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Subzero Temperature Chromatography and Top-Down Mass Spectrometry for Protein Higher-Order Structure Characterization: Method Validation and Application to Therapeutic Antibodies

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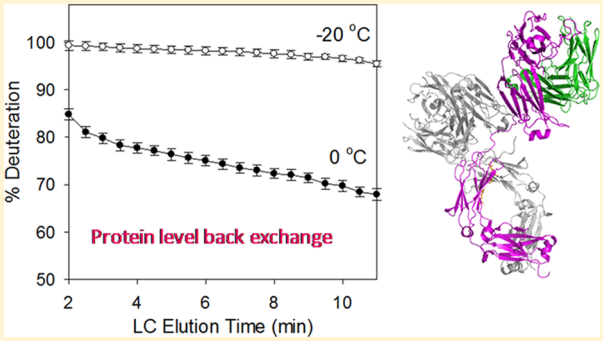
Supporting Information

ABSTRACT: Characterization of the higher-order structure and structural dynamics of proteins is crucial for in-depth understanding of their functions. Amide hydrogen/deuterium exchange (HDX), monitored by mass spectrometry (MS), is now a popular technique for measuring protein higher-order structural changes. Although the proteolysis-based HDX-MS approach is most commonly used, the "top-down" approach, which fragments intact proteins directly using electron-based dissociation, is becoming an important alternative and has several advantages. However, the commonly used top-down strategies are direct-infusion based and thus can only be used with volatile buffers. This has meant that the "top-down" approach could not be used for studying proteins under physiological conditions—the very conditions which are often very important for preserving a protein's native structure and function. More complex proteins such as those with disulfide bonds present another challenge. Therefore, there is significant interest in developing novel top-down HDX methods that are applicable to all types of protein samples. In this paper, we show how top-down electron capture dissociation and subzero temperature HPLC can be combined and used for this purpose. This method keeps the back-exchange level as low as 2% and has no limitations in terms of protein type and sample solution conditions. Close to single-residue level protein structural information can be generated. The new method is validated through comparison with NMR data using calmodulin as a model protein. Its capability of determining structural changes in therapeutic antibodies (Herceptin) is also demonstrated.



INTRODUCTION

Accurate characterization of the higher-order structure and structural dynamics of proteins is essential for in-depth understanding of their functions.[1] This is of particular importance for protein therapeutics and their generic versions—biosimilars—because even a subtle change in these structural elements can dramatically affect their therapeutic activities and cause unexpected adverse effects on patients.[2] Amide hydrogen/deuterium exchange (HDX) is a widely used means of characterizing protein higher-order structure and dynamics, as the exchange of amide hydrogens with the solvent molecules is dependent on the hydrogen-bonding status and solvent accessibility of the corresponding amides in the protein. Although 2D-NMR has long been the method of choice for HDX measurements, mass spectrometry (MS) is now becoming increasingly popular as it needs a much smaller amount of protein and can cope with proteins that are beyond NMR size limit.[3−5]

The key to HDX-MS studies is to locate the deuterium atoms that have been incorporated into the protein. Traditionally, the location of the deuterium atoms is determined through limited proteolysis under cold acidic conditions, followed by liquid chromatography (LC)-MS analysis of the resulting peptides.[3,4,6] While this experimental strategy is well-established and has provided important insights into the role of conformational dynamics in protein function, it also has some disadvantages. The spatial resolution acquired in this manner is limited by the length of the peptides (~10 amino acid residues), although this can be improved somewhat by analyzing overlapping peptides or by inducing further fragmentation in the gas phase.[7−9] The sequence coverage is also often incomplete. Significant deuterium loss (back-exchange) during proteolysis and HPLC is unavoidable and is typically 10−50%.[10]

Alternatively, the deuterium-labeled sites can be determined by directly dissociating the intact labeled protein in the gas phase. Since the successful demonstration of negligible H/D

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scrambling for a peptide[11] and a small protein[12] using electron capture dissociation (ECD), this top-down approach has been used by several groups to produce amino acid-level HDX information with complete sequence coverage.[13−21] Also, because this approach bypasses both in-solution digestion and HPLC, the back-exchange level can be kept very low (≤6%) by using two stage online mixing,[18,21] protein supercharging,[15] or a subzero-cooled nanomate apparatus.[17] Despite the aforementioned advantages, these direct infusion-based top-down strategies are only compatible with volatile buffers. In addition, proteins with disulfide bonds represent a challenge for these direct infusion methods because disulfide bonds make the protein difficult to cleave by ECD or electron transfer dissociation (ETD).[22,23] Probably for stability reasons, the vast majority of therapeutic proteins contain multiple disulfide bonds. For example, there are a total of 16 intra- and interchain disulfide bonds in the clinically utilized monoclonal immunoglobulin 1 (IgG1) and IgG4 antibodies and 18 in IgG2 antibodies.[24] Hence, there is a significant need for the development of novel top-down HDX methods that have no limitation on protein type, and which can be used with a variety of solution conditions, including physiological conditions, which are often very important for preserving a protein's native structure and function. Recently, we successfully combined ECD with LC for top-down HDX measurements.[25] However, the LC method was the one commonly used for HDX and resulted in an elution window of only 0.5 min. Such a short time-window is a serious problem for ECD data accumulation and greatly limits the application and scope of the approach. The goal of this study was to develop an approach that could overcome these limitations. The measurement of intact protein HDX using top-down ECD is combined with LC at subzero temperature, in order to achieve more complete fragmentation but without significant back exchange. This results in the ability to determine deuterium incorporation at better spatial resolution.

