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Spatially Resolved Protein Hydrogen Exchange Measured by Subzero-Cooled Chip-Based Nanoelectrospray Ionization Tandem Mass Spectrometry

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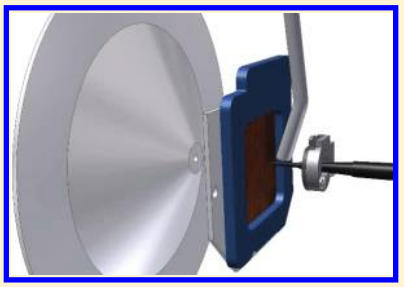
Supporting Information

ABSTRACT: Mass spectrometry has become a valuable method for studying structural dynamics of proteins in solution by measuring their backbone amide hydrogen/deuterium exchange (HDX) kinetics. In a typical exchange experiment one or more proteins are incubated in deuterated buffer at physiological conditions. After a given period of deuteration, the exchange reaction is quenched by acidification (pH 2.5) and cooling (0 °C) and the deuterated protein (or a digest thereof) is analyzed by mass spectrometry. The unavoidable loss of deuterium (back-exchange) that occurs under quench conditions is undesired as it leads to loss of information. Here we describe the successful application of a chip-based nanoelectrospray ionization mass spectrometry top-down fragmentation approach based on cooling to subzero temperature (−15 °C) which reduces the back-exchange at quench conditions to very low levels. For example, only 4% and 6% deuterium loss for fully deuterated ubiquitin and β_2-microglobulin were observed after 10 min of back-exchange. The practical value of our subzero-cooled setup for top-down fragmentation HDX analyses is demonstrated by electron-transfer dissociation of ubiquitin ions under carefully optimized mass spectrometric conditions where gas-phase hydrogen scrambling is negligible. Our results show that the known dynamic behavior of ubiquitin in solution is accurately reflected in the deuterium contents of the fragment ions.



The conformational dynamics of proteins are essential for their function. For example, molecular recognition depends on conformational plasticity in the binding interface when a protein adapts its structure upon binding to different binding partners.[1] Allosteric regulation of protein activity also relies on structural flexibility.[2−4] Hydrogen/deuterium exchange monitored by mass spectrometry has recently become an important technique for characterizing protein structural dynamics.[5,6] The basis of this method is that backbone amide hydrogens that are engaged in stable hydrogen bonds in the natively folded conformation are protected against exchange with the solvent.[7] According to the thermodynamic principle that proteins unfold and refold continuously, the protecting hydrogen bonds will break when the protein cycles through locally (and globally) unfolded states thereby allowing exchange to occur. The exchange kinetics of amide backbone hydrogens is therefore a direct probe for the structural dynamics of the protein backbone. When a natively folded protein is incubated in D_2O, it will gradually exchange its amide hydrogens with deuterium. The protein mass increases by 1 Da for each exchange event, and the global deuterium uptake is thus readily measured by mass spectrometry.[8] The traditional approach[9] to localize the incorporated deuterium is based on proteolytic cleavage of the deuterium-labeled protein at cold acidic conditions where the isotopic exchange rate is slowed down several orders of magnitude (i.e., quench conditions). Pepsin or other proteases with a broad specificity are used to digest the labeled protein yielding a relatively complex mixture of labeled peptides which are typically analyzed by liquid chromatography−mass spectrometry (LC−MS).[6,10] Importantly, the chromatographic separation of the labeled peptides must be carried out at quench conditions and as fast as possible to minimize the unavoidable loss of deuterium caused by back-exchange with the HPLC solvents. The occurrence of back-exchange has been described as "The single biggest problem with solution-phase H/D exchange" by Emmett et al.[11] Direct fragmentation of the labeled protein in the mass spectrometer by electrons[12−21] or matrix-assisted laser desorption ionization in source decay (MALDI ISD)[22] is an attractive alternative approach to the traditional protease-based bottom-up method. The top-down approach omits the need for in solution digestion as well as chromatographic separation. Therefore, it opens for the possibility to attain very low levels of back-exchange. One analytical strategy is based on minimizing the back-exchange time by having a continuous flow of the protein in the labeling buffer where the quench solution is mixed into the flow just prior to entering the ion source.[15,16] The acidification upon quenching causes the protein ions to attain higher charge states in the electrospray process, and this is highly beneficial for the electron-based fragmentation as higher charge states fragment more efficiently than lower charge states.[23,24] This continuous flow strategy is, however, only compatible with volatile buffers as the flow of the exchange buffer mixed with quench buffer enters the ion source without a

