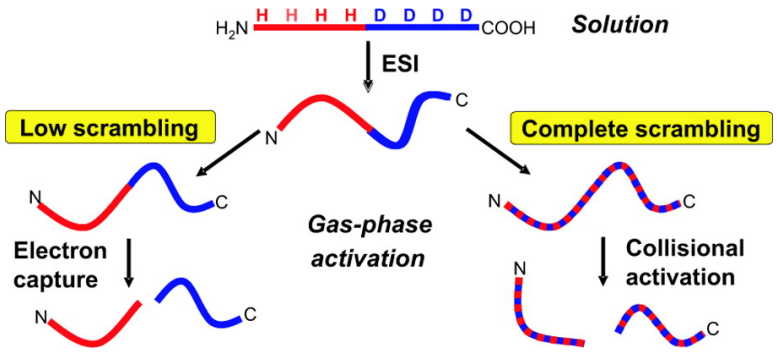
Article

Electron Capture Dissociation Proceeds with a Low Degree of Intramolecular Migration of Peptide Amide Hydrogens

Kasper D. Rand, Christopher M. Adams, Roman A. Zubarev, and Thomas J. D. Jrgensen

J. Am. Chem. Soc., **2008**, 130 (4), 1341-1349 • DOI: 10.1021/ja076448i



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 6 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

J|A|C|S
ARTICLES
Published on Web 01/03/2008

Electron Capture Dissociation Proceeds with a Low Degree of Intramolecular Migration of Peptide Amide Hydrogens

Kasper D. Rand,[†] Christopher M. Adams,[‡] Roman A. Zubarev,[‡] and Thomas J. D. Jørgensen*,[†]

Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark, and Laboratory of Biological & Medical Mass Spectrometry, Uppsala Biomedical Centrum, Husargatan 3, Box 583, SE-75 123 Uppsala, Sweden

Received August 27, 2007; E-mail: tjdj@bmb.sdu.dk

Abstract: Hydrogen (^{1}H/^{2}H) exchange combined with mass spectrometry (HX−MS) has become a recognized method for the analysis of protein structural dynamics. Presently, the incorporated deuterons are typically localized by enzymatic cleavage of the labeled proteins and single residue resolution is normally only obtained for a few residues. Determination of site-specific deuterium levels by gas-phase fragmentation in tandem mass spectrometers would greatly increase the applicability of the HX−MS method. The biggest obstacle in achieving this goal is the intramolecular hydrogen migration (i.e., hydrogen scrambling) that occurs during vibrational excitation of gas-phase ions. Unlike traditional collisional ion activation, electron capture dissociation (ECD) is not associated with substantial vibrational excitation. We investigated the extent of intramolecular backbone amide hydrogen (^{1}H/^{2}H) migration upon ECD using peptides with a unique selective deuterium incorporation. Our results show that only limited amide hydrogen migration occurs upon ECD, provided that vibrational excitation prior to the electron capture event is minimized. Peptide ions that are excessively vibrationally excited in the electrospray ion source by, e.g., high declustering potentials or during precursor ion selection (via sideband excitation) in the external linear quadrupole ion trap undergo nearly complete hydrogen (^{1}H/^{2}H) scrambling. Similarly, collision-induced dissociation (CID) in the external linear quadrupole ion trap results in complete or extensive hydrogen (^{1}H/^{2}H) scrambling. This precludes the use of CID as a method to obtain site-specific information from proteins that are labeled in solution-phase ^{1}H/^{2}H exchange experiments. In contrast, the deuteration levels of the c- and z-fragment ions generated from ECD closely mimic the known solution deuteration pattern of the selectively labeled peptides. This excellent correlation between the results obtained from gas phase and solution suggests that ECD holds great promise as a general method to obtain single residue resolution in proteins from solution ^{1}H/^{2}H exchange experiments.

Introduction

Monitoring the backbone amide hydrogen exchange rates in proteins has provided valuable insight into the conformational properties of the native state and it has long been an important tool in structural biology.[1−3] In recent years, mass spectrometric measurement of the time-resolved mass increase in proteins following solution ^{1}H/^{2}H exchange (HX−MS) has become a recognized method for the analysis of structural dynamics of proteins.[4−6] In a typical global hydrogen (^{1}H/^{2}H) exchange experiment, as introduced by Zhang et al.,[7] isotopic exchange is initiated by dilution of the protein of interest into deuterated buffer. The incorporation of deuterium with time is usually monitored by harvesting aliqouts where the isotopic exchange reaction is quenched by acidification and cooling. Subsequently, the deuterium content is determined by mass spectrometric analysis. Localization of incorporated deuterons is achieved by enzymatic cleavage of the labeled protein with pepsin, followed by reversed-phase chromatographic separation and mass spectrometric analysis of resulting peptic peptides. The residue resolution of this approach with respect to location of the deuterated sites is limited by the sizes of the peptic peptides. Single-residue resolution can typically only be obtained for a few residues in a protein.

At first glance, gas-phase fragmentation appears to be an attractive alternative to improve the residue resolution by tandem mass spectrometry. A central premise for this approach, however, is that the solution ^{1}H/^{2}H labeling pattern must be retained during gas-phase fragmentation. Several lines of theoretical and experimental evidence suggest that gas-phase fragmentation by collision-induced dissociation (CID) is ac-

[†] University of Southern Denmark.
[‡] Uppsala Biomedical Centrum.

(1) Hvidt, A.; Nielsen, S. O. *Adv. Protein Chem.* **1966**, *21*, 287−386.
(2) Englander, S. W.; Krishna, M. M. G. *Nat. Struct. Biol.* **2001**, *8*, 741−742.
(3) Englander, S. W.; Sosnick, T. R.; Englander, J. J.; Mayne, L. *Curr. Opin. Struct. Biol.* **1996**, *6*, 18−23.
(4) Hoofnagle, A. N.; Resing, K. A.; Ahn, N. G. *Annu. Rev. Biophys. Biomolec. Struct.* **2003**, *32*, 1−25.
(5) Konermann, L.; Simmons, D. A. *Mass Spectrom. Rev.* **2003**, *22*, 1−26.
(6) Wales, T. E.; Engen, J. R. *Mass Spectrom. Rev.* **2006**, *25*, 158−170.
(7) Zhang, Z.; Smith, D. L. *Protein Sci.* **1993**, *2*, 522−531.

