

Increased Coverage in the Transmembrane Domain with Activated Ion ECD for Top-down FTMS of Integral Membrane Proteins

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Introduction

Nearly one third of the proteome is believed to consist of integral membrane proteins of biological membrane bilayers that compartmentalize living cells and as such have been identified as important drug targets (Lehnert et al., 2004). Because of their unique chemical nature, these proteins are difficult to characterize with conventional enzymatic digestion and LC-MS/MS analyses of the subsequent peptides. As an alternative approach, “top-down” protein characterization has been developed. Top-down protein characterization relies on high-resolution Fourier-transform mass spectrometry (FT-MS) to accurately measure the mass of intact protein molecular ions, followed by their fragmentation in the mass spectrometer without prior digestion. This makes it possible to obtain primary structure information for unambiguous identification and characterization of protein covalent modifications (Kelleher et al., 1999; Kelleher, 2004).

Incorporating the “top-down” approach, a suite of techniques has been developed using convenient purification and online electrospray-ionization (ESI) mass spectrometry to study integral membrane proteins (Whitelegge et al., 1998; 1999; 2002; Whitelegge, 2004). Whitelegge and colleagues have applied FT-MS to measure the mass of the seven transmembrane domain bacteriorhodopsin apoprotein with better than 10 ppm mass accuracy (Whitelegge et al., 2003). They have also used collisionally activated dissociation (CAD) with low resolution mass analyzers to investigate small, single transmembrane helix, integral subunits of the cytochrome *b₆f* complex (Whitelegge et al., 2002).

Described herein are experiments demonstrating a top-down analysis of the *c*-subunit of the ATP synthase (ATPH) from chloroplasts of *Arabidopsis thaliana*, an integral membrane protein with two transmembrane domains. The experiment was conducted (see Thermo Fisher Scientific AN30081) using a Thermo Scientific LTQ FT equipped with electron capture dissociation (ECD) to achieve a consistent mass accuracy of better than 5 ppm for product ions from the protonated protein molecules. By activating membrane protein ions *in vacuo* using an IR laser, the efficiency of ECD was enhanced over CAD or conventional ECD so that backbone cleavage was observed frequently in transmembrane α -helical domains. This activated ion ECD technique permits analysis of covalent modifications present in the transmembrane domains of hydrophobic proteins which are otherwise difficult to detect.

Experimental Methods

Protein Samples

A suspension of *Arabidopsis thaliana* thylakoid membranes (50 μ L of 1 mg chlorophyll/mL) (Whitelegge et al., 1992) was diluted 0.25:1:3:1 with water, methanol and chloroform respectively and mixed prior to the addition of 400 μ L of water to induce phase separation. After vigorous mixing and centrifugation (5 minutes at 10,000 \times g) the lower chloroform enriched phase was recovered. An aliquot was injected into an HPLC system for immediate size-exclusion chromatography with a buffer containing chloroform/methanol/1% formic acid in water; 4/4/1, v/v) at 250 μ L/minute and 40°C to purify *c*-subunit of ATPH from small molecule contaminants. One minute fractions were collected into glass vials and stored at -20°C until they were analyzed by static-nanospray experiments. For direct infusion, sequential LC fractions were individually loaded into 2 μ m i.d. externally-coated nanospray emitters (New Objective Inc., Woburn, MA) and desorbed using a spray voltage of between 1.2-1.4 kV (*versus* the inlet of the mass spectrometer). These conditions produced a flow rate of 20-50 nL/min.

