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Journal of Chromatography A, 1013 (2003) 203–213
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of post-translational modifications of proteins by high-sensitivity, high-resolution Fourier transform ion cyclotron resonance mass spectrometry

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Abstract

The response of a cell to its extracellular environment is a multi-step process beginning with signal transduction that is governed by ''subtle changes'' often resulting in protein expression. Proteomics is the tracking of this protein expression. Post-translational modification (PTM) is a ''subtle change'' that has a major influence on signal transduction. Phosphorylation and glycosylation propagate signals by sequential, reversible modifications. High-sensitivity, high-resolution and multiple MS capabilities of Fourier transform ion cyclotron resonance mass spectrometry permit localization of the PTM(s) with electron-capture dissociation, and then structural determination of the PTM with infrared multiphoton dissociation.

Keywords: Mass spectrometry; Electron-capture dissociation; Infrared multiphoton dissociation; Ion cyclotron resonance; Proteins; Glycoproteins; Lectins

1. Introduction

The awarding of the Nobel Prize to John Fenn for the discovery of electrospray [1] should be the wakeup call for the biomedical sciences to realize that analytical mass spectrometry is an indispensable tool in biological research. Mass spectrometry is not only useful in academic research, but also plays an important role in diagnosis and disease research. The basic element of multiple ion charging as described in Fenn's invention [2] allows the extension of the mass analyzer's molecular mass range. The increased mass range permits the analysis of large biomolecules such as proteins. As mass range increases, there is a much greater need for increased resolution. In his Nobel acceptance speech, Dr. Fenn discussed Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) as the highest resolution, highest mass accuracy mass spectrometric analysis available. Comisaro and Marshall introduced FT-ICR-MS in 1974 [3]. Many refinements to FT-ICR-MS have been made since its debut, but the introduction of electrospray ionization (ESI) to FT-ICR-MS by McLafferty's group [4] truly opened the door to high resolution biomedical analysis. Coupled with high-sensitivity modifications of the electrospray technique such as microelectrospray (micro-ESI) [5] or nanoelectrospray [6], FT-ICR-MS can analyze bio-samples at true biological levels (nanomolar) [7,8].

Currently, the field of proteomics occupies a predominate role in biomedical research. Mass spectrometry [mainly matrix-assisted laser desorption

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0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)01127-0

ionization time-of-flight (MALDI-TOF)] plays a major role in the determination of proteins after they have been separated and digested into specific enzymatic fragments. Separation is necessary to reduce sample complexity and digestion is used to reduce the size of the analyte and to provide specific cleavage, which is useful in subsequent database searching. The relatively low resolution (<10 000) and low mass accuracy (100–200 ppm) mass analyzers typically used in proteomics analysis can induce ambiguity into protein identification. Another major complication in proteomic analysis is dealing with post-translational modifications (PTMs) of proteins. PTM of proteins alters the molecular mass of the protein/fragment(s) further exacerbating the assignment of absolute mass and thus protein identification. Furthermore, localization of the PTM is impossible with conventional mass spectrometry, due to the loss of labile post-translational modifications in MS–MS techniques. Conventional MS–MS techniques induce primary cleavage of the weakest bonds in the molecule [9]. Once the PTM has been cleaved, all localization information is lost. High throughput analysis is championed as the most important aspect of proteomics, in reality the most important aspect is in accuracy of results and localization and identification of PTMs.

High-resolution FT-ICR-MS can fill the gaps in today's proteometric analysis. FT-ICR-MS can unambiguously identify/confirm protein identification by providing multiple sequence tags from multiple MS (MS^n) analysis with unmatched mass accuracy. The multiple and unique fragmentation techniques of FT-ICR-MS such as collisional activated dissociation (CAD) [10], infrared multiphoton dissociation (IRMPD) [11], and electron-capture dissociation (ECD) [12–14], allow the determination of the position of the post-translational modification(s); as well as, the structure of the modification [15]. ECD cleaves the amino acid backbone without removing the PTM [15–18], often resulting in near complete sequence coverage and absolute localization of the PTM. IRMPD then cleaves the PTM permitting structural information of the PTM to be acquired [15]. Currently, the only other method to perform these identifications of PTMs involves multiple, time-consuming, expensive and laborious wet chemistry manipulations requiring large amounts of highly purified analyte.

