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# Investigation of amide hydrogen back-exchange in Asp and His repeats measured by hydrogen ($^1H/^2H$) exchange mass spectrometry

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ABSTRACT

Hydrogen/deuterium exchange (HDX) monitored by mass spectrometry (HDX-MS) has become a valuable tool in studies of protein dynamics and protein interactions. Isotopic exchange is typically initiated by diluting a protein solution into deuterated buffer at physiological conditions. For MS analysis, the exchange reaction is quenched by acidification and cooling and the labeled protein (or a digest thereof) is analyzed by mass spectrometry. An inevitable deuterium loss occurs during quench conditions (*i.e.*, back-exchange). The primary structure of the peptide influences the back-exchange rate due to steric hindrance by bulky side groups and by inductive and charge effects. Here we show that the back-exchange in histidine repeats ($His_6$) at HDX-MS quench conditions is complete within a few seconds using either acetic acid or formic acid in the quench solution, while aspartic acid repeats ($Asp_6$) retain deuterons for several minutes using formic acid. We employ electron transfer dissociation to obtain residue-specific deuterium levels of the Asp repeat in $K_2D_6$IIKIIK using a hybrid linear ion trap-Orbitrap mass spectrometer. Our results show an unexpected uneven distribution of deuterium in the Asp repeat. The implication of the rapid back-exchange of His repeats for HDX-MS protein hydrogen exchange studies is discussed. We also discuss the implications of retained deuterons in the Asp repeat of $K_2D_6$IIKIIK when this peptide is used as a probe for the occurrence of hydrogen scrambling.



## 1. Introduction

Hydrogen/deuterium exchange (HDX) monitored by mass spectrometry (MS) has become a recognized method for probing the conformational properties of proteins and protein complexes [1–6]. Isotopic exchange is typically carried out by incubating a protein in deuterated buffer at physiological conditions. After various periods of deuteration, aliquots are harvested and the exchange reaction is slowed down (quenched) several orders of magnitude by cooling (0 °C) and acidification (pH ~2.5). The subsequent analysis is carried out at quench conditions to retain as much as possible of the deuterium that was incorporated into the backbone amides. The deuterated protein (or a pepsin digestion thereof) is typically desalted by reversed-phase chromatography and mass analyzed by electrospray ionization mass spectrometry. The back-exchange that inevitably occurs for backbone amides under quench conditions is unwanted and must be minimized by, *e.g.*, having short chromatographic retention times. Nevertheless, the deuterium loss during chromatography at quench conditions is still significant and 30% back-exchange is not unusual. In fact, the occurrence of back-exchange has been denoted "The single biggest problem with solution-phase H/D exchange" by Emmett et al. [7].

The exchange rate for an amide hydrogen depends on its neighboring side chains and their effect is additive. This allows for prediction of exchange rates of amide hydrogens in unstructured polypeptides (*i.e.*, intrinsic chemical exchange rates), which are used to calculate protection factors for individual residues in NMR studies of folded proteins [8]. The protection factor is the exchange rate measured in a protein relative to the intrinsic chemical exchange rate, and it is a direct measure of the level of protection against isotopic exchange imposed by protein structure. The predicted intrinsic chemical exchange rate for polyalanine at quench conditions in $^1H_2O$ (pH 2.5 and 0 °C) is $1.8 \times 10^{-2}$ min$^{-1}$ corresponding to a half-life of ~40 min. The bulky isobutyl side chain of isoleucine sterically blocks for solvent exposure and reduces the exchange rate for polyisoleucine by a factor of 20, relative to that of polyalanine, yielding a predicted half-life of ~780 min at quench conditions. On the other hand, polar side chains increase the acidity of the amide hydrogen by an inductive effect [8] and this accelerates the base catalyzed exchange rate, as the first step in this mechanism is abstraction of the amide proton by $OH^-$ (or $OD^-$). In particular, the acidic side chain of aspartic acid accelerates the exchange rate approximately 5-fold relative to alanine and polyaspartic acid has a predicted half-life of ~8 min at quench conditions. Note that the predicted intrinsic chemical exchange rates pertain to the experimental conditions used in the NMR measurements,

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*i.e.*, buffered aqueous solvent with non-volatile salts. In HDX-MS experiments, the quench conditions are somewhat different, as organic solvent is present and non-volatile salts are excluded. The prediction of intrinsic rates under HDX-MS solvent conditions would be of considerable value, but the lack of site-specific exchange rates in standard HDX-MS experiments has hampered this effort.