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possibility for desalting. Another strategy[25] is to avoid quenching in solution altogether by electrospraying the exchange solution and thereby monitoring the protein hydrogen exchange kinetics in real time[20] (the isotopic exchange reaction in solution is quenched when the protein ions are desolvated in the ion source). To ensure efficient fragmentation, the charge states are increased by having a supercharging reagent (*m*-nitrobenzyl alcohol, m-NBA)[26] in the exchange buffer. The presence of this reagent at a relatively high concentration (1%) may, however, affect the protein stability and hence its dynamics. The real-time strategy is also limited to volatile buffers. In the continuous flow strategy, the protein should be exposed to quench conditions as short time as possible to minimize back-exchange, whereas the real-time strategy completely omits the quench step. Here we propose an alternative analytical strategy for minimizing back-exchange in top-down fragmentation studies by quenching at subzero temperature. We utilize a chip-based nanoelectrospray ionization setup (Advion TriVersa NanoMate) equipped with a device that has been especially adapted to minimize the level of back-exchange by cooling the quenched sample to −15 °C. Under these conditions, the level of back-exchange is reduced to a very low level even after the protein has been stored in the quench solution for a prolonged period (e.g., only 4% deuterium loss for fully deuterated ubiquitin after 10 min of back-exchange). This allows for relatively long acquisition times to accumulate signals from low-abundant deuterium-labeled fragment ions. Our setup offers the same advantages as nanoelectrospray needles over conventional microflow electrospray, such as lower sample consumption, higher sensitivity, and no risk of carry over. Moreover, the chip-based setup offers some additional advantages over nanoelectrospray needles such as high spray reproducibility and spray stability and the possibility for automation.[27,28] Furthermore, a desalting step is easy to implement in the hydrogen/deuterium exchange mass spectrometry (HDX-MS) workflow if nonvolatile salts are present in the exchange buffers. The protein sample could be rapidly desalted by reversed-phase chromatography at normal quench conditions and eluted by an acidic methanolic solution directly into the 96-well plate of the NanoMate.

EXPERIMENTAL SECTION

Materials and Reagents. Bovine ubiquitin (BioUltra, purity ≥98%), methanol (LC−MS grade), formic acid (p.A. grade), ammonium-d_4 acetate-d_3 (99 atom % D), acetic-d_3 acid-*d* (99.5 atom % D), and methyl alcohol-*d* (99.5 atom % D) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). D_2O (99.9% atom% D) was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.). Water was purified by a Millipore Milli-Q system (Bedford, MA, U.S.A.). Human β_2-microglobulin was obtained from Innovative Research (Novi, MI, U.S.A.).

Hydrogen/Deuterium Exchange. Bovine ubiquitin (500 μM) was fully deuterated in 3% acetic-d_3 acid-*d* in a 1:1 (v/v) mixture of D_2O and methyl alcohol-*d* for 3 days at 37 °C, followed by lyophilization, dissolution in 50 mM ammonium-d_4 acetate-d_3, and subsequent incubation for 3 days at 37 °C. Native state D-to-H exchange was initiated by 50-fold dilution into ^1H_2O at 25.0 °C, pH 6.6. After the desired exchange time, the exchange reaction was quenched by mixing 1:1 (v/v) with ice-cold 0.40 M formic acid in methanol. An aliquot of the quenched sample solution (20 μL) was directly pipetted into a 96-well plate cooled to 4 °C by the TriVersa NanoMate system (Advion BioSciences, Inc., Ithaca, NY, U.S.A.) for subsequent analysis by mass spectrometry (see below for a further description of the system). To measure the initial deuterium content of ubiquitin (i.e., at t_{ex} = 0 min), an aliquot of fully deuterated ubiquitin was transferred by 100-fold dilution directly to the ice-cold quench solution (1:1 (v/v) water/methanol mixture with 0.20 M formic acid) and analyzed immediately. As a reference, equilibrium D-to-H exchanged ubiquitin was prepared by 50-fold dilution of fully deuterated ubiquitin into ^1H_2O at 55 °C for 3 days. This sample represents a homogeneously labeled protein with 2% deuterium at all labile sites. To measure the level of back-exchange, an aliquot of fully deuterated ubiquitin was transferred by 100-fold dilution directly to the ice-cold quench solution (1:1 (v/v) water/methanol mixture with 0.20 M formic acid) and immediately analyzed with or without cooling of the front end of the NanoMate pipet tip (see below). The dead time in these measurements with the NanoMate system was 30 s (i.e., the time from loading the sample solution into the 96-well plate until the sample is spraying). Without cooling, the sample solution will reach room temperature (22 °C). Ubiquitin contains 76 residues and 72 backbone amide hydrogens (76 residues − 1 N-terminal residue − 3 Pro residues = 72 backbone NH). Fully deuterated β_2-microglobulin was prepared by dissolving lyophilized β_2-microglobulin (1.0 mM) in deuterated buffer (100 mM ammonium-d_4 acetate-d_3) and incubating for at least 6 h at 37 °C as previously described.[29] Global back-exchange kinetics was measured by a 100-fold dilution of fully deuterated β_2-microglobulin into ice-cold quench solution (1:1 (v/v) water/methanol mixture with 0.20 M formic acid) and immediately analyzed with or without cooling of the front end of the NanoMate pipet tip. β_2-Microglobulin has a total of 93 exchangeable backbone amide hydrogens (99 residues − 1 N-terminal residue − 5 Pro residues = 93 backbone NH). The global back-exchange measurements measure predominantly the deuterium content of the backbone amides. However, the ε-NH in the side chain of arginine contributes to the deuterium content at short exchange times, as its exchange rate is only ∼10-fold higher than that of the poly-D/L-alanine backbone amides at quench conditions.[30]