In the FT-ICR-MS analysis of biological PMTs, maximization of the protein separation and specific targeting of PTM proteins is accomplished with two-dimensional (2D) gel electrophoresis. 2D gel electrophoresis is deemed superior to other separation techniques in speed, efficiently of separation and identification of proteins with specific PTM(s). PTM staining and differential staining techniques [19] permit specific targeting of proteins in normal vs. diseased states [20,21]. Targeted proteins are in-gel digested and FT-ICR-MS is used for absolute protein identification based on accurate mass and MS^n analysis. Tandem ECD–IRMPD analyses are used to first localize the PTM with ECD and then elucidate structural information of the PTM with IRMPD.

2. Experimental

2.1. Sample preparation

Peptides, acetic acid, and formic acid (FA) were obtained from Sigma (St. Louis, MO, USA). Peptide stock solutions (0.1 μ*M*) were prepared by dissolving them in HPLC-grade water (J.T. Baker, Philipsburg, NJ, USA). Stock solutions were diluted for electrospray (100 fmol/μL–10 pmol/μl) in either methanol (Baker)–water (1:1) with 2.5% acetic acid, or acetonitrile (Baker)–water (1:1) with 0.1% FA. The *Erythrina corallodendron* lectin (Sigma) was dissolved in 500 μl of 0.1 m*M* $CaCl_2$ and 0.1 *M* NH_4HCO_3 to a final concentration of 10 fmol/μl. The lectin was tryptic digested by addition of 100 pmol modified trypsin (Promega, Madison, WI, USA) at 38 °C for 4 h. Peptides were lyophilized in a Savant speedvac (Savant, Farmingdale, NY, USA). Peptides were resuspended in HPLC-grade water to ~100 pmol/μl. Further dilution to ~5 pmol/μl in methanol–water (50:50) with 2.5% acetic acid was made for electrospray analysis.

2.2. 2D Gel electrophoresis

The first dimension was carried out using immobiline dry strip gels (IPG), pH 5–8, 7 cm with the Protean IEF cell from Bio-Rad (Hercules, CA, USA). The second dimension separation was carried out using the Nu-PAGE mini-gel system. The mini-gels were stained with SYPRO-Ruby protein stain

[glycoproteins were detected by modification of periodic acid-Schiff (PAS) methods] [20]. Excised spots from gels were digested with modified trypsin. Trypsin digested spots were purified by Zip Tip (Millipore, Bedford, MA, USA) and initially analyzed by MALDI-TOF-MS (Proteomics Facility, Göteborg University, Gothenburg, Sweden). Left over samples from MALDI-TOF-MS analysis were dried and reconstituted in 20 μl of 0.1% FA in acetonitrile–water (50:50, v/v) for analysis by micro-ESI-FT-ICR-MS (FT-ICR User Facility, National High Magnetic Field Laboratory [NHMFL], Tallahassee, FL, USA).

2.3. FT-ICR-MS at 9.4 T

High-resolution analysis were performed in the positive ion mode with a laboratory-built mass spectrometer positioned in a passively shielded 9.4 T superconducting magnet [22]. A laboratory-available modular ICR data acquisition system (MIDAS) and analysis workstation controlled the FT-ICR-MS system [23]. Charged analyte droplets were generated by microelectrospray [5] at 300 nl/min and subsequently desolvated to multiply charged ions with a Chait-style heated metal capillary atmosphere-to-vacuum interface [24]. Ions were externally accumulated in a linear octopole trap [25]. Prior to accumulation, ions could be selected as they passed through quadrupole ion selection device [26]. External accumulation (either full mass range or selected m/z) was performed for the entire experimental sequence except during ion transfer through an octupole ion guide to an open cylindrical cell. Ions were collected in the cell by gated trapping. Ions were often further isolated after gated trapping by application of stored waveform inverse Fourier transform (SWIFT) [27,28].