RESULTS AND DISCUSSION

Protein Back Exchange at Subzero Temperature. LC has long been used in the proteolysis-based HDX workflow to desalt and separate the tens to hundreds of peptides produced from each protein after pepsin digestion. In top-down experiments, the composition of the sample is much simpler, so a brief LC desalting step might be sufficient for sample cleanup prior to MS analysis. There are, however, two potential problems that must be solved before LC can be used for top-down HDX measurements: (1) the time window of protein elution needs to be wide enough so that sufficient ECD signal can be accumulated; and (2) the deuterium content of the protein must not undergo a significant change during HPLC.

In this present study, calmodulin (CaM) was chosen as the model system for method validation. The problem of the short time window became apparent when a high flow rate (200 μL/min) was used for the LC run and the elution window for CaM was only 0.5 min (Figure S1). ECD within this time window only gave good fragmentation at the two termini, namely up to fragment ion c41 from the N-terminus and z52 from the C-terminus (data not shown). This resulted in a sequence coverage of only 63% and a limited number of ECD fragments. This is understandable considering that previous top-down HDX studies used an accumulation time ranging from minutes to about 1 h.[15,17,18,20,21] This result is also in line with our recent study on interferons, where a sequence coverage of 58% was obtained.[25] To prolong the time available for ECD, the HPLC flow rate during protein elution was reduced from 200 to 10 μL/min. This resulted in an elution time increase from 0.5 min to >8 min (Figure S1). The accumulation of ECD data over this time window resulted in fragment ions covering the entire protein and a spatial resolution of <2 residues (Figures S2 and S3). Also of note is that the protein MS signal intensity of each scan is approximately the same at all the flow rates, suggesting that the ECD signal can be enhanced dramatically at low flow rate while consuming the same amount of protein.

To determine the extent of the second problem, the back-exchange level of the fully deuterated CaM was measured at 0 °C using the low-flow LC condition described above. It was found that ∼123 deuteriums were retained at 2 min and that this fell to ∼101.1 at 10 min. This corresponds to a deuteration level of 84.8% to 69.7% (Figure 1), i.e., a back-exchange level of

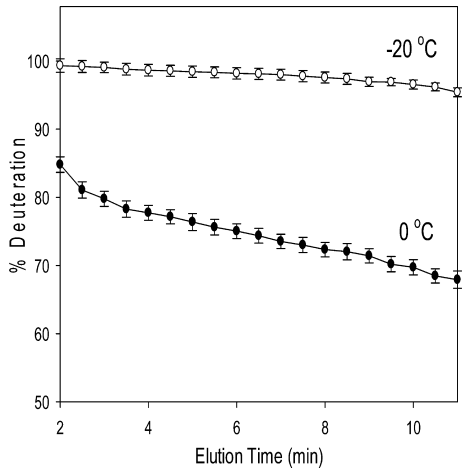

Figure 1. Comparison of protein back-exchange level at different LC elution temperature. Fully deuterated CaM was eluted at 0 °C (filled circle, ●) and −20 °C (open circle, ○), respectively. Error bars represent standard deviation for measurements made in triplicate.

15.2% to 30.3%, considering that CaM contains a total of 145 amides. This high level of, and significant variation in, back-exchange is not amenable to top-down HDX measurements. Our idea was to limit the unfavorable back-exchange by cooling the LC elution system to subzero temperature (−20 °C) using a well-controlled deep freezer (see Supporting Information for details). The exchange rates of amides are dependent on temperature,[26] and a temperature below 0 °C did help retain the deuterium content of a protein[17] and peptide,[27] compared to 0 °C. Using this strategy, the deuteration level of CaM was measured as 99.3% at 2 min and 96.5% at 10 min (Figure 1). This corresponds to an average back-exchange level of only ∼2%.

