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Development of a Peptide Probe for the Occurrence of Hydrogen ($^1H/^2H$) Scrambling upon Gas-Phase Fragmentation

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Hydrogen ($^1H/^2H$) exchange combined with mass spectrometry (HX-MS) has become a valuable method for the analysis of protein structural dynamics. Currently, localization of the incorporated deuterons is made by enzymatic cleavage of the labeled proteins, and single-residue resolution is typically only achieved for a few residues. Determination of site-specific deuterium levels by gas-phase fragmentation would greatly increase the applicability of the HX-MS method. It is, however, mandatory for this gas-phase approach that hydrogen ($^1H/^2H$) scrambling in the gaseous peptide is negligible. Thus, it is important to have a simple reference system where the onset of scrambling processes is readily detected. Here we describe a simple well-characterized set of peptides with a unique regioselective labeling that ensures an inherent high sensitivity for the detection of scrambling. This selective labeling is achieved by utilizing differences in the intrinsic exchange rates between various amino acid residues. We demonstrate that our peptides can be infused directly into an electrospray ion source by means of a cooled glass syringe, while maintaining their selective labeling in solution. We further show that the selective labeling is completely erased upon low-energy collisional activation in a tandem mass spectrometry experiment as a result of extensive hydrogen ($^1H/^2H$) scrambling.

Amide hydrogen ($^1H/^2H$) exchange monitored by mass spectrometry (HX-MS) has become a recognized method for the characterization of structural dynamics of proteins and their complexes.[1−4] This method presents some distinct advantages compared to more traditional spectroscopic techniques such as NMR and X-ray crystallography. In particular, the ability of HX-MS to observe EX1-type exchange directly by the appearance of bimodal peak distributions has been used to study transient unfolding processes,[5−8] folding intermediates,[9] and dynamics of peptide−protein complexes.[10] Furthermore, aggregation-prone proteins or proteins not amenable to crystallization can be studied in solution at low concentrations (μM).[8,11] A typical global exchange experiment is carried out by incubating the protein of interest in deuterated buffer. At regular intervals, aliquots are withdrawn and the isotopic exchange of backbone amide hydrogens is quenched by acidification and cooling. Subsequently, the deuterium content of the labeled protein is determined directly by mass spectrometric analysis. To obtain information about local deuterium incorporation, the labeled protein is proteolytically cleaved with pepsin and the resulting peptides are separated by reversed-phase chromatography and analyzed by mass spectrometry. By carefully analyzing the deuterium content of peptides with overlapping sequences, it is possible to extract site-specific deuterium levels. However, single-residue information can usually only be obtained for a few residues in a protein.

Gas-phase fragmentation appears as an attractive alternative to obtain site-specific information and has in recent years been used in the attempt to localize sites within proteins or peptides that have been isotopically labeled during solution $^1H/^2H$ exchange experiments.[12−30] Furthermore, gas-phase fragmentation of proteins labeled in solution avoids the inevitable deuterium loss

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caused by back exchange during proteolytic cleavage and chromatographic separation. An essential prerequisite for this approach, however, is that the level of hydrogen ($^1H/^2H$) scrambling is minimal in the gaseous proteins or peptides prior to their dissociation. That is, the labeled sites must not interchange their labels with nonlabeled sites in the gaseous peptide upon activation. Otherwise, the information gained from such fragmentation experiments will not be relevant for the labeling pattern in solution, and incorrect conclusions regarding the protein structure will inevitably be drawn. The degree of hydrogen ($^1H/^2H$) scrambling upon collision-induced dissociation of protonated peptides is currently a matter of some controversy. Recent reports have indicated that hydrogen scrambling is minimal for b-type fragment ions generated by low-energy collisional activation.[18,21,22] A similar observation was reported for fragments ions derived from in-source collisional activation of multiply protonated proteins.[19,20] In contrast, we[31,32] and others[17,33−37] have consistently found that extensive scrambling occurs upon collisional activation of protonated peptides. Similar findings have been observed for proteins.[38,39] Several experimental differences exist between the abovementioned studies, such as the type of mass spectrometer, the ion source design, and the conditions used for collisional activation as well as the investigated peptides and proteins. Some of these differences and their significance in relation to the process of hydrogen scrambling have been discussed in detail elsewhere.[31] The detection of the onset of scrambling processes in a typical protein is complicated by the rather small difference in the fragment ion's deuterium content between the two limiting cases of zero and complete scrambling.[40] It is therefore clear from the conflicting results in the literature that it is important to have a common set of reference peptides from which the degree of hydrogen scrambling upon gas-phase fragmentation can be accurately determined. We have previously investigated the occurrence of hydrogen scrambling using peptides selectively labeled with deuterium in their C-terminal half, while the N-terminal half is nondeuterated.[31,32] This unique polarized labeling ensures an inherent high sensitivity for the detection of scrambling. In particular, the fragment ions derived from the N-terminal half are very sensitive reporter ions as they exhibit a large difference in their deuterium content between the limiting cases of 0 and 100% hydrogen scrambling. Thus, for 0% scrambling, the deuterium content will be zero, while the onset of intramolecular migration of amide hydrogens is readily detected by a mass shift due to the presence of deuterons. The selective deuterium labeling was achieved by having a high-affinity receptor-binding sequence in the C-terminal half of the peptide.[10] When this peptide binds to its specific receptor, the amide hydrogens in its C-terminal half become strongly protected against isotopic exchange with the solvent, while the N-terminal half is not engaged in the interaction and thus undergoes rapid isotopic exchange. This approach is, however, less suitable as a common reference method for measuring the degree of scrambling for the following practical reasons. The receptor protein (urokinase plasminogen activator receptor, uPAR) is unfortunately not readily commercially available. Futhermore, a specialized cooled HPLC setup is required to desalt the labeled peptides prior to their tandem mass spectrometric analysis.