Mass Spectrometry. Samples were introduced into a Thermo LTQ Orbitrap XL equipped with electron-transfer dissociation (ETD) (Thermo Fisher Scientific, Waltham, MA, U.S.A.) via a TriVersa NanoMate system operated in the infusion mode. An amount of 5 μL of sample solution was picked up from the 96-well plate by a disposable conductive pipet tip which subsequently docked onto the electrospray ionization (ESI) chip (A-chip with a nozzle of i.d. 5.5 μm). For spraying a voltage of 1.25 kV and a nitrogen pressure of 0.3 psi were applied. During the spraying process the pipet tip of the NanoMate was cooled in order to minimize back-exchange. This was achieved by means of a flow of cooled nitrogen (see below for a further description). The ion source parameters of the mass spectrometer were kept at gentle declustering conditions in order to avoid hydrogen scrambling during the ionization process (capillary voltage, 30 V; capillary temperature, 100 °C; tube lens potential, 100 V). MS and MS/MS spectra were acquired with a resolution of 100 000 and an automated gain control of 1×10^6 for MS and 2×10^5 for MS/MS spectra. The maximum allowed fill time for MS spectra was 500 ms, and for MS/MS spectra it was 1000 ms. The scan range was set to m/z 500−2000 for full scan spectra and m/z

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200−1800 for MS/MS spectra. MS/MS experiments of ubiquitin were carried out on charge state 12+, where the precursor isolation window was set to 20 m/z in order to avoid side band activation resulting in hydrogen scrambling. ETD was performed at a reaction time of 10 ms using a target population of fluoranthene anions of 2×10^5 at a maximum fill time of 100 ms. The acquisition time was 0.5 min for MS spectra and 1 min for MS/MS spectra (35 scans), respectively. Fragmentation of deuterated ubiquitin by ETD yielded 44 c and 32 z fragment ions with sufficiently high signal-to-noise ratios (S/N) (>5) to allow a reliable determination of their deuterium content (Figure S1, Supporting Information). The signal intensity of the base peak (z_{17}^{3+}) in the ETD spectra of [ubiquitin + 12H]$^{12+}$ was approximately 10^5. The signal intensity of the fragment ions that were used for the calculation of deuterium content was 10^4 or higher.

Cooling Device. We modified the NanoMate by introducing a flow of cold nitrogen gas to the front of the pipet tip that is in direct contact with the ESI chip (as shown in Figure 1). The flow of cold nitrogen gas was generated by passing nitrogen through a coil of cooled copper tubing (length 20 m, i.d. 2 mm). The tubing was cooled by dry ice in an insulated container (inner dimensions approximately 30 × 25 × 20 cm^3). The copper tubing outlet was mantled with thermal insulation tubing (EPDM rubber tube, wall thickness 2 cm). A short noninsulated Teflon tubing (10 cm, i.d. 2 mm) directed the cold nitrogen gas flow to the NanoMate pipet tip containing the sample solution for electrospraying. The tubing was carefully positioned approximately 2 mm above the pipet tip. A flexible dial indicator holder (from a lathe) mounted on the NanoMate allowed easy positioning of the Teflon tubing. The nitrogen flow rate was 3 L/min, and the temperature of the gas was approximately −15 °C measured at the location of the tip. This experimental setup ensures efficient cooling of a sample volume of approximately 2 μL, sufficient for approximately 15−20 min of measuring time.