We have recently utilized differences in intrinsic chemical exchange rates to prepare selectively labeled synthetic peptides having a protiated N-terminal half and a deuterated C-terminal half [9]. This polarised labeling pattern was achieved by having fast exchanging amide hydrogens in the N-terminal half and slowly exchanging amide hydrogens in the C-terminal half. The fast exchanging residues in the N-terminal half were either an Asp repeat comprised of six Asp residues (–DDDDDD–) [10] or a His repeat [9,11] comprised of six His residues (–HHHHHH–). The slowly exchanging residues in the C-terminal half were Ile and Lys (–IIKIIK). The peptides were selectively labeled by diluting fully deuterated peptide into a cold mixture of $^1H_2O$ and methanol acidified with acetic acid (*i.e.*, D to H exchange). The selectively labeled peptides were infused directly into the electrospray ion source using a glass syringe cooled with dry-ice. In these experiments, we observed that the His and Asp repeats were devoid of deuterium at the first measuring point (~1 min), while the residues in the C-terminal half retained their deuterium even after prolonged exposure (~15 min) to the protiated solvent. The complete back-exchange of the His and Asp repeats occurred significantly faster than we expected, as the predicted half-life for an amide hydrogen in a His or Asp repeat is 4 or 8 min at pH 2.5 and 0 °C, respectively. It should be noted that having a N-terminal half devoid of deuterium is an absolute advantage for using these peptides as probes for the occurrence of intramolecular migration of amide hydrogens ($^1H/^2H$) (*i.e.*, hydrogen scrambling) upon gas-phase fragmentation by various mass spectrometric techniques (*vide infra*). However, a rapid back-exchange of such repeats would be more problematic if they appear in proteins which are under investigation by HDX-MS. If the repeats are completely devoid of deuterium any correction for back-exchange is not possible and they will remain uncharacterized. In this regard, it is relevant to note that the occurrence of amino acid repeats consisting of the acidic residues Asp or Glu is relatively common in proteins. For example, 15% of all proteins in yeast (>200 residues) have at least one amino acid repeat which is five or more residues long and 14% of these proteins have one Asp repeat [12]. Although His repeats occur somewhat less frequently in eukaryotic proteins, 1.6% of all proteins in the Drosophila genome (>200 residues) have a His repeat which is five or more residues long [12]. To further explore the back-exchange kinetics of Asp, Glu and His repeats, we have undertaken a systematic study of the back-exchange kinetics of the peptides $H_6$IIKIIK, $K_2D_6$IIKIIK and $K_2E_6$IIKIIK at quench conditions which resembles those typically employed in HDX-MS studies. Furthermore, we have used electron transfer dissociation to determine site-specific deuterium levels in the Asp repeat of $K_2D_6$IIKIIK.

## 2. Experimental

### 2.1. Materials

The synthetic peptides P1 ($H_6$IIKIIK), P2 ($K_2D_6$IIKIIK), P2$^{Met}$ ($K_2D_6{}^{Met}$IIKIIK), P3 ($K_2E_6$IIKIIK) were obtained from Genscript Corp. (Piscataway, NJ, USA). The peptide sequences are shown in single letter amino acid code and the superscript "Met" indicates methyl esterified side chains of the $Asp_6$ sequence. $D_2O$ (99.9 atom% D) was purchased from Cambridge Isotopic Laboratories (Andover, MA, USA) and $CH_3OD$ (99.5 atom% D) was obtained from Aldrich Chemicals (Milwaukee, WI, USA). All other reagents and chemicals were of the highest grade commercially available.

