Collisional Activation by MALDI Tandem Time-of-flight Mass Spectrometry Induces Intramolecular Migration of Amide Hydrogens in Protonated Peptides*

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Considerable controversy exists in the literature as to the occurrence of intramolecular migration of amide hydrogens upon collisional activation of protonated peptides and proteins. This phenomenon has important implications for the application of CID as an experimental tool to obtain site-specific information about the incorporation of deuterium into peptides and proteins in solution. Using a unique set of peptides with their carboxyl-terminal half labeled with deuterium we have shown unambiguously that hydrogen (1H/2H) scrambling is such a dominating factor during low energy collisional activation of doubly protonated peptides that the original regioselective deuterium pattern of these peptides is completely erased (Jørgensen, T. J. D., Gårdsøll, H., Ploug, M., and Roepstorff, P. (2005) Intramolecular migration of amide hydrogens in protonated peptides upon collisional activation. J. Am. Chem. Soc. 127, 2785–2793). Taking further advantage of this unique test system we have now investigated the influence of the charge state and collision energy on the occurrence of scrambling in protonated peptides. Our MALDI tandem time-of-flight experiments clearly demonstrate that complete positional randomization among all exchangeable sites (i.e. all N- and O-linked hydrogens) also occurs upon high energy collisional activation of singly protonated peptides. This intense proton/deuteron traffic precludes the use of MALDI tandem time-of-flight mass spectrometry to obtain reliable information on the specific incorporation pattern of deuterons obtained during exchange experiments in solution. Molecular & Cellular Proteomics 4:1910–1919, 2005.

Mass spectrometry-based proteomics is currently one of the key technologies for the identification of protein interaction networks in the cell (1–3). This technology has provided a wealth of information unraveling the complexity of these biological processes at the protein level. A detailed understanding of the function of these protein networks at the molecular level requires, however, characterization of the dynamics and structural properties of the interacting proteins. Structural analyses by NMR spectroscopy or x-ray crystallography require relatively large amounts of pure proteins (typically milligram quantities), and many proteins are furthermore not inherently amenable to analysis by these traditional techniques (e.g. modular proteins or proteins with heterogeneous glycosylation patterns are often reluctant to yield well diffracting crystals).

An alternative approach for the characterization of protein complexes without these limitations is provided by carefully monitoring by mass spectrometry changes in the rates of amide hydrogen (1H/2H) exchange upon complex formation (4, 5). In a typical experimental set-up for investigation of conformational properties of a protein complex, the unligated proteins as well as the preformed protein complex are incubated separately in deuterated buffer under physiological conditions. Global deuterium incorporation kinetics are subsequently established by monitoring deuterium contents in these protein samples withdrawn at appropriate intervals with their amide hydrogen isotopic exchange quenched by acidification and cooling. Information about local deuterium incorporation is subsequently collected after pepsin digestion followed by reversed-phase LC-MS. The chromatography is carried out with cold protiated solvents allowing effective back-exchange at all exchangeable sites with protium (1H) with the sole exception of the amide groups forming the polypeptide chain. The actual number of individual amide hydrogens that can be resolved by this method, however, is limited by the number and sizes of peptides generated by pepsin. Overlapping sequence coverage is often provided by the broad specificity of pepsin, and although this promiscuity in substrate recognition increases the resolution in the local assignment of deuterium incorporation, single residue information can generally be obtained for only a few positions (6). For a first hand view, gas-phase fragmentation in the mass spectrometer may appear to be the logical choice for an auxiliary method providing the desired site-specific informa-
tion. Accordingly it has been widely adopted in the attempt to identify specific sites that have become deuterated in solution $^{1}$H/$^{2}$H exchange experiments (7–21). It is, however, mandatory for this approach that the level of intramolecular hydrogen ($^{1}$H/$^{2}$H) migration upon ion activation is negligible. If hydrogen migration (i.e. scrambling) occurs upon collisional activation it is thus impossible to retain any information on the original deuterium incorporation from the isotopic exchange in solution.