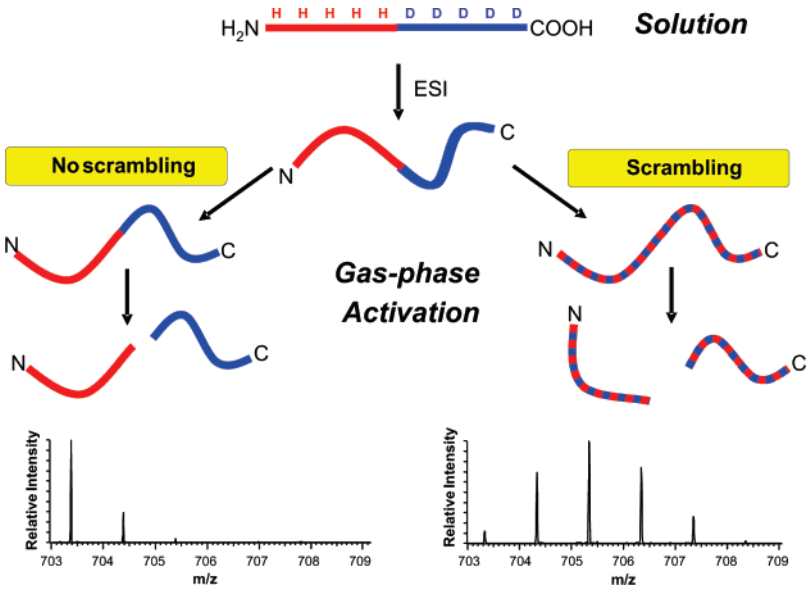


Figure 1. N-terminal fragment ions of a selectively labeled peptide constitute a sensitive probe for the occurrence of scrambling. In solution, the backbone amides in the C-terminal half are deuterated (shown in blue), while the amides in the N-terminal half are protiated (shown in red). The selectively labeled peptides are ionized and transferred to the gas phase by electrospray ionization. At the occurrence of scrambling, amide deuterons will migrate from the C-terminal half to the residues in the N-terminal half. This migration is readily detected as a mass increase of the fragment ions derived from the N-terminal half. These fragment ions are thus sensitive reporters of intramolecular migration of amide hydrogens (^{1}H/^{2}H) in tandem mass spectrometry experiments. In the absence of scrambling, the mass spectrum will display an isotope distribution that closely resembles the natural abundance isotope distribution of the fragment (lower left panel). When scrambling has occurred, the presence of deuterons shifts the isotopic envelope to higher masses (lower right panel).

companied by extensive intramolecular hydrogen (^{1}H/^{2}H) migration due to an induced mobility of protons/deuterons in the vibrationally excited protonated peptide ions.[8−15] This proton/deuteron traffic (i.e., hydrogen scrambling) within the peptides redistributes the incorporated deuterons among all exchangeable sites and thereby erases the original solution deuteration pattern.[16] In contrast, electron capture dissociation (ECD) cleaves the polypeptide backbone with minimal vibrational excitation[17] and is, therefore, likely to limit the possibility for intramolecular amide hydrogen (^{1}H/^{2}H) migration during dissociation. To investigate the applicability of ECD as a method to obtain site-specific information about amide hydrogen exchange rates in solution ^{1}H/^{2}H experiments, we have undertaken a systematic study of the occurrence of hydrogen (^{1}H/^{2}H) scrambling during electron capture dissociation of selectively labeled peptides. We find that ECD of selectively labeled peptides proceeds with a low level of scrambling.

To arrive at this result, several issues must be solved. A major experimental difficulty in probing the extent of scrambling is to prepare peptides with selective labeling at backbone amides that enables an accurate assessment of the degree of scrambling. In the present study, we have utilized peptides with a uniquely well-defined deuterium incorporation (Figure 1).[18] To preserve this selective labeling during the mass spectrometric experiment that can last several minutes, the temperature of the spraying solution was kept as low as possible using dry-ice cooling.

Another important issue to address was the undesired ion excitation in the mass spectrometer prior to ECD fragmentation. Ions produced by electrospray ionization typically undergo declustering in a heated ion guide, as well as heating in the collision region between the nozzle and the skimmer of the atmosphere−vacuum interface. Furthermore, ion storage and isolation in a storage multipole may lead to excessive vibrational excitation. Such excitation phenomena had to be avoided or minimized.

A further complicating issue is the hydrogen atom migration that can occur between the complementary ECD fragments when these fragments do not separate immediately upon N−C_α bond dissociation but instead form a long-lived "reduced species" complex.[19] Left unchecked, this migration may contribute to hydrogen (^{1}H/^{2}H) scrambling. To minimize the risk of this effect, which is particularly prominent in the ECD of dications, we chose to fragment triply charged ions. Unlike the ECD of dications that give singly charged reduced species held together by attractive ion−dipole interactions, singly charged complementary fragments of +3 precursors repel each other, which leads to very short lifetimes of the doubly charged reduced species and thus to a minimum of hydrogen migration.

We demonstrate here that the degree of scrambling as measured by ECD reaches low levels only when all of the above concerns are addressed. This may represent a major difference

(8) Harrison, A. G.; Yalcin, T. *Int. J. Mass Spectrom.* **1997**, *165*, 339−347.
(9) Jørgensen, T. J. D.; Gårdsvoll, H.; Ploug, M.; Roepstorff, P. *J. Am. Chem. Soc.* **2005**, *127*, 2785−2793.
(10) Jørgensen, T. J. D.; Bache, N.; Roepstorff, P.; Gårdsvoll, H.; Ploug, M. *Mol. Cell. Proteomics* **2005**, *4*, 1910−1919.
(11) Demmers, J. A. A.; Rijkers, D. T. S.; Haverkamp, J.; Killian, J. A.; Heck, A. J. R. *J. Am. Chem. Soc.* **2002**, *124*, 11191−11198.
(12) Ferguson, P. L.; Pan, J. X.; Wilson, D. J.; Dempsey, B.; Lajoie, G.; Shilton, B.; Konermann, L. *Anal. Chem.* **2007**, *79*, 153−160.
(13) Johnson, R. S.; Krylov, D.; Walsh, K. A. *J. Mass Spectrom.* **1995**, *30*, 386−387.
(14) McLafferty, F. W.; Guan, Z. Q.; Haupts, U.; Wood, T. D.; Kelleher, N. L. *J. Am. Chem. Soc.* **1998**, *120*, 4732−4740.
(15) Mueller, D. R.; Eckersley, M.; Richter, W. J. *Org. Mass Spectrom.* **1988**, *23*, 217−222.
(16) We note, however, that low levels of scrambling has been reported for in-source collisional activation of multiply protonated proteins: Hoerner, J. K.; Xiao, H.; Dobo, A.; Kaltashov, I. A. *J. Am. Chem. Soc.* **2004**, *126*, 7709−7717.
(17) Zubarev, R. A. *Mass Spectrom. Rev.* **2003**, *22*, 57−77.
(18) Rand, K. D.; Jørgensen, T. J. D. *Anal. Chem.* **2007**, *79*, 8686−8693.
(19) Savitski, M. M.; Kjeldsen, F.; Nielsen, M. L.; Zubarev, R. A. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 113−120.

between this study and earlier works that failed to clearly demonstrate the absence of scrambling in ECD.[20,21]

Experimental Section

Materials. Peptides were synthesized and purified by Genscript Corporation (Piscataway, NJ) and D_2O (99.9 atom % D) was obtained from Sigma-Aldrich (St.Louis, MO). All other chemicals were of the highest grade commercially available.