### 2.2. Hydrogen/deuterium exchange

Fully deuterated peptides were prepared by a 10-fold dilution of a peptide stock solution (5.0 mM, DMSO) into a 1:1 (v/v) mixture of $D_2O$ and $CH_3OD$ followed by 2 h incubation at 45 °C. D to H exchange was initiated by a 50-fold dilution of the deuterated peptide into a cold (0 °C) 1:1 (v/v) mixture of $H_2O$ and $CH_3OH$ acidified with either 0.20 M formic acid or 1.90 M acetic acid. At appropriate time intervals an aliquot (100 μL) was injected with an ice-cold glass syringe into the 100 μL sample loop via a Rheodyne injection valve (both immersed into an ice-water bath). Fully equilibrated samples were prepared by prolonged exchange (3 h at 40 °C). All measurements of the intact peptide were carried out in triplicate. The pH values of the acetic and formic acid solutions containing methanol were identical within experimental uncertainty (pH 2.5). The pH glass electrode was calibrated with aqueous buffers.

### 2.3. Mass spectrometric analysis

The samples were analyzed by flow injection analysis. Samples loaded via an injection valve were carried directly to the electrospray ion source by an isocratic flow of 100 μL/min 1:1 (v/v) mixture of $H_2O$ and $CH_3OH$ with 0.06 M formic acid at 0 °C. The flow was delivered by an HPLC pump (Dionex Ultimate ISO-3100SD) and the dead time of this set-up was 12 s. Mass spectra were acquired on a Q-TOF mass spectrometer (model "Micro" from Micromass, Manchester, UK) using the following ion source settings, spray (capillary) voltage +3500 V, cone voltage 30 V, ion source temperature 80 °C, desolvation gas flow 500 L/h, desolvation gas temperature 200 °C.

ETD mass spectra were acquired on a LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The ion source conditions were optimized to avoid gas-phase hydrogen scrambling as previously described [10,11]. Hydrogen scrambling was reduced to a negligible level with the following ion source settings, heated capillary temperature 50 °C, heated capillary voltage 30 V, tube lens voltage 100 V. ETD spectra were acquired with the following settings, precursor ion (3+) isolation window width 16 $m/z$ units, reaction time 50 ms, spectra were recorded with a resolution of 60 000 and summed up over 1 min.

### 2.4. Data analysis

The deuterium content of the intact peptides was determined from the difference in average mass between the isotope distribution of the non-deuterated peptide and that of the deuterated peptide. The software HX-Express [13] for Excel2007 greatly facilitated the calculations for the intact peptides. The values in the plots represent the average of three independent measurements. For reasons of clarity, error bars are only displayed if the length of the error bar is larger than the data marker symbol. The total length of the error bar represents two standard deviations. The deuterium contents of the c- and z-type fragment ions from the ETD experiments were determined from the difference in average mass between the isotope distribution of the fragment ion obtained from a fully equilibrated peptide and that of the deuterated peptide. Residue-specific deuterium levels were determined by subtraction of the deuterium content of consecutive c- or z-type fragment ions. Note that the amide hydrogen of residue $n$ is contained in the $c_{n-1}$ fragment ion.

The predicted exchange curves in Fig. 1 were calculated using 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. [8]. The curves were calculated