Previously we have investigated the extent of intramolecular hydrogen ($^{1}$H/$^{2}$H) migration in two doubly protonated peptides (AE133 and AE138, see Table I) (22). These peptides represent a unique model system that is ideally suited to subject gas-phase fragmentation to close scrutiny for the occurrence of scrambling. The virtue of these peptides is their capability to be labeled regioselectively by deuteriums. This property is accomplished due to their high affinity interaction with a specific receptor protein, the urokinase-type plasminogen activator receptor (uPAR) (23). The tight engagement with uPAR protects all amide hydrogens in the carboxyl-terminal half of the peptide against isotopic exchange with the solvent, whereas the amino-terminal half exchanges without restraints (24). Particularly b2 and b3 fragment ions constitute a very sensitive probe for the onset of scrambling as these ions arise from cleavages within the amino-terminal half of the regioselectively labeled peptides. If such b-ions contain close to zero deuteriums the level of scrambling upon ion activation is negligible. Conversely if a significant deuterium content is indeed detected it signifies that scrambling involving the amide hydrogens has occurred. This test system have previously unraveled the complete scrambling for all N- and O-linked hydrogens upon low energy collisional activation of doubly protonated peptides (22). The main objective of the present study was to explore which factors determine the onset of amide hydrogen scrambling in protonated peptides. It seems reasonable to assume that proton mobility in a singly protonated peptide is significantly lower than that of the corresponding doubly protonated peptide, and this pertains in particular to peptides containing an Arg residue (25, 26). Furthermore it has been asserted that higher collision energies should diminish hydrogen scrambling (27). We consequently investigated the extent of hydrogen scrambling upon high energy collisional activation of two singly protonated peptides, one of which actually contains an internal Arg residue.

**EXPERIMENTAL PROCEDURES**

Materials—$^{2}$H$_2$O (99.9 atom % $^{2}$H) was obtained from Cambridge Isotope Laboratories (Andover, MA). Ammonium acetate-$d_7$ (98 atom % $^{2}$H) and the MALDI matrices 2,5-dihydroxy benzoic acid (DHB) and $\alpha$-cyano-4-hydroxy cinnamic acid ($\alpha$-CHCA) were from Sigma. All other chemicals and reagents were of the highest grade commercially available. The peptides AE133 and AE138 were synthesized, purified, and characterized as reported previously (23, 28). The sequences of AE133 and AE138 are KGSGGD- Cha-FsARLYWS and KGSGGD-Cha-FsARLYWS, respectively. Lowercase letters denote D chirality, and Cha is $\beta$-cyclohexyl-L-alanine. Soluble, recombinant human uPAR was produced in Chinese hamster ovary cells and immunoaffinity-purified as described previously (29).

**Hydrogen/Deuterium Exchange**—Deuterated uPAR-peptide complexes were prepared by mixing a 2-fold molar excess of deuterated uPAR with deuterated peptide (22). In brief, deuterated uPAR was prepared by incubating protiated uPAR in deuterated buffer for 12 h at 6 °C. After lyophilization and redissolution in deuterated buffer the deuterated peptide ligands were added to a uPAR solution.

Peptide ligands labeled selectively with deuterium in their uPAR binding region were prepared by a 50-fold dilution of a solution of deuterated uPAR-peptide complexes into protiated buffer (i.e. exchange-out): 1 μl of a 20 μM solution of the peptide ligand in the presence of 400 μM uPAR in deuterated buffer (50 mM $d_7$-ammonium acetate, pH 8.0, 0.10 M NaCl) was added to 49 μl of the correspondingly protiated buffer (i.e. 50 mM ammonium acetate buffer, pH 8.0, 0.10 M NaCl). Solutions maintained at 0 °C were subjected to exchange for 30 s. The $^{1}$H/$^{2}$H exchange solution was quenched by the addition of an equal volume of 0.5 μM phosphate buffer (pH 2.2, 0 °C) and subjected immediately to rapid desalting.