We have therefore undertaken a study to develop a set of peptides with the unique property of having a polarized labeling as described above, but without the requirement of a receptor protein as well as specialized HPLC equipment. The concept of this new approach is based on the intrinsic exchange properties of the various amino acid residues. Harnessing these properties, we synthesized a set of peptides with an inherently high sensitivity for the detection of the onset of amide hydrogen ($^1H/^2H$) scrambling and that allows an accurate determination of the degree of scrambling. These peptides are selectively labeled with deuterium in their C-terminal half, while the N-terminal half is nonlabeled. With these peptides, the occurrence of scrambling for different gas-phase fragmentation methods at various experimental settings can be readily evaluated, e.g., for collision-induced dissociation (CID), electron capture dissociation (ECD), electron-transfer dissociation (ETD), electron detachment dissociation (EDD), and others.

Here, we show that the peptides are readily selectively labeled and that they can be introduced into an electrospray ion source using a simple direct infusion setup, thus making them widely applicable to monitoring hydrogen scrambling processes in various ESI mass spectrometers. Furthermore, we show that low-energy collisional activation in a quadrupole time-of-flight mass spectrometer results in complete positional randomization (i.e., 100% scrambling) of the selective labeling from solution.

EXPERIMENTAL SECTION

Materials. Peptides were synthesized and purified in-house or obtained from Genscript Corp. (Piscataway, NJ). D_2O (99.9%

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D) was purchased from Cambridge Isotopic Laboratories (Andover, MA). All other reagents and chemicals were of the highest grade commercially available.

Hydrogen (^{1}H/^{2}H) Exchange Experiments. To monitor the protium (^{1}H) uptake over time, peptides were either infused directly from the exchange solution into the electrospray ion source or desalted by reversed-phase chromatography prior to mass spectrometric (MS) analysis. Samples that required desalting prior to their MS analysis were made as follows. Fully deuterated peptides were prepared by dissolution of lyophilized peptide (100 μM) in deuterated phosphate buffer (20 mM NaD_2PO_4, pH 2.6 uncorrected value, 99% D_2O), followed by incubation for 18 h at 25 °C. Isotopic exchange was initiated by a 50-fold dilution into phosphate buffer (20 mM NaH_2PO_4, pH 2.6) at 0 °C. Aliquots were harvested at defined times, snap frozen in liquid N_2, and stored at −80 °C until they were desalted and analyzed by mass spectrometry. A fully deuterated control (100% D) was prepared by injecting a fully deuterated peptide sample directly into the rapid desalting setup.

Peptide samples for direct infusion into the electrospray ion source were fully deuterated by incubation in 99.9% D_2O for 18 h at 4 °C. Isotopic exchange was initiated by a 50-fold dilution into a cold buffer (50% MeOH, 0.5 M acetic acid, pH 2.6) and immediately quenched by freezing on dry ice.

Setup for Rapid Desalting. Deuterated peptide samples were desalted by reversed-phase liquid chromatography using a cooled setup for rapid desalting as described previously[11] with only minor modifications. Samples loaded via the injection valve were flushed onto a microcolumn (~2-μL bed volume of Poros 20R2)[41] and desalted for 3 min at 250 μL/min in 0.05% trifluoroacetic acid. Subsequently, the microcolumn was automatically switched inline with the electrospray ion source and the peptide eluted at 50 μL/min by 80% acetonitrile in 0.05% trifluoroacetic acid. The time for desalting, elution, and mass analysis of a peptide sample was 4 min.

Setup for Direct Infusion. Peptide samples in direct infusion buffer (i.e., 50% MeOH, 0.5 M acetic acid, pH 2.6) were thawed and immediately transferred to a precooled syringe (810RN or 825RN, Hamilton Co., Reno, NV). The use of 800-series Hamilton syringes equipped with reinforced plungers ensured a tight seal even at low temperatures. The syringe was mounted on a sample pump (Linton Instrumentation, Norfolk, UK) and cooled by placing dry ice contained in a small reclosable plastic bag directly on the glass syringe barrel. In order to avoid freezing of the solution, no direct contact was made between the dry ice and the metal needle of the syringe. Direct infusion of the cooled solution through a short fused-silica capillary (50 cm in length with a internal diameter of 100 μm or by PEEK tubing with an internal diameter of 175 μm) into the electrospray source was performed at 5−15 μL/min flow rates. The C-terminally truncated peptides were analyzed by the direct infusion setup, and N-terminally truncated peptides were analyzed by the rapid desalting setup.

Mass Spectrometric Analyses. Positive ion electrospray ionization mass spectra were acquired on a LCT mass spectrometer (Waters Inc.) calibrated with sodium iodide cluster ions $[(NaI)_nNa]^+$. The capillary voltage was kept at 3 kV and the cone voltage at 45 V. N_2 desolvation gas was used at 550 L/h and a temperature of 250 °C. The ion source temperature was set to 100 °C.