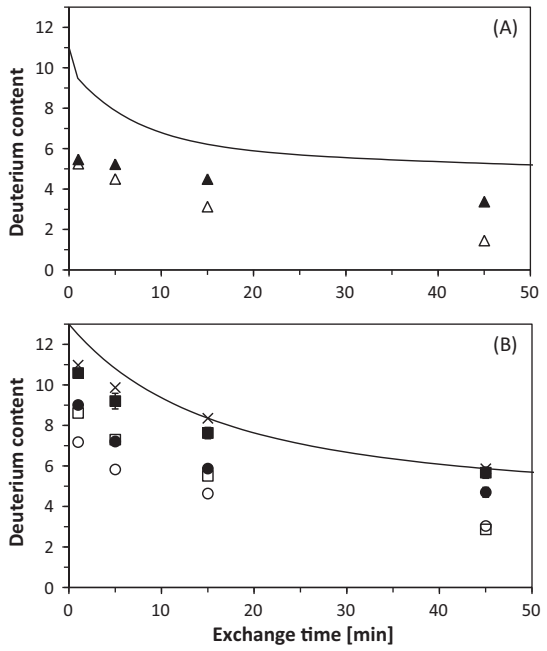


**Fig. 1.** Plots showing the deuterium content of peptide P1 ($H_6$IIKIIK, triangles) in (A), and in (B) peptide P2 ($K_2D_6$IIKIIK, circles), $P2^{Met}$ ($K_2D_6{}^{Met}$IIKIIK, squares) and P3 ($K_2E_6$IIKIIK, crosses) as a function of exchange time at 0 °C in a 1:1 (v/v) mixture of $H_2O$ and $CH_3OH$ with 0.20 M formic acid (closed symbols and crosses) or 1.90 M acetic acid (open symbols). The lines show the predicted exchange curves for (A) peptide P1 ($H_6$IIKIIK) and in (B) peptide ($K_2D_6$IIKIIK).

using the following parameters: aqueous solution at 0 °C, pH 2.2, reference data from poly-D/L-alanine, low salt conditions. Note that pH 2.2 is equal to the pH of an aqueous solution of 0.20 M formic acid or 1.90 M acetic acid. Also, note that the presence of $CH_3OH$ is expected to decrease the exchange rate, as it effectively lowers the concentration of the exchange catalyst ions of water, *i.e.*, $H^+$ and $OH^-$. In pure methanol, the intrinsic chemical exchange rates are ~20-fold lower than in water due to the suppressed ionization constant of $CH_3OH$ [14]. The predicted exchange curves in the present study are not adjusted for the presence of organic solvent.

## 3. Results and discussion

### 3.1. Accelerated back-exchange of His and Asp repeats

Fig. 1A shows the deuterium content of $H_6$IIKIIK as a function of exchange time in two different solvents, 0.2 M formic acid and 1.9 M acetic acid both in a 1:1 mixture of $H_2O$:$CH_3OH$. The theoretical maximum deuterium content is 11, as this peptide contains 11 backbone amide hydrogens. Only five deuterons are, however, retained after 1 min exchange in both solvents. This number agrees well with our previous results and we have shown that these deuterons are located in five C-terminal residues [9]. It is clear from Fig. 1A that the deuterium loss after 5 min is significantly higher in the acetic acid solvent. Although the pH of the acetic acid solvent and formic acid solvent is identical within experimental uncertainty (pH 2.5), the exchange kinetics is markedly faster in the acetic acid solvent. The exchange data obtained from the formic acid solvent agrees well our previous results [9]. There are, however, some notable differences in the experimental conditions between the present and previous experiments. In our previous measurement, the shortest exchange time was 0.5 min incubation in phosphate buffer (0 °C, pH 2.6) and the subsequent analysis was carried out by desalting with 0.05% TFA by reversed-phase chromatography and elution with 80% acetonitrile and 0.05% TFA. Since the dead time of this chromatographic set-up was 4 min, the total time for back-exchange for the first measuring point (*i.e.*, 0.5 min) was in fact 4.5 min under varying solvent conditions. In the present flow injection analysis experiments (Fig. 1), the dead time is only 0.2 min and the solvent composition is more uniform throughout the experiment. In the attempt to monitor the exchange kinetics of the N-terminal His repeat, we shortened the exchange time to 10 s and decreased the analysis dead time to 4 s by increasing the flow rate to 300 μL/min, but only a marginal increase in the D-content was observed (0.2 D, data not shown) relative to the 1 min exchange time in formic acid solvent (*i.e.*, 5.5 D, see Fig. 1A). Our present data strongly suggest that His repeats in proteins will escape characterization by HDX-MS due to their very rapid back-exchange kinetics at typical quench conditions in HDX-MS studies. A similar phenomenon may occur when histidine periodic patterns occur in proteins, *e.g.*, the two-histidine pattern $(HX)_8 = H_2HQHSHIHSHLHLHQ$ in the dentatorubral-pallidoluysian atrophy protein [12]. The functional role of His repeats in human proteins has recently been investigated in a genome-wide analysis and it was found that proteins containing stretches of five or more histidines are predominantly localized to the subnuclear organelle known as nuclear speckles, where proteins involved in RNA processing accumulate [15].