**Rapid Desalting and MALDI Sample Preparation**—The equipment used for rapid desalting has been described previously (24). It consists of two HPLC pumps, an injection valve, and a 10-port vacuum valve equipped with a C18 microcolumn (30). One HPLC pump delivers the solvent for desalting, and the other delivers the solvent for elution. Solvent precleaning coils, the injection valve with loop, and the 10-port valve with microcolumn were all immersed into an ice/water slurry (0 °C) to minimize amide hydrogen ($^{1}$H/$^{2}$H) back-exchange with protiated solvents. The desalting removes deuterium incorporated into side chains and amino/carboxyl termini because these exchangeable hydrogens (i.e. attached to nitrogen, oxygen, and sulfur) exchange much faster than peptide backbone amide hydrogens at acidic pH (31). Any mass increase observed after isotopic exchange and desalting thus reflects deuteration of the peptide mainchain amide groups. An ice-cold glass syringe was used to load the acid-quenched samples onto the C$_18$ microcolumn followed by desalting for 45 s using a flow of H$_2$O containing 0.05% (v/v) trifluoroacetic acid (pH 2.2). The sample was eluted within 1 min by addition of 70% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid (20 μl/min) into a precooled Eppendorf tube, snap frozen, and stored in liquid nitrogen until MALDI sample preparation. To minimize deuterium losses during matrix embedment the MALDI target plate was cooled to −80 °C prior to depositing the MALDI matrix solution and the sample solution (32). 1 μl of matrix solution ($\alpha$-CHCA, 4 mg/ml in 70% CH$_3$CN containing 0.1% TFA) was applied to the cold target plate. The low temperature caused freezing of the droplet, but the frozen matrix melted instantaneously upon addition of 1 μl of cold sample thawed just prior to this step. Triplicate spots of each deuteron-labeled sample were prepared in this manner. Evaporation of the solvent and concomitant crystallization of the matrix peptide solution was completed within 2 min in vacuo, and MALDI mass spectra (MS and MS/MS) of labeled peptides were recorded as soon as possible. Spectra (MS and MS/MS) of non-labeled peptides were obtained without cooling the target plate. “Pure” high energy MS/MS experiments were recorded using DHB (20 mg/ml in 70% CH$_3$CN containing 0.1% TFA) as matrix.

The deuterium content of the selectively deuterated peptides was significantly lower in the MALDI experiments than in our previous ESI.
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experiments (22). This is an unavoidable consequence of the higher deuterium losses encountered during quench conditions in the former experiments due to the longer duration of sample preparation. In this context, it should be noted that the deuterium loss for AE133 was slightly higher than that of AE138 as the latter peptide was analyzed before AE133 on the MALDI target plate. When peptides are analyzed by ESI-MS the deuterium loss caused by back-exchange under quench conditions is ~12% (22), whereas the corresponding deuterium loss encountered in the MALDI experiments is 50–60%. Similar losses have been reported by other investigators using MALDI MS and amide hydrogen (H/$^2$H) exchange (32). It is, however, important to emphasize that this deuterium loss does not affect the selectivity of the labeling. The vast molar excess of protiated solvent ensures that loss of deuterons is a virtually irreversible process that does not allow peptides to undergo scrambling by intermolecular isotopic exchange at quench conditions.

MALDI-TOF and -TOF/TOF Mass Spectrometry—Positive ion MALDI-TOF and -TOF/TOF mass spectra were recorded on an Applied Biosystems (Framingham, MA) 4700 Proteomics Analyzer equipped with a 200-Hz neodymium:yttrium-aluminum-garnet laser operating at a wavelength of 355 nm. A prototype version of this instrument has been described previously (33). MALDI-TOF mass spectra were acquired in reflectron mode (20-kV accelerating voltage) with 470-ns delayed extraction, accumulating 5000 laser shots. For MS/MS experiments, the acceleration voltage was 8 kV, and the collision energy ($E_{coll}$) was 1 keV; 5000 laser shots were accumulated. Argon was used as a collision gas at an indicated manifold pressure of ~1 x 10$^{-7}$ millbar (note that the pressure is not measured directly in the CID collision chamber). The assignment of fragment ions was confirmed by CID of amino-terminal acetylated AE138 and AE133. All a-, b-, and d-ions displayed a mass shift of 42 Da. The assignment of the $w_8$ fragment from AE133 (i.e. cleavage of the $\beta$-$\gamma$-bond causing loss of the cyclohexyl side chain) was verified by the absence of this particular type of fragment upon CID of a peptide analogue in which the cyclohexyl group is replaced by a phenyl group (i.e. KGSGGDF-FsrYLWS) as $\beta$-$\gamma$-bond cleavage does not occur for aromatic residues (34).