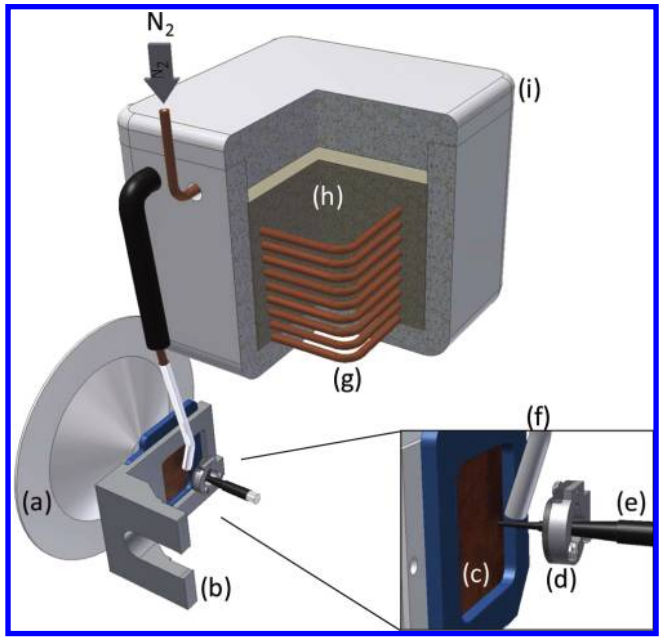

Figure 1. Schematic drawing of the cooling device adapted to a chip-based nanoflow electrospray ionization (ESI) setup. A flow of nitrogen gas cooled to subzero temperature is generated by passing it through a coil of copper tubing immersed in dry ice. The inset shows how the cold gas flow is directed to the front of the pipet tip (containing the sample solution) that is in direct contact with the ESI chip. The labeled parts are (a) entrance cone of Orbitrap mass spectrometer, (b) holder for ESI chip, (c) ESI chip, (d) holder for conductive pipet tip, (e) conductive pipet tip, (f) outlet tubing for cold nitrogen gas, (g) copper tubing, (h) dry ice, and (i) thermoinsulated box. Only the front end parts of the NanoMate are shown.

Data Analysis. Mass spectra and peak lists were processed by Xcalibur version 2.1 (Thermo Fisher Scientific). Isotopic patterns of the fragment ions were extracted and the intensity-weighted average mass for each fragment ion was calculated by an in-house written spreadsheet macro (Microsoft Excel, Redmond, WA). The deuterium content of the fragment ions was calculated by the mass difference between the fragment ion obtained from deuterium-labeled ubiquitin and the corresponding fragment ion obtained from equilibrium D-to-H exchanged ubiquitin (i.e., homogeneously labeled ubiquitin with 2% deuterium). The values in the plots represent the average of at least 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.

RESULTS AND DISCUSSION

Back-Exchange for Intact Proteins. Top-down fragmentation of deuterium-labeled proteins from solution-phase HDX experiments holds great promise as an analytical tool to investigate protein structural dynamics with high spatial resolution. One of the benefits of this approach is that the labeled residues are localized by fragmentation in the solvent-free gas phase where back-exchange does not occur. However, it is not possible to avoid the undesired loss or gain of deuterium due to back-exchange with the solvent in the quenched sample solution (i.e., before the sample is transferred to the gas phase). Our approach to limit the undesired back-exchange in the quenched sample solution is to cool it to subzero temperature (−15 °C) while it is electrospraying. We cooled the NanoMate pipet tip containing the quenched sample solution by directing a flow of cold nitrogen gas to the front end of the pipet tip. The efficiency of the cooled NanoMate setup in regard to minimizing the deuterium loss was investigated by monitoring the global back-exchange kinetics of fully deuterated ubiquitin in a quench solution with and without cooling. Figure 2 shows that cooling is highly efficient in reducing the back-exchange of ubiquitin to a very low level (4% deuterium loss after 10 min of back-exchange). A close inspection of the data points reveal that ∼71 deuterons (out of a total number of 72) are retained in the first half minute. Even after 10 and 15 min of back-exchange, ∼69 and ∼68 deuterons are retained, respectively. Without cooling, the level of back-exchange is dramatically increased, and after just half a minute significantly less deuterons are retained (∼63) than after 15 min with cooling (∼68). Further deuterium depletion occurs rapidly without cooling and after 10 and 15 min, only ∼27 and ∼22 deuterons are retained, respectively. Clearly, our cooling device efficiently reduces the level of back-exchange and allows ample time for accumulating ETD spectra of the labeled protein. Although ubiquitin rapidly looses deuterons without cooling at quench conditions, it is known to attain a partially folded conformation (called the A state) in water/alcohol mixtures at acidic conditions.[31−34] This partially folded state will slow down the global back-exchange kinetics relative to a random coil structure. Therefore, we also investigated the back-exchange kinetics of β_2-microglobulin

