Abstract

Complete protein characterization becomes a major challenge in both general proteomics as well as quality control of recombinant proteins. ETD/PTR is a valid method to generate sequence information in top-down experiments. The high sensitivity, the perfect combination of scan speed and resolution as well as robust and fast ETD/PTR make the amaZon™ the instrument of choice for those applications.

Introduction

The direct analysis of intact proteins gains highly increased interest because of the possibility of a complete protein characterization including termini and modifications – in particular in combination with bottom-up approaches. As well, protein systems like histones where modifications on highly conserved amino acid sequences decide about biological functions are analyzed at best on the intact species. Collision induced dissociation (CID) has drawbacks in terms of a limited applicable molecular weight and the dependence of the fragmentation efficiency on the individual bond strength and amino acid sequence particularly for the fragmentation of highly charged precursor ions. Electron transfer dissociation (ETD) and its related proton transfer reaction (PTR) are alternative ways of fragmenting peptides and proteins. ETD became the preferred method for the analysis of post-translational modifications (PTM) in proteins since it preserves the bonds of modifications. PTR is a most useful addition for the fragmentation of larger peptides or even small proteins in the top-down approach. Intact proteins can be identified and/or sequenced without any prior enzymatic digestion. While ETD is still used for the dissociation itself, the charge stripping with PTR anions lowers the charge states of the highly charged product ions towards a higher m/z. The resulting PTR-product is easier to resolve and much more suitable for the detection with an ion trap. However, this limitation is the main reason for the sequence read-out in ion traps so far, because a highly charged ETD fragment ion can be shifted out of the m/z range of the mass detector when to many PTR-steps are involved. Therefore, to increase sequence coverage and reachable fragment mass range, a high resolution is in general required to observe “less stripped” higher fragment charge states. This should be achieved, however, at no cost of time, i.e. at a still highest possible scan speed.

The amaZon ion trap is equipped with the latest ETD/PTR setup. Its improved electronics and trap design allows a truly high resolution at a scan speed of still 4,600 u/s. Presented here are applications of this new design on the MS/MS analysis of various intact proteins.
Fig. 1: Spectrum extension with the 3+ charge state of intact Ubiquitin (MW 8565 Da). The mass resolution here is more than 20,000, allowing to observe the full isotope pattern of this protein.

Fig. 2: ETD/PTR fragment spectrum of Ubiquitin. Up to 6+ charge states can be resolved in the complete MS/MS spectrum.
**Experimental**

ETD/PTR-MS/MS was performed on the amaZon ETD ion trap. The highly improved control of the non-linear ejection process and the further developments in the trap enable a fast scan mode with 4,600 u/s speed at peak width of < 0.1u for fragments. Performing ETD and PTR is particularly easy in the amaZon setup since only a single reservoir filled with the neutral ETD/PTR-reagent compound is needed. Switching between ETD and PTR is just a matter of nCI lens parameters and can be done within a few msec.

The purified proteins were injected into the ion trap by offline nanospray (Triversa Nanomate). ETD/PTR of the isolated protein is performed with reagent anions dedicated for either ETD or PTR. The formation of the required different reagent anions is accomplished from only one neutral compound by altering the voltage settings of the negative chemical ionization source. For the protein marker identification and sequence characterization, the tissue lysate was ultra-sonificated in an ice bath. The extract was centrifuged with a Vivaspin 5000 ultra-filtration unit. The supernatant was collected and separated with an Agilent mRP protein column into a 96-well plate.

Fractions were measured with MALDI-TOF MS for peak localization, and the respective protein fractions subsequently measured with the amaZon ETD coupled to a TriVersa Nanomate.

**Results**

The high mass resolution of the amaZon allows for a mass resolution even of intact proteins. Figure 1 shows a spectrum extension with the 3+ charge state of intact Ubiquitin (MW 8,565 Da). The mass resolution here is more than 20,000 FWHM, allowing to observe the full isotope pattern of this protein. The scan speed of 4,600 u/s enables the spectra acquisition in a seamless scan across the entire m/z range of 50 – 3,000 in a reasonable time. Using ETD/PTR to fragment Ubiquitin results in data presented in figure 2. Up to 6+ charge states can be resolved in the complete MS/MS spectrum. The Bruker patented peak recognition software SNAP II™ picks the peaks within the isotopic patterns and determines the monoisotopic fragment masses with high confidence. The subsequent database search with MASCOT returns a complete sequence coverage of the intact protein from the full mass of 8,565 Da down to di- and tri-peptide fragments (Fig. 3).

Figure 4 shows the same for an even higher mass protein, Myoglobin. Here, 200 fmol/μL were injected by the Triversa nanomate at a flow rate of ca. 50 nL/min. The MASCOT result show the sequence assignment up to ca. 11,000 Da MW with an remarkable average mass accuracy for an ion trap of 23 ppm.
Table 1 shows typical MASCOT scores for ETD/PTR on highly charged protein species with the amaZon. Even with an isolated charge state of 26+ for Myoglobin, ETD and effective PTR in combination with the high mass resolution of the amaZon generate a Top-Down MASCOT score of nearly 400 for the protein identification.