Electrons for ECD were generated with a 10 mm diameter dispenser cathode (No. 1109; Heat Wave, Watsonville, CA, USA). The cathode was mounted on axis of the system, inside the passive shielding and 73 cm from the center of the ICR cell. A molybdenum grid was located 8 mm from the emitting surface. ECD was performed by application of −5.5 V to the cathode, and raising the grid potential to +100 V for 3–51 ms while holding the trap plates at 5–10 V. Excess electrons were scavenged by changing the potentials to: trap plates to 2 V, grid to +5 V and cathode to +10 V for 10 ms. At all other times, the cathode bias voltage was −0.1 V and the grid potential was −200 V.

IRMPD was accomplished with a 40-W, 10.6 μm CO_2 laser (Synrad, Mukilteo, WA, USA). Photons were generated off axis outside the shield of the magnet passed through a $BaFl_2$ window. The arrangement was designed to allow the photon beam to clear the cathode emitter, but still fully irradiate ions trapped in the ICR cell allowing simultaneous/tandem ECD and IRMPD events [21]. Normal photon irradiation was performed at 450–800 ms at 30–90% power.

Ions were subjected to chirp excitation [48–480 kHz (or between) at 150 Hz/μs] and direct-mode broadband detection (512 Kword or 1 Mword data points). Hanning apodization and one zero fill were applied to all data prior to fast Fourier transform (FFT) and subsequently magnitude transformed. Spectra were normally internally calibrated (Ledford equation) [29] with three ions of known m/z.

2.4. FT-ICR-MS at 7 T

Experiments were performed with a laboratory-built mass spectrometer positioned in an unshielded 7 T superconducting magnet [30] operating under the control of a MIDAS data station. Charged analyte droplets were generated by microelectrospray at 300 nl/min and subsequently desolvated to multiply charged ions with a Chait-style heated metal capillary atmosphere-to-vacuum interface and externally accumulated within a modified linear octopole trap for improved ion ejection along the z-axis [31]. External accumulation was performed for the entire experimental sequence except during the transfer through a hexapole ion guide to an open orthorhombic cell. Ions were captured with gated trapping. Ions were isolated after gated trapping by application of SWIFT.

Electrons for ECD were generated with a heated metal filament located behind the cell, off axis and external to the bore of the magnet. SWIFT isolated ions were irradiated for 30 s under conditions previously described [15]. IRMPD was accomplished with a 40-W, 10.6 μm CO_2 laser (Synrad). Photons were generated on axis, at 20% power and passed through a $BaFl_2$ window to irradiate SWIFT isolated ions for 200 ms. Fragment ions were subjected to

chirp excitation (43–540 kHz at 350 Hz/μs) and direct-mode broadband detection (256 or 512 Kword data points). Hanning apodization and one zero fill were applied prior to FFT and magnitude transformation.

2.5. Data processing

To identify peptides, monoisotopic mass lists were submitted to Mascot (Matrix Science, London, UK) to be searched against the NCBI database. Phosphorylation and methionine oxidation were selected as variable modifications. Monoisotopic masses (or m/z ratios) were calculated using IsoPro version 3.1 (MS–MS Software). Identification of glycosylations were made with the help of GlycoMod tool (http://www.expasy.ch).

3. Results and discussion

3.1. Protein identification

Proteomics identification of proteins by enzymatic digestion and mass analysis at mass accuracy of 100 ppm often is not sufficient for absolute protein identification. Even mass accuracy of <5 ppm is not suitable if the fragments are large and there are not many of them. Absolute identification must involve MS–MS with identification of sequence tags or some other way to unambiguously identify amino acids in the sequence to aid in the database search.