Data Analysis—The data analysis was carried out as described previously (22). To calculate the theoretical deuterium content for a given fragment ion in case of 100% scrambling among all exchangeable sites in the precursor ion, the experimental deuterium content of the precursor ion is divided by the total number of its exchangeable sites, and this ratio is subsequently multiplied with the number of exchangeable sites in the given fragment ion. A number of fragment ions generated from deuterated AE133 had overlapping isotope distributions, e.g. $w_6$ and $v_6$, $w_4$ and $a_{10}$. It was thus not possible to accurately determine the deuterium content for these ions.

### RESULTS

In the present investigation, formation of synthetic peptides with defined polarized deuterium labeling of their amide groups represents a decisive factor for the scrutiny of hydrogen migration during MALDI-MS/MS. The structural basis for this unique property has recently been unraveled by the exploration of the three-dimensional structure of an analogous receptor-peptide complex (uPAR-AE147) solved at 2.7 Å by x-ray crystallography (35). Importantly the crystal structure reveals that the uPAR binding sequence of the peptide is confined to the carboxyl-terminal half of AE147, and this region furthermore adopts a stable $\alpha$-helical structure upon complex formation. Because the two peptides used in the present study (AE133 and AE138) have comparable receptor binding sequences (see Table I) it is implicit from the present x-ray structure that the high level of protection against isotopic exchange is achieved by the similar formation of several intramolecular hydrogen bonds stabilizing a corresponding carboxy-terminal $\alpha$-helix in these peptides. Specific deuterium labeling of only these $\alpha$-helical amide hydrogens in the peptide is accomplished by incubating deuterated uPAR-peptide complexes in protiated buffer. This exchange-out experiment allows all amide hydrogens situated in the flexible amino-terminal region to exchange rapidly with the solvent protium, while the carboxy-terminal amide hydrogens engaged in the $\alpha$-helix formation are resilient to this process and thus retain their deuterium. An outline of this experiment is depicted schematically in Fig. 1.

The peptide antagonist AE133 differs from AE138 by a single substitution of Ala to Arg (amino acid sequences are listed in Table I). This substitution does not affect the number of protected amide hydrogens of the peptide when it participates in complex formation with uPAR (22, 24). The presence of an internal Arg residue, however, does have a pronounced impact on the gas-phase fragmentation pattern of that particular peptide (AE133). Accordingly the MS/MS spectrum of [AE133 + H]$^+$ is dominated by a site-selective cleavage at Asp$^6$ yielding the very abundant $y_6$ fragment ion and the somewhat less abundant complementary $b_6$ ion (Fig. 2, upper panel). In contrast, the MS/MS spectrum of [AE138 + H]$^+$ displays a continuum of abundant b-type fragment ions (from $b_6$ to $b_{13}$), which are associated with satellite peaks representing the corresponding a-type fragments (Fig. 2, lower panel). Although these MS/MS spectra were obtained with argon as collision gas we anticipate that decomposition of metastable ions contributes significantly to the fragment ion yield in the MS/MS experiments due to the ionizing properties of $\alpha$-CHCA during laser desorption. This process is commonly known as PSD. To investigate the contribution of PSD to the observed gas-phase fragmentation, MS/MS spectra were ob-

| Ligand | Sequence | $k_{off}$ |
|--------|----------|----------|
| AE133  | KGSGGD-Cha-FsrYLWS | 0.9  |
| AE138  | KGSGGD-Cha-FsaYLWS | 6.3  |
| AE147  | KSD-Cha-FskYLWSSK | 19.7  |

* Amino acids are shown in the single letter code where capitals denote $\alpha$ chirality and lowercase denotes $\beta$ chirality. Peptides have unmodified amino and carboxyl termini. Bold residues highlight the amide hydrogens that gain a high degree of protection against isotopic exchange when the peptide is bound to uPAR (22). Residues of AE147 shown in italics adopt an $\alpha$-helical structure upon binding to uPAR (35).