The top-down analysis provides in many cases complementary information to bottom-up fragmentation. One example shown here is the sequence analysis of recombinant interferon beta (Fig. 5). Bottom-up fragmentation could confirm large parts of the core sequence, but failed to provide information on both termini. In order to confirm the termini the intact protein was analysed by ETD-PTR and the generated spectrum was matched against the theoretical sequence in BioTools™.

An excellent sequence coverage is obtained for both the C- and N-terminus. The confirmed sequence range comprises 30 resp. 25 amino acids. Disulfide bridges are not cleaved in ETD, therefore no fragments are obtained beyond the cysteines. This fact can be used to localize the crosslink, here between C31 and C141.

Another interesting example for the usefulness of ETD/PTR for top-down sequencing is the histone system whose biological function strongly depends on the attached modification. Histons are highly basic lysine and arginine-rich DNA-binding proteins. Conventional bottom-up proteomic analysis including CID MS/MS and trypsin hydrolysis is less promising for a complete characterization of histones including their multiple modifications. Figure 6 shows part of the processed ETD/PTR MS/MS data.
of a human histon ([M+16H]16+; m/z 709) after charge deconvolution. Biotools reveals two sequence series: one including H18 being phosphorylated, the other one non-phosphorylated – indicating ca. 50% phosphorylation on H18.

MALDI Imaging is a rather new technology where molecular information is retrieved directly from tissue sections by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry. It allows histology researchers to measure spatially resolved peptide, protein and lipid profiles in tissue sections. Tissue-type specific molecular signatures (e.g. from tumors) can be generated and used for biomarker discovery and molecular histology. After potential biomarker candidates are detected on the tissue, they need to be identified. Shown in figure 7 is a potential protein marker discovered by a MALDI-TOF MS Imaging experiment of breast cancer tissue (Fig 3a). The tissue was then extracted and purified. ETD/PTR of the isolated intact multiply charged protein lead to successful biomarker identification with a Mascot Score of 126.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MWmono</th>
<th>m/z (precursor)</th>
<th>charge state</th>
<th>Mascot Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitin</td>
<td>8560</td>
<td>714</td>
<td>12</td>
<td>375</td>
</tr>
<tr>
<td>RNAse A*</td>
<td>13682</td>
<td>978</td>
<td>14</td>
<td>194</td>
</tr>
<tr>
<td>Lysozym C*</td>
<td>14303</td>
<td>842</td>
<td>17</td>
<td>207</td>
</tr>
<tr>
<td>Myoglobin**</td>
<td>16941</td>
<td>653</td>
<td>26</td>
<td>393</td>
</tr>
</tbody>
</table>

* treatment with DTT for the reductively opening of the cystein SS-bridges
** data base search with Mascot TD, surpass Mascot's precursor mass limit > 16kDA

Fig. 5: ETD/PTR fragment spectrum of Interferon beta with complete sequence information on both termini.
Conclusion

The amaZon’s maximum resolution scan mode (4,600 u/sec) allows the seamless and fast detection of a plethora of multiply charged ETD-fragments up to 6+. Data processing with SNAP IITM generates a list of monoisotopic ETD-fragment masses, enabling the ID of the intact protein by a Mascot data base search. This enables a complete characterization of proteins up to MW of 20 kDa including post-translational modifications. ETD/PTR with the amaZon ion trap reaches new levels for Top-Down proteomics and is ready to become a powerful tool for characterization of intact protein biomarkers.

Fig. 6: Part of the ETD/PTR spectrum of Histone pH4. Sequence coverage with either phosphorylated (top, significant fragments marked with red stars) or non-phosphorylated H18 (bottom, fragments marked with green stars). Both fragment ion series are present, indicating ca. 50% phosphorylation.

Fig. 7 (following page):

a) MALDI Imaging results of a breast cancer tissue; HER2 positive/negative cells

b) workflow for sample processing
   i) Lysate of entire tissue cells, ii) LC-separation and fractionation,
   iii) biomarker identification, iv) sequence characterization
   with ETD MS/MS

c) ETD/PTR spectrum of an intact protein from a cell lysate (top);
   maximum resolution scan mode (4,800 u/sec, resolution up to 5+ ions)
   data processing with SNAP IIITM

d) BioTools annotation of Mascot search result of the processed spectrum
Top-Down ID and Sequence characterization of an intact biomarker protein

**HER2 positive**

**HER2 negative**

4000 6000 8000 10000

Tissue lysis
(0.1% TFA)
ultrasonic centrifugation dilution

**Cap LC**

**MALDI TOF MS Autoflex** to identify fractions including biomarker

Agilent mRP column
Sum 0,5x100mm, 12 uL/min

**Fractionating**

96 well plate
4,0 uL/vial (2x)

**ETD/PTR**

of 3 combined fractions offline nanospray amaZon ETD

Maximum Resolution
Dual ion funnel transfer.

For research use only. Not for use in diagnostic procedures.

Authors
Andrea, Schneider, Christian Albers, Andreas Brekenfeld, Christoph Gebhardt, Eckhard Schwabe, Ralf Hartmer, Arnd Ingendoh; Bruker Daltonik, Bremen, Germany

Keywords
ETD
PTR
intact protein
PTM
high resolution
high scan speed

Instrumentation & Software
amaZon series