FT-ICR-MS has the resolution, mass accuracy and MS^n capabilities to make it the perfect tool to complement and completely finalize a modern proteomics facility. Fig. 1 shows the preliminary data from a MALDI-TOF-MS analysis of a protein targeted from a 2D gel. These samples were generated by comparison 2D gels of nuclear proteins from normal and differentiated mouse microglia cell lines. Three proteins that were upregulated in the differentiated cell line were targeted. All were excised, tryptic digested and preliminary screened by MALDI-TOF-MS at the Göteborg University Proteomics facility. Remaining samples were analyzed by micro-ESI-FT-ICR-MS^n. Fig. 1 shows the MALDI-TOF-MS spectrum of spot 54. The protein was initially identified as proliferation associated gene A. The lower spectrum shows the broadband FT-ICR of the same sample and the middle spectrum shows the IRMPD of a SWIFT isolated tryptic fragment. The IRMPD generated four sequence tags in the isolated tryptic fragment and also determined that the methionine residue in that fragment was oxidized. Of the other two proteins targeted, one was identified by MALDI-TOF-MS and was confirmed by accurate mass FT-ICR and MS^n. The other protein was not identified by MALDI-TOF-MS, but was identified by FT-ICR through accurate mass identification of 14 tryptic fragments and MS^n (data not shown).

3.2. Glycoprotein carbohydrate structure elucidation

IRMPD like other slow heating fragmentation techniques cleave the weakest bonds in the molecule first and thus the first cleavage is at the PTM. In most mass analysis loss would be detrimental, but in FT-ICR this can be used to the analyst/researcher's benefit. SWIFT isolation is used to isolate the proposed PTM labeled fragment from the broadband tryptic digest spectrum. Once isolated, the fragment is irradiated with photons, which causes the PTM to break away from the peptide fragment. In the case of glycoproteins as in Fig. 2, the carbohydrate continues to fragment providing valuable structural information on the carbohydrate. Fig. 2 also demonstrates the high sensitivity of the technique. The spectrum shown was generated from a single targeted glycoprotein spot from a 2D gel loaded with 300 μl of human cerebrospinal fluid (CSF). The protein was identified as α-antitrypsin and the structure of the carbohydrate was determined from this data.

3.3. Complementary data from ECD and IRMPD

ECD fragments the amino acid backbone, but leaves the labile PTM(s) intact. IRMPD breaks the weakest bonds and thus removes the PTM(s) first. In the case of glycosylation, IRMPD continues to fragment the PTM providing structural information on the sugar that was attached. Fig. 3 shows a schematic representation of the data that can be acquired when these two techniques are used together. The data comes from initial studies (7 T