Hydrogen ($^1H/^2H$) Exchange. The isotopic exchange rate of backbone amide hydrogens in an unstructured peptide depends on pH, temperature, and neighboring side chains.[22] The neighboring side chains affect this intrinsic exchange rate by steric and inductive effects. To illustrate this, the bulky isobutyl side chain of isoleucine decreases the exchange rate by blocking the solvent accessibility to the amide hydrogen. We have very recently utilized differences in intrinsic exchange rates among residues to develop a set of peptides that can be labeled with deuterium exclusively at the backbone amides in their C-terminal half.[18] This unique property was achieved by having residues with a rapid intrinsic amide hydrogen exchange rate in the N-terminal half, while the C-terminal half comprised residues with a slow intrinsic exchange rate. In the present study, we have used the synthetic peptides P1 (HHHHHHHIIKIIK) and P2 (HHHHHHHIITIIT). They contain a fast exchanging N-terminal half (HHHHHH−) and a slow exchanging C-terminal half (−IIKIIK or −IITIIT). The selective labeling is achieved by diluting a fully deuterated peptide into a cold acidic ^1H_2O solution. At these conditions, the amides in the N-terminal half are completely protiated within a few minutes, whereas the amides in the C-terminal half retain the deuterons for a much longer period with a half-life exceeding 100 min.[18]

The synthetic peptides were fully deuterated by dissolution of lyophilized peptide in 99.9% D_2O (100 μM) followed by incubation for 18 h at 4 °C. The selective labeling was carried out by a 50-fold dilution into cold electrospray ^1H_2O buffer (50% MeOH, 0.5 M acetic acid, pH 2.5), followed by quenching of the isotopic exchange by freezing on dry ice. After manually thawing the sample, it was immediately transferred to a precooled syringe (810RN or 825RN, Hamilton Company, Reno, NV) and infused directly into the electrospray ion source of the mass spectrometer via a fused-silica capillary (length 50 cm, ID 100 μm). The use of the 800-series Hamilton syringes for infusion ensured a tight seal at low temperatures. The syringe was mounted on a sample pump (Linton Instrumentation, Norfolk, UK) and cooled with a small plastic bag containing dry ice. The flow rate was 5−15 μL/min. To avoid sample freezing, only the glass components of the syringe was in direct contact with the enclosed dry ice. This setup facilitated continuous infusion of selectively labeled peptides thereby enabling us to quantitatively investigate gas-phase scrambling processes during systematic variations of the instrumental settings. Nondeuterated peptide samples were prepared by a 50-fold dilution of concentrated peptide stock solution into electrospray buffer.

Mass Spectrometry. Positive ion ESI mass spectra, collision-induced dissociation (CID) spectra and electron capture dissociation (ECD) spectra were recorded on an 7-T LTQ FT mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The spectra were recorded at a spray voltage of 3.6 kV and the settings for the declustering potentials (tube lens and heated capillary), the heated capillary temperature, and the width of precursor isolation window are indicated in the text. ECD was performed as described previously[23] using a standard Thermo ECD setup based on an indirectly heated dispenser cathode (HeatWave, CA). Irradiation time was set at 70 ms, and the electron energy was set to 5% of full scale. This corresponds to an electron energy less than 1 eV. CID was performed in the linear ion trap of the LTQ-FT at a default collision energy setting of 30, as well as a lesser energy setting of 15 to avoid total precursor ion depletion. No nebulizer gases were used in the ESI source.

Data Analysis. All of the mass spectra used for data analysis were the sum of 120 to 240 scans, accumulated over 1 to 2 min. Note that the deuterium content of the selectively labeled peptides remain constant for at least 10 min.[18] Average masses of isotopic envelopes were determined from centroided data (processed by Xcalibur software, Thermo) using an Excel spreadsheet. The deuterium content of deuterated peptides and their fragment ions was determined from the difference in average mass between deuterated and nondeuterated species. The experimental uncertainty of the measurement of the degree of scrambling was assessed in a series of identical ECD fragmentation experiments on replicate deuterated samples, where the standard deviation for the c_5 fragment ion was 3% ($n = 4$). The scrambling analysis was carried out as described previously.[9] Note, however, that the residual deuterium content in the present exchange-out solution is somewhat higher due to the presence of 50% (v/v) methanol, which has only one exchangeable hydrogen per molecule. The residual deuterium content (2.5%) was determined by the mass shift of peptide P1 after equilibration in the exchange-out solution.

Results

Electron Capture Dissociation of Selectively Labeled Peptides. Electron capture dissociation (ECD) of the triply protonated peptide P1, with the sequence HHHHHHIIKIIK, yielded an almost complete series of c- and z-type fragment ions (i.e., c_2-c_9 and z_3-z_{10}) as shown in Figure 2A. A largely similar fragmentation pattern was observed for peptide P2 (sequence: HHHHHHIITIIT, data not shown). Next, we subjected the selectively labeled peptide P1 to electron capture dissociation using standard instrumental settings for peptide analyses (Figure 2B). Accordingly, the isotopic envelope of the parent ion shifted to a higher *m*/*z* value corresponding to an average deuterium content of 3.6 (left insert, Figure 2B). In solution, these deuterons are exclusively located at the amides in the C-terminal half (i.e., -IKIIK), whereas the N-terminal half is nondeuterated. The small c-ions (c_2-c_6) are derived from the N-terminal half and they should therefore contain ∼0 deuterons in the absence of hydrogen ($^1H/^2H$) scrambling. Inspection of the isotopic envelope of, e.g., the c_5-ion clearly reveals, however, the presence of deuterons (right insert in Figure 2B). The deuterium content of the c_5 ion is 1.5 corresponding to ∼74% scrambling among all exchangeable sites in the parent ion. In conjunction, the complementary z_7-fragment ion had an average deuterium content of 2.1, which is consistent with the fact that the summed deuterium content of a complementary fragment ion pair should be equal to the deuterium content of the parent ion. The deuterium content for all other complementary ion pairs was consistent with this (c_2/z_{10}, c_3/z_9, c_4/z_8, c_5/z_7, c_6/z_6, c_8/z_4, c_9/z_3). The occurrence of amide hydrogen ($^1H/^2H$) scrambling is critically dependent upon the internal energy of the peptide ion. Using the standard instrumental settings, the internal energy of the peptide ions is likely to exceed the threshold energy for the onset of scrambling. The ECD spectra shown in Figure 2A & B were recorded using standard declustering potentials in the electrospray ion source (tube lens = 160 V, heated capillary = 140 V and 50 °C) and using a precursor ion isolation width in the linear ion trap of 6 Da as summarized in Table 1 (the isolation width defines the *m*/*z* window that selects precursor ions for MS/ MS experiments).