Mass Spectrometry

In this study, all of the proteins were analyzed using a linear trap/FTICR hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Ion transmission into the linear trap and further to the FTICR cell was automatically optimized for maximum ion signal. The ion count targets for the full scan FTICR and MS/MS FTICR experiments were 2×10^6 . The *m/z* resolving power of the FTICR mass analyzer was set at 100,000 (defined by $m/\Delta m_{50\%}$ at *m/z* 400). Individual charge states of the protonated protein molecules were selected for isolation and collisional activation in the linear ion trap followed by the detection of the resulting fragments in the FTICR cell. For the CAD studies, the precursor ions were activated using 35% Normalized Collision Energy™ at the default activation *q*-value of 0.25. Alternative studies were conducted in which the precursor ions were guided to the FTICR cell and further fragmented using ECD. The ECD fragmentation efficiency was optimized to maximize fragment ion signal. Activated ion ECD experiments were also done in which precursor ions were excited with an infrared laser to an energy level just below their dissociation threshold, while simultaneously being irradiated with electrons via ECD using the same conditions as previously

Key Words

- LTQ FT™
- ECD
- IRMPD
- Top-down analysis

mentioned for conventional ECD. All FTICR spectra were processed using Thermo Scientific Xtract to produce monoisotopic mass lists. For clarity, the mass difference (in units of 1.00235 Da) between the most abundant isotopic peak and the monoisotopic peak is denoted in italics after each M_r value. FT-MS data was derived from an average of between 50-500 transient signals. The fragment mass sequence assignments consistent with the sequence of ATPH were made using ProSight™ PTM software (LeDuc et al., 2004) with a 5 ppm mass accuracy threshold.

Results and Discussion

Electrospray spectra of the intact membrane protein

A spectrum containing signals for the multiply-protonated and adducted ATPH molecules is presented in the Figure 1. The charge state distribution of this 8003.9-4 Da ion was suppressed with the 5+ charge state being the dominant signal. The number of oxidized and sodiated adduct signals are identified (Figure 1, inset a). In contrast, spectra of proteins of the similar size but lower hydrophobicity typically show signals indicative of twice as many charges (Figure 1, inset b, top spectra) resulting in m/z signals with a distribution at significantly lower m/z values. It is likely that the protonated molecules of ATPH are tightly folded and held by tertiary noncovalent interactions that are preserved in the gas phase. This assumption is supported by published studies on gaseous structures of different charge states of ubiquitin, which is a protein of a similar size to ATPH (Oh et al., 2002). The lower number of charges in ATPH results in decreased sensitivity and lowered m/z resolution in the ICR mass analyzer (resolution decreases with increasing m/z).

Collisionally Activated Dissociation of ATPH

CAD fragmentation of 5+ precursor ion resulted in sixteen amide bond cleavages (Figure 2, top), with a mass accuracy of 2.9 ppm (RMS) for the corresponding *b* and *y* fragments. Fifteen of those cleavages were observed in two transmembrane domains (Figure 3, color) resulting in a cumulative coverage of 34%. Since CAD breaks the weakest amides first, the number of the primary *b* and *y* fragments is difficult to increase to improve the efficiency of this dissociation technique.

Electron Capture Dissociation of ATPH

The fragmentation by ECD of the 5+ protonated molecular ion (Figure 2, middle spectrum) produced a spectrum that is dominated by signals from reduced charge odd electron species thus confirming that electron capture occurred. Surprisingly, only three *c*- and z^* -type fragments ions (Figure 3) were detected, and of that, only one fragment formed by cleavage in the transmembrane domain was observed. Such low efficiency in producing *c* and z^* fragments ions might result from a tightly folded conformation such that the inner sequence regions are not exposed to electrons for capture and subsequent fragmentation.

Activated Ion Electron Capture Dissociation of ATPH

Previous studies (Horn et al., 2000; Oh et al., 2002) indicate that activation of the gas-phase protein protonated molecules by heat, collisions with neutrals or IR photons led to temporary unfolding. The simultaneous electron capture dissociation of such “activated” ions produces many more primary *c*- and z^* -type fragment ions which lead to significantly increased sequence coverage. In this study, we irradiated the precursor ions with the