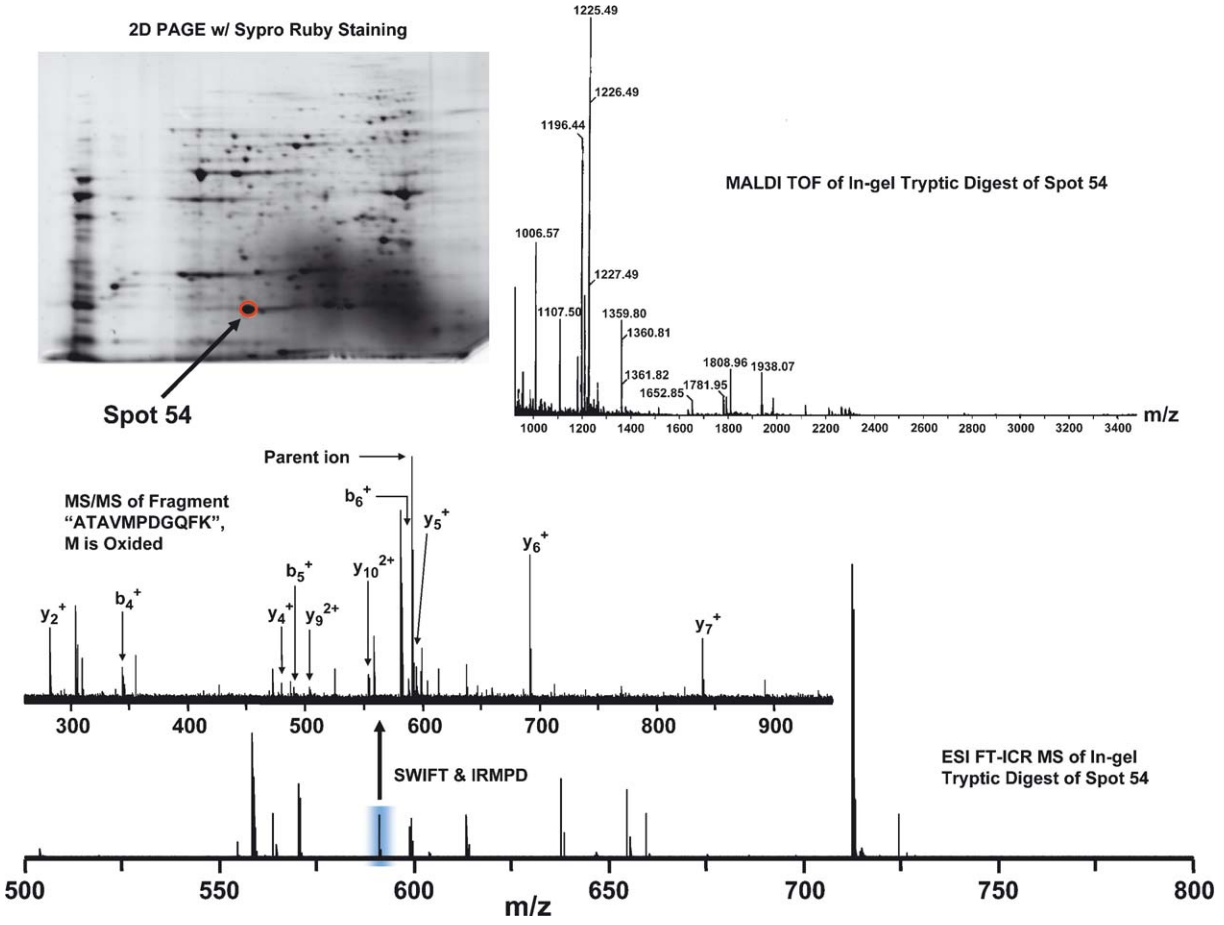

Fig. 1. Protein verification by micro-ESI-FT-ICR-MS. An upregulated microglia cell line nuclear protein (spot 54) was targeted, excised and tryptic digested from a 2D gel. Initial MALDI-TOF-MS analysis preliminarily identified the protein as proliferation gene A. Remaining sample was analyzed by micro-ESI-FT-ICR-MS. The bottom spectrum shows the broadband FT-ICR spectrum. The middle spectrum shows the IRMPD fragmentation of a SWIFT isolated fragment ion, easily identifying four sequence tags, verifying protein identification and determination that the fragment contained an oxidized methionine residue.

FT-ICR) performed on the unfractionated tryptic digest of the lectin of the coral tree, *Erythrina corallodendron*. After SWIFT isolation of a suspected tryptic fragment, IRMPD was performed which released and fragmented the sugar, enabling the structure to be predicted as seen in the top of the figure. Next, the fragment was again SWIFT isolated, but this time was fragmented by ECD. The extensive amino acid cleavage without loss of PTM permitted the localization of the PTM to a specific amino acid (bottom of Fig. 3).

