Site-specific amide hydrogen exchange in melittin probed by electron capture dissociation Fourier transform ion cyclotron resonance mass spectrometry

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Received 15th August 2005, Accepted 28th September 2005
First published as an Advance Article on the web 14th October 2005
DOI: 10.1039/b511565e

Electron capture dissociation (ECD) has been proposed to be a non-ergodic process, i.e. to provide backbone dissociation of gas-phase peptides faster than randomization of the imparted energy. One potential consequence could be that ECD can fragment deuterated peptides without causing hydrogen scrambling and thereby provide amino acid residue-specific amide hydrogen exchange rates. Such a feature would improve the resolution of approaches involving solution-phase amide hydrogen exchange combined with mass spectrometry for protein structural characterization. Here, we explore this hypothesis using melittin, a haemolytic polypeptide from bee venom, as our model system. Exchange rates in methanol calculated from consecutive c-type ion pairs show some correlation with previous NMR data: the amide hydrogens of leucine 13 and alanine 15, located at the unstructured kink surrounding proline 14 in the melittin structure adopted in methanol, appear as fast exchangers and the amide hydrogens of leucine 16 and lysine 23, buried within the helical regions of melittin, appear as slow exchangers. However, calculations based on c-type ions for other amide hydrogens do not correlate well with NMR data, and evidence for deuteron scrambling in ECD was obtained from z-type ions.

Introduction

Backbone amide hydrogen/deuterium (H/D) exchange1 in combination with proteolytic digestion and electrospray ionization mass spectrometry (ESI-MS)2 has been widely used to probe protein conformation, interaction, and folding.3,7 In comparison to other detection techniques, such as nuclear magnetic resonance (NMR), mass spectrometry has the great advantages of high sensitivity and speed, and the ability to characterize large proteins. Structural resolution is generally limited by the size of detected proteolytic peptides and tandem mass spectrometry (MS/MS) has been suggested as a means to increase resolution. However, most MS/MS strategies involve heating, which may cause deuterium scrambling prior to or during dissociation and thereby loss of site-specific information.

Some reports have shown that collision-activated dissociation (CAD) can be used to assess site-specific amide hydrogen exchange rates of proteolytic peptides,8–13 particularly for b-type14 product ions.15,16 However, other studies have demonstrated various degrees of both inter- and intramolecular deuterium scrambling, precluding determination of site-specific information.17–21 For example, Kaltashov and co-workers presented evidence for minimal scrambling at relatively high collision energy, achieved via nozzle-skimmer dissociation.22 However, the same paper showed that more significant, but not random, internal exchange occurred at lower collision energies (achieved via sustained off-resonance irradiation (SORI) CAD). Recently, Jorgensen et al. found that complete randomization of all hydrogen atoms attached to nitrogen and oxygen occurred in low energy CAD.23 Thus, CAD MS/MS data from deuterated peptides must be interpreted with great caution.

Electron capture dissociation (ECD)24–27 involves irradiation of gas-phase multiply charged cations with low energy electrons, resulting in unique fragmentation pathways via a radical ion intermediate. The mechanism of fragmentation is drastically different from CAD although still debated.28–31 ECD has been suggested to occur faster than energy randomization, i.e. to be a non-ergodic process.32 That hypothesis suggests that site-specific hydrogen exchange information (as a result of minimal scrambling) could be preserved. Some initial evidence for limited scrambling was presented in the initial ECD publication.32 Two main hypotheses for ECD fragmentation involve hydrogen rearrangement: the ‘hot hydrogen atom’ mechanism,33 a hydrogen atom is relocated from a protonated site (e.g. arginine or lysine side chain) to a backbone carbonyl oxygen and in the recently proposed ‘amide superbase’ mechanism,34 a proton is transferred to a backbone amide radical site. However, neither of these mechanisms suggests rearrangement of the original backbone amide hydrogens, rendering it plausible to envision derivation of site-specific amide hydrogen exchange information from ECD. O’Connor and co-workers have proposed a radical cascade mechanism in which an alpha carbon radical propagates along the protein backbone.35 Such radical migration may cause deuterium scrambling.

Kelleher and co-workers have shown that 13C and 15N depletion prior to H/D exchange results in signal enhancement,
allowing detection of an increased number of ECD backbone fragments and faster experiments, which limits deuterium back exchange.\textsuperscript{3,4} However, no definite conclusion concerning the degree of amide hydrogen scrambling in ECD has yet been obtained. In the work presented here, we show that some ECD product ion pairs appear to successfully provide site-specific amide hydrogen exchange rates for melittin, a 2.8 kDa haemolytic polypeptide from bee venom, in methanol. NMR data from the helical structure adopted in methanol\textsuperscript{37,38} is used for comparison.