We have not previously investigated in detail the back-exchange kinetics of $K_2D_6$IIKIIK. In our previous experiments, this peptide was selectively labeled by diluting the fully deuterated peptide into a 1:1 mixture of $CH_3OH$ and $H_2O$ with 0.5 M acetic acid cooled in dry-ice [10]. This solution was infused directly into an electrospray ion source using a glass syringe cooled with a small plastic bag filled with dry-ice. Under these experimental conditions, the peptide retained a maximum of ~6 deuterons which were exclusively located in the C-terminal half (-IIKIIK) [10]. The N-terminal half ($K_2D_6$–) thus underwent a rapid deuterium loss under these experimental conditions. We have now investigated the exchange kinetics of $K_2D_6$IIKIIK using flow injection analysis and experimental conditions which resembles those typically employed in HDX-MS studies. After 1 min exchange in the formic acid solvent, $K_2D_6$IIKIIK contains ~9 deuterons, while it only contains ~7 deuterons in the acetic acid solvent at the present experimental conditions (Fig. 1B). Interestingly, the N-terminal half ($K_2D_6$–) thus clearly retains several deuterons (~3) in the formic acid solvent after 1 min exhange, while approximately one deuteron is retained using the acetic acid solvent. At prolonged exchange times, the deuterium content of $K_2D_6$IIKIIK is consistently lower in the acetic acid solvent reflecting faster exchange kinetics in this solvent. In contrast to our previous experiments (using direct infusion with a dry-ice cooled glass syringe), the N-terminal half ($K_2D_6$–) clearly retains deuterons at the short exchange times. But there are several differences in the experimental conditions that explain why the N-terminal half was devoid of deuterium in our previous experiments. Although the temperature of the bulk solution in the syringe in our previous experiments was lower than 0 °C, the solution has been heated in the transfer line capillary as well as in the metal capillary of the electrospray ion source and the residence time in these capillaries was longer than in our current experiments as a lower flow rate (5 μL/min) was used.

The present results highlight the importance of the experimental set-up and the choice of acid for achieving a rapid selective labeling of $K_2D_6$IIKIIK. More importantly, however, our results emphasize the experimental difficulty in obtaining information on deuterium levels of Asp repeats in proteins. Unless the exposure to protiated solvents at quench conditions is short (<5 min) it will be very difficult to gain information on the exchange kinetics of Asp repeats in proteins (*vide infra*). To investigate whether a similar situation applies to the case of Glu repeats, we have probed the exchange kinetics of the peptide $K_2E_6$IIKIIK in the formic