(20) Kweon, H. K.; Hakansson, K. *Analyst* **2006**, *131*, 275−280.
(21) Charlebois, J. P.; Patrie, S. M.; Kelleher, N. L. *Anal. Chem.* **2003**, *75*, 3263−3266.
(22) Bai, Y.; Milne, J. S.; Mayne, L.; Englander, S. W. *Proteins* **1993**, *17*, 75−86.
(23) Adams, C. M.; Zubarev, R. A. *Anal. Chem.* **2005**, *77*, 4571−4580.

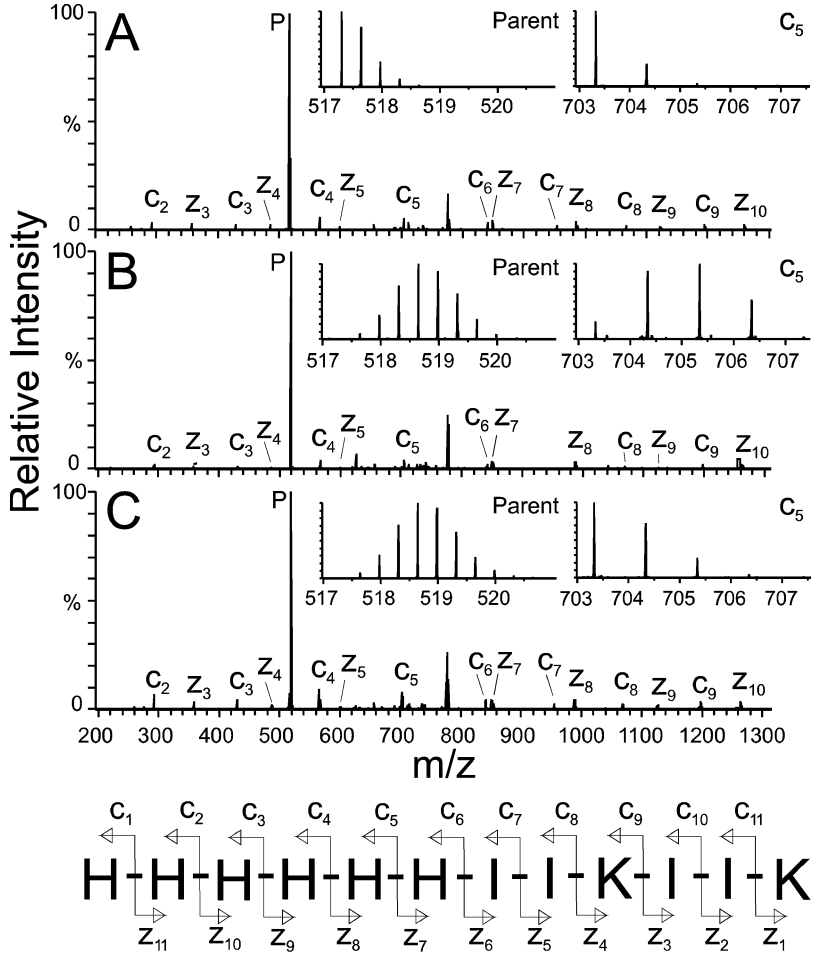


Figure 2. ECD has a low occurrence of amide hydrogen (^{1}H/^{2}H) scrambling. ECD spectra of the triply protonated nonlabeled peptide P1 (A), and peptide P1 labeled with deuterium exclusively in its C-terminal half (B,C). Mass spectrum B was obtained with a narrow precursor isolation window and high declustering potentials. Spectrum C was obtained with a wide precursor isolation window and low declustering potentials. The precursor ion is denoted P. The isotopic distribution of the precursor and the c_5-ion in each ECD spectrum is shown in the inserts. The ECD fragmentation scheme displays the sequences of fragment ions of Peptide P1. The low deuterium content of c_5 in spectrum C reveals that the extent of intramolecular migration of amide hydrogens (^{1}H/^{2}H) prior to fragmentation was low.

Table 1. Overview of ECD and CID MS/MS Experiments on Selectively Labeled Peptide P1 (HHHHHHIIKIIK)

	electrospr ay ion source settings			ion trap settings			
exp.	cap. temp (°C)[a]	cap. voltage (V)[b]	tube voltage (V)[c]	isolation width[d]	charge state	fragment ion	hydrogen scrambling (%)[e]
ECD	50	140	160	6	3	c_5	74
						c_4	69
						c_3	51
						c_2	43
ECD	50	20	50	18	3	c_5	8
						c_4	<5
						c_3	<5
						c_2	<5
ECD	50	20	100	6	3	c_4	17
						c_3	<5
ECD	50	20	100	6	4	c_4	55
						c_3	40
CID	50	140	160	6	3	b_4	92
CID	50	20	100	18	3	b_4	91

[a] Heated capillary temperature. [b] Heated capillary voltage. [c] Tube lens voltage. [d] Width (Da) of the precursor isolation window in the linear ion trap. [e] Calculations were based on the average of at least two identical experiments ($n = 2$). Determination of the hydrogen scrambling level in a c_5 fragment ion of replicate samples ($n = 4$) showed a standard deviation of 3% (absolute value) using the described experimental setup.

To minimize the extent of vibrational excitation of the intact peptide prior to ECD, the declustering potentials in the electrospray ion source were decreased (tube lens = 50 V, heated capillary = 20 V and 50 °C) and the isolation width in the linear ion trap was increased to 18 Da. With these settings, a dramatic reduction in the deuterium content of the diagnostic c_5 ion could be observed (compare the right insert of Figure 2, part C with that of part B). Note that the deuterium content of the parent ion was unchanged between these experiments (Figure 2, parts B and C, left inserts). The