Experiment

Deuterium/hydrogen exchange and electrospray ionization

Deuterated bee venom melittin was prepared by dissolving lyophilized melittin (Sigma, St. Louis, MO) in methanol d-4 (99.8\% Aldrich, Milwaukee, WI) and incubating at 37 °C for 24 h. Deuterium/hydrogen (D/H) exchange was initiated by a 20-fold dilution into regular methanol with 0.1\% formic acid (pH = 2.6) to a final concentration of 6.5 × 10\(^{-6}\) M. The pH was selected to promote the slowest possible exchange for more accurate mass spectrometric monitoring.\textsuperscript{39} The diluted melittin solution was immediately vortexed, loaded into a 25 μL syringe, and microelectrosprayed\textsuperscript{40} in positive ion mode at 300 nL min\(^{-1}\) (flow rate maintained by a Harvard Apparatus syringe pump (Holliston, MA)) via an electrospray emitter consisting of a 50 μm id fused silica capillary (total length 20 cm) that had been mechanically ground to a uniform thin-walled tip.\textsuperscript{41} Standard electrospray parameters were used (needle potential of 1800 V, 3.8 A heating current for the inlet capillary, capillary exit potential of 70 V, tube lens of 350 V, and skimmer potential of 12 V). D/H exchange was monitored on-line for up to one hour by acquiring ECD spectra at defined points in time.

Electron capture dissociation mass spectrometry

All mass spectra were acquired with a 9.4 T quadrupole (Q)-Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer,\textsuperscript{42} located at the National High Magnetic Field Laboratory in Tallahassee, FL. The most abundant quadruply charged melittin molecular ions were mass-selectively externally accumulated\textsuperscript{43,44} for a total of 200 ms: 100 ms accumulation in the first octopole, followed by transfer to the second octopole (modified to allow improved ejection of ions along the z axis\textsuperscript{45} and located behind the quadrupole mass filter) was repeated twice. The quadrupole mass window was set to 30 amu to ensure efficient accumulation throughout the experiment as the melittin mass decreased. Ions were transferred to the ICR cell through an octopole ion guide and captured by gated trapping in an open cylindrical cell.\textsuperscript{46} ECD was performed for 20 ms with a –1 V dispenser cathode (10 mm diameter, Heat Wave, Watsonville, CA) bias voltage at 10 V trapping potential. The extraction grid was set to 3 V. Immediately following the ECD event, the trap plates were changed to 2 V, the grid to 5 V, and the cathode to 10 V for 10 ms to remove remaining electrons. For the rest of the experiment, the cathode was biased at –0.1 V and the grid at –200 V. The cathode heating power was 11 W. Following ECD, ions were subjected to chirp excitation (69 to 720 kHz at 150 Hz μs\(^{-1}\)) and direct-mode broadband detection (512 Kword data points) at 2 V trapping voltage. The experimental event-sequence was controlled by a MIDAS data acquisition system.\textsuperscript{47} Each spectrum was summed over 10 scans. The experiments were repeated three times for error analysis.

Data analysis

All spectra were processed with the MIDAS analysis free-ware.\textsuperscript{47,48} A Hanning window function was applied and the data were zero-filled once prior to fast Fourier transformation followed by magnitude calculation. Internal frequency-to-m/z calibration was performed with a two-point calibration equation.\textsuperscript{49} The calculated values of the most abundant isotope of the precursor [M + 4H]\textsuperscript{4+} melittin ions and the charge-reduced [M + 4H]\textsuperscript{3+} ions were used for calibration. Average m/z values for ECD product ions were assigned with H_D helper in the MIDAS “Find Peak” menu, which computes the center of mass of a selected set of isotopic peaks. Amino acid residue-specific deuterium content was assessed by first subtracting the average m/z of c\(_n\) (or z\(_m\)) ions from the average m/z of c\(_{n+1}\) (or z\(_{m+1}\)) ions (Roepstorff nomenclature)\textsuperscript{14} of the same charge state (z) and multiplying by z to achieve the average mass of amino acid residue (n + 1) (or (m + 1)). Second, the calculated average mass of non-deuterated amino acid (n + 1) (or (m + 1)) was subtracted to obtain the total deuterium content, D\(_t\). Due to the structure of c- and z-type ions,\textsuperscript{52} the mass difference between c\(_{n+1}\) and c\(_n\) defines the amide deuterium content of the amino acid (n + 2) together with any side chain deuteriums on amino acid (n + 1), counting from the N-terminus. For z-type ions, the mass difference between z\(_{m+1}\) and z\(_m\) corresponds to the amide deuterium content of amino acid (m) along with any side chain deuteriums on amino acid (m + 1), counting from the C-terminus.