* Dissociation rate constants, $k_{off}$ $\times (10^{-4}$ s$^{-1}$), were measured by amide hydrogen exchange for uPAR$^{H_2}$ (24).

* Measured at 12 °C by surface plasmon resonance using a Biacore$^{TM}$ 2000 with uPAR produced by Drosophila S2 cells as the immobilized receptor on the sensor chip (3800 response units) and 2-fold dilution series of AE147 as analytes (4–500 nM).
tained with and without collision gas. In the absence of collision gas, the fragment ions are exclusively formed by decomposition of metastable precursor ions within the collision cell of the MALDI tandem time-of-flight mass spectrometer (i.e. PSD). In the presence of collision gas, the fragment ions result from two different processes, namely high energy CID and PSD. It should be emphasized that PSD yields peptide fragments, which are similar to those generated by low energy collisional activation (36), whereas the characteristic high energy CID fragments are generally not observed with PSD (37).

The occurrences of fragment ions involving cleavage of side-chain as well as main-chain peptide bonds (i.e. v-, w-, and d-type ions) are thus spectral signatures for the existence of high energy CID processes. The MS/MS spectrum of AE133 acquired with collision gas (Fig. 3, upper panel) contains several of these characteristic high energy fragment ions. Note that these ions are not observed in the absence of collision gas (Fig. 3, middle panel). The presence of these characteristic fragments was further investigated by acquiring a CID spectrum with DHB as MALDI matrix and a laser intensity close to the threshold for ion production (Fig. 3, lower panel). These gentle conditions for desorption generated precursor ions possessing insufficient internal energy to support metastable fragmentation. Accordingly nearly pure high energy CID spectra without PSD fragments can be recorded under such conditions (38, 39). Unfortunately the yield of the “cold” precursor ions is very low compared with the yield of “hotter” precursors ions obtained at higher laser intensities. This precludes the use of cold precursor ions in CID experiments with deuterium-labeled precursors as the abundance of fragment ions becomes too low to allow an accurate determination of their deuterium content. Due to these limitations, we chose to investigate the intramolecular migration of amide hydrogens under conditions where both PSD and high energy CID processes are operative. Fig. 4, middle panel, shows the CID + PSD spectrum of AE133 selectively labeled
Fig. 2. The effect of arginine on the fragmentation pattern of singly protonated peptides. The MALDI MS/MS spectrum of AE133 containing an internal Arg residue is shown in the upper panel, and the corresponding spectrum of AE138 in which Ala has replaced this Arg is shown in middle panel. Both spectra were obtained under identical conditions with argon as collision gas and α-CHCA as MALDI matrix. The fragmentation scheme shown in the lower panel provides an inventory of the b and y sequence ions derived from AE138 and AE133, respectively. x is either D-Ala (AE138) or D-Arg (AE133). Lowercase single letter code denotes D chirality of amino acid residue. Rel. Int., relative intensity.

Fig. 3. Fragment ions of AE133 are formed by a combination of high energy collision-induced dissociation and postsource decay. MALDI MS/MS spectra of AE133 obtained in the presence (upper and lower panels) or absence (middle panel) of collision gas are shown. In the absence of collision gas the fragments result from postsource decay. The spectra were obtained using α-CHCA as MALDI matrix with the exception of the spectrum shown in the lower panel where DHB was used as matrix. Rel. Int., relative intensity.

Fig. 4. Prevalence of hydrogen scrambling during gas-phase fragmentation of an Arg-containing peptide. MALDI MS/MS spectra of non-labeled AE133 and AE133 labeled with deuterium in the uPAR binding sequence are shown in the upper and middle panel, respectively. The lower panel shows the isotopic distributions of fragment ions \( b_6, y_8, \) and \( d_{12} \). Rel. Int., relative intensity.
with deuterium in the uPAR binding region. Also shown in Fig.
4 is the isotopic envelope of the b6 fragment ion, which
constitutes a sensitive probe for the occurrence of amide
deuterium atom scrambling. The amino-terminal residues en-
compassed by this particular fragment belong to the non-
binding region of AE133, and these residues do not contain
any deuterium prior to the MS/MS analysis (22). Thus, in case
of 0% scrambling the b6 ion should not contain deuterium
atoms, while the onset of scrambling inevitably will increase
its deuterium content. Inspection of the mass-shifted isotopic
envelope of the b6 ion from selectively deuterated AE133
reveals immediately that considerable scrambling does in-
deed occur under these conditions. The average deuterium
content of this fragment ion is 1.0, which is consistent with a
situation causing 100% scrambling (1.0). Consistently the
deuterium content of all fragment ions listed in Table II mim-
icked closely the predicted values for 100% scrambling. This
was also true for the specific high energy fragment ions (i.e. v7
and d12).

