

Unfolding of Intact Ubiquitin in the Gas Phase by IRMPD and Fragmentation with ECD

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Key Words

- Finnigan™ LTQ FT™
- Intact proteins
- Post-translational Modifications (PTMs)
- Top-down proteomics

Introduction

The study of *in vivo* folding of proteins into their biologically active forms has been a mainstay of research for decades. Different mass spectrometric techniques, such as H/D exchange^[1] and electron capture dissociation (ECD)^[2] can be used to study protein folding in the gas phase. Protein folding in the gas phase has an often underestimated influence in the recently emerged field of top-down proteomics with ECD: Tight folding prevents efficient cleavage by ECD and limits therefore its use.

The forces that are responsible for the stable secondary structure of proteins are hydrogen bonding, dipole-dipole bonding (van-der-Waals bonding) and hydrophobic interactions (all non-covalent bonds).

In solution, basic side chains on protonation sites of the protein are solvated into the surrounding water to denature the protein; in the gas phase, these protonated sites are solvated internally. Removal of water during the electrospray process can greatly decrease hydrophobic bonding and van-der-Waals bonding while enhancing hydrogen bonding; however, reversible folding and unfolding between stable gaseous conformers is still observable.

The high amount (~6 eV) of energy released upon capture of an electron by a multiply charged protein ion in ECD can result in immediate (<10⁻¹² s), nonergodic dissociation of backbone bonds, producing mainly c-type and z-type fragment ions. Even with randomization of the remainder of this 6 eV energy over the thousands of degrees of freedom of the protein ion, other bonds will not be appreciably energized.

This phenomenon has two effects. Firstly, any weak non-covalent bond between the newly formed complementary c- and z-type ions will keep those ions together. This complex product exhibits the same mass as the reduced molecular ion. Secondly, tight folding of the protein in the gas phase can prevent further fragmentation and can therefore limit the fragmentation efficiency. This can be overcome by activating the protein in the gas phase prior to ECD irradiation.

A number of approaches have been published for ion activation prior to ECD, such as activated ion electron capture dissociation (AI ECD),^[3] plasma electron capture dissociation,^[4] ICR cell heating^[5] and infra-red multiphoton dissociation (IRMPD).^[5] Of these methods, only IRMPD is available on commercial FTICR mass spectrometers. The other approaches require extensive modifications of the instrument hardware (such as extra trapping plates for the simultaneous storage of positive ions and electrons) and have been implemented on only few academic research instruments. Table 1 shows the different activation methods and their working principles.

Activation method prior to ECD	Principle
AI-ECD	Activation by collision with background gas
"Plasma"-ECD	Activation by collision with background gas and concurrent introduction of electrons and analyte ions
ICR cell heating	Activation by heating the ICR cell (from ambient room temperature up to 175 °C)
IRMPD	Activation by absorption of photons

Table 1: Activation methods prior to ECD and working principle

The common result of the application of an ion activation method prior to ECD is the significant increase of bond cleavages observable in ECD product ion spectra. This results in a more complete determination of the protein sequence in top-down proteomic approaches.

This application note describes the use of IRMPD as an activation method prior to ECD on the protein ubiquitin, a small (8.6 kDa) protein. IRMPD is available on the Finnigan LTQ FT in addition to an ECD cathode positioned slightly off-axis to facilitate the simultaneous use of ECD and IRMPD.

The secondary structure of ubiquitin is well characterized in solution. Ubiquitin forms one alpha helix and five beta sheets in solution (see Figure 1)

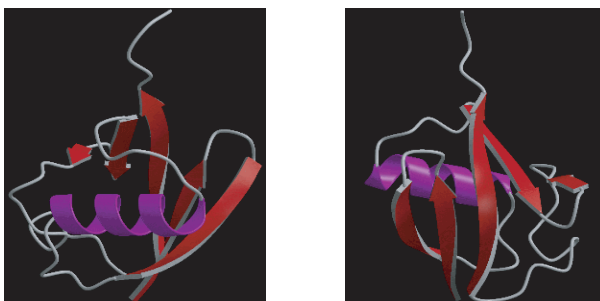


Figure 1: Structure of ubiquitin in solution^[6]

McLafferty and coworkers showed that the structure of ubiquitin in the gas phase however depends on the charge state.^[5] They demonstrated that removal of the aqueous solvent from the protein ubiquitin gives an extensive variety of conformational structures, in contrast to the singular native form in solution.