3.4. Complex glycosylation structure determination from human CSF

With the high mass accuracy of FT-ICR-MS it is often unnecessary to perform MS^n experiments to identify the sugars that are attached to a glycoprotein. Using accurate mass assignments of tryptic fragments of identified glycoproteins is often sufficient to identify the carbohydrate on a fragment by using database searching. GlycoMod tool and FindMod tool (http://www.expasy.ch) are used to fit

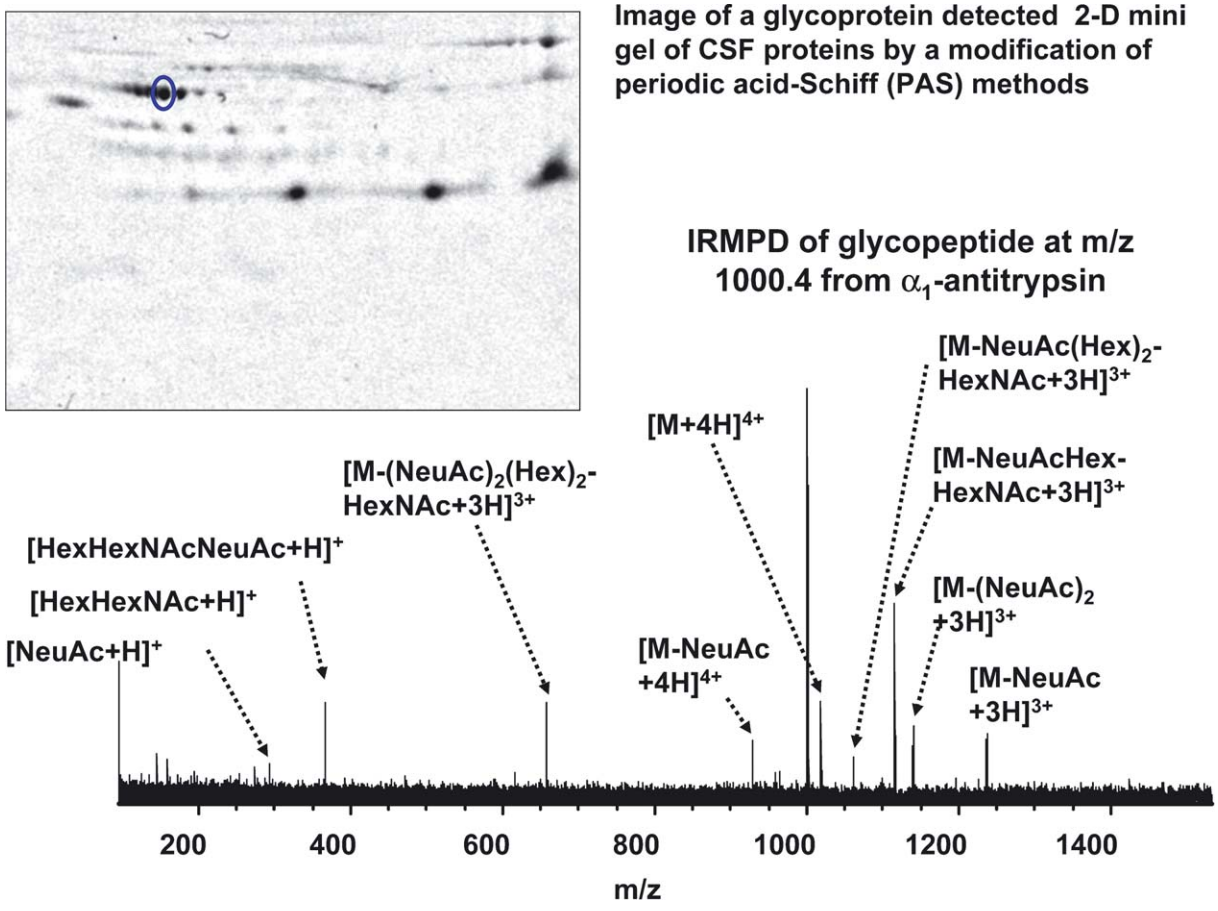

Fig. 2. Carbohydrate structure identification by micro-ESI-FT-ICR-MS–MS. A glycosylated protein isolated from 300 μl CSF by 2D gel electrophoresis and specifically targeted with glycospecific staining methods (upper left) was targeted, excised and tryptic digested. The spectrum shows the IRMPD fragmentation of a SWIFT isolated glycosylated fragment. The IRMPD cleaves the glycosylation from the peptide and fragments it; thereby, providing structural information on the carbohydrate.