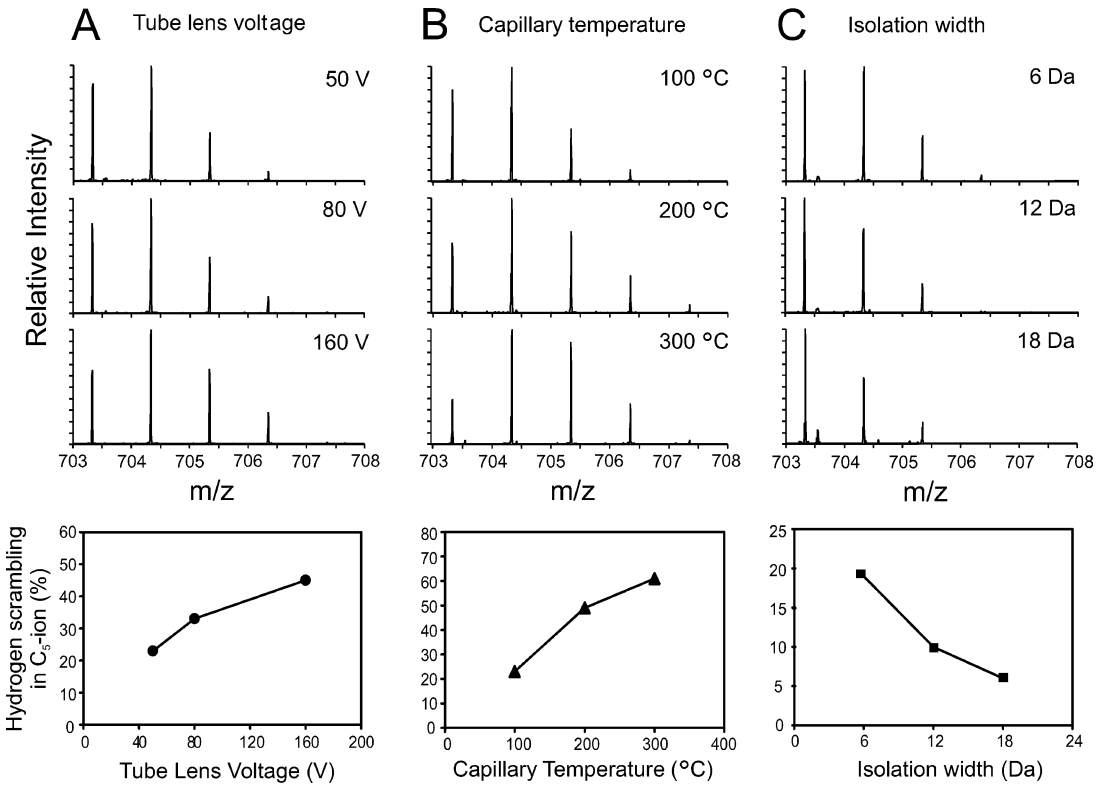


Figure 3. Minimizing vibrational excitation in the electrospray ion source and during precursor isolation decreases amide hydrogen ($^1H/^2H$) scrambling. The isotopic distributions (top panel) and the degree of hydrogen scrambling (lower panel) of the c_5-ion obtained from ECD of selectively labeled peptide P1 as a function of the following instrumental parameters: (A) declustering potential (tube lens) in the electrospray ion source, (B) heated capillary temperature in the electrospray ion source; and (C) the width of the precursor isolation window. The deuterium content of the precursor ion was constant in (A), (B), and (C).

deuterium content of the c_5 ion was 0.4, corresponding to ~8% scrambling (Table 1). Interestingly, the smaller c-fragments (i.e., c_2-c_4) obtained from ECD experiments acquired at standard instrumental settings (Figure 2B) or at low-scrambling settings (Figure 2C) had, in both cases, lower levels of scrambling than the corresponding c_5-fragment (Table 1). For example, the c_2 and c_3 ions exhibited scrambling levels below 5% at low-scrambling settings. Similar low levels of scrambling were found for the corresponding fragments obtained from ECD of the selectively labeled P2 peptide at these conditions (data not shown).

To further investigate the effect of the declustering conditions and the isolation width on the occurrence of scrambling, a detailed investigation was carried out by monitoring the deuterium content of the c_5 ion from the selectively labeled peptide P1, $[M+3H]^{3+}$, while the instrumental settings were systematically varied. The Thermo electrospray ion source consists of a heated capillary, where the ions are desolvated before they enter a tube lens/skimmer region. A focusing multipole guides the ions from the skimmer region to the linear ion trap. In the electrospray ion source, the degree of scrambling increased significantly when the tube lens voltage was raised from 50 V to the standard value of 160 V (Figure 3A; the other settings were, heated capillary = 20 V and 50 °C, isolation width 6 Da). A similar effect was observed when the temperature of the heated capillary was increased from 100 to 300 °C (Figure 3B; the other settings were tube lens = 50 V, heated capillary = 20 V, isolation width 6 Da; note that the standard temperature for the heated capillary is 50 °C). Increasing the voltage of the heated capillary also caused an increase in the degree of scrambling but only at a high tube lens potential (>160 V, data not shown). Increasing the isolation width for precursor ion selection in the linear trap from 6 to 18 Da reduced dramatically the degree of scrambling (Figure 3C; the other settings were tube lens = 50 V, heated capillary = 20 V and 50 °C). The effect of electron energy on the occurrence of scrambling was also investigated. ECD experiments of selectively labeled peptide P1 were carried out at elevated electron voltage (E_{kin} = 21 eV), referred to a *hot ECD*.[24] No significant difference in the degree of scrambling between normal ECD (<1 eV) and hot ECD (21 eV) could be observed (data not shown). This supports the model of hot ECD in which the electron capture follows electronic excitation, and the fragmenting species are electronically (but not yet vibrationally) excited.

The lowest degree of hydrogen ($^1H/^2H$) scrambling in triply protonated peptide P1 was attained at the following *low scrambling conditions*: tube lens = 50 V, heated capillary = 20 V and 50 °C, isolation width = 18 Da, while nearly complete scrambling was induced at the following *high scrambling conditions*: tube lens = 160 V, heated capillary = 140 V & 50 °C, isolation width = 2 Da. Figure 4 shows the deuterium content of c-fragment ions generated upon ECD fragmentation of polarized deuterium labeled peptide P1 at *high scrambling conditions* (Figure 4A) and *low scrambling conditions* (Figure 4B).

To investigate the influence of charge on scrambling, the quadruply protonated P1 peptide was subjected to ECD. Figure 5A and 5B show the ECD spectra of triply and quadruply

(24) Kjeldsen, F.; Haselmann, K. F.; Budnik, B. A.; Jensen, F.; Zubarev, R. A. *Chem. Phys. Lett.* **2002**, *356*, 201−206.

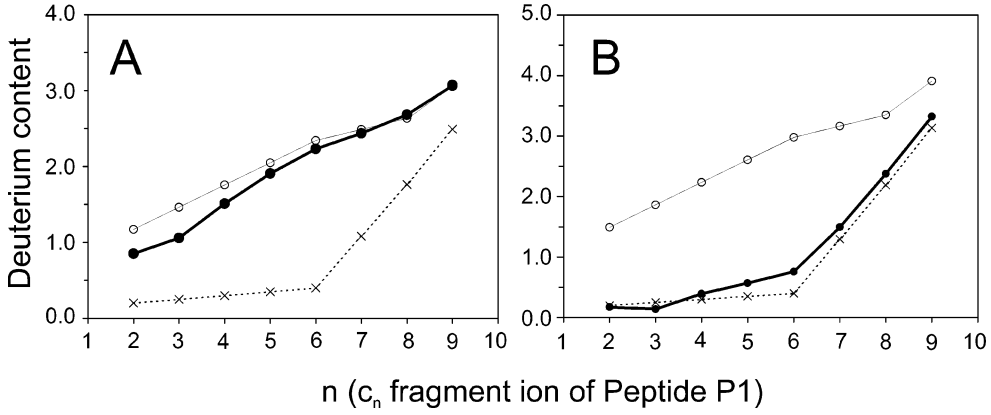


Figure 4. The solution deuteration pattern is readily recognized from the deuterium levels of the fragment ions generated by ECD at the instrumental settings for low scrambling. Deuterium content of c-fragment ions (filled circles, bold line) upon ECD of the selectively labeled peptide P1 at high scrambling settings (A) and low scrambling settings (B) (see text for details). The theoretical deuterium content in the case of 100% scrambling (open circles, thin line) and 0% scrambling (crosses, dotted line) is shown. The deuterium content of the precursor ion was 4.0 and 5.0 in (A) and (B), respectively.