Ubiquitin of lower charge states (6-9) appears to be bent in an antiparallel helical conformer stabilized by non-covalent tertiary bonding. Higher charge states appear to be fully α -helical and show extensive ECD fragmentation.

Results and Discussion

Protein unfolding in the gas phase by IRMPD at first may seem to be of theoretical interest, but it is of high impact in top-down proteomics experiments using ECD.

A priori, the gas phase conformation of an unknown protein is also unknown, and best characterization of the protein can only be achieved with a sufficient number of cleavage events. Therefore, it is highly desirable to either isolate the charge state with the most promising gas phase conformation or to use activation of the precursor by IRMPD prior to ECD to unfold the gas phase conformers and in that way enhance ECD fragmentation.

Figure 3 shows the ECD spectra of the 7+ charge state of ubiquitin. The left figure shows the ECD spectrum without prior activation. The right figure shows the ECD spectrum with prior activation by a short IRMPD pulse. The energy of the IRMPD pulse was adjusted so as not to induce ergodic fragmentation (i.e. b- and y-type fragment ions). The bottom spectra are the deconvolution of the respective top spectra.

It can clearly be seen that activation by IRMPD prior to ECD results in far more cleavage events.

Figure 4 shows the ProSight™ PC search results. Activation with ECD only results in few identified fragment ions whereas many fragment ions, distributed almost evenly throughout the protein sequence, were identified with mild IRMPD activation prior to ECD.

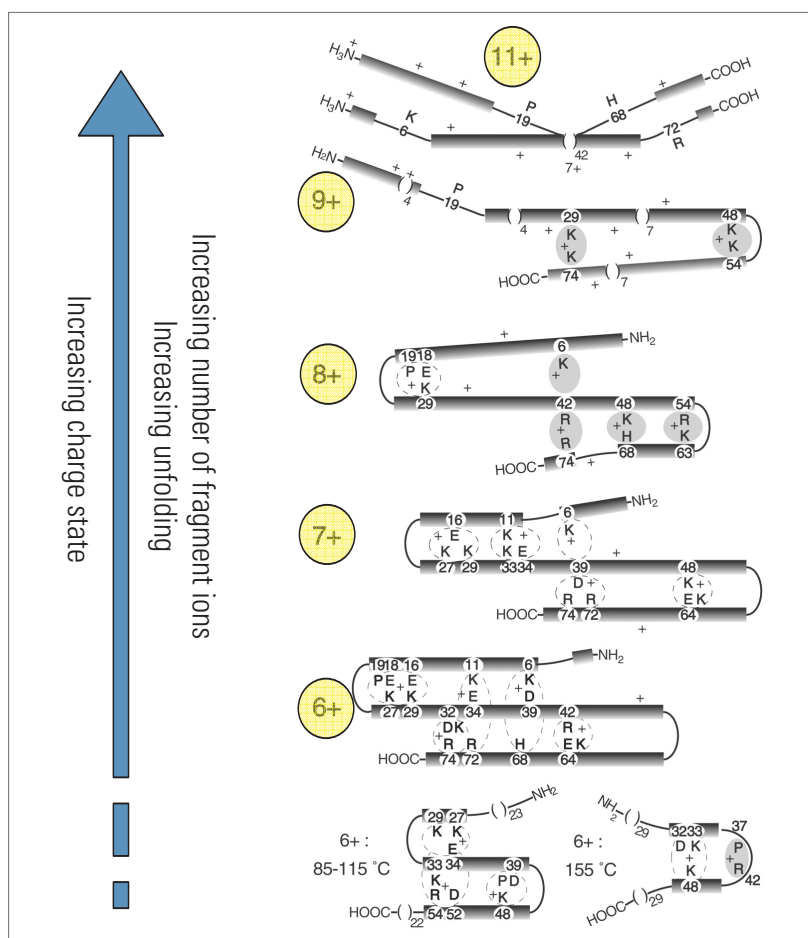


Figure 2: Proposed gas phase structures of ubiquitin, depending on charge state (reproduced and adapted with kind permission of the authors.^[7] Copyright (2002) National Academy of Sciences, U.S.A)