Results and discussion

Melittin was chosen for this study for several reasons: First, both the structure and amide hydrogen exchange rates in methanol have been established by NMR\textsuperscript{37,38} providing excellent references for comparison with our ECD data. Second, melittin is small enough to not require proteolytic digestion or ion activation\textsuperscript{50} (i.e. heating) for extensive ECD fragmentation. Those factors are likely to promote back exchange and/or scrambling, rendering data difficult to interpret, as was observed in the earlier Kelleher study.\textsuperscript{36} Finally, melittin adopts a defined structure in organic solvent, precluding the use of buffer salts during exchange and thereby subsequent desalting, which can also enhance back exchange. The sequence and structure of the melittin dimer are shown in Fig. 1. Melittin contains 26 amino acid residues and the structure in organic solvent consists of two alpha-helices, one at each terminus, separated by a non-helical kink around proline (Pro) 14.

The ECD spectrum obtained from non-deuterated melittin under the present experimental conditions is shown in Fig. 2. Mainly c-, z-, and γ-type product ions were observed, corresponding to cleavage at 18 of 25 interresidue amine bonds. However, only 14 out of 32 identified product ions had

function of the D/H exchange time. c distributions of two consecutive corresponding to cleavage at 18 of 25 interresidue amine bonds.

Fig. 1 Sequence and Brookhaven Protein Databank structure (Macromolecular Structure Database, entry 2MLT from X-ray crystallography) of the melittin dimer. The spheres at the C-terminal ends of melittin represent the nitrogen atoms of the amidated C-termina. Two sulfate ions, used as crystallization reagent, are also shown.

Fig. 2 ECD product ion spectrum from quadruply protonated non-deuterated melittin (20 ms irradiation, −1 V cathode potential, 10 scans). Mainly c-, z-, and y-type product ions are observed, corresponding to cleavage at 18 of 25 interresidue amine bonds.

[Image 56x85 to 291x217]
[Image 56x370 to 291x516]
[Image 88x604 to 259x721]

a neighboring consecutive ion and sufficient signal-to-noise (S/N) ratios for calculation of site-specific D/H exchange. Exchange rates were only determined for consecutive product ions of identical ion type and charge state. An example is shown in Fig. 3 for the time evolution of the resolved isotopic distributions of the c_{22}^{2+} and c_{23}^{2+} ions. Due to the structure of c-type ions (see above), the mass difference between these two ions is representative of the amide deuterium content of arginine (Arg) 24 along with any remaining deuterium on the side chain of lysine (Lys) 23.

Site-specific D/H exchange rates of amide hydrogens were determined by plotting the natural logarithm (ln) of the deuterium content (D, calculated as defined above) minus the expected deuterium content after an infinite exchange period (D_{inf}) as a function of the exchange time (t) for each observable amino acid residue, based on a first order reaction scheme for the exchange:

\[ D_t - D_{inf} = Ce^{-kt} \]

in which k_{ex} is the first order exchange rate constant and C corresponds to (D_t - D_{inf}) prior to exchange, i.e. at time zero.51 D_{inf} is simply the deuterium content in the spraying solution, in our case 5%. A linear fit (y = Ax + B) of ln(D_t - D_{inf}) vs. time results in a line with a slope corresponding to k_{ex}.