Spatially Resolved Hydrogen Exchange Measurements. With these two problems solved, we applied this subzero temperature LC system to top-down ECD of CaM for spatially resolved protein HDX measurements. HDX was carried out at pD 7.4 for 2 min in 90% D_2O buffer containing 100 mM NaCl in the absence and presence of 5 mM $CaCl_2$. ECD was conducted for ∼8 min during subzero temperature (−20 °C) LC conditions. As can clearly be seen in Figure 2, apo-CaM (without calcium) and holo-CaM (with calcium) experienced a different mass shift after HDX (Figure 2A and B), suggesting that calcium binding has caused substantial

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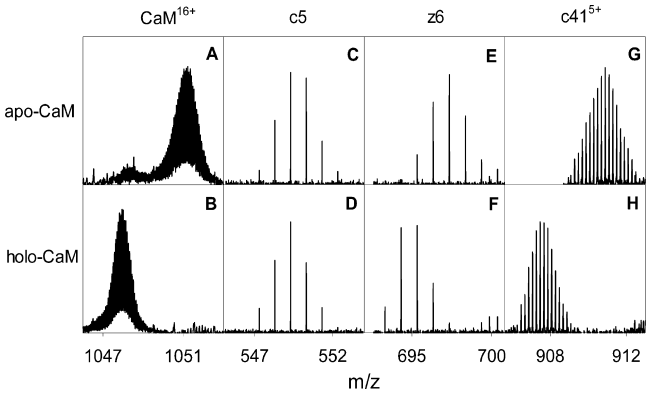

Figure 2. Mass spectra of representative top-down ECD fragments from apo-CaM (top row) and holo-CaM (bottom row) after HDX for 2 min. Note that both the N-terminal ion c5 and C-terminal ion z6 contain five amide hydrogens, but their mass shifts are clearly different, indicating that calcium binding-induced structural dynamics change is not evenly distributed in the protein.

structural and/or dynamics changes in CaM. Based on the mass determination, apo-CaM acquired ∼110 D atoms, whereas holo-CaM acquired only 51 D atoms. Hence, there were 59 amide hydrogens that became protected because of binding of Ca^{2+}.

To obtain spatially resolved exchange information, a detailed analysis of the deuteration status of all of the ECD fragments was performed (see Experimental Section for details). Because the deuterium atoms on the side chains and charge carriers are all washed away during the HPLC step, the data analysis in this method is much simpler than those described previously.[18] The determined amide deuteration levels (D) for apo-CaM are shown in Figure 3A. Because each residue (except for proline) contains one amide hydrogen, the D value for a given residue normally falls between 0 (complete protection) and 1 (no protection).

A total of 39 c ions and 40 z ions were used for the analysis of the HDX data, indicating an average spatial resolution of ∼2 residues (Figure S3), while single-residue resolution was achieved in several portions of the protein. It can be seen from Figure 3A that the strongly protected residues (low D values) were mostly located in α-helices such as H1 and H3, whereas the residues in the loop regions were less protected (high D value). This is consistent with previous observations that the HDX rate can be reduced by several orders of magnitude for sites that are hydrogen bonded and/or shielded from the solvent.[3−5,26] The amide deuteration status of apo-CaM, as determined by top-down MS, was also compared to that obtained from NMR data.[28] The amide deuteration level from NMR (D_{NMR}) was calculated based on $D_{NMR} = 1 - \exp(-kt)$. The reported k values were multiplied by $10^{0.64}$ to account for the pD difference between the NMR experiments and the current work. The D_{NMR} values obtained in this manner are shown as pink + signs in Figure 3A, revealing fairly good agreement with the D values from the ECD measurements for most of the residues. The minor differences observed for some residues might come from the small differences in experimental conditions. This general agreement between our ECD data and previous NMR data also provides evidence that H/D scrambling[29] in our study is insignificant.

Conformational Change in Calmodulin. The amide deuteration status of holo-CaM was also determined using the same analytical strategy (Figure 3B). To better visualize these changes, the amide D values for apo- and holo-CaM were mapped onto the three-dimensional structure of calcium loaded CaM (PDB entry 1cll),[31] using a color coding of blue for D < 0.33, green for D = 0.33−0.66, and red for D > 0.66 (Figure 4).

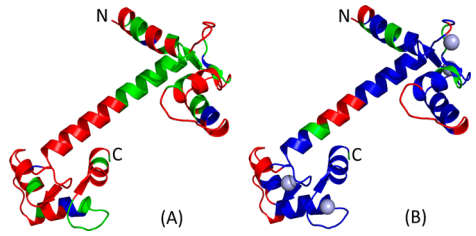

Figure 4. Amide deuteration level D mapped onto the crystal structure of CaM (PDB entry 1cll). (A) apo-CaM and (B) holo-CaM. The color coding is as follows: blue for D < 0.33, green for D = 0.33−0.66, and red for D > 0.66. The light blue spheres denote calcium ions.