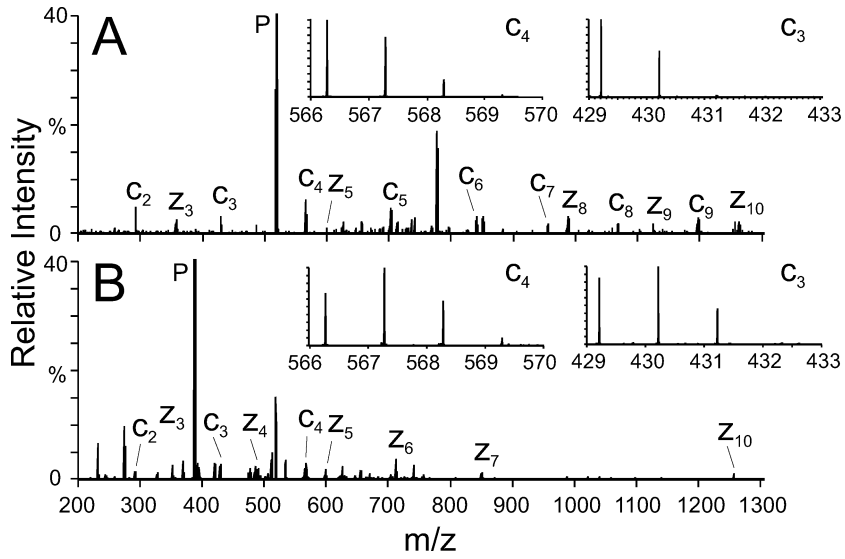


Figure 5. Effect of charge state on amide hydrogen ($^1H/^2H$) scrambling. ECD spectra of triply protonated (A) and quadruply protonated (B) selectively labeled peptide P1. The spectra were acquired using the following instrumental settings: tube lens = 100 V, heated capillary = 20 V and 50 °C, precursor isolation window = 6 Da. The parent ion is denoted P. The isotopic distribution of the c_4-ion and the c_3-ion is shown in inserts. The deuterium content of the parent ion was identical in both (A) and (B).

protonated P1 peptide with its C-terminal half labeled with deuterium. The ECD spectra were acquired using the following settings: tube lens = 100 V, heated capillary = 20 V and 50 °C, isolation width = 6 Da. A tube lens voltage of 100 V and an isolation width of 6 Da was necessary to obtain sufficient signal intensity and a specific selection of the +4 charge state, respectively. Several c-fragments (c_2-c_4) and z-fragments (z_3-z_7, z_{10}) results from electron capture of the quadruply protonated peptide. The isotopic envelopes for the c_3 and c_4 fragments ions depicted in Figure 5, parts A and B clearly show that the deuterium content for these ions are greater when they are derived from the quadruply protonated precursor. Thus, the deuterium level of the c_3 and c_4 fragments obtained from the triply protonated peptide were 0.2 (<5%) and 0.5 (17%), respectively, while these levels increased to 0.6 (40%) and 0.9 (55%) in the corresponding fragments from the quadruply protonated peptide (numbers in parentheses denote the degree of scrambling). As the deuterium content of the parent ion was identical in both experiments it is clear that the extent of intramolecular hydrogen ($^1H/^2H$) migration was substantially higher for the 4+ precursor peptide (Table 1). ECD of the doubly protonated peptide P1 yielded only four z-fragment ions (z_7-z_{10}, data not shown) thereby preventing a direct comparison with c-fragment ions from the other two charge states. Inspection of the deuterium content of the z_7 fragment ion from ECD of +2, +3, and +4 charge states recorded at identical conditions (as in Figure 5) revealed that the lowest level of hydrogen scrambling was observed for the +3 charge state (39%), while the highest level of scrambling was observed for +4 charge state (77%). Interestingly, the +2 charge state yielded an intermediate level of scrambling (70%) (data not shown).

It was generally observed that the peptide ion abundance decreased when the ion source parameters were adjusted to *low scrambling conditions*. In more quantitative terms, we observed, e.g., a 2-fold decrease in ion abundance when the tube lens voltage was changed from 100 to 50 V (the other parameters were: heated capillary 50 °C and 20 V, precursor isolation width 18 Da). Expectedly, a concomitant decrease in the degree of scrambling was also observed (24% to 8% as measured by the c_5 ion).

The instrumental settings for low scrambling are specific to the LTQ-FT mass spectrometer. For other types of instruments,

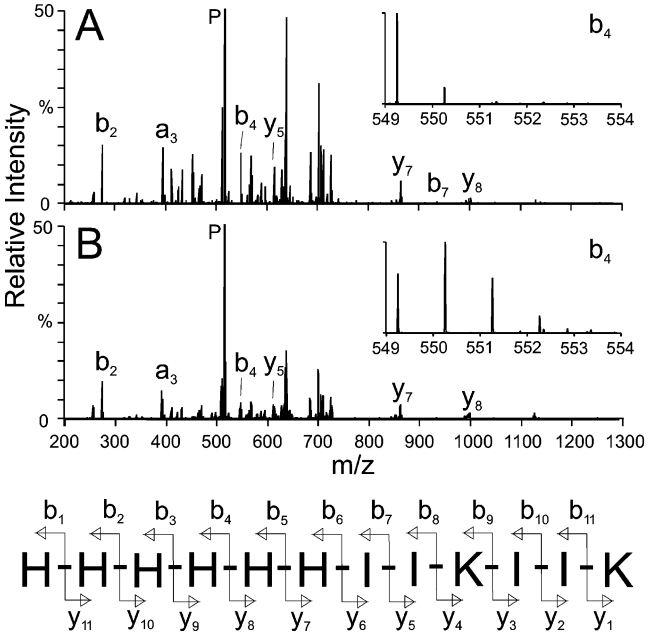


Figure 6. Collision-induced dissociation (CID) causes nearly complete hydrogen ($^1H/^2H$) scrambling. CID spectra of triply protonated nonlabeled peptide P1 (A), and selectively labeled peptide P1 (B). The CID spectra were obtained at the instrumental settings which yield a low degree of scrambling upon ECD. The precursor ion is denoted P. The isotopic distribution of the b_4-ion is shown in inserts. The CID fragmentation scheme shows the fragment ion sequences of peptide P1.

the declustering conditions in the ion source and the parameters for precursor ion isolation have to be carefully adjusted to minimize the extent of vibrational activation prior to the electron capture event. Owing to our recently developed selectively labeled peptides, the degree of scrambling can be readily monitored during this optimization procedure. In general, the critical ion source parameters for minimizing scrambling are most likely those used to generate in-source fragmentation.