Gas-Phase Fragmentation by Collision-Induced Dissociation. CID experiments were carried out using a quadrupole time-of-flight mass spectrometer equipped with an electrospray ion source (Q-TOF 1, Micromass, Manchester, UK). The instrument was calibrated with sodium iodide cluster ions $[(NaI)_nNa]^+$. The ion source parameters were as follows: electrospray voltage 3.1 kV, cone 40.0 V, extraction cone 4.0 V, hexapole 1.1 V, ion source block temperature 80 °C, nebulizer gas flow 20 L/h (25 °C), and desolvation gas flow 400 L/h (200 °C). Nitrogen was used as nebulizer and desolvation gas. In the CID experiments, the isotopic envelopes of the selectively labeled peptides were transmitted by the quadrupole (LH = HM = 7) and then accelerated into the hexapole collision cell. The laboratory collision energies for doubly protonated peptides were 62 eV. Argon was used as a collision gas at an indicated manifold pressure of 3×10^{-5} mbar. To obtain good fragment ion statistics, four separate injections of selectively labeled peptides were accumulated in each CID spectrum (signal-to-noise ratio for the weakest diagnostic fragment ion was better than 50).

Data Analysis. Average masses of peptide isotopic envelopes were determined from lockmass-corrected centroided data (processed using MassLynx software, Waters Inc.) using an Excel spreadsheet. The deuterium content was determined from the mass difference between the isotope natural-abundance distributions and the isotope distribution obtained from the deuterated sample. Exchange rates and the predicted exchange curves in Figure 1 were calculated with the HXPEP software kindly provided by Dr. Z. Zhang. This program calculates amide hydrogen-exchange rates in unstructured peptides based on the work by Bai et al.[42] For the predicted exchange curves in Figure 1, the exchange time is offset by 4 min to allow direct comparison between experimental data and predicted values. Note that the exchange rates of hydrogens attached to N or O in the side chains and the N-terminal amino group are orders of magnitude faster than the exchange rates of backbone amide hydrogens at low pH. Similarly, for C-terminally amidated peptides, the primary amide hydrogens at the C-terminus exchange much faster than backbone amide hydrogens. Furthermore, the exchange rate of the N-terminal amide hydrogen is accelerated by the presence of the nearby positive charge of the N-terminal ammonium ion. These fast exchanging hydrogens exchange too fast to be monitored by HX-MS. The scrambling analysis was carried out as described previously.[31] Briefly, to calculate the theoretical deuterium content for a given fragment ion in the case of complete positional randomization for all labile hydrogens in the precursor ion (i.e., 100% scrambling), the experimental deuterium content of the precursor ion is divided by the total number of its labile hydrogens and this ratio is subsequently multiplied with the number of labile hydrogens in the given fragment ion. Note, that labile hydrogens (i.e., exchangeable hydrogens) are those bonded to nitrogen, oxygen, or sulfur.

RESULTS AND DISCUSSION

The isotopic exchange rate for backbone amide hydrogens in a random chain peptide depends on pH, temperature, and the

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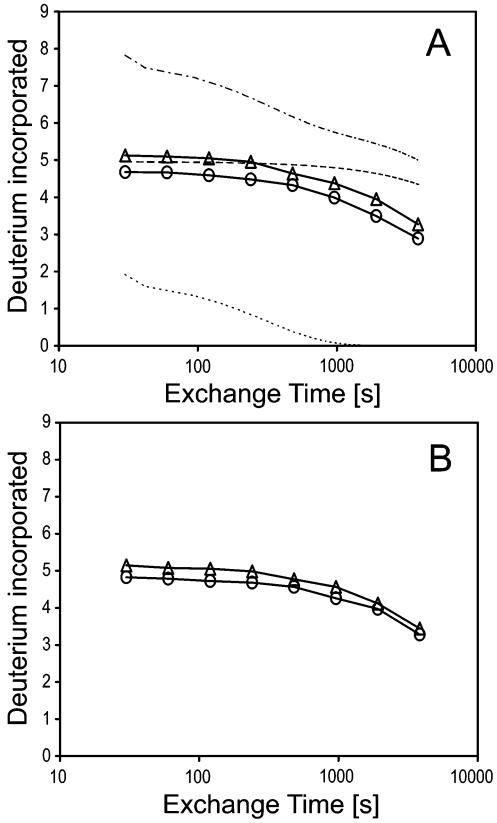

Figure 1. Plots showing the deuterium content of (A) peptides P1 (HHHHHHIIKIIK, triangles) and P2 (HHHHHHIITIIT, circles) and in (B) the C-terminally amidated forms, P1A (triangles) and P2A (circles) as a function of exchange time at 0 °C and pH 2.6. The plots show that the full-length peptides contain five slowly exchanging amide hydrogens. The predicted exchange curves are shown in (A) for the following peptide sequences: HHHHHHIIKIIK (dash-dotted line), HHHHHH (dotted line), IIKIIK (dashed line).

Table 1. Predicted Intrinsic Amide Exchange Rates[a] of Peptide P1

residues	amide HX rate, k (min^{-1}) × 10^3	amide HX half-life, $t_{1/2}$ (min)
$H_{(1)}$		
$H_{(2)}$	8280	0.1
$H_{(3)}$	200	3.5
$H_{(4)}$	200	3.5
$H_{(5)}$	200	3.5
$H_{(6)}$	200	3.5
$I_{(7)}$	6	110
$I_{(8)}$	0.8	811
$K_{(9)}$	3	211
$I_{(10)}$	2	378
$I_{(11)}$	0.8	811
$K_{(12)}$	4	187

[a] HX rates of peptide P1 (HHHHHHIIKIIK) were calculated at pH 2.6 and 0 °C using the HXpep software, based on an algoritm derived from Bai et al.[42]

nature of the neighboring side chains. This so-called intrinsic chemical exchange rate has been investigated in detail by Bai et al.[42] using model peptides and NMR spectroscopy. The neighboring side chains affect the exchange rate by steric and inductive effects. For example, bulky side chains, such as the isobutyl group of isoleucine, retard the exchange rate by blocking the solvent accessibility. Thus, an amide hydrogen flanked by two isoleucine