It is evident that many residues shown green and red in apo-CaM become blue (protected) in holo-CaM, and most of these are located close to the bound calcium ions. This indicates that the binding of Ca^{2+} has significantly decreased the flexibility of the region surrounding these metal ions. Another interesting

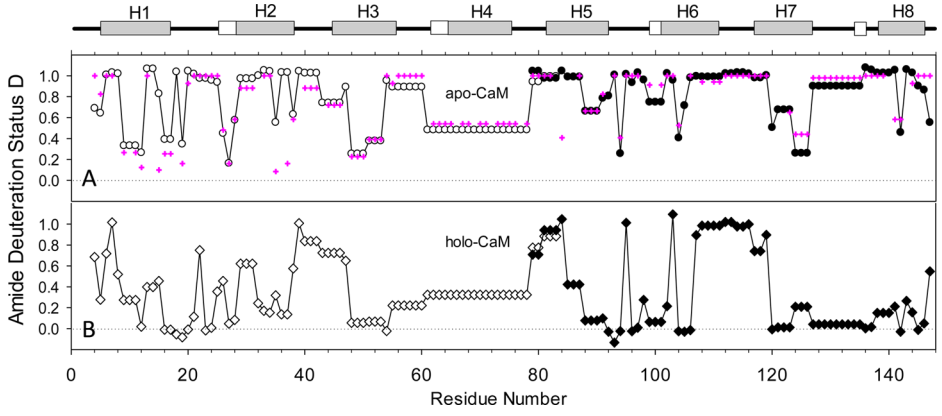

Figure 3. Amide deuteration level of each residue after HDX for 2 min as obtained from c ions (open symbol) and z ions (filled symbol). (A) apo-CaM and (B) holo-CaM. The D values represent an average of two independent measurements. D_{NMR} of apo-CaM are shown in panel (A) as pink cross. The secondary structure of apo-CaM is shown at the top of the figure.[30] Filled gray bars denote α-helices, open bars denote short β-strands.

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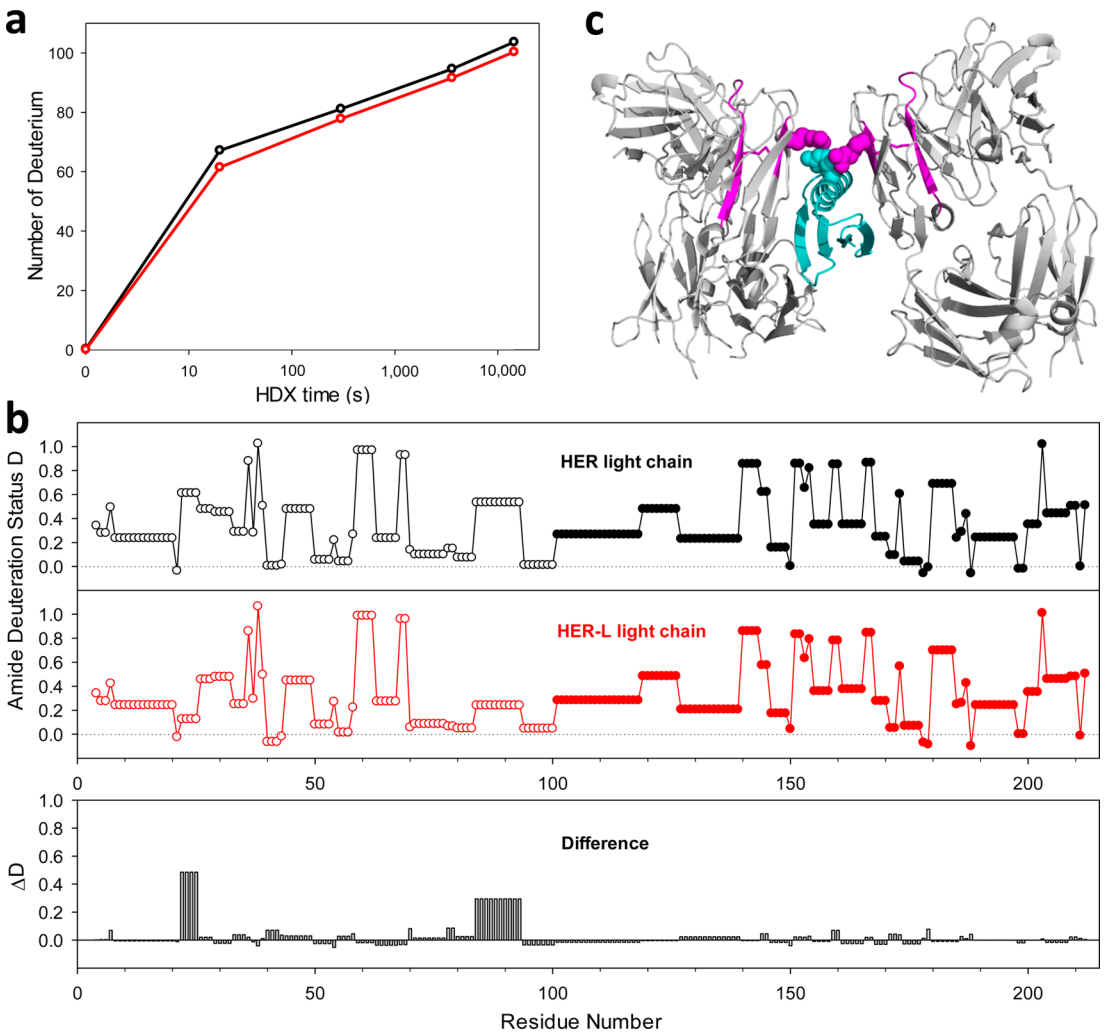