Collision-Induced Dissociation (CID) of Selectively Labeled Peptides. We have previously performed CID of selectively labeled peptides using a quadrupole-time-of-flight instrument, observing complete hydrogen ($^1H/^2H$) scrambling.[9,18] In these experiments, it was not known whether scrambling already had occurred in the electrospray ion source, i.e., before the peptide had entered the collision cell. In the present work, we have optimized the instrumental settings so that only low levels of scrambling are induced in the electrospray ion source and during precursor ion selection in the linear trap. With the present CID experiments, the precursor ions will thus retain their selective labeling until the point where they are collisionally activated in the linear ion trap. The resulting fragments are subsequently transferred to the ICR cell and mass-analyzed. Figure 6 shows CID spectra of unlabeled and selectively labeled P1 peptide acquired at *low scrambling conditions*. CID of peptide P1 yielded several abundant b-ions (b_2, b_4, b_7) and y-ions (y_5, y_7, y_8). The b_4 ion is derived from the N-terminal half, which is nondeuterated in solution, the isotopic envelope of this ion (Figure 6B), however, reveals a substantial deuterium content. This indicates that deuterons from the amides of the C-terminal half have migrated to the residues of the N-terminal half prior to or during dissociation of the peptide ion. The deuterium content of the b_4 ion (and the complementary y_8 ion) correspond to nearly complete scrambling (Table 1). Similar levels of scrambling were measured for the other fragment ions (b_2, b_7, y_5, y_7, y_8). Decreasing the collision energy did not lower the extent of scrambling (data not shown). Evidently, CID causes extensive hydrogen scrambling, in obvious contrast to ECD.

Discussion

In the present work, we have utilized peptides with a unique deuterium labeling to probe the extent of intramolecular migration of amide hydrogens ($^1H/^2H$) upon electron capture dissociation (ECD). In solution, the amides in the N-terminal half of the peptides are protiated, while the amides in the C-terminal half are deuterated.[18] The labeled peptides are ionized and transferred to the gas phase by electrospray ionization. At the advent of hydrogen ($^1H/^2H$) scrambling in the gaseous peptide ion, the amide deuterons in the C-terminal half will migrate to the residues in the N-terminal half. This intramolecular migration is readily detected as a mass increase of N-terminal fragment ions. Note, that scrambling is extremely unlikely to occur while the peptides are in solution.[25] Our results show that by carefully minimizing vibrational excitation of the labeled peptide during ion formation and during precursor ion selection, a low level of hydrogen scrambling (<10% for c_2-c_5) upon ECD is observed. At these *low scrambling settings*, the original solution deuteration pattern is readily recognized from the gas-phase data (Figure 4B). The observed level of scrambling can originate from two sources. First, although great care was exercised in minimizing vibrational excitation prior to electron capture, the desolvation process and/or the precursor isolation may result in internal energy distributions where the hottest ions have sufficient internal energy to mobilize the amide hydrogens ($^1H/^2H$). Second, although backbone cleavage by ECD is likely to occur by a nonergodic pathway with a priori no scrambling,[17,26] the presence of long-lived radical intermediates may facilitate amide hydrogen ($^1H/^2H$) scrambling (see below).[27,28]

Interestingly, the selectively labeled peptides act as molecular thermometers, in the sense that they report on the history of the internal energy of the gaseous peptides. In the ion source, the peptides are desolvated by collisional activation. The degree of collisional activation is controlled by the magnitude of the declustering potentials and the temperature of the heated capillary. When the peptides were excessively activated by, e.g., raising the temperature of the heated capillary, a dramatic increase in the level of scrambling was observed (Figure 3B). Similarly, the level of scrambling increased as a result of sideband excitation when the precursor isolation was carried out with narrow isolation windows in the linear ion trap (Figure 3C). The increased level of scrambling for the quadruply protonated peptide relative to the triply protonated peptide most

(25) When the peptides are solvated, the amide hydrogens cannot intramolecularly migrate, as the exchange mechanisms for amide hydrogens in solvated peptides involve an intermolecular transfer of a H^+ between solvent and amide group. In a microdroplet, the abstraction of a deuteron from an amide group to the protiated solvent is virtually an irreversible process as the solvent is present in a huge molar excess. Thus, any exchange process occurring while the peptides are solvated will merely reduce the deuterium content of the peptide.

(26) Zubarev, R. A.; Kelleher, N. L.; McLafferty, F. W. *J. Am. Chem. Soc.* **1998**, *120*, 3265−3266.
(27) Lin, C.; O'Connor, P. B.; Cournoyer, J. J. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 1605−1615.
(28) O'Connor, P. B.; Lin, C.; Cournoyer, J. J.; Pittman, J. L.; Belyayev, M.; Budnik, B. A. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 576−585.

likely results from the more energetic collisions of +4 charge state when it is accelerated by the declustering potentials and by the oscillating potentials in the linear trap during precursor isolation[29] (i.e., sideband excitation). Interestingly, the +2 charge state scrambled more than the +3 charge state. The +2 charge state is less collisionally activated than the +3 and +4 charge states by the declustering potentials and by sideband excitation. This indicates that the increased level of scrambling for the +2 charge state has occurred during the ECD process and it most likely reflects an increased life time of the singly charged radical intermediate.

$$[\text{peptide} + 3\text{H}]^{3+} + e^- \rightarrow [c' + z^\bullet]^{2+}_{\text{complex}} \rightarrow c'^{+} + z^{\bullet +} \quad (1)$$

$$[\text{peptide} + 2\text{H}]^{2+} + e^- \rightarrow [c' + z^\bullet]^{+}_{\text{complex}} \rightarrow c' + z^{\bullet +} \quad (2)$$

$$[\text{peptide} + 2\text{H}]^{2+} + e^- \rightarrow [c' + z^\bullet]^{+}_{\text{complex}} \rightarrow [c^\bullet + z']^{+}_{\text{complex}} \rightarrow c^\bullet + z'^{+} \quad (3)$$