Table 2. List of Synthetic Peptides

peptide	sequence
P1	HHHHHHIIKIIK
P2	HHHHHHIITIIT
P1A	HHHHHHIIKIIK[a]
P2A	HHHHHHIITIIT[a]
$P1A_{(2-12)}$	HHHHHIIKIIK[a]
$P1A_{(3-12)}$	HHHHIIKIIK[a]
$P1A_{(4-12)}$	HHHIIKIIK[a]
$P1A_{(5-12)}$	HHIIKIIK[a]
$P1A_{(6-12)}$	HIIKIIK[a]
$P1_{(1-6)}$	HHHHHH
$P1_{(1-7)}$	HHHHHHI
$P1_{(1-8)}$	HHHHHHII

[a] C-Terminally amidated.

side chains exchanges ~10 times slower than an amide hydrogen between two alanine side chains. As the steric and inductive effects are additive and only depend on the side chains of the nearest neighbors, site-specific amide hydrogen-exchange rates in an unstructured peptide can be predicted.[42] We have utilized the intrinsic differences in exchange rates among amino acid residues to design peptides that can be labeled with deuterium exclusively in their C-terminal half. This unique property is achieved by having residues with an intrinsic slow exchange rate (e.g., isoleucine) in the C-terminal half, while the N-terminal half contain residues with a relatively fast intrinsic exchange rate (e.g., histidine). Two such peptides are P1 and P2 with the sequences HHHHHHIIKIIK and HHHHHHIITIIT, respectively. Note, that the C-terminal half contains, in addition to isoleucine, either two lysine residues or two threonine residues. These residues are included to prevent possible aggregation of the hydrophobic C-terminal half during peptide synthesis. Table 1 shows that the predicted exchange rates for the amide hydrogens in the C-terminal half are ~2 orders of magnitude lower than those of the N-terminal half. Thus, if isotopic exchange is initiated by transferring fully deuterated peptide into normal buffer at pH 2.6 and 0 °C, then the N-terminal half will lose its deuterium atoms on a minute time scale, while the C-terminal half will retain its deuterium atom for much longer time as the predicted half-lives of its amides are on the order of hours.

To investigate the accuracy of the predicted exchange rates and to test the feasibility of our approach, we carried out deuterium exchange-out experiments for peptide P1 and P2 and their C-terminally amidated forms P1A and P2A, respectively. Figure 1 shows the deuterium content of these peptides as a function of exchange time. Evidently, only ~5 amides remain deuterated in both peptides at the first time point. The remainder of the time course shows that these amides exchange quite slowly, effectively causing the deuterium content of both peptides to remain constant for several minutes. At prolonged incubation, a gradual deuterium loss is observed, but even after 60-min incubation both peptides retain ~70% of their initial deuterium content. To verify that the slowly exchanging amides were confined to the C-terminus, we determined the exchange profiles for a series of N-terminally truncated peptides. The sequences of these truncated peptides are listed in Table 2. If the deuterated amides are exclusively located in the C-terminal half, then removal of the residues in the N-terminal half should not affect the