Figure 5. Intact subunit-level HDX and amino acid-level deuteration status of antibodies. (a) Time course of deuterium uptake for the light chain of HER (black) and HER-L (red). These data represent an average of three replicates. The error bars (smaller than the symbols) are not shown. (b) The amide deuteration level of each residue on the light chain after HDX for 20 s as obtained from *c* ions (open symbol) and *z* ions (filled symbol). (c) Location of affected residues (pink) on the antibody Fab fragment (gray) (PDB entry 1hez). Pink spheres denote residue R24 of HER light chain, while cyan spheres denote residue R852 of protein L (cyan).

feature occurs in the long exposed helix that connects the two domains, the center of which (residues 79−84) is in red for both apo- and holo-CaM. Although crystallography has shown that these residues are involved in hydrogen-bonding in holo-CaM, our top-down HDX-MS data suggest that these residues are highly dynamic in solution. This conclusion is supported by another crystallographic study where residues 79−81 were found to have very high temperature factors.[32]

Application to Therapeutic Antibodies. Compared to direct infusion-based top-down approaches, the use of LC has the advantage of being able to use physiological buffers which *cannot* be used in direct infusion experiments. It also enables the use of protein chemistry techniques, such as the reduction of disulfide bonds, because LC provides efficient sample cleanup prior to MS analysis, which is imperative for obtaining high-quality MS/MS spectra. Disulfide bond reduction is important for ECD MS/MS experiments in order to achieve the highest possible sequence coverage of disulfide containing proteins.[22] This has been the main problem preventing the use of top-down HDX for structural characterization of antibodies, which are used as biotherapeutics for the treatment of a variety of diseases including cancer.[33−35] To determine if our new method can be applied to these important large proteins, the HDX behavior of Herceptin (HER), a monoclonal antibody currently in clinical use for treating breast cancer, was studied before and after ligand binding.

The time-course HDX plots of for the intact light chain (Figure 5a) showed that the presence of protein L (its binding partner) reduced HER's deuterium uptake by up to ∼5 Da at 20 s. In contrast, the heavy chain remained the same (Figure S7). This indicates that protein L binding only provided extra protection for the light chain. To further localize these protected residues, the deuteration status of individual amides from the light chain was obtained at an HDX time of 20 s using the top-down ECD method and data analysis strategy developed for CaM as well as a difference plot between HER and the complex (HER-L). As shown in Figure 5b, the affected residues were identified as T22-A25 and A84-T93. Since there is no crystal structure available for the HER-L complex, the location of these residues is visualized using a homologous structure (PDB entry 1hez) (Figure 5c). Interestingly, the two affected regions of HER are found to be connected by a conserved disulfide bond (C23−C88), and the conserved arginine residue (R24) strongly interacts with R852 from

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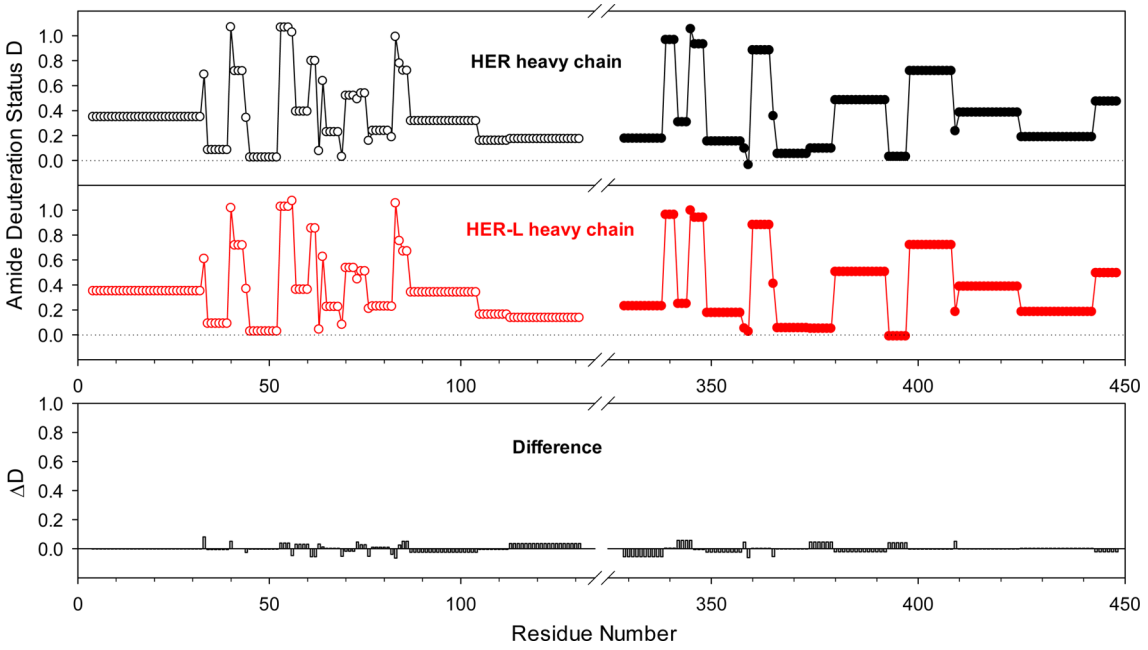

