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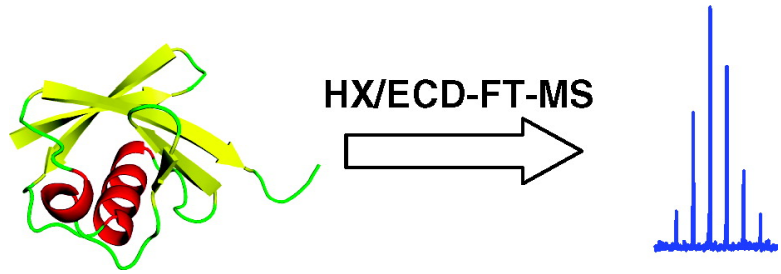

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Electron Capture Dissociation of Electrosprayed Protein Ions for Spatially Resolved Hydrogen Exchange Measurements

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Amide hydrogen exchange (HX) measurements have become an indispensable tool for monitoring structural aspects of proteins in solution.[1] Mass spectrometry (MS)-based techniques are being widely used for this purpose,[2,3] typically employing acid quenching and limited proteolysis, followed by mass analysis of the resulting peptides.[4,5] It is an interesting question whether the gas-phase fragmentation of peptides or proteins offers an alternative avenue for determining deuteration patterns in a spatially resolved manner. The feasibility of this approach remains unclear, mainly due to the possibility of intramolecular hydrogen migration. "Scrambling" processes of this kind can randomize or distort the spatial $^1H/^2H$ pattern along the polypeptide backbone.[6,7] This work demonstrates that top-down electron capture dissociation (ECD)[8] of an intact protein after HX results in deuteration patterns that are in excellent agreement with previous NMR data.

Gas-phase fragmentation methods hold tremendous promise for HX studies in the context of protein folding, dynamics, and binding. Unfortunately, previous experiments employing collision-induced dissociation (CID) resulted in conflicting observations. Some reports suggest that spatial deuteration patterns can be retained to varying degrees,[9−15] whereas others found complete scrambling regardless of the conditions used.[6,7,16,17] Hoerner et al.[18] concluded that $^1H/^2H$ migration is most pronounced when using slow heating such as SORI-CID. Thus, strategies leading to very rapid bond cleavage should be best suited for this type of application.

One of these techniques is ECD, where capture of an electron by a multiply charged gas-phase polypeptide cation leads to the "nonergodic" formation of c and $z^{\bullet}$ fragments on a time scale of 10^{-12} s with a minimum degree of vibrational excitation.[8,19] ECD FT-MS is already being successfully applied in various research areas.[20] Although bond cleavage during ECD is believed to proceed faster than intramolecular H migration,[8] Kweon et al.[21] found significant scrambling for $z^{\bullet}$ ions derived from melittin. Jørgensen and co-workers[22] provided a possible explanation for this observation, by noting that H migration can occur as a result of collisional activation during ion sampling and transfer, *i.e.*, before the analyte reaches the ICR cell. When conducting experiments without imparting excess vibrational energy to the ions, ECD fragments of regioselectively labeled 12-mer oligopeptides showed excellent agreement with the expected solution-phase deuteration pattern.[22]

Here we extend the use of ECD to spatially resolved HX measurements on ubiquitin (76 residues, 8565 Da). The native structure of this protein encompasses five β-strands, a three-turn α-helix, a single turn of 3_{10} helix, six short loops, and a highly flexible C-terminal tail (residues 71−76).[23] NMR studies have mapped the HX behavior of ubiquitin,[24,25] providing a solid foundation against which MS experiments can be compared. An earlier HX/ECD study on this protein did not determine the extent of scrambling, and comparisons with NMR data were not made.[26]

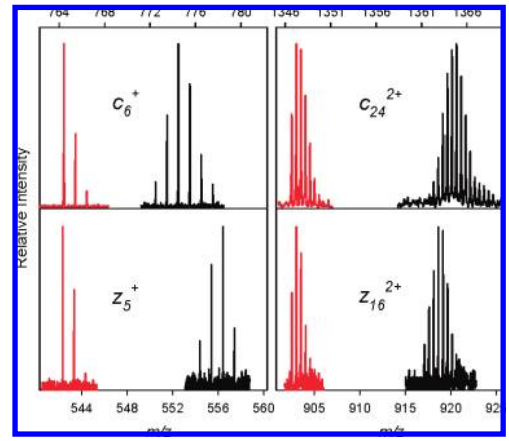

Figure 1. Mass spectra of selected ubiquitin ECD fragments. Red, unlabeled control; black, after 30 min of D_2O labeling. Note the different mass shifts of c_6^+ and z_5^+, both of which possess 14 exchangeable hydrogens (the m/z axes in both panels cover 20 units). c_6^+ represents an extensively H-bonded section, whereas z_5^+ is part of the unprotected tail. c_{24}^{2+} and z_{16}^{2+} possess 43 and 33 exchangeable hydrogens, respectively.

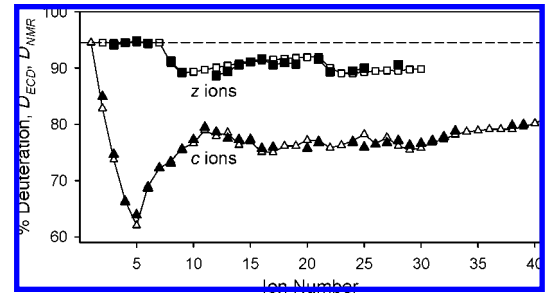

Figure 2. Solid symbols: Deuteration levels D_{ECD} of ubiquitin ECD fragments after 30 min of labeling in 95% D_2O at 22 °C. Open symbols: Expected levels D_{NMR}. Repeat measurements of D_{ECD} for z_6^+ over a 7 h period agree within ± 0.3% ($n = 3$). Dashed line: 94.5% deuteration level of the bradykinin control. The intact protein deuteration level was 84%.