Methods and Materials

All spectra were acquired on a Finnigan LTQ FT, a 7 Tesla hybrid linear ion trap-FTICR mass spectrometer, equipped with an indirectly heated dispenser cathode (Heatwave, Watsonville, CA, USA) and a 25 W continuous CO₂-laser (Synrad, Mukilteo, WA, USA; 10.6 μ m). Both the ECD cathode and the IRMPD laser introduce electrons and photons from the back into the ICR cell. The ECD cathode is positioned slightly off-axis to allow the simultaneous use of ECD and IRMPD.

Bovine ubiquitin was obtained from Sigma-Aldrich®.

Precursor ions were isolated in the linear ion trap and transferred into the ICR cell. A short IRMPD pulse (typically 40 msec at 40% energy) was applied before ECD irradiation (typically 30 msec at 3% energy). ECD spectra were deconvoluted using Xtract™. The deconvoluted and deisotoped spectrum was searched with ProSight PC in single protein mode against the sequence provided by SwissProt (P62990).

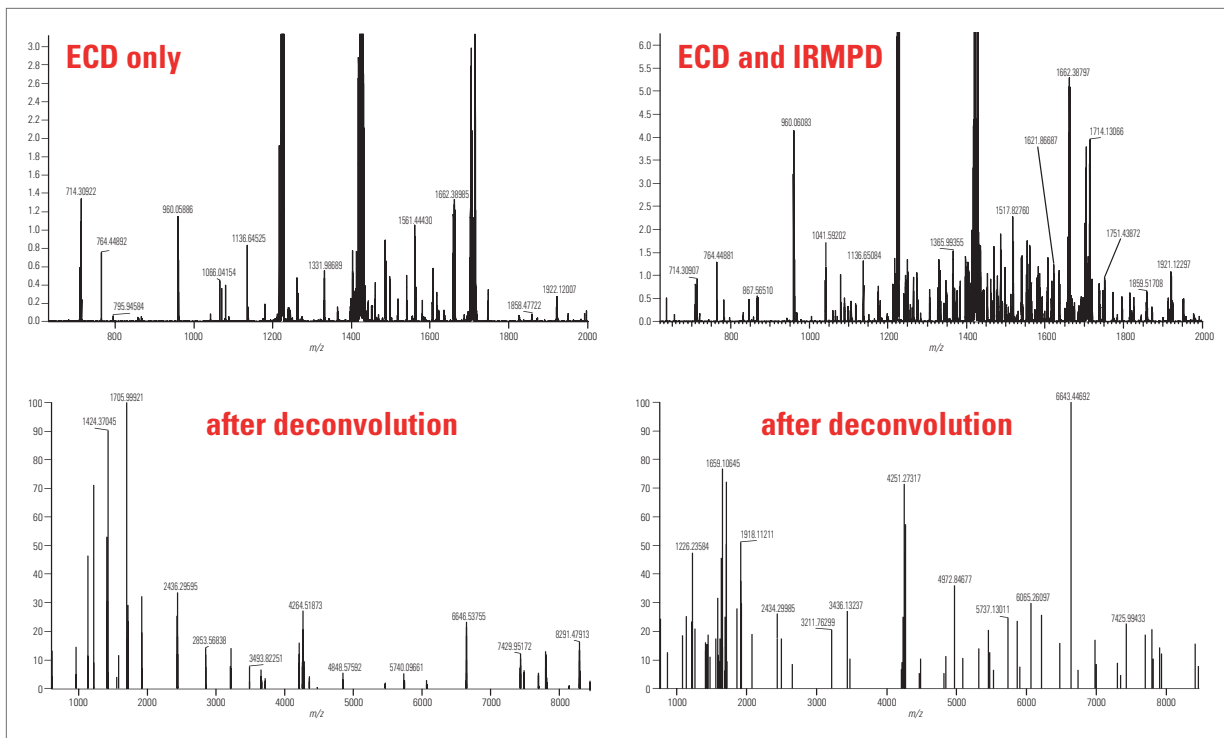


Figure 3: ECD spectra of 7+ charged molecular ion of ubiquitin with and without IRMPD pulse