Figure 6. Amide deuteration status of the heavy chain after HDX for 20 s, as obtained from *c* ions c ions (open symbol) and *z* ions (filled symbol). (Top) HER; (middle) HER-L; and (bottom) the difference spectrum between HER and HER-L.

protein L. Based on these results, we propose that HER interacts with protein L *only* through the light chain, with probably a similar binding mode as other IgGs.[36]

Although no differences were observed in the heavy chains, LC-ECD experiments were also carried out on them for both HER and HER-L. The largest protein studied by top-down HDX-ECD so far has been 29 kDa carbonic anhydrase, and in that study the data were acquired for 30 min using direct-infusion.[37] With the subzero LC method, we were able to measure amide deuteration information for N-terminal residues 1−132 and C-terminal residues 328−449 of the antibody heavy chain (50 kDa) on the LC time scale. The average amino acid resolution for the heavy chain sequenced regions is about 4.5 residues, with close to single-residue resolution in several regions such as residues 39−44 and 52−86 in the N-terminal. The mass shifts of the ECD fragments (Figure S8) and the amino-acid level deuteration information (Figure 6) all agree with the intact subunit level HDX data (Figure S7), suggesting that protein L binding has little effect on the structure and dynamics of the heavy chains of HER. It is worth noting that the N-terminal residues that we are able to sequence with this method (residues 1−132) cover all of the complementarity-determining regions (CDRs) of the heavy chain paratope (antigen binding site), including CDR1, CDR2, and CDR3, which are crucial for HER's function and therapeutic activities.[38] The HER paratope residues are within amino acids 1−100 from the N-terminus for both the heavy and light chains and are responsible for human epidermal growth factor receptor 2 (HER2) binding.[38]

■ CONCLUSIONS

We have developed an approach that allows the use of LC-MS/MS for determining the HDX behavior of intact proteins accurately at nearly single amino acid resolution. Subzero temperatures significantly reduce back exchange (to ∼2%) and therefore allow longer LC run-times which permit more complete fragmentation, similar to that achieved with direct infusion. Moreover, the LC desalting step provides efficient sample cleanup prior to MS detection, so pretreatment of the protein samples, with methods such as buffer exchange, is no longer necessary. The amide deuteration information on CaM was obtained with complete sequence coverage and an average resolution of two residues, also with single-residue resolution in several regions. The determined deuteration pattern of apo-CaM was validated through comparison with NMR data. Further, calcium binding-induced conformational changes in solution conditions mimicking the physiological environment were uncovered from the amino acid level information.

The incorporation of protein desalting with subzero LC also makes top-down structural characterization of disulfide proteins possible. Using the monoclonal antibody HER as an example, it was demonstrated that even subtle structural differences (only 5 Da compared to the 148 kDa molecular weight of the antibody in this work) between antibodies can be precisely determined after combining with rapid disulfide reduction at low pH and low temperature. Although the amide deuteration status was not obtained for every amino acid in the heavy chain of this particular antibody, sequence-level information was obtained for 56% of the heavy chain, including the paratope region, in a single 17 min experiment. We believe that it is the subzero-temperature chromatography that has made it possible to obtain high-quality HDX-ECD data on the LC time scale for antibody heavy chains as well as for antibody light chains. It would be interesting to see if more complete sequence coverage can be obtained for other antibodies or by incorporating the subzero approach to newer generation mass spectrometers. This new method should be readily applicable for higher-order structural characterization of other types of antibodies and for structural comparability assessment of antibody biosimilars. The capability of sequencing the important paratope regions on both the heavy chain and light chain by top-down HDX-ECD may also help to provide insights for immunologists and people designing and modeling antibody drugs. The LC separation at subzero temperature also opens up the possibility of using top-

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down mass spectrometry to characterize higher-order structure of more complex systems such as large multicomponent protein complexes. We believe that this will widen the application and scope of the top-down HDX-MS approach, and we anticipate that the new top-down HDX method presented here will be widely employed by researchers in these areas in the near future.