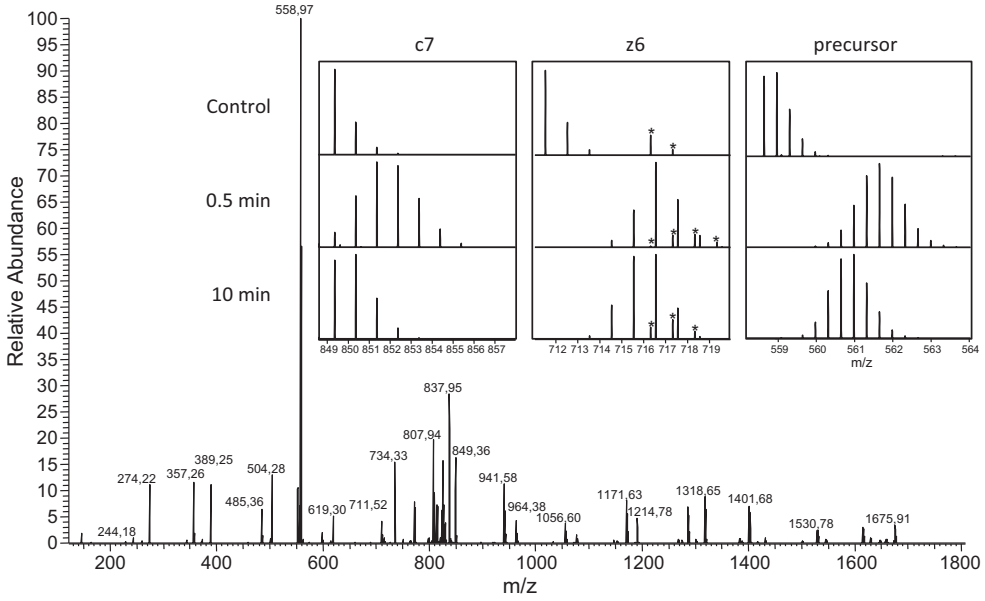


**Fig. 2.** ETD mass spectra of triply charged peptide P1 ($K_2D_6$IIKIIK) obtained from fully equilibrated sample (large spectrum and upper insets marked "control") and labeled samples obtained after 0.5 min and 10 min isotopic exchange at quench conditions in a mixture of 1:1 (v/v) $H_2O$ and $CH_3OH$ with 0.2 M formic acid (middle and lower insets). The isotopic patterns of the precursor ion and the fragment ions $c_7$ and $z_6$ are displayed in the insets (the asterisked isotope peaks correspond to another fragment ion).

acid solvent. This peptide retains significantly more deuterium than $K_2D_6$IIKIIK (Fig. 1B), for example, ~10 vs. ~7 deuterons after 5 min exchange, respectively. This finding strongly suggests that Glu repeats in proteins have relatively slow exchange kinetics at quench conditions which should make them amenable for analysis by HDX-MS.

To shed some light on the mechanism for the rapid exchange kinetics of the Asp repeat at quench conditions, we included the methyl esterified analogue, $K_2D_6{}^{Met}$IIKIIK, in our exchange experiments. In this peptide, all six acidic hydrogens in the Asp side chains are replaced with methyl groups. The methyl groups considerably retard the isotopic exchange in both acidic solvents (Fig. 1B). For the formic acid solvent, this effect is most pronounced having ~2 additional deuterons retained in the peptide after 1 min exchange. The decreased exchange rate upon methyl esterification could indicate that the acidic hydrogen of the Asp side chain is directly involved in an acid catalyzed mechanism of isotopic exchange. However, the replacement of the acidic hydrogen by the much larger methyl group is also likely to reduce the exchange rate by sterically blocking access to the solvent. Interestingly, the exchange curve for $K_2D_6{}^{Met}$IIKIIK is quite similar to that of $K_2E_6$IIKIIK.

The deuterium loss for all peptides in the present study was higher in the solvent with acetic acid than in the solvent with formic acid although the pH of both solvents were similar. The increased back-exchange rate when using acetic acid most likely reflects the fact that the acetate anion is a somewhat stronger base than the formate anion. The acetate anion is thus more efficient in abstracting an amide deuteron, thereby facilitating the isotopic exchange reaction. In this regard, it is worth noting that at quench conditions, a pH-independent neutral water-catalyzed exchange accounts for 25% of the observed exchange rate [8]. It is believed that the pH-independent water reaction occurs via a water molecule acting as a base to directly extract the amide proton [8]. Burkitt and O'Connor [16] also found that the level of back-exchange was significantly higher in acetic acid than in formic acid, and the large deuterium loss (~70%) for the peptide Angiotensin I in the mass spectrometric measurements of back-exchange by Feng et al. [17] is, at least in part, due to the use of acetic acid in their experiments. It should be noted that strong acids like trifluoroacetic acid are efficient quenchers for isotopic exchange as their corresponding base is very weak, but they tend to suppress the ionization of peptides in ESI-MS [16]. Although acetic acid results in a higher level of back-exchange, it has not escaped our attention that this acid is occasionally used among the practitioners of HDX-MS.