The MS/MS spectra of AE138 obtained with and without
collision gas are almost indistinguishable (Fig. 5). Even a close
inspection of the “gas on” spectrum (Fig. 5, upper panel) does
not reveal any sequence ions different from those observed in
the “gas off” spectrum (Fig. 5, middle panel). In this particular
case the high energy CID of singly protonated AE138 thus
does not lead to the formation of any characteristic high
energy CID fragments. The absence of these fragments is
corroborated by the pure CID spectrum using DHB as MALDI
matrix and a laser intensity close to the threshold for ion
production (Fig. 5, lower panel). Because these characteristic
fragment ions are absent upon high energy CID conditions for
AE138 it is evident that the occurrence of side-chain cleavage
is directly linked to the presence of the arginine residue in
AE133. A similar observation of the ability of arginine residues
to induce side-chain cleavage in smaller peptides was re-
cently reported using a prototype version of the present in-
strument (33). This effect has been ascribed to the high proton
affinity of arginine, which effectively sequesters the ionizing
proton thus facilitating charge-remote fragmentation of spe-
cific side chains (40). Furthermore the preference for site-
specific cleavages at Asp and Glu residues has also been
ascribed to the immobilization of the ionizing proton by argi-
nine (25, 26). Fig. 6, middle panel, shows the MS/MS spectr
of AE138 selectively labeled with deuterium in the uPAR
binding region. As for AE133, the deuterium content for the b6
fragment ion of AE138 provides the real acid test for occur-
cence of amide hydrogen atom scrambling during collisional
activation. The isotopic envelope of this ion is also displayed
in Fig. 6, and the deuterium content of the b6 ion (1.5) is once
again identical to the theoretical value (1.5) calculated for
100% scrambling among all N- and O-linked hydrogens.
A summary of the corresponding analysis for the whole b-ion
series of AE138 is shown in Fig. 7. Unequivocally the scram-

| Ion   | Sequencea | $^{2}$H | Exp. c | 100%c | 0%d |
|-------|-----------|--------|--------|-------|-----|
| b6    | KGSGGD    | 1.0    | 1.0    | 0.0   |
| a7    | KGSGGD-Cha| 1.2    | 1.1    | 0.0   |
| d12   | KGSGGD-Cha-FlsY | 2.1 | 2.1    | 1.9   |
| v8    | FlsYLS    | 1.9    | 1.8    | 2.7   |
| v7    | FlsYL     | 1.6    | 1.6    | 2.5   |

a Amino acid sequences for fragment ions formed by high energy
collision-induced dissociation of singly protonated AE133 (denoted
parent). Amino acids are shown in the single letter code where cap-
mitals denote L chirality and lowercase denotes D chirality. Residues in
italics are those that undergo side-chain fragmentation during CID.

b Experimental deuterium content of the fragment ions. This number
is derived from the difference in the average mass between a
fragment ion generated from AE133 selectively labeled with deute-
rion in the uPAR binding region and the corresponding fragment
generated from non-labeled AE133.

c Theoretical deuterium content of the fragment ions assuming 100% scrambling among all N- and O-linked hydrogens.

d Theoretical deuterium content of the fragment ions assuming 0% scrambling.