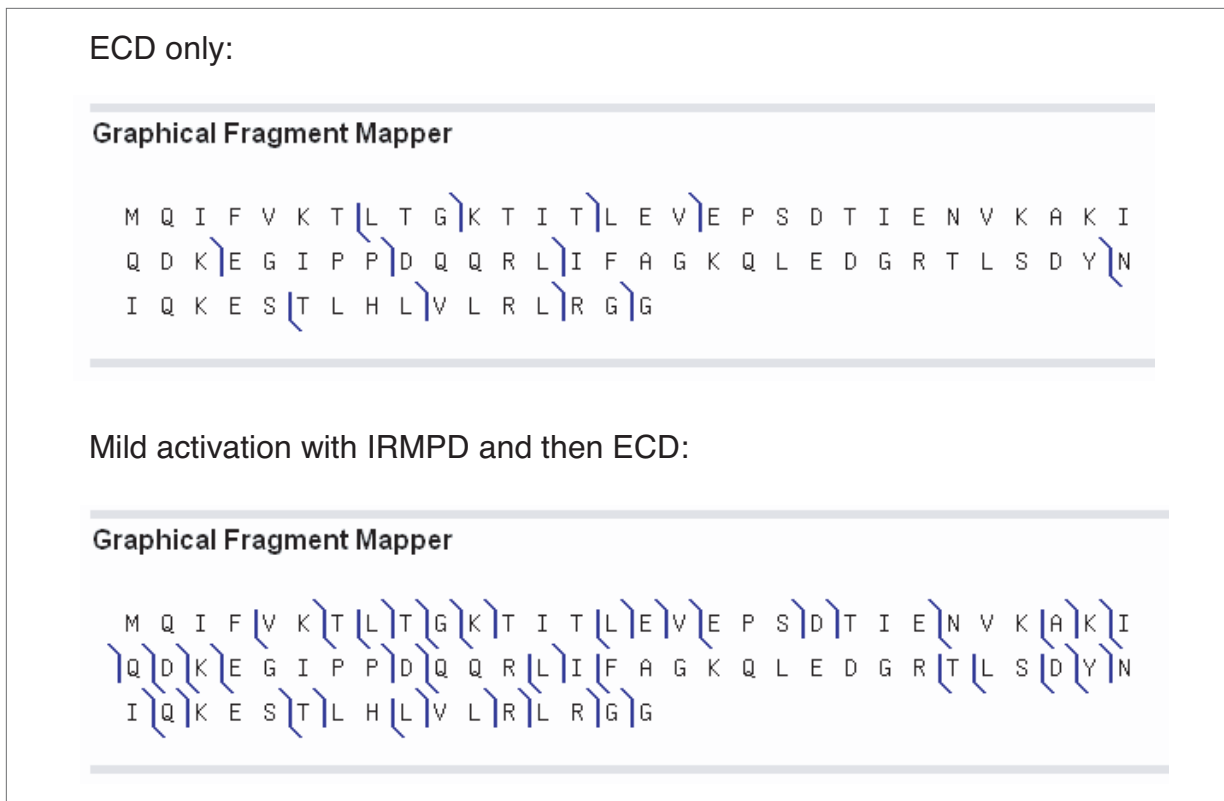


Figure 4: ProSight PC search results of 7+ charged molecular ion of ubiquitin

Conclusion

We have shown that top-down proteomics with ECD and IRMPD is available on a commercial FTICR instrument. Furthermore, mild activation by IRMPD prior to ECD breaks weak non-covalent bonds and greatly enhances the number of identified fragment ions.

This is especially important when little is known about the analyte ions and the different fragmentation result of each individual charge state.

Often, sample amount is also limited. Thus, it can be a straightforward approach to isolate the most intense charge state irrespective of its gas phase structure and use IRMPD for unfolding of its tertiary structure to achieve the most complete ECD fragmentation. Then, if time and sample amount permits, other charge states can be analyzed.

We have also demonstrated that ProSight PC software is a suitable tool for data-mining of top down proteomic data.

Abbreviations

AI ECD	Activated ion electron capture dissociation
ECD	Electron capture dissociation
FT	Fourier transformation
ICR	Ion cyclotron resonance
IRMPD	Infrared multiphoton dissociation

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