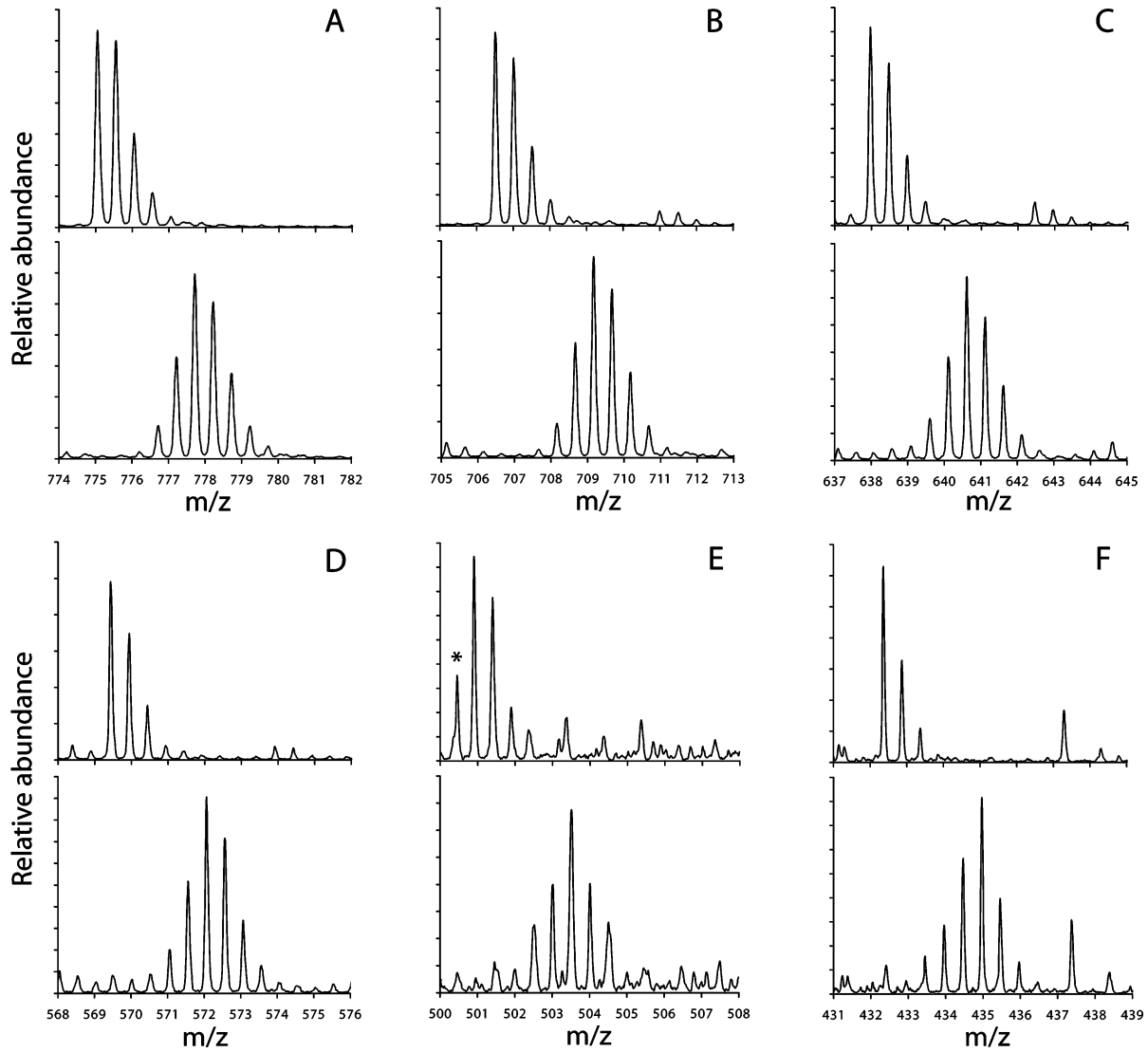

Figure 2. Mass spectra obtained after 4 min of deuterium exchange-out for the full-length P1A peptide and various N-terminally truncated variants at 0 °C and pH 2.6. Displayed are the isotopic envelopes for the doubly protonated peptides. The upper spectrum in each panel shows the isotope natural-abundance distribution. (A) Full-length peptide P1A, HHHHHHIIKIIK−NH_2, *4.7*, (B) $P1A_{2-12}$, HHHHHIIKIIK−NH_2, *4.8*, (C) $P1A_{3-12}$, HHHHIIKIIK−NH_2, *4.8*, (D) $P1A_{4-12}$, HHHIIKIIK−NH_2, *4.8*, (E) $P1A_{5-12}$, HHIIKIIK−NH_2, *4.5*, and (F) $P1_{6-12}$, HIIKIIK−NH_2, *4.5*. The italicized numbers denote the deuterium content. Note, the asterisked peak is an impurity. The isotopic envelopes show that the slowly exchanging amide hydrogens in peptide P1A are exclusively located in the C-terminal half.

deuterium content. Figure 2 shows a mass spectrum of P1A and the truncated peptides obtained after they have undergone isotopic exchange together in exchange-out buffer for 4 min. Evidently, the deuterium levels of peptide P1A and the five truncated forms are nearly identical, demonstrating that the slow-exchanging deuterated amides are indeed localized in the C-terminal half of peptide P1A. Furthermore, the deuterium content after prolonged exchange was also similar for the aforementioned peptides (data not shown). Comparable results were obtained for peptides P1, P2, and P2A (data not shown). These findings demonstrate that a unique polarized deuterium labeling of the peptides is achieved after a short period of isotopic exchange. The results also show that isoleucine side chains confer an effective intrinsic protection against isotopic exchange with the solvent. To further illustrate this, a control experiment was carried out where the glufibrinogen peptide (EGVNDNEEGFFSAR) was included in the exchange experiment together with peptides P1 and P2. The glufibrinogen peptide retained less than 25% of its initial deuterium label after 60 min of exchange-out (data not shown).

Table 1 shows that the predicted half-lifes for six amides in peptide P1 are greater than 100 min. On the basis of this, we would expect to observe six retained deuterons after a short period (~4 min) of exchange-out. However, we consistently find that only five deuterons are retained. To localize the exact position of these five deuterons in the C-terminal half, we analyzed a series of C-terminally truncated peptides: $P1_{1-6}$, $P1_{1-7}$, and $P1_{1-8}$ (sequences are listed in Table 2). After exchange-out for 4 min, the deuterium content of $P1_{1-6}$ and $P1_{1-7}$ was very close to zero, while $P1_{1-8}$ retained ~0.7 deuteron (Figure 3). As expected, the N-terminal half ($P1_{1-6}$) does not retain any deuterons, and this corroborates the aforementioned results obtained from the N-terminally truncated peptides. Surprisingly, the amide group of isoleucine in position 7 has not retained its deuteron, despite having a predicted half-life of 110 min. The amide group of isoleucine in position 8