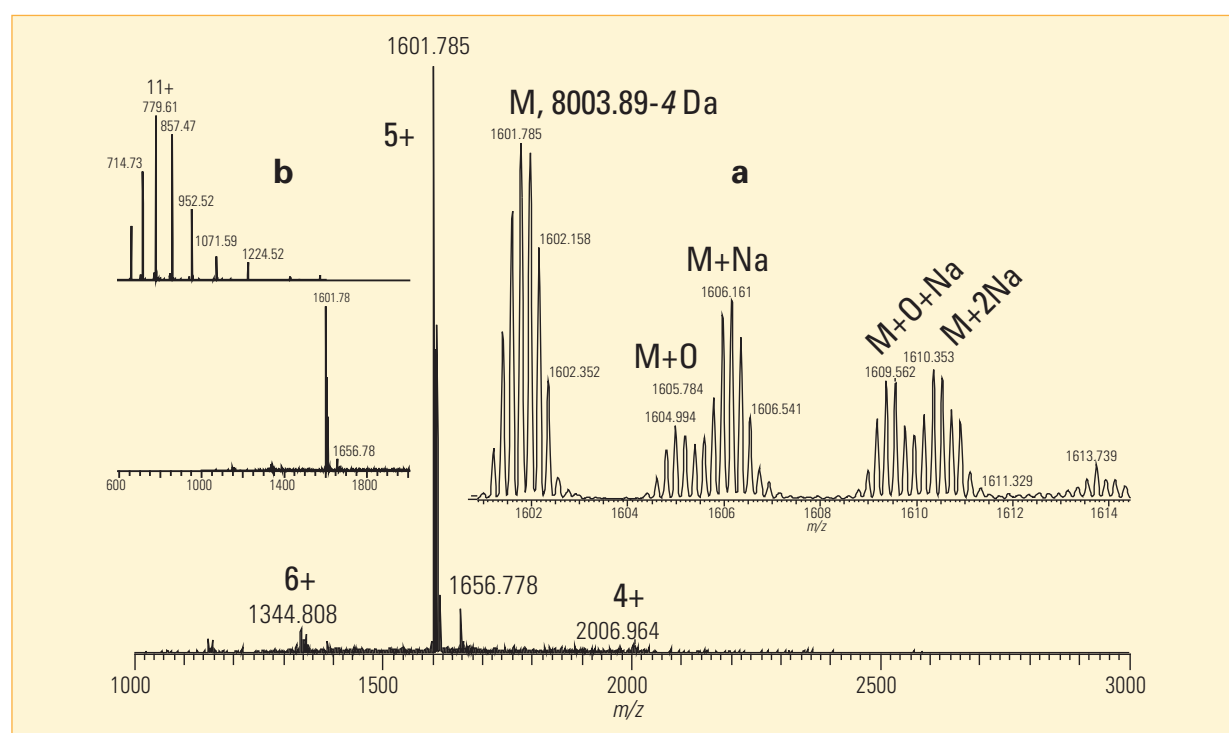


Figure 1: Molecular ion spectrum of ATPH. Molecular ion adducts (Inset a); Comparison of the charge state distribution between ubiquitin (8.6 kDa, Inset b Top) and ATPH (Inset b Bottom)

output from an infrared laser (the laser power was kept at a level below the dissociation threshold for the precursor ions of interest) while simultaneously performing ECD (Figure 2, bottom spectrum). The resulting spectrum contained signals that were assigned as *c*-type (19 observed) and *z*^{*}-type (16 observed) fragment ions based on their accurate mass values (2.5 ppm RMS). Of these fragments, 23 were indicative of cleavages in the transmembrane region (52% coverage) (Figure 3).

Conclusion

Clearly, we have shown that activated ion ECD is a powerful tool for sequencing hydrophobic proteins of the type isolated from cellular membranes. Conventional electron capture dissociation has been shown to preserve labile post translational modifications including phosphorylation and glycosylation (Mirgorodskaya et al., 1999; Shi et al., 2001). This study suggests that ECD when combined with IR laser activation can be a powerful tool to characterize covalent modifications in the transmembrane loops of the hydrophobic proteins.