### 3.2. Electron transfer dissociation of $K_2D_6$IIKIIK

The selectively labeled peptides used in this study have been developed for determining the occurrence of hydrogen scrambling upon gas-phase activation. It is important to note that the N-terminal half should be completely devoid of deuterium, while the C-terminal half should be deuterated when the peptide is to be used as a hydrogen scrambling probe. If hydrogen scrambling is prevalent then the deuterons in the C-terminal half are statistically redistributed over all exchangeable sites in the peptide ion and this is readily detected as a mass increase of the fragment ions from the N-terminal half. When $K_2D_6$IIKIIK is used as a scrambling probe then any retained deuterons in the N-terminal half (due to incomplete back-exchange) could erroneously be interpreted as scrambling. It is therefore important to ensure that the N-terminal half is devoid of deuterium before scrambling levels are measured. Also, the selective labeling with an N-terminal half devoid of deuterium ensures a maximal sensitivity for detecting and measuring the level of gas-phase hydrogen scrambling. Using such peptides, we have demonstrated that CID [9,18–20] cause extensive hydrogen scrambling, while electron capture dissociation (ECD) [11], electron transfer dissociation (ETD) [10] and MALDI in-source-decay [21] proceed with a negligible level of hydrogen scrambling. These fragmentation techniques are thus available as experimental tools in either a "bottom-up" [22] or a "top-down" [23–25] approach to determine site-specific deuterium levels of proteins from solution H/D exchange experiments provided that any excessive vibrational excitation is avoided prior to the fragmentation event. The use of ETD and ECD for this particular purpose requires that the declustering conditions in the electrospray ion source are optimized to avoid excessive vibrational excitation which induces scrambling.

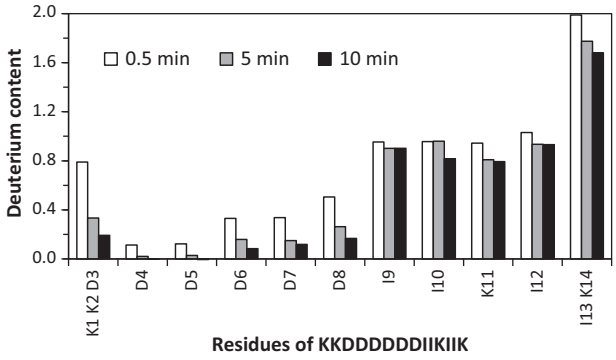


**Fig. 3.** Residue-specific deuterium levels for $K_2D_6IIKIIK$ after 0.5, 5 and 10 min exchange in 0.2 M formic acid in a 1:1 mixture of $H_2O$ and $CH_3OH$ at 0 °C. The deuterium levels were determined from consecutive c-type fragment ions generated by electron transfer dissociation (ETD).