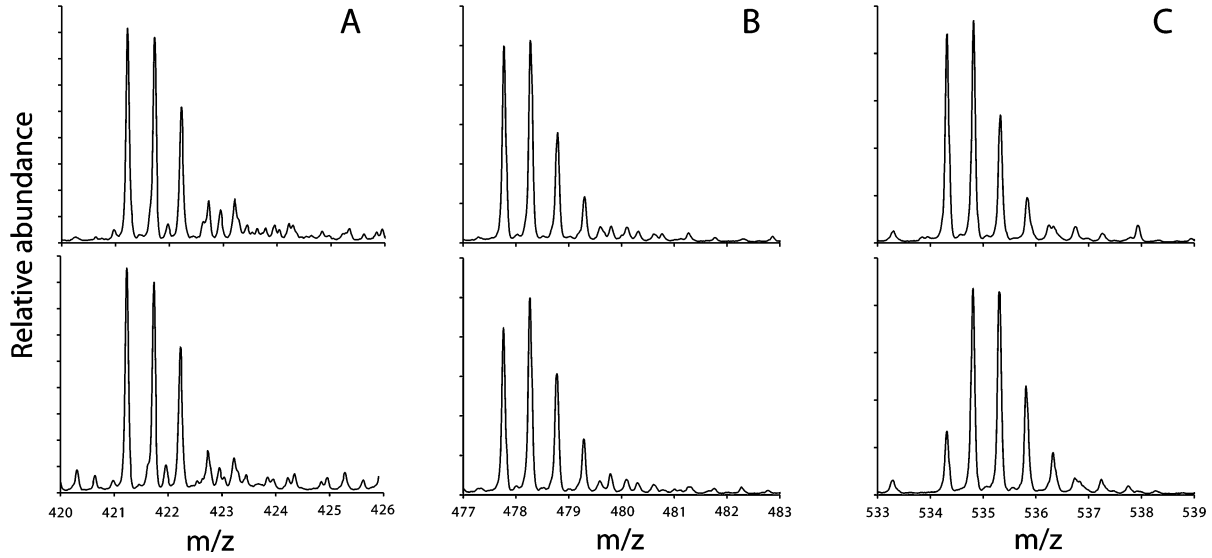

Figure 3. Mass spectra obtained from a deuterium exchange-out experiment of a mixture of C-terminally truncated P1 peptid. Displayed are the isotopic envelopes of the doubly protonated ions. (A) $P1_{1-6}$, HHHHHH, (B) $P1_{1-7}$, HHHHHHI, and (C) $P1_{1-8}$, HHHHHHII. The upper traces display the isotope distributions of fully exchanged peptides, i.e., after prolonged deuterium exchange-out at elevated temperature (2 h, 37 °C). Lower traces, after ~4-min exchange-out at low pH and low temperature. Note, the deuterium contents remained constant during 10 min of exchange-out at low pH and low temperature.es. The isotopic envelopes show that the amide hydrogens of the histidine residues and that of the isoleucine in position 7 of peptide P1 exchange rapidly with the solvent.

has, however, retained its deuteron. Thus, the amide group of the isoleucine residue in position 8 exhibits slow exchange, while the amides of the histidine residues and that of the isoleucine in position 7 undergo rapid exchange with the solvent. In conjunction, these findings strongly indicate that the five retained deuterons in our model peptides are located at the amides of residues from Ile8 to Lys12 (i.e., -IKIIK). Clearly, the observed exchange rate of Ile7 does not correlate well with the predicted exchange rate. Similarly, while the predicited exchange rates of the six N-terminal histidine residues are much larger than those of the residues in the C-terminal half, one would still expect to observe residual deuterons in the amides of the His residues at the first time points in the exchange-out experiment. This is clearly illustrated in Figure 1, where the predicted deuterium content for His_{1-6} is close to 2 at 30 s and then it levels out at ~1000 s. These apparent discrepancies between experimental and predicted exchange rates indicate that some primary structure effects are presumably not completely accounted for in the work by Bai et al.[42] The deviation from the predicted exchange rates could result from a high charge density of the N-terminal half, which attracts counterions, thereby increasing the local hydroxide ion concentration and enhancing the exchange rates.[43] Note, however, that the predicted deuterium content for the theoretical peptide IIKIIK was in good agreement with the observed values for the full-length peptides up to ~1000 s exchange (Figure 1). The accelerating effect of the N-terminal ammonium ion was omitted in the calculation of exchange rates for this theoretical peptide.

To illustrate the applicability of our model peptides for systematic studies of hydrogen ($^1H/^2H$) scrambling upon gas-phase fragmentation, we subjected the selectively labeled peptides to collisional activation in a quadrupole time-of-flight mass spectrometer. Figure 4 shows collision-induced dissociation mass spectra of labeled and nonlabeled peptide P1A. The CID spectra are dominated by several abundant b fragment ions (i.e., b_{11}^{2+}, b_{10}^{2+}, b_9^{2+}, b_8^{2+}, b_7^{2+}, b_2^{1+}). The deuterium content of these ions, obtained from peptide P1A labeled with deuterium exclusively in its C-terminal half, is plotted in Figure 5. Also shown is the theoretical deuterium content of these ions assuming either 0 or 100% scrambling among all labile hydrogens. The interpretation of this experiment is exemplified by the b_2^+ ion, which only contains a single amide group that does not retain any deuterons after isotopic exchange in solution. The experimental deuterium content of the b_2^+ ion is, however, 0.9. Similarly, the b_7^{2+} comprises residues that are nondeuterated in solution. Nevertheless, this fragment ion contains 2.8 deuterons. It is thus clear that amide deuterons from the C-terminus have migrated to residues in the N-terminal half in the gaseous peptide ion prior to or upon collisional activation. Note that if the level of scrambling was 0%, then both of these fragment ions should contain ~0 deuterons. In the case of 100% scrambling among all labile sites, the theoretical deuterium content of the fragment ions b_2^+ and b_7^{2+} is 1.0 and 2.7, respectively. These theoretical values are very close to the experimentally observed values of 0.9 and 2.8. A similar good agreement between theoretical and experimental values is observed for the other b ions (Figure 5). In quantitative terms, the deviation is only $\chi^2_{100\%} = 0.04$, while the correlation between experimental data and theoretical values for 0% scrambling is very poor ($\chi^2_{0\%} = 4.8$). The nearly perfect agreement between the experimental data and the theoretical values for 100% scrambling shows that all labile hydrogens have undergone complete positional randomization prior to or during dissociation of the gaseous peptide. This finding is in line with our previous results with urokinase receptor ligands where ~100% scrambling was observed.[31,32]

To make our selectively labeled peptides easily adaptable to the various mass spectrometers equipped with electrospray ion