Consider the capture of an electron by the triply protonated peptide (eq 1). After capture, an N−C_α bond is cleaved (possibly non-ergodical), but the incipient complementary fragments (c'^{+} and $z^{\bullet +}$) remain noncovalently associated by hydrogen bonds in a doubly charged complex. This complex is destabilised by Coulombic repulsion between the two protons thereby shortening its lifetime. Such a Coulombic destabilization is absent when a doubly protonated peptide captures an electron (eq 2) and the longer lifetime of the singly charged complex allows more intermolecular amide hydrogen migration to occur before the complex dissociates. The additional presence of z′ ions for the +2 charge state[30] further supports the occurrence of hydrogen migration during ECD as this particular ion appears to be formed by a hydrogen atom transfer from the incipient neutral c fragment (eq 3).[19] Similarly, O'Connor et al.[28] reported radical migration of C_α-linked deuterium atoms in glycine residues upon formation of $c^\bullet$ and z′ fragments from doubly protonated precursors (eq 3). The higher charge states +3 and +4 of our peptides, however, yield exclusively $z^\bullet$ and c′ fragments indicating that radical migration is absent. The hydrogen atom donors in the hydrogen radical transfer mechanism in the singly charged radical intermediates (eq 3) are most likely α-carbons[28] and side chains,[19] whereas the amide hydrogen migration most likely occurs by a mechanism where amide nitrogens participate in reversible proton/deuteron transfer reactions.[9] Our results suggest that doubly protonated peptides are not optimal targets for ECD in the light of minimizing scrambling. Higher charge states are the preferred targets provided that vibrational excitation is minimized prior to ECD. Interestingly, the occurrence of scrambling at the given instrumental settings is *not* constant among the c-ions. The extent of scrambling appears to depend on the size of the c-fragment ion with the smallest c-ions having the lowest degree of scrambling (Table 1). This phenomenon is most likely related to the lifetime of the metastable complex $[c' + z^\bullet]^{n+}$. During the existence of this complex, amide hydrogens (^{1}H/^{2}H) may migrate between the incipient fragments and cause scrambling. In the complex, a small c-ion is likely to have fewer intermolecular hydrogen bonds than a larger c-ion. This means that the dissociation kinetics will be slower for the formation of a large c-ion relative to that of smaller c-ions (given a constant energy input, i.e., the recombination energy). In other words, the lifetime of the radical intermediate of the larger c-ions is longer than that of the intermediate of the smaller c-ions. As the probability for the occurrence of scrambling is known to increase with the ion lifetime this model provides a plausible explanation for the observed increase in the degree of scrambling with c-ion size.

In strong contrast to our ECD results, nearly complete scrambling (~90%) was observed upon CID of the selectively labeled peptides in the linear ion trap, even at the instrumental settings for minimal scrambling in the ion source and during precursor isolation. This finding is in line with our previous CID results obtained from a quadrupole-time-of-flight (Q-TOF) mass spectrometer[9,18] and a MALDI tandem time-of-flight instrument.[10]

Recently, Kweon et al.[20] attempted to correlate amide hydrogen exchange rates, obtained from deuteration levels in c-fragment ions generated by ECD of deuterated melittin, with solution exchange rates obtained by NMR spectroscopy. Their gas phase measurements show that 4 out of a total of 7 amide hydrogens are slowly exchanging with rates close to 0.006 min^{-1}. However, according to the NMR data, two of these amides are categorized as fast exchanging amides, whereas the other two are slow exchangers. The remaining 3 amides are categorized as exchanging with a fast/medium exchange rate by NMR. The gas phase measurements for these amides yield exchange rates close to 0.05 min^{-1}. Unfortunately, no quantitative assessment of the extent of amide hydrogen scrambling was carried out. That said, the apparent lack of correlation between the gas phase measurements (ECD) and the solution data (NMR) suggests that scrambling was prevalent.

The decreased signal intensity at the present ion source settings for low scrambling may compromise the acquisition of ECD spectra of less abundant peptic peptides from on-line chromatographic separations in an HX−MS experiment. To obtain ECD spectra of such peptides, it may be necessary to use higher amounts of proteins. Alternatively, the signal intensity can be increased by accepting an increase in the degree of scrambling at somewhat higher declustering potentials in the ion source. In this case, an accurate determination of site-specific solution deuterium levels may require a mathematical deconvolution procedure.

For a typical HPLC setup for hydrogen exchange studies of proteins, the chromatographic separation of peptic peptides is hampered by a short gradient and low temperature (0 °C). In this setting, the wide precursor isolation window may include more than one precursor ion for ECD, thereby complicating the mass spectral interpretation. The chromatographic separation can be greatly improved, however, by employing the recently developed ultra-performance LC (UPLC) which has superior chromatographic resolution of peptic peptides at short gradients

(29) McClellan, J. E.; Murphy, J. P.; Mulholland, J. J.; Yost, R. A. *Anal. Chem.* **2002**, *74*, 402−412.

(30) ECD of the nondeuterated +2 charge state clearly revealed the presence of z_7' ions (~30%). To calculate the deuterium content, the average mass of the nondeuterated isotopic distribution (containing z_7' and $z_7^\bullet$ ions) was substracted from the average mass of the deuterated isotopic distribution. It is thus implicitly assumed that deuteration is not altering the branching ratio of the z_7' and $z_7^\bullet$ ion formation pathways. There was no experimental evidence of any z_7' ions upon ECD of the nondeuterated +3 and +4 charge state, only $z_7^\bullet$ ions were observed.

and at low temperature (0 °C).[31] We thus anticipate that ECD holds great promise as a general method to obtain single residue resolution in hydrogen exchange experiments of proteins.

Conclusion

Using selectively labeled peptides, we have demonstrated that ECD proceeds with a low level of amide hydrogen ($^1H/^2H$) scrambling. We have further shown that the extent of scrambling prior to ECD could be minimized by lowering the declustering potentials in the electrospray ion source and increasing the width of the precursor isolation window. At these instrumental settings, the site-specific labeling from solution $^1H/^2H$ exchange is readily recognized from the gas-phase data. This shows that ECD holds great promise as a method to obtain site-specific deuteration levels from peptic peptides derived from proteins deuterated in solution. In contrast, CID performed in the linear ion trap produced nearly complete hydrogen ($^1H/^2H$) scrambling, thereby precluding the use of CID as a method to gain information about single residue deuteration levels for proteins in solution.

Acknowledgment. We thank Henning R. Stennicke and Mette D. Andersen for constant interest in this work and Novo Nordisk A/S for purchasing the synthetic peptides. We also thank Frank Kjeldsen, Anne J. Kleinnijenhuis, Jesper V. Olsen, and Martin Zehl for valuable discussions. This work was supported by a scholarship from the Danish Ministry of Science, Technology and Innovation (to KDR) and by a grant (No. 272-06-0493) from The Danish Natural Science Research Council (FNU).

JA076448I

(31) Wu, Y.; Engen, J. R.; Hobbins, W. B. *J. Am. Soc. Mass Spectrom.* **2006**, *17*, 163−167.