While the N-terminal half of $K_2D_6IIKIIK$ is relatively fast exchanging at quench conditions, it may retain deuterons at short exchange times, in particular when formic acid is used to acidify the exchange solution (Fig. 1B). To investigate how the retained deuterons are distributed in the N-terminal half, we fragmented the deuterated peptide by electron transfer dissociation (ETD) at conditions where hydrogen scrambling was negligible. Fig. 2 shows ETD spectra from a fully equilibrated sample, and labeled samples after 0.5 min and 10 min exchange. The retained deuterons in the N-terminal half are clearly observed as a mass increase of the $c_7$ fragment ion after 0.5 min exchange relative to the control. The $c_7$ fragment ion contains the amide hydrogens of the residues KKDDDDDD. After 10 min, the N-terminal half is nearly devoid of deuterium, as shown by the mass decrease of the $c_7$ fragment ion, while the deuterium content of $z_6$ ion is only slightly decreased. The $z_6$ ion contains the slowly exchanging amide hydrogens of the five last residues in the C-terminal half (IKIIK). Surprisingly, the deuterons are not distributed evenly among the Asp residues in the N-terminal half (Fig. 3). The two Asp residues D4 and D5 contain significant less deuterium than the following three Asp residues D6, D7 and D8 at all three measuring time points. A very similar pattern was consistently observed for the site-specific deuterium levels obtained from the complementary z-type fragment ion series (data not shown). Notably, this phenomenon is not a result of any gas-phase processes (*e.g.*, scrambling) but it is due to differences in the exchange rates of the Asp residues in solution. An uneven deuterium distribution among D4 to D8 is not expected from the known effect of primary structure on backbone amide hydrogen exchange, where only nearest-neighbor side chains affect the intrinsic chemical exchange rate (with the exception of the N- and C-terminal amides) [8]. D4 to D8 is thus predicted to have identical intrinsic chemical exchange rate, but our site-specific measurements show that D4 and D5 exchange significantly faster than D6, D7 and D8. It is unlikely that this effect is due to a structural protection of D6, D7 and D8, as Asp has a very low helix propensity [26]. But through-space electrostatic effects could be responsible for the observed acceleration of the exchange rate for D4 and D5. The electrostatic field from positively charged side chains attract negatively charged counter ions, including $OH^-$, and this may increase the local pH around the exchangeable sites and thereby increase their exchange rate [27]. Strong electrolytes, such as sodium chloride, can be added to suppress this effect [8,27]. The present study, however, was carried out without any strong electrolytes, as our aim is to investigate back-exchange under salt-free quench conditions mimicking those typically encountered in HDX-MS experiments. We speculate whether the two positively charged lysine side chains along with the N-terminal ammonium ion may create a local environment with a higher pH that accelerates exchange of the adjacent D4 and D5 residues. A similar phenomenon is almost certainly accelerating the back-exchange kinetics of the polycationic His repeat in the $H_6IIKIIK$ peptide. We attribute this counterion condensation mechanism as the main cause for the observed difference between predicted and measured exchange curves. In this regard, it is relevant to point out that single His and Asp residues in a protein are, in general, not completely back-exchanged during HDX-MS analysis. We have previously determined the level of back-exchange for numerous residues in $\beta_2$-microglobulin by ETD and the following list gives the residue-specific deuterium levels for the fully deuterated protein (*i.e.*, an "100% D control"): His13, 0.60; His31, 0.55; Asp34, 0.50; His51, 0.80; Asp53, 0.14; Asp76, 0.54; Asp96, 0.92 [9].

The selective deuterium depletion of the N-terminal half with exchange time is evident in Fig. 3 (compare white and black bars). It is, however, clear that some of the Asp residues have retained a small amount of deuterium even after 10 min exchange in the formic acid quench solvent. As mentioned above, an N-terminal half which is not completely back-exchanged complicates the use of the peptide as a probe for the occurrence of scrambling. We therefore recommend the use of acetic acid for this particular peptide for scrambling measurements.

## 4. Conclusion

In the present study, we have probed the back-exchange kinetics of His and Asp repeats at conditions which are similar to quench conditions used in HDX-MS protein hydrogen exchange experiments. Our results show that the His repeat in $H_6IIKIIK$ is completely exchanged within ~14 s suggesting that His repeats in proteins are not amenable for analysis by the standard HDX-MS method. The Asp repeat in $K_2D_6IIKIIK$ exchanged more slowly and several deuterons were retained after 1 min exchange using formic acid in the quench solvent. Fewer deuterons were retained when acetic acid was used to quench the exchange reaction indicating that the acetate anion promotes exchange. This finding suggests that acetic acid should not be used to quench the exchange reaction in traditional HDX-MS experiments. The peptides $H_6IIKIIK$ and $K_2D_6IIKIIK$ were developed to probe the level of hydrogen scrambling [9]. For this particular purpose, it is important that their N-terminal half is devoid of deuterium. Otherwise, it might be erroneously interpreted as scrambling has occurred. Our present results highlight the experimental conditions for obtaining an N-terminal half that is devoid of deuterium, while the C-terminal half remains deuterated. Electron transfer dissociation (ETD) using a hybrid linear ion trap-Orbitrap instrument was successfully employed to obtain residue-specific deuterium levels of deuterated $K_2D_6IIKIIK$. Surprisingly, an uneven distribution of deuterons was observed within the Asp repeat of this peptide. We attribute the non-uniform distribution to the presence of the three ammonium ions of the N-terminal lysine residues which may accelerate the exchange of the adjacent Asp residues by attracting counter ions and thereby locally increasing the pH.

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