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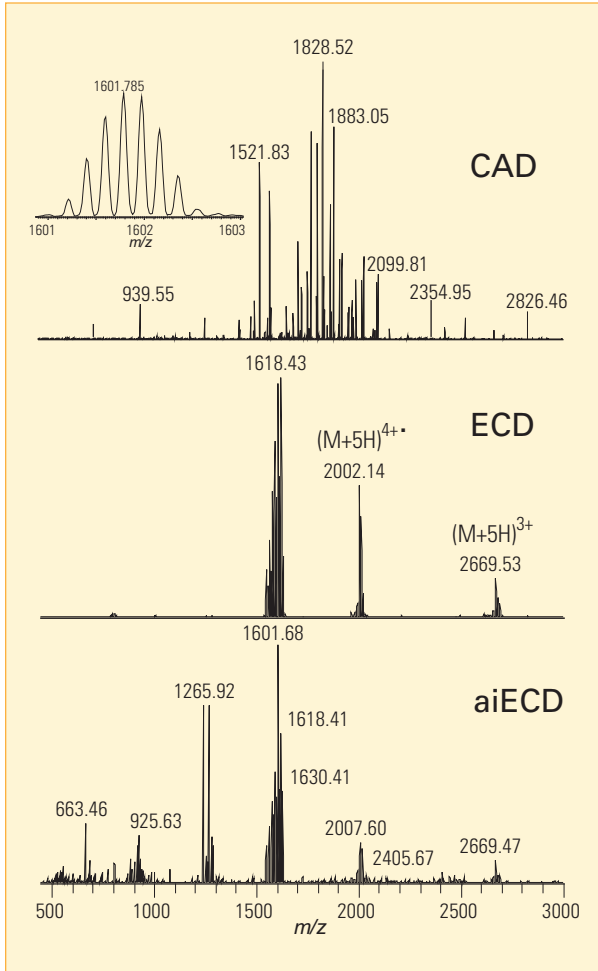


Figure 2: Fragmentation mass spectra of 5+ protonated molecule of ATPH (Inset top). Collisionally activated dissociation spectrum (Top). Electron capture dissociation (no activation) spectrum (Middle). Activated ion electron capture dissociation (aiECD) spectrum (Bottom).

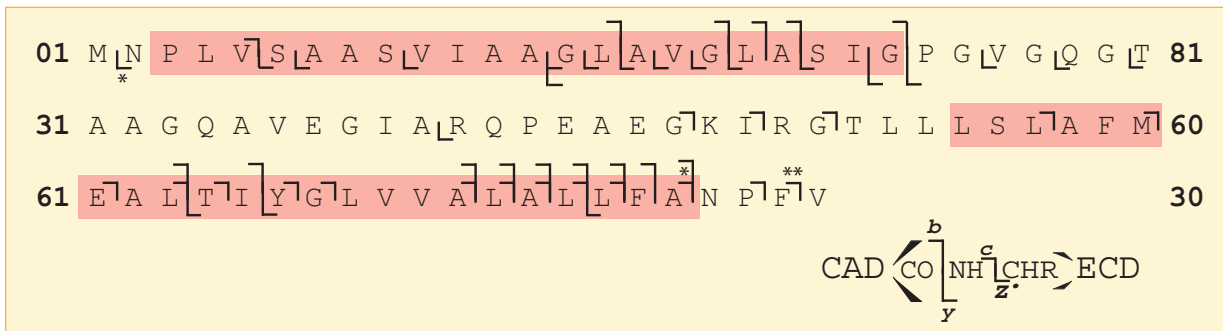


Figure 3: Fragment assignments from CAD (*b* and *y* fragment ions), ECD and aiECD (*c* and *z*^{*} fragment ions) experiments to the sequence of ATPH. The *c* and *z*^{*} fragment ions marked by single star are present in conventional ECD and activated ion ECD spectra. The *c* and *z*^{*} fragment ions marked by double star are present only in conventional ECD spectra.

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