EXPERIMENTAL SECTION

Hydrogen/Deuterium Exchange. Lyophilized recombinant human calmodulin CaM (apo-CaM), expressed in *E. coli*, was dissolved in 10 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. HDX was performed under exchange-in (H to D) conditions at room temperature (22 °C). The protein sample (100 μM) was mixed with a D_2O buffer (pD = pD_{read} + 0.4 = 7.4, 100 mM NaCl) at a ratio of 1:9 (v/v), resulting in a deuterium content of 90%. After incubation for 2 min, 15 μL aliquots were removed and quickly quenched by adding 15 μL of a solution containing 1% formic acid. The final pH was 2.4, as determined from a test sample. These samples were flash frozen in liquid nitrogen and stored at −80 °C. HDX samples for holo-CaM were prepared similarly except for the presence of 5 mM $CaCl_2$ in both the initial protein sample and the D_2O buffer. Fully deuterated CaM was prepared by dissolving lyophilized apo-CaM in 100% D_2O at a concentration of 250 μM, followed by incubation for 3 days at 37 °C, and then for another day in the presence of 1% deuterated acetic acid. Prior to subzero temperature LC-MS analysis, 0.5 μL fully deuterated CaM was quickly diluted into 24.5 μL ice-cold 35% methanol containing 0.1% formic acid and instantly injected onto the column.

HDX samples for the monoclonal antibody Herceptin (HER, trastuzumab) were prepared using a similar workflow. The stock solution of HER from Genscript (Piscataway, NJ, USA) had a concentration of 100 μM. A complex of HER and protein-L (HER-L) was prepared by adding 2 μL protein L (1 mM) to 20 μL solution of stock HER, followed by vortexing and incubation for 2 h at room temperature. Based on the very high-affinity binding between protein L and various immunoglobulins ($\sim 1.0 \times 10^9$ M^{-1}),[36] the concentrations in this study would ensure close to 100% complex formation. HDX was carried out by mixing HER or HER-L with D_2O buffer at a ratio of 2:8 (v/v). After incubation for various time periods (20 s, 5 min, 1 h, 4 h), 15-μL aliquots were removed and quickly quenched by reducing the pH to 2.5 with 30 μL of 0.5 M TCEP and 8 M guanidine hydrochloride in H_2O, pH 2.4. These samples were flash frozen in liquid nitrogen and stored at −80 °C.

Liquid Chromatography. LC-MS at subzero temperature was achieved by placing the HPLC column (C4, 5 μm, 30 × 2 mm, Phenomenex Inc., Torrance, CA, USA) in a portable deep freezer (Twinbird Corporation, Tsubame city, Japan) which was set at −20 °C. This freezer is able to control the temperature from +10 to −40 °C in 1.0 °C increments. To avoid freezing of the solvents inside the column and solvent delivery lines, 35% methanol was added to solvent A, along with 0.1% formic acid. Solvent B contained 100% acetonitrile and 0.1% formic acid. It was noted that the column pressure almost doubled for every 20 °C that the temperature was lowered. For this specific column, the column pressure with 100% solvent A at a flow rate of 200 μL/min was measured as follows: room temperature (22 °C), 36 bar; 0 °C, 73 bar; −20 °C, 140 bar. To obtain efficient cooling of the column, both solvent A and B bottles were kept in an Styrofoam cooler filled with ice, and all the solvent delivery lines except those in the freezer were wrapped with insulation. The sample injector (Rheodyne Model 7125, loop volume 20 μL) was embedded in an ice bath close to the deep freezer. LC-MS at 0 °C was performed similarly, except that the column was placed in an ice-bath.

Mass Spectrometry. All MS experiments were conducted on a Bruker 12T hybrid FT-ICR mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The ion optics and parameter settings used have been described previously.[18,37] The ESI interface parameters were as follows: capillary voltage 3600 V, spray shield voltage 3200 V, nebulizer gas flow 3 L/min, drying gas flow 5 L/min, and drying gas temperature 130 °C. The ECD experiments of CaM were performed on charge states 13+ to 18+ within the ICR cell using a precursor selection window of 380 m/z units. For HER light chain, ECD was performed on charge states 22+ to 27+ using a precursor selection window of 280 m/z units. ECD settings were as follows: electron pulse length 10 ms, electron beam bias 1.3 V, grid potential 13 V, and heater current through the dispenser cathode filament 1.2 A. Mass calibration was performed using the ECD fragments of ubiquitin.

For HDX experiments of CaM, the protein aliquots were quickly thawed on ice, injected onto the column, and analyzed by LC-MS. The column eluent was diverted from the ESI source for the first 1.5 min to prevent salts from entering the instrument. The protein samples were eluted with an 18 min binary solvent gradient with a 1.8 min desalting time. CaM was eluted as a single peak starting from 2 min using 50% solvent B. The width of the elution time window was highly dependent on the flow rate used during protein elution, which ranged from 200 μL min^{-1} to 10 μL min^{-1} (Figure S1). Up to 480 scans were accumulated for each ECD spectrum over the m/z range 250−2500, at HPLC elution time of 2−10 min. It should be noted that all of the salts, including the calcium ions, were removed by the LC, so the mass spectra of CaM and its ECD fragments are independent of the initial calcium binding state of CaM.