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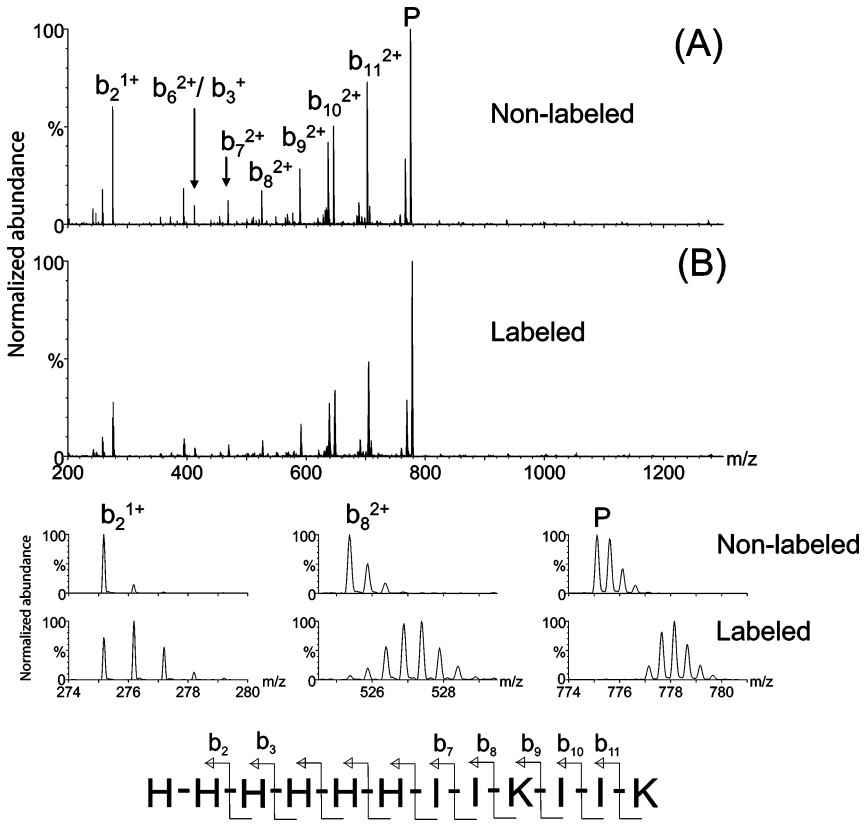

Figure 4. CID spectra of doubly protonated peptide P1A (HHHHHHIIKIIK−NH_2). Spectrum A was obtained from nonlabeled P1A and spectrum B from P1A labeled with deuterium exclusively in its C-terminal half. The precursor ion is denoted P. The lower panel shows isotopic envelopes of the b_2 and b_7 fragment ions derived from nonlabeled and labeled P1A precursor ions, which are also displayed. The fragmentation scheme shows sequences of the fragment ions. The isotopic envelopes show that deuterons migrate from the amides of the C-terminal half to the residues in the N-terminal half upon activation of the gaseous peptide.

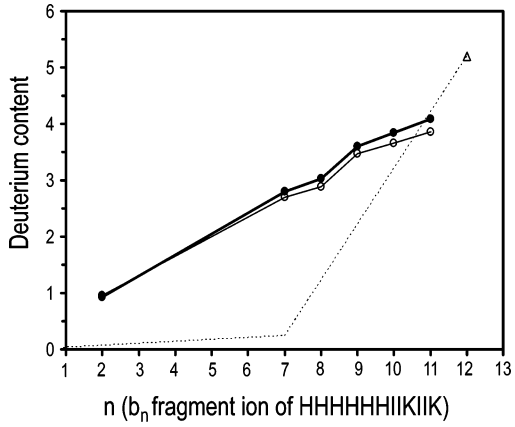

Figure 5. Deuterium content of b-fragment ions (filled circles, thick line) generated by CID of doubly protonated P1A (HHHHHHIIKIIK−NH_2) labeled with deuterium exclusively in its C-terminal half. The dotted lines indicate the theoretical deuterium content in the case of 0% scrambling. Also shown is the theoretical deuterium content of b-fragment ions in the case of 100% scrambling among all exchangeable hydrogens (open circles, thin line). The open triangle indicate the deuterium content of the precursor ion. Collisional activation causes complete positional randomization among all labile sites in peptide P1A.

sources and to circumvent the need for a specialized cooled HPLC instrumentation, we have further devised a simple setup for direct infusion of selectively labeled peptides into an electrospray ion source. Importantly, such a setup should be capable of maintaining a low temperature of the sample to minimize the inevitable deuterium loss from the intrinsically protected deuterated C-terminus. We found that an effective and practical method for cooling of the exchange buffer could be obtained by placing a small plastic bag with dry ice directly on the glass syringe, while it infuses exchange buffer into the ion source. The use of acidic buffers containing organic solvent (i.e., methanol or acetonitrile) enabled cooling by dry ice to subzero temperatures, which minimized the deuterium loss without freezing the solution. The deuterium levels of peptides from direct infusion of exchange-out buffers with organic solvent were comparable to those obtained with the cooled HPLC system for rapid desalting of peptide samples (Figure 6). Importantly, the deuterium content remained virtually constant for at least 10 min of continuous direct infusion, indicating that the combined effect of cooling to subzero temperatures and the presence of methanol significantly reduced the deuterium loss from the intrinsically protected C-terminal half. For studies of hydrogen atom scrambling upon gas-phase activation, this direct infusion approach has the advantage of a simple experimental setup combined with the utility of continuous infusion of selectively labeled peptides in a prolonged time span. We expected that a high concentration of acetonitrile in the exchange-out buffer would further reduce the isotopic exchange rates as acetonitrile does not contain any exchangeable hydrogens and its presence therefore effectively lowers the concentration of the exchange catalyst ions (H^+ and OH^-). Indeed, using an exchange-out buffer with 90% acetonitrile further minimized the deuterium loss by ~15% relative to the 50% methanol buffer