Site-specific exchange from c-type ions

Site-specific D/H exchange obtained from c-type ions is shown in Fig. 4. For c ion pairs whose mass difference includes an amino acid side chain that does not contain labile hydrogens, the calculated deuterium content is solely due to the amide hydrogen differing between the two fragments. In our experiments, such situations were encountered for three ion pairs: c_{11}^{+}\text{Lc}_{11}^{+} (amide hydrogen of leucine (Leu) 13 and side chain of glycine (Gly) 12, Fig. 4b), c_{12}^{+}\text{Lc}_{14}^{+} (amide hydrogen of alanine (Ala) 15 (Pro 14 does not have an amide hydrogen) and side chains of Leu 13 and Pro 14, Fig. 4c), and c_{14}^{+}\text{Lc}_{15}^{+} (amide hydrogen of Leu 16 and side chain of Ala 15, Fig. 4d). The deuterium content for the differing amino acid residue at time zero should be 0.998 (equal to the purity of our deuterated methanol) for these c ions pairs. Thus, ln(D_t - D_{inf}) at time zero, i.e. the y axis intercept, should be −0.053. However, only the data for one amino acid residue, the one containing the amide hydrogen of Leu 16 (Fig. 4d), is close to such behavior (an intercept of −0.058). That amide hydrogen is buried within the C-terminal, more stable, alpha-helix of melittin.8,38 Kaltashov and Fenselau have shown that triply charged melittin retains its helical structure in the gas phase.52 Although, to our knowledge, corresponding data for the quadruply charged ions is not available, the stability of the C-terminal helix should render its constituent amide hydrogens less likely to undergo exchange during transfer to the gas phase and into the mass spectrometer. The amide hydrogens of Leu 13 and Ala 15, on the other hand, are located in the hinge region surrounding Pro 14 and are therefore more exposed and likely to undergo gas-phase exchange. Accordingly, the y axis intercept is less than −0.053 in both those cases (−0.58 and −0.43, respectively). The latter values correspond to an initial deuterium content of 61% for Leu 13 and 70% for Ala 15, significantly differing from the expected value of 99.8%. We propose the discrepancy is caused by gas-phase exchange during ionization and transport through the mass spectrometer. This rather high degree of gas-phase exchange is likely related to our electrospray conditions. We used standard operating parameters (see the Experimental section) and did
not attempt to, e.g., lower the capillary heating current because such alterations result in reduced precursor ion signal, which is detrimental to ECD. However, gas-phase exchange should influence each measured time point equally, shifting the lines in Fig. 4 along the y axis but not affecting the slope of the lines. Thus, site-specific exchange rates may still be derived from these lines. The hypothesis of enhanced gas-phase exchange in the exposed hinge region in melittin is supported by the larger standard deviations observed for Gly 12, Leu 13, and Ala 15 (Fig. 4a–c).

For c ion pairs whose mass difference includes an amino acid side chain that does contain labile hydrogens, two situations can be envisioned: First, the observed exchange behavior is solely due to the amide hydrogen differing between the two fragments because the side chain labile deuteriums are exchanging instantly upon dilution into regular methanol. In such cases, behavior similar to that discussed above should be seen, which is observed for c10+/e11+ (amide hydrogen of Gly 12 and side chain of threonine (Thr) 11, Fig. 4a). Here, the y axis intercept is –0.35, corresponding to an initial deuterium content of 75%. Second, if deuterium is remaining on an amino acid side chain throughout the experiment, the extrapolated deuterium content at time zero should be larger than 0.998, i.e. \( \ln(D_t - D_{\text{inh}}) \) should be larger than –0.053. The latter behavior is observed for c21+/e22+ (amide hydrogen of Lys 23 and side chain of Arg 22, Fig. 4e), c22+/e23+ (amide hydrogen of Arg 24 and side chain of Lys 23, Fig. 4f), and c24+/e25+(amide hydrogen of glutamine (Gln) 26 and side chain of Gln 25, Fig. 4g). For arginine, NMR experiments have shown that acid catalysis is significantly more effective for the \( \eta \)-protons than for the c-proton at low ionic strength. Thus, it is not surprising that some additional deuterium is observed for that particular residue. The excess deuterium observed on the side chain of Gln 25 is more difficult to explain but may suggest that this side chain is hydrogen bonded, e.g., to the C-terminal carbonyl oxygen of melittin.

Site-specific amide hydrogen exchange rate constants obtained from ECD and NMR data sets. For example, larger exchange constants were obtained from ECD for the two amide hydrogens surrounding Pro 14 (Leu 13 and Ala 15). These two hydrogens were also classified as fast exchangers with NMR. In addition, smaller rate constants were obtained from ECD for the amide hydrogens of Leu 16 and Lys 23, correlating with their classification as slow exchangers by NMR.