LC-MS analysis of HER samples was done using a similar procedure, except that the HDX samples containing 0.33 M TCEP and 5.33 M guanidine hydrochloride (pH 2.5) were kept on ice for 3 min before injection, in order to reduce the disulfide bonds in the antibody. Under these conditions, TCEP was able to reduce all the disulfide bonds in the antibody, including the inter- and intrachain disulfides, to facilitate ECD fragmentation. Nonetheless, the efficiency of the TCEP reduction was not known due to the difficulty in determining the percentage of reduced HER molecules. The H/D back exchange was negligible (<2%) during disulfide bond reduction under quench conditions.[38] To measure the deuterium content of the intact light chain and heavy chain of HER, 30−35% solvent B was used for protein elution at a flow rate of 200 μL min^{-1}. For the ECD experiments, the light chain was eluted using 30% solvent B at a flow rate of 30 μL min^{-1}. Protein L in the HER-L samples was washed off by 27% solvent B before 3.5 min. Top-down ECD data were acquired from 4 to 10 min of the HPLC run. The ECD experiments on the heavy chain were carried out using 35% solvent B for elution and an ion selection window of 5 m/z units centered on charge state 49+ (m/z 1013 for the unlabeled heavy chain, m/z 1015 for the labeled heavy chain).

Data Analysis. CaM is composed of 148 amino acid residues with a total of 145 amide hydrogens on the backbone (there are two prolines and a free N-terminal amine). HER contains two light chains and two heavy chains and 16 disulfide bridges. The light chain of HER contains 214 residues and 201 amide hydrogens in total (there are 12 prolines and a free N-terminal amine). Top-down ECD data were processed using the Bruker Data Analysis software (version 4.0). The ECD fragment ions of the unlabeled CaM were identified through ProteinProspector (http://prospector.ucsf.edu) using an m/z uncertainty constraint of 10 ppm. The centroid m/z values for the unlabeled ECD fragment ions were obtained from ProteinProspector, and the centroid m/z values for fragments after HDX were determined using HX-Express.[39] Only ECD fragment ions which had a signal-to-noise ratio (S/N) > 5 were used for deuteration content determination. The normalized number of exchanged hydrogens (N_{exch}) for each fragment ion can be calculated from eq 1:

$$N_{exch} = \frac{n(R - R_0)}{P \times 1.0063} \quad (1)$$

where R_0 and R are the centroid m/z values for a particular ECD fragment before and after HDX, respectively. The parameter n is the charge state of the fragment ion, and 1.0063 is the atomic mass difference between deuterium and hydrogen. Considering that the back-exchange level during the LC is 2%, the P value for the HDX experiments in 90% D_2O is determined as 90% × 98% = 0.882. In the case of HER, P is determined as 80% × 98% = 0.784. Because ECD was performed *after* the HPLC step, the deuterium atoms on the side

chains and charge carriers were all washed off. This makes this data analysis much simpler than those described previously.[18] The amide deuteration level D of each residue can be determined from the difference in N_{exch} values and the number of amide hydrogens between two ECD fragments. When consecutive ECD ions are observed, the amide deuteration level (D) of residue $n + 1$ was determined from the following equation:

$$D_{n+1} = \frac{N_{exch}(c_n) - N_{exch}(c_{n-1})}{N_{amide}(c_n) - N_{amide}(c_{n-1})} \quad (2)$$

where $N_{exch}(c_n)$ is the normalized number of exchanged hydrogens for fragment ion c_n, while $N_{amide}(c_n)$ is the number of amide hydrogens included in the same fragment. In cases where consecutive ECD ions were not found, an average deuteration value was obtained ($n > m$) according to

$$D_{m+2} = D_{m+3} = \ldots = D_{n+1} = \frac{N_{exch}(c_n) - N_{exch}(c_m)}{N_{amide}(c_n) - N_{amide}(c_m)} \quad (3)$$

The D values in the plots represent an average of at least two independent measurements. The variation in the D values obtained from replicate experiments was found to be within ±0.1. The D value of the C-terminal residues was determined similarly using $z^{\bullet}$ ions, bearing in mind that in CaM the ion z_m contains the amide hydrogens of residues ($148 - m + 2$) to 148. The NMR HDX rate for a given residue can be obtained according to $k = \ln 2/\tau_{exch}$, where τ_{exch} is from the literature.[28] To make a direct comparison, the D_{NMR} values were displayed in Figure 3A in the same way as for the top-down ECD data. For residues where single-residue resolution was achieved in our top-down measurements, the D_{NMR} values were displayed directly. For residues where consecutive ECD ions were not found, an average D_{NMR} value was used.

■ ASSOCIATED CONTENT

Supporting Information

Details of experimental procedures and data analysis, LC-MS elution profiles, and ESI mass spectra. 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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