Fig. 5. Fragment ions of AE138 are formed by a combination of high energy
collision-induced dissociation and postsource decay. MALDI MS/MS
spectra of AE138 obtained in the presence (upper and lower panels) or ab-

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Bbling model taking all $N$- and $O$-linked hydrogen atoms into account exhibits a nearly perfect fit to the experimental data. This model mimics accurately even the fine structure of the experimental data (i.e. the non-linearity of the graph). In more quantitative terms, the deviation was only $0.017$, whereas the scrambling model excluding $O$-linked hydrogens correlates less well with the data ($\chi^2 = 0.15$). Evidently a very large deviation was observed between experimental data and the theoretical values for 0% scrambling ($\chi^2 = 4.4$). In all experiments we consistently found that the $b_6$ ion from AE138 contains more deuterium than the corresponding $b_6$ ion from AE133. Two factors pertain to this. First, the deuterium content of AE133 is lower than that of AE138 because the former experienced a higher deuterium loss in the solid $\alpha$-CHCA matrix (see "Experimental Procedures"). Second, AE133 has four additional exchangeable hydrogens compared with AE138 because of the presence of the internal arginine, and this reduces the deuterium fraction of AE133 and, in turn, decreases the deuterium content of its $b_6$ ion (22).

**DISCUSSION**

The degree of hydrogen ($^{1}\text{H}/^{2}\text{H}$) scrambling induced by collisional activation of protonated peptides is currently a matter of some controversy. Recently both Deng et al. (7) and Kim et al. (8, 9) reported that the deuterium content retained in $b$-type fragment ions derived from low energy collisional activation still pertains to the original deuteration pattern that was incorporated during preceding solution-phase exchange experiments. In these studies, the degree of hydrogen scrambling upon collisional activation was accordingly claimed to be negligible. In apparent agreement with this observation, Hoerner et al. (13) reported a low degree of scrambling when a protein was fragmented by low energy collisional activation in an electrospray ion source (i.e. by nozzle-skimmer fragmentation). On the other hand, we (22) and others (41, 42) have consistently found extensive hydrogen scrambling upon low energy collisional activation of protonated peptides and proteins (43). Similarly migration of $N$-linked hydrogens upon $y$-ion formation has been observed upon high energy (44) and low energy (45) CID of singly charged peptides. Several experimental differences exist between the above mentioned studies, and the significance of these differences in relation to the process of hydrogen scrambling have been discussed in detail elsewhere (22). Because of our recent development of a
unique set of peptides that can be selectively deuterium-labeled through a controlled exchange reaction in solution, the occurrence of hydrogen scrambling induced by various ion activation conditions can now be investigated rigorously. The amides in the carboxyl-terminal half of these peptides are labeled with deuterium, whereas the amides in the amino-terminal half are protiated. This polarized labeling ensures an inherent high sensitivity for the detection of scrambling. Particularly the b₆ and b₇ fragments, both of which are derived from the non-binding amino-terminal region of the parent peptide, function as efficient and sensitive reporters of positional randomization because these fragments display a large difference in their theoretical deuterium content between the two extremes of no and 100% scrambling. As the size of the b-ion increases this difference becomes smaller, and for the b₁₂ ion the two extreme cases have almost identical deuterium content (see Fig. 7). In conclusion, it is very important to have a sensitive model system that exhibits a large difference in deuterium content for the two limiting scenarios of 0 and 100% scrambling if the extent of hydrogen scrambling shall be determined accurately.

According to the generally accepted “mobile proton” model for gas-phase peptide fragmentation, the proton mobility is highly dependent upon the presence of Arg residues and the number of added protons. This phenomenon is ascribed to the high proton affinity of Arg residues, which effectively sequester protons and hence promote charge-remote fragmentation pathways (25, 26, 40, 46). The effect is most pronounced when the number of added protons is less than or equal to the number of Arg residues (26). In our previous study, we demonstrated that low energy collisional activation of doubly protonated AE133 caused complete scrambling among all its N- and O-linked hydrogens (22). As AE133 contains a single Arg residue, we anticipate that the proton mobility will be reduced in the singly protonated molecule. A mass spectrometric signature for reduced proton mobility is the occurrence of a site-selective cleavage at Asp residues (25, 26). As predicted, this cleavage is much more prominent in the MS/MS spectra of singly protonated AE133 compared with that of doubly protonated AE133 (e.g. compare Fig. 2, upper panel, with Fig. 4B in Ref. 22). Nonetheless our results clearly show that the degree of intramolecular proton/deuteron migration is still sufficient to cause complete scrambling between all N- and O-linked hydrogens (see Table II).