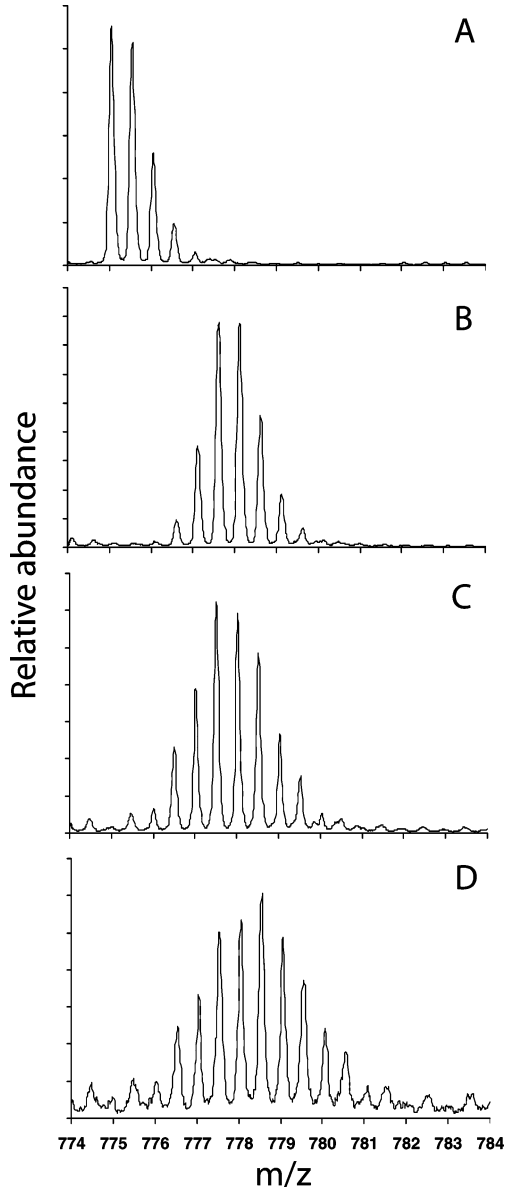

Figure 6. Direct infusion of peptide P1A into the electrospray ion source while maintaining selective labeling. Mass spectra obtained from deuterium exchange-out experiments for the P1A peptide (HHHHHHIIKIIK−NH_2) using desalting by HPLC or direct infusion with a glass syringe. Displayed are the isotopic envelopes of the doubly protonated peptide. (A) Isotope natural-abundance distribution; (B) 30-s deuterium exchange-out in 0.5 M acetic acid followed by ice-cooled HPLC desalting; the peptide eluted after 4 min; (C) 2-min deuterium exchange-out in 50% methanol, 0.5 M acetic acid in a dry ice-cooled glass syringe; (D) 2-min deuterium exchange-out in 90% acetonitrile, 0.5 M acetic acid buffer in a dry ice-cooled glass syringe. Note, the deuterium content of the P1A peptide in (C) and (D) remained virtually constant during 10 min.

(compare Figure 6D and C). This effect was, however, achieved at the expense of a significant loss in the signal intensity of the peptide ions, making such buffers less suitable. The effect of different flow rates on deuterium level was also investigated, and we found that the deuterium content of infused peptides was reduced upon decreasing the flow rate (lowering of the sample pump flow rate by 33%, reduced the deuterium content by ~20%, data not shown). This indicates that part of the deuterium loss takes place in the transfer line, ion source, or both, which were at ambient temperature. From a practical point of view, however, such effects are less important as the deuterium loss was relatively small and the deuterium content of the infused peptides using our present setup remained constant in a sufficient time frame (~10 min).

CONCLUSION

We have developed a set of peptides with a unique selective labeling that makes them highly suitable for detecting and measuring the onset of amide hydrogen ($^1H/^2H$) scrambling in tandem mass spectrometry experiments. The peptides are labeled with deuterons exclusively at the amide groups in their C-terminal half, while the N-terminal half is nondeuterated. This selective labeling occurs as a result of differences in the intrinsic exchange rates between the residues of the N- and C-terminal halves. In the absence of scrambling, fragment ions derived from the N-terminal half will contain zero deuterons. When, however, scrambling processes do occur, the deuterons from the C-terminal half will migrate to the N-terminal half and this is readily detected as a mass increase. In the present work, we have demonstrated that complete scrambling among all exchangeable sites occurs upon low-energy CID of our doubly protonated peptides. Currently, conflicting results exist in the literature with respect to the extent of scrambling upon CID of protonated peptides. Our selectively labeled peptides may help in resolving this pending controversy, as the extent of scrambling is readily and accurately measured with our peptides and they can easily be adapted to any electrospray ionization mass spectrometer. They should also be suitable for MALDI MS/MS analyses with minor modifications of the MALDI sample preparation protocol.[32] It is of particular interest to investigate the occurrence of amide hydrogen ($^1H/^2H$) scrambling for the recently introduced gas-phase fragmentation techniques based on the interaction between electrons and peptide ions, i.e., ECD, ETD, and EDD. Cleavage of the polypeptide backbone with, for example, ECD is believed to occur extremely fast and without concomitant vibrational excitation of the peptide ion.[44] Consequently, these electron-based fragmentation techniques hold great promise for obtaining site-specific solution data by means of gas-phase dissociation.

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