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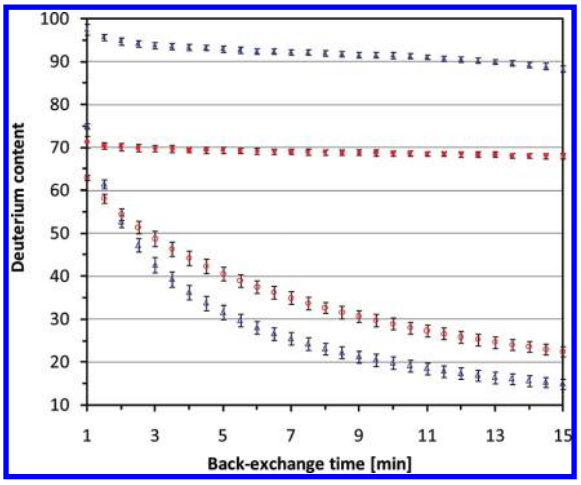

Figure 2. Time course of back-exchange. The deuterium content of ubiquitin (circles) and β_2-microglobulin (triangles) is shown as a function of exchange time in quench solution with (closed symbols) or without (open symbols) cooling to subzero temperature (−15 °C). Error bars represent average ± SD for measurements made in quadruplicate. Note: the back-exchange time includes the instrumental dead time (0.5 min) and the acquisition time (0.5 min).

which is more highly unfolded at low pH as well as upon addition of organic solvent.[35−37] With cooling, the deuterium loss from β_2-microglobulin is only slightly higher than that of ubiquitin (2% point higher loss after 10 min of back-exchange). Without cooling, the back-exchange curve of β_2-microglobulin crosses that of ubiquitin already after 1 min clearly reflecting a higher degree of unfolding of β_2-microglobulin at quench conditions (Figure 2). This underscores the efficiency of cooling to subzero degrees with respect to limiting back-exchange at quench conditions in top-down fragmentation experiments.

Importantly, the subzero temperature cooling does not have any adverse effect on the stability of the electrospray (Figure S2, Supporting Information). The cooled front end of the pipet tip contains approximately ∼2 μL which allows for approximately 15 min of measurement time for mass spectrometric experiments. After ∼15 min of spraying, the remainder of sample solution which was initially not cooled enters the electrospray nozzle and this is readily detected by the appearance of a bimodal peak pattern (Figure S3, Supporting Information). The high-mass peak in the bimodal distribution represents the labeled protein molecules which were initially present in the cooled volume, whereas the low-mass peak represents protein molecules that were initially outside the cooled volume and hence have undergone a higher degree of back-exchange. Note that such a bimodal peak pattern was also observed for peptides (data not shown).

Spatially Resolved Hydrogen/Deuterium Exchange Measurements of Ubiquitin. Ubiquitin is a well-characterized protein, and its backbone amide hydrogen exchange rate constants under various conditions are known from NMR studies.[32,38,39] Therefore, we selected ubiquitin as a model protein for our present HDX ETD top-down fragmentation study. The subzero cooling did not affect the charge state