In our MS/MS experiments, the fragment ions formed by PSD prior to the collision region are not transmitted to the collision cell, and therefore they do not contribute to the MS/MS spectra. The metastable precursors of these fragments represent the highest internal energy of the population of peptide ions that survived the MALDI process and subsequent acceleration. The precursor ion population that actually enters the collision cell is thus depleted of the hottest metastable ions. Nevertheless a significant proportion of the fragment ions in our high energy CID experiments are formed by postsource decay as demonstrated by the presence of relatively abundant fragment ions in the “gas-off” MS/MS spectra obtained with α-CHCA as MALDI matrix. For AE138, PSD and high energy CID yielded identical fragments, whereas high energy CID of AE133 gave a series of fragments that are distinct for this activation process (i.e. d-, v-, and w-ions). It should be noted that the internal energy distributions of the precursor ion populations after low energy or high energy collisional activation are rather similar with the exception of a high energy tail in case of high energy activation (47). It has been hypothesized that high energy CID should be less prone to cause hydrogen scrambling as the heating process occurs on a much faster time scale (27). Analogously the prevalence of certain gas-phase peptide rearrangements is indeed diminished when the ion activation time is shortened (48, 49). This phenomenon can be rationalized as rearrangement reactions usually have lower activation energies and lower frequency factors than competing cleavage reactions (47). In the present study, the deuterium content of the characteristic high energy CID fragments, listed in Table II, was in close agreement with theoretical values calculated for 100% scrambling. This signifies that high energy collisional activation causes hydrogen scrambling in the present setting. This, in turn, suggests that the activation energies for the scrambling processes are lower than the activation energies for the charge-remote fragmentation pathways and that the frequency factors for the scrambling processes are at least comparable to or probably higher than those of the cleavage reactions. However, we cannot rigorously rule out the possibility that the hydrogen scrambling observed for the charge-remote fragments has occurred already during the heating processes in the MALDI ion source. The gain in internal energy of ions generated by MALDI is determined by several processes (50): 1) collisional activation, as the ions are accelerated by the electrical field in the ion source they may collide with neutrals in the expanding plume; 2) thermochemistry of gas-phase reactions involving the ionized analyte, e.g. the exothermicity of a gas-phase proton transfer reaction between MALDI matrix and analyte; and finally 3) photon absorption. These processes may have imparted sufficient energy to the peptides forcing them to undergo complete hydrogen scrambling. Furthermore our choice of MALDI matrix (α-CHCA) causes a significant degree of heating as evidenced by the relatively abundant PSD fragments. As PSD makes a considerable contribution to the abundance of fragment ions in our MS/MS experiments, it is evident that complete scrambling has occurred in the metastable precursors that undergo PSD. In this context, we note that PSD has been used previously in the attempt to localize...
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the sites of incorporation of deuterium in solution (19, 20). In these studies, it was implicitly assumed that the level of scrambling was negligible, but no experimental evidence was presented to support this assumption.

CONCLUSIONS

The presence of an Arg residue in AE133 induces a number of fragmentation pathways that are believed to represent charge-remote dissociation channels. These include the site-selective cleavage at Asp (25, 26) as well as the formation of fragment ions with side-chain cleavage (i.e. d-, v-, and w-ions) (40). The promotion of charge-remote fragmentation pathways by the presence of Arg is believed to reflect attenuated proton mobility within the precursor ion. However, our experimental data showed that a high level of protons/deuteron traffic persists in the collisionally activated peptides, and this results in complete positional randomization among N- and O-linked hydrogens prior to dissociation of the gaseous peptide. With a view to our current results, we recommend that extreme caution should be exercised if PSD or CID is chosen as the method to obtain information on protein structure in solution. However, we believe that electron capture dissociation (ECD) may still hold some promise for gas-phase fragmentation without the concomitant induction of hydrogen scrambling in the gaseous peptides. Peptide backbone cleavage by ECD is believed to occur on a much faster time scale than the unimolecular fragmentation reactions of collisionally activated peptides (51). If ECD thus passes the present acid test for scrambling it should be possible to achieve single amide resolution for nearly every residue within a deuterium-labeled protein.

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