### Table 1 Summary of site-specific amide hydrogen exchange rates obtained from ECD and NMR

<table>
<thead>
<tr>
<th>Amide hydrogen</th>
<th>Ion type and charge state</th>
<th>( k_a ) (D/H) from ECD/min(^{-1} )</th>
<th>( k_{13} ) (H/D) from NMR/min(^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly 12</td>
<td>c11+ - c10+</td>
<td>0.007 ± 0.0054</td>
<td>Fast</td>
</tr>
<tr>
<td>Leu 13</td>
<td>c12+ - c11+</td>
<td>0.039 ± 0.0084</td>
<td>Fast</td>
</tr>
<tr>
<td>Ala 15</td>
<td>c14+ - c12+</td>
<td>0.05 ± 0.0033</td>
<td>Fast</td>
</tr>
<tr>
<td>Leu 16</td>
<td>c15+ - c14+</td>
<td>0.007 ± 0.0034</td>
<td>Slow</td>
</tr>
<tr>
<td>Lys 23</td>
<td>c22+ - c21+</td>
<td>0.004 ± 0.0037</td>
<td>Slow</td>
</tr>
<tr>
<td>Arg 24</td>
<td>c23+ - c22+</td>
<td>0.059 ± 0.0046</td>
<td>Medium</td>
</tr>
<tr>
<td>Gln 26</td>
<td>c23+ - c24+</td>
<td>0.006 ± 0.0024</td>
<td>Fast</td>
</tr>
<tr>
<td>Ala 4</td>
<td>z23+ - z22+</td>
<td>Not available</td>
<td>Medium</td>
</tr>
<tr>
<td>Val 8</td>
<td>z20+ - z19</td>
<td>0.09 ± 0.0024</td>
<td>Slow</td>
</tr>
</tbody>
</table>

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![Fig. 4 Site-specific exchange rates obtained from c-type ion pairs for seven different amide hydrogens in melittin. The deuterium content, \( D_t \), of the particular amide hydrogen was calculated from two consecutive solution, is plotted vs. the D/H exchange time. The slopes of the fitted lines represent the site-specific exchange rate constants (see text).](image-url)
were used to calculate the exchange rate. These extra protons may promote hydrogen scrambling.

Site-specific exchange from z'-type ions

Two sets of z' ion pairs were investigated: \( z_{19}^{2+} \) and \( z_{20}^{2+} \) (amide hydrogen of valine (Val) 8 and side chain of Lys 7) and \( z_{23}^{3+} \) and \( z_{24}^{3+} \) (amide hydrogen of Ala 4 and side chain of Gly 3). For the Val 8 amide hydrogen, a rate constant could be obtained using the same procedure as for the c ion pairs described above. However, the resulting value does not correlate well with NMR data, see Table 1. Again, this behavior may be related to the high charge state (proton content) of these z' ions. For the amide hydrogen of Ala 4, a rate constant could not be obtained because the observed deuterium content of \( z_{23}^{3+} \) (with fewer exchangeable hydrogens) was always higher than for \( z_{24}^{3+} \), indicative of hydrogen scrambling that could be related to the radical character of z ions.24 The initial radical site of \( z_{23} \) is an alanine alpha carbon whereas the initial radical site of \( z_{24} \) is a glycine alpha carbon. The former is a more stable radical, which may explain the observed preferential deuterium loss in \( z_{24} \). We attempted to investigate this hypothesis by examining the isotopic distributions of the \( z_{24} \) and \( z_{23} \) ions in the ECD spectra from non-deuterated melittin. However, all isotopic distributions, including the one for the precursor ion, are skewed following ECD, rendering a comparison difficult.

Conclusions

Although an indirectly heated dispenser cathode, which provides millisecond ECD, was used, only a limited number of c- and z' ion pairs had sufficient S/N ratios to allow assessment of site-specific amide hydrogen exchange rates within a reasonable spectral acquisition time (\( < 2 \) s). More concentrated melittin solutions are not likely to provide higher ECD conversion efficiency because the precursor ion abundance was already optimized (higher abundance results in lower ECD efficiency). Thus, our results raise some concerns on the general applicability of ECD for determination of site-specific amide hydrogen exchange rates. However, pepsin digestion generally produces rather small peptides, for which the total product ion signal is spread over fewer peaks than for melittin, so these concerns may prove unjustified. For the consecutive c-type ion pairs investigated, we observed some agreement between exchange rates measured by ECD and previous results from NMR data. By contrast, evidence of deuterium scrambling in ECD was obtained from the limited number of z' ion pairs characterized. The latter observation may be a result of the radical character of z ions. Further experiments with other model systems are needed to elucidate whether amide hydrogen exchange rates from c-type ions can provide reliable site-specific structural information.

Acknowledgements

Generous financial support was provided by the University of Michigan. We also thank Prof. Alan G. Marshall and the NSF National High Field FT-ICR Mass Spectrometry Facility in Tallahassee, FL (CHE-99-09502) for supporting data collection with their instrumentation.

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