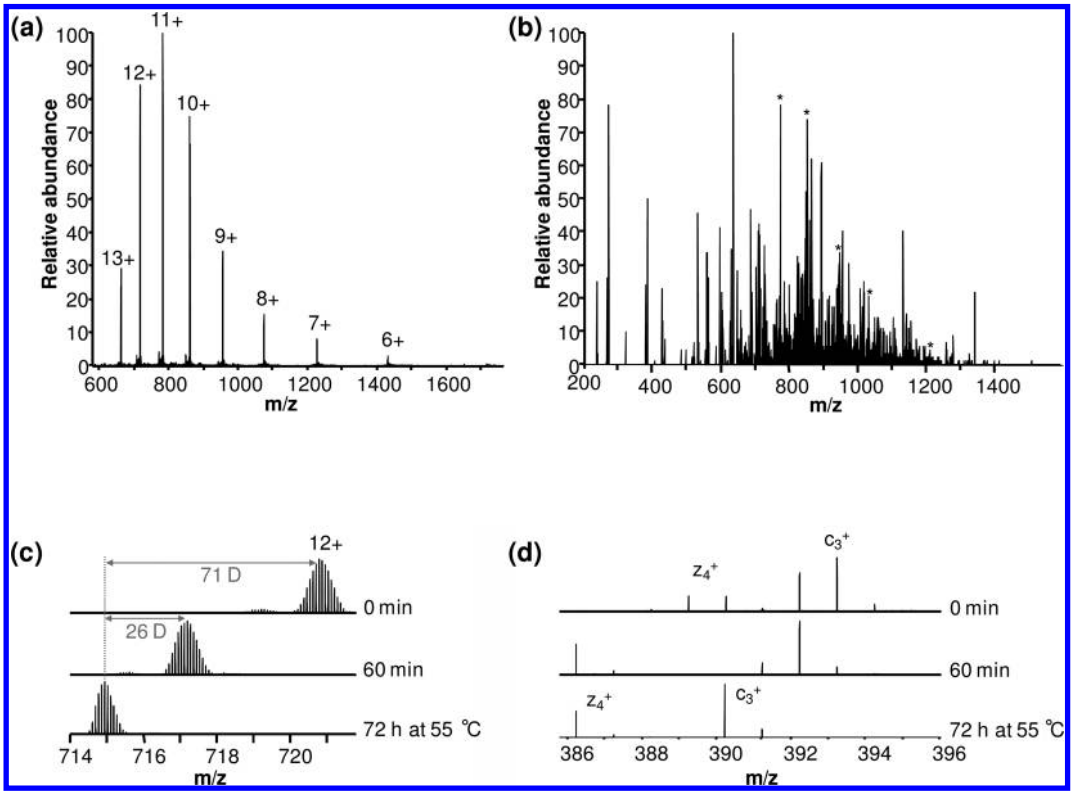

Figure 3. Electrospray ionization mass spectra of ubiquitin sprayed from a solution at subzero temperature (−15 °C). The charge state distribution of ubiquitin is shown in panel a. Electron-transfer dissociation (ETD) mass spectrum of the +12 charge state of ubiquitin is shown in panel b; the asterisked peaks correspond to charge-reduced protein ions. Panel c shows the +12 charge state after D-to-H exchange for various periods of time, while panel d shows the corresponding ETD spectra displaying the isotopic envelopes of fragment ions z_4^+ and c_3^+ originating from a fast and slow exchanging region in ubiquitin, respectively.

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distribution of ubiquitin or the signal intensity (Figure S2, Supporting Information). The ESI spectrum of ubiquitin in Figure 3a shows a charge state distribution dominated by highly charged ions which are characteristic for proteins electrosprayed at denaturing conditions. Fragmentation of the four highest charge states (+13 to +10) of ubiquitin by ETD yielded similar fragmentation patterns (data not shown). This was also observed by Sterling and Williams.[20] Figure 3b shows the ETD spectrum of the +12 charge state which yielded 59 backbone bond cleavages representing 82% of the cleavable peptide bonds. This allows for a detailed analysis of the deuterium levels of backbone amides in the protein. Native-state D-to-H exchange was carried out by incubating fully deuterated ubiquitin in ^1H_2O at neutral pH. Under these conditions, the most stable regions in ubiquitin are protected against isotopic exchange with ^1H_2O and they will retain their deuterium, whereas the more dynamic and flexible regions undergo exchange. The initial deuterium content of ubiquitin was 71 D (i.e., at t_{ex} = 0 min) (Figure 3c, upper spectrum). After 60 min of D-to-H exchange, ubiquitin retained 26 deuterium atoms (Figure 3c, middle spectrum). The retained deuterium can be assigned to individual residues by the mass shift of the fragment ions obtained from ETD fragmentation. To illustrate this, Figure 3d displays the isotopic envelope of the c_3 and z_4 ion obtained from ubiquitin after D-to-H exchange for 60 min. Also shown are the isotopic envelopes for t_{ex} = 0 min displaying the initial deuterium content (2.5 and 3.4 D for c_3 and z_4) and for t_{ex} = 72 h at 55 °C displaying the exchange end point (i.e., homogeneously labeled ubiquitin with ~2% deuterium). The c_3 ion encompasses the first three N-terminal backbone amides (i.e., Q2, I3, and F4) of which the latter two are highly protected against exchange as they are part of a stable β-sheet in the natively folded protein (protection factors are larger than 10^5 as determined by NMR).[38,39] In contrast, the three C-terminal backbone amides of the z_4 ion are rapidly exchanging as they belong to the highly dynamic C-terminal region of ubiquitin.[38,40,41] Accordingly, Figure 3d shows that the c_3 ion has retained ~1.9 D after 60 min of exchange, whereas the z_4 ion is devoid of deuterium. These values are in excellent agreement with those calculated from known NMR exchange rates, i.e., 2.0 and 0.0 D (values are not corrected for back-exchange). The deuterium content of the c ion series up to and including c_{40} is plotted in Figure 4. A nearly perfect correlation is observed between the deuterium content of the c ion series and the graph showing the cumulative deuterium content obtained from known NMR exchange rate constants[39] (compare filled circles with red graph in Figure 4). The cumulative deuterium graph exhibits a distinctive profile, where the horizontal region of the graph from c_6 to c_{11} corresponds to a flexible region in ubiquitin that is devoid of deuterium (a turn between β-strands 1 and 2), whereas the neighboring protected regions (β-strands 1 and 2) retain deuterium and exhibit a nearly linear increase. The known solution behavior of ubiquitin is thus readily apparent by the top-down fragmentation data. A nearly horizontal region from c_{30} to c_{40} corresponds to the loop between the central α-helix α1 and β-strand 3. Interestingly, the first horizontal region (i.e., turn $\beta1-\beta2$) has recently been reported to undergo a large amplitude pincer-like movement toward the loop $\alpha1$-$\beta3$ in solution.[41] This motional mode is essential for molecular recognition by enabling ubiquitin to adapt to different protein binding partners.[41]

