the thus formed amide anion radical.[34−37] However, even in these cases it is very likely that the resulting negative charge on the c ions is neutralized by proton transfer.

Finally, one should be aware of processes that might cause hydrogen scrambling *after* the backbone $N-C_\alpha$ bond cleavage. It is well established that the c′ and z• fragments obtained by ETD and ECD can stay noncovalently associated in a hydrogen-bonded intermediate. The lifetime of these intermediate complexes is sufficiently long to facilitate hydrogen radical migration processes between the c′ and z• fragments to form, e.g., c• and z′ fragment ions.[30,43,57−59] So far, only C_α-groups and side chains have been found to donate hydrogen radicals, which obviously does not interfere with the localization of backbone amide deuterons but might interfere with the analysis of side-chain labeled peptides.[57,59] With our model peptides, interfragment hydrogen radical migration did not seem to contribute significantly to the ETD mass spectra of the triply protonated ions but is clearly observed upon ETD of quadruply protonated ions. Several singly charged z′ fragment ions were observed in high abundance in the latter case. The mass resolution of the ion trap was insufficient to separate the z′ from the ^{13}C isotopes of the corresponding z• ions. However, the deuterium content of the mixed singly charged z•/z′ fragment ions was indistinguishable from the deuterium content of the corresponding doubly charged z• ions, which did not show a significant contribution from hydrogen radical migration (data not shown). This indicates that hydrogen radical abstraction from amide nitrogens does not play a significant role in ETD.

In addition, proton mobility in vibrationally excited intermediate complexes has been supposed to contribute to higher levels of hydrogen scrambling upon ECD of doubly protonated peptide ions.[26] We have not analyzed the $[M + 2H]^{2+}$ ions in this study

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due to their very low abundance under the employed experimental conditions. However, the lower recombination energy upon ETD and the dramatically increased cooling efficiency in the ion trap can be expected to significantly decrease the risk of hydrogen scrambling by vibrational excitation in the intermediate complex.

HX-MS at Single Residue Resolution. Despite the fact that extensive hydrogen scrambling precludes the localization of incorporated deuterium in peptide and protein ions, several attempts have been made to use CID as an experimental tool to obtain single-residue deuterium levels. Surprisingly, a significant fraction of these studies have reported no or only low levels of hydrogen scrambling.[14−20] Different explanations have been put forward to resolve the discrepancy of these results with the above-mentioned experimental and theoretical findings. For instance, it has been suggested that hydrogen scrambling can be limited by the formation of sufficiently stable gas-phase structures that reduce the flexibility of the backbone chain upon activation.[20] Another recent finding might also serve to explain some of the apparent discrepancies regarding the localization of labile deuterium by CID. Ferguson et al. have recently shown that hydrogen scrambling upon CID does not necessarily lead to the previously expected uniform distribution of deuterons in a peptide and protein ions.[24,25] It cannot be excluded that such a nonuniform redistribution of the incorporated deuterons, which can be easily misinterpreted as retained regioselective labeling, might have occurred in previous studies.[60]

A detailed discussion of the existing CID results is outside the scope of this work, and we refer to recent articles regarding this topic.[5,19,20,22−25] The main conclusion that can be drawn after 20 years of research is that the occurrence of hydrogen scrambling complicates the localization of labile deuterons by CID to such an extent that no widely applicable and generally accepted CID-based method has been established so far.

Initial experiments indicating that ECD might facilitate backbone fragmentation of protein ions without inducing extensive hydrogen scrambling were reported in the pioneering ECD study by Zubarev et al. in 1998.[27] Despite this promising finding, only four studies have further investigated ECD as a tool to localize labile deuterium in a peptide or protein ion.[26,61−63] The reasons that ECD has so far not been used routinely as a method to obtain site specific deuterium levels of peptides and proteins are probably the comparably low accessibility of this fragmentation technique and that sensitive peptide probes, which enable the minimization of hydrogen scrambling prior to electron capture, were just recently developed.[26,44]

We show in the present study that ETD in a 3D-quadrupole ion trap retains the site-specific solution-phase deuterium incorporation pattern and allows for localization of incorporated deuterium with single residue resolution. ETD is readily implemented on a large variety of mass spectrometers, ranging from rather cheap benchtop ion traps to high-resolution hybrid instruments that facilitate top-down approaches.[39−41,43] Furthermore, ETD is easily performed on a chromatographic time scale.[39,40,43] Due to this high accessibility and flexibility, we are convinced that ETD will become a highly utilized method for the localization of deuterium atoms in peptide and protein ions.

Conclusions

Overall our results clearly demonstrate that ETD of multiply charged peptides can be performed without inducing significant hydrogen scrambling. Considering that this technique typically yields very high amino acid sequence coverage, even for large polypeptides, and can be implemented in a variety of instrument types, our results hold promise that ETD is well-suited to measure the deuterium distribution in polypeptides at single residue resolution. We have demonstrated that the combination of collisional activation with ETD in an ion trap experiment provides energy-resolved data on proton migration in peptide ions, thus showing that ETD of deuterium-labeled peptides is an excellent tool for fundamental studies of gas-phase reaction mechanisms. Furthermore, our findings will hopefully encourage further investigations to answer whether ETD can be routinely applied in the HX-MS workflow to accurately measure site-specific deuterium incorporation rates, thereby significantly improving the study of the structural dynamics of proteins.

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Supporting Information Available: Detailed experimental procedures; additional data for uniformly labeled peptide P1, quadruply protonated peptide P1, peptides P2−P6; further data regarding the validity of our theoretical models and the effect of instrumental parameters on the level of scrambling. This material is available free of charge via the Internet at http://pubs.acs.org.

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(60) We have excluded the occurence of a similar nonuniform redistribution during our measurements by perfoming ETD of the homogenously labeled model peptide P1 (see Figure S4 in the Supporting Information).

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