Native ubiquitin was incubated in D_2O/H_2O (95:5, v/v) for 30 min at pD 7.2 and 22 °C. After 1:1 mixing with acetonitrile containing 0.4% formic acid for 1 min, the protein was electrosprayed and analyzed by FT-MS at a final concentration of 10 μM. Bradykinin was added as a rapidly exchanging internal standard,[3] exhibiting a deuteration level of $C_{BK} = 94.5\%$. This value represents the maximum possible extent of labeling under the conditions used. The instrument settings were adjusted in separate control experiments to ensure "soft" ionization, resulting in no detectable heme loss from holo-myoglobin.[27] The entire ubiquitin ion population, charge states 5+ to 12+, was subjected to ECD (Figure 1). This

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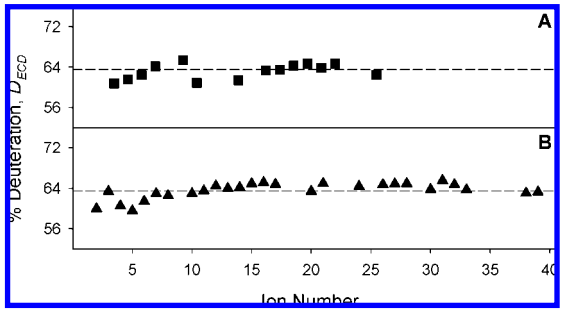

Figure 3. Deuteration levels D_{ECD} of ubiquitin ECD fragments after 3 days of labeling in 62.5% D_2O at 70 °C. (A) $z^{\bullet}$ ions; (B) c ions. The dashed line indicates the 63.5% deuteration level of the intact protein.

resulted in extensive sequence coverage, yielding fragment ions from z_3^{+} to z_{48}^{5+} and c_2^{+} to c_{54}^{5+}, with a few even larger species such as c_{75}^{5+}. However, only 30 c and 19 $z^{\bullet}$ fragments had sufficiently high *S/N* ratios for a reliable determination of their HX properties. The percent deuteration, D_{ECD}, of each of these fragments was determined as $D_{ECD} = (R - R_0)/N$, where R is the centroid mass of the isotope distribution (measured by HX-EXPRESS[4]), R_0 is the centroid of the unlabeled control (UCSF ProteinProspector), and N is the number of exchangeable hydrogens (those in N−H and O−H bonds, including side chains and charge carriers). Analysis of this data set results in very distinct progressions of D_{ECD} values for c and $z^{\bullet}$ ions (Figure 2, solid symbols).

The key question is whether the deuteration levels of the ECD fragments are compatible with the known isotope exchange behavior of ubiquitin. Expected deuteration levels D_{NMR} were calculated based on previous NMR data[25] according to

$$D_{NMR} = \frac{C_{BK}}{N} \times \left(N - \sum_{i=1}^{M} \exp(-k_i t)\right) \quad (1)$$

where M is the number of amide hydrogens undergoing HX with rate constants k_i and $t = 30$ min. The reported values of k_i[25] were multiplied by 10^1 to account for the one pD unit difference between the NMR experiments[25] and our study. $(N - M)$ represents the number of hydrogens that undergo instantaneous exchange on the time scale of the NMR experiment. D_{NMR} values for $t = 30$ min based on ref 25 are very similar to those obtained for an older data set[24] (not shown). The expected deuteration levels calculated from eq 1 are shown as open symbols in Figure 2, revealing gratifying agreement with the D_{ECD} values.

To provide additional evidence that the D_{ECD} progressions indeed reflect the regioselective solution-phase deuteration of ubiquitin, "homogeneously" labeled protein was generated by incubation in 62.5% D_2O for 3 days at pD 7.2 and 70 °C. Ideally, this procedure should provide 62.5% labeling at *every* exchangeable site. The measured ubiquitin deuteration level under these conditions is 63.5%, which is close to the D_{ECD} values of most fragments, although a few short c and $z^{\bullet}$ ions exhibit small deviations (Figure 3). These nonuniformities, as well as the slightly elevated deuteration of the intact protein, may be caused by $^1H/^2H$ fractionation.[28] In addition, a very small degree of residual protection cannot be completely excluded. In any case, the HX conditions used for Figure 3 result in D_{ECD} patterns that are dramatically different from those in Figure 2. This behavior is in contrast to CID experiments, where homogeneous and regioselective labeling can result in qualitatively similar data.[7]

In summary, this study demonstrates for the first time the successful application of ECD for spatially resolved HX studies on an intact protein, with validation through detailed comparisons with previous NMR data. Our results are further solidified by contrasting experiments under varying solution-phase HX conditions, which lead to drastically different fragment deuteration patterns (Figures 2, 3). We conclude that ECD provides a viable strategy for monitoring solution-phase HX patterns of proteins in top-down experiments, with minimal interference from scrambling or other gas-phase artifacts. Our work on an intact protein extends the findings of Jørgensen et al.,[22] who demonstrated the retention of regioselective deuteration for small peptides. The combination of HX and top-down ECD will be most useful for exploring the folding and dynamics of small proteins (<100 residues), many of which are important model systems for biophysical studies. It remains to be seen in how far this approach can be extended to even larger systems, since the ECD efficiency decreases with increasing protein size, especially when using "native" ESI conditions.

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Supporting Information Available: Experimental procedures and ESI mass spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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