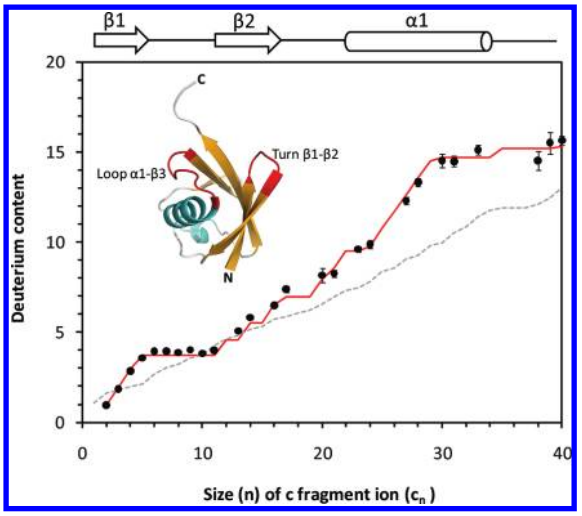

Figure 4. Dissecting the solution structural dynamics of the N-terminal half of ubiquitin. Deuterium levels of the c fragment ions of ubiquitin after native state D-to-H exchange for 60 min measured by electron-transfer dissociation mass spectrometry (filled circles) and NMR (red line). Error bars represent average ± SD for measurements made in triplicate. NMR deuterium levels were obtained from exchange rate constants determined by Johnson et al. (ref 39). The theoretical deuterium content in the case of 100% gas-phase H/D scrambling is indicated (dotted gray line). The inset displays the crystal structure of ubiquitin (PDB 1UBQ). The turn between β-strands 1 and 2 and the loop between the central α-helix α1 and β-strand are highlighted in red (see text for further information). Regions of secondary structure of the N-terminal half are illustrated above the figure for reference.

Hydrogen scrambling is a major concern when using mass spectrometric fragmentation as an experimental tool to determine deuterium levels of individual residues in proteins and peptides.[22,29,42−49] The level of hydrogen scrambling in our measurements is clearly negligible, as our experimental data closely resembles the known solution deuteration profile and not the theoretical graph for 100% scrambling (Figure 4). The deuterium content of the z ions correlates well with that of the c ions (which is in excellent agreement with the known solution behavior) corroborating that hydrogen scrambling is negligible (Figure S4, Supporting Information).

To measure the site-specific level of back-exchange, fully deuterated ubiquitin was analyzed by ETD top-down fragmentation under quench conditions at subzero degrees. The measured deuterium levels exhibit a nearly linear increase with residue number (Figure 5), as expected for a fully deuterated uniform labeling at all backbone amides. The black graph in Figure 5 shows the theoretical cumulative deuterium content of all backbone amides assuming an average deuterium loss of 3% per residue (except for the N-terminal amide at residue Q2 where the loss was 45% due to the accelerating effect of the positively charged N-terminus).[30] The very low level of back-exchange at individual residues (3%) corroborates the efficiency of our chip-based setup with subzero cooling. The small horizontal segments of the graph correspond to proline residues which lack amide hydrogens.

The spatial resolution (the ability to obtain site-specific deuterium levels) in top-down fragmentation HDX experiments of small proteins approaches single-residue resolution. For electron-based fragmentation of proteins, however, the proportion of observed cleaved interresidue bonds decreases

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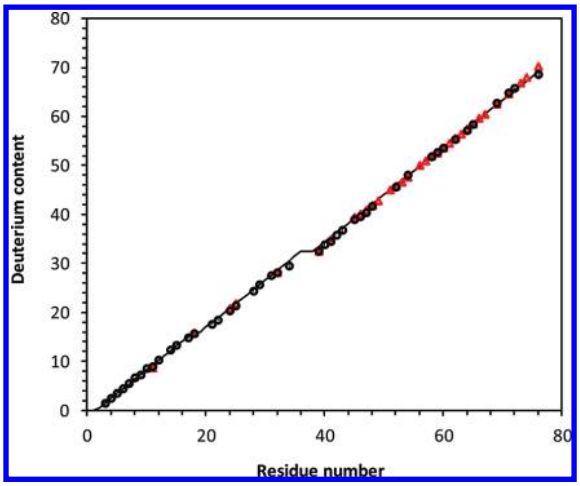

Figure 5. Spatially resolved deuterium content of fully deuterated ubiquitin at subzero temperature. Deuterium levels of the c fragment ions (black circles) and z fragment ions (red triangles) obtained from fully deuterated ubiquitin are plotted with the graph indicating the cumulative deuterium content of backbone amides assuming an average deuterium loss of 3% per residue. Error bars represent average ± SD for measurements made in triplicate.

with protein size.[50] Consequently, the spatial resolution in top-down fragmentation HDX experiments decreases with protein size. For larger proteins, supplemental activation is required in order to observe the fragment ions generated by ETD or electron capture dissociation (ECD).[51] Importantly, supplemental activation of proteins should not be expected to induce scrambling as supplemental activation of peptides (after having received electrons) appears to be sufficiently gentle to avoid the occurrence of scrambling.[52]

CONCLUSION

The deuterium loss caused by back-exchange at quench conditions is of major concern in HDX-MS analyses as it leads to loss of information. Here we demonstrate that very low levels of back-exchange can be obtained by cooling the quenched sample to subzero temperature (−15 °C) while it is analyzed with a chip-based nanoelectrospray ionization setup. It ensures a nearly constant deuterium content of the protein throughout the measurement, and this allows for prolonged acquisition time of ETD spectra at quench conditions so that good fragment ion statistics can be obtained. This is essential for an accurate determination of the deuterium content of the fragment ions. Furthermore, with a prolonged acquisition time the effect of various instrumental settings on the level of scrambling is readily explored. Our approach is particularly useful for electron-based top-down fragmentation of deuterium-labeled proteins. For the traditional bottom-up approach (based on pepsin digestion) subzero cooling is not feasible as the acidic aqueous mobile phase has a freezing point close to zero degrees. We note, however, that transient supercooling can occur in refrigerated HPLC systems.[53] As the exchange reaction is efficiently quenched at subzero temperature in our setup, it is possible to acquire and average scans for up to 15 min for a sample corresponding to a single exchange time point. In the real-time kinetic data measuring method,[20] native state exchange proceeds while ETD spectra are acquired and averaged for 2 min for each exchange time point (the experimental dead time is also ∼2 min). This compromises kinetic analysis of fast exchanging amides by the real-time method;[20] however, fast exchanging amides are amenable for analysis by our approach as the exchange reaction is efficiently quenched during ETD spectrum acquisition. If higher charge states are desired for ETD fragmentation then a supercharging reagent[26] could be added to the quench solution. Importantly, any denaturing effect of the supercharging reagent[54] would not affect the native state kinetic measurements as the reagent is only present in the quench buffer. Although it is not important for our method whether a protein partially retains its structure or completely unfolds at the present quench conditions, it is nevertheless interesting to speculate how these conditions affect protein structure. The acidic pH and the presence of organic solvent are in general destabilizing conditions for the native structure. Furthermore, cold denaturation can also occur at low temperature. For example, cold denaturation of natively folded ubiquitin (at pH 5.0) is expected to occur around −50 °C[55] and the presence of denaturant (guanidinium hydrochloride) increases the cold denaturation temperature.[56] On the other hand, subzero temperature has been reported to preserve enzyme activity in methanolic solutions[57] and several organic solvents have been used as cryosolvents to study slowed-down enzyme-catalyzed reactions at temperatures below 0 °C.[58] In our setup, the presence of methanol serves several important purposes. It is an antifreeze agent, and it enhances the electrospray ionization process by reducing surface tension. Methanol also reduces the back-exchange rate as it lowers concentration of the exchange catalyst ions of water, i.e., H^+ and OH^-.[59] In this regard, the back-exchange rates at quench conditions could be even further reduced by decreasing the temperature and increasing the concentration of methanol. For example, a 90% methanol solution with 0.2 M formic acid (v/v) can be cooled to the temperature of dry ice (−78 °C) without freezing. As back-exchange is very efficiently reduced by subzero cooling, we envision that this will be used as a general method for acquiring top-down fragmentation MS spectra of deuterium-labeled proteins. In this regard, we note that it should be possible to mount our cooling device on other types of nanoESI sources.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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