modifications to tryptic fragments based on mass alone. In some cases (as in Fig. 4), there is more than one possibility for the structure of carbohydrate that could be attached based solely on accurate mass alone. In the example shown in Fig. 4 there were three possibilities. To determine which of the possibilities was correct, IRMPD was performed on the SWIFT isolated tryptic fragment. The sample came from another 2D gel separation of human CSF from a total 300 μl sample. Glycospecific staining targeted the spot. The protein was identified as β-trace. IRMPD of the isolated fragment produced the spectrum in Fig. 4 and permitted the positive identification of the carbohydrate attached to the fragment.

3.5. Enhancement of tandem ECD–IRMPD

Initial experiments with ECD dictated long electron irradiation times (upwards of 30 s). Recent conversions to a cathode dispenser [32] in lieu of a conventional filament style electron gun have demonstrated greatly reduced electron irradiation times. Another problem in most systems is the inability to perform both ECD and IRMPD in rapid succession or simultaneously. In most cases the on axis electron emitter blocks the path of the photon beam from the laser. Tandem experiments presented above were performed on our unshielded 7 T FT-ICR-MS. Due to the unshielded magnet, it was possible mount the

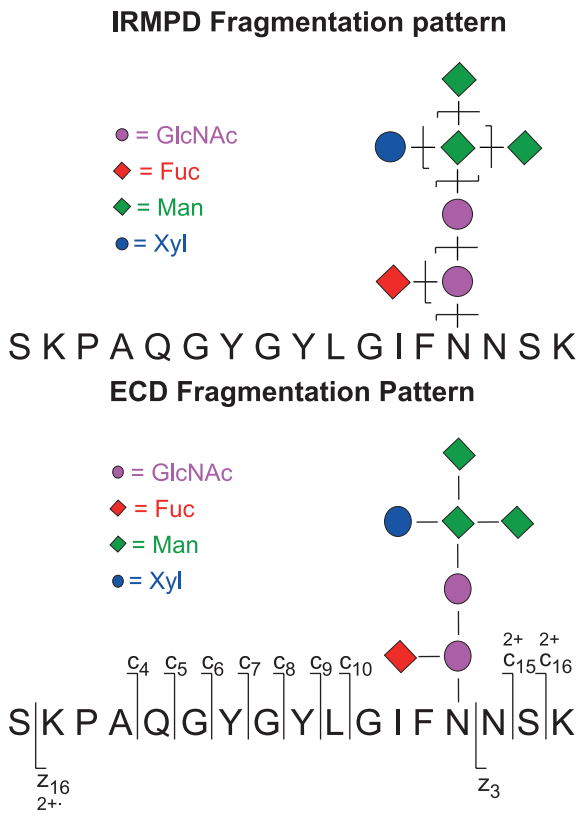

Fig. 3. Effects of IRMPD or ECD on glycosylated peptide. The upper panel demonstrates the fragmentation induced by IRMPD, which is limited to only fragmenting the labile glycosylation from the peptide and the carbohydrate that was removed, permitting the structural determination of the carbohydrate. The lower panel shows the amino acid backbone fragmentation of the glycopeptide. The glycosylation remains intact permitting localization of the carbohydrate attachment.

CO_2 laser on-axis and the electron gun off-axis external to the magnet bore. The electron beam followed the field lines to the center of the cell permitting tandem ECD–IRMPD experiments. The main drawbacks were long (30 s) ECD irradiation times and minimal fragmentation.

To enhance the ability to perform tandem ECD–IRMPD, the 9.4 T was modified as described in Fig. 5. A cathode dispenser was installed on axis, in the magnet bore, located only 73 cm from the center of the ICR cell. The CO_2 laser was located outside the passive shield of the magnet and the beam was brought in off-axis, skirting the cathode dispenser and into the center of the cell. Typical 30 s ECD experiments dropped to 30 ms experiments along with the generation of more fragments both in abundance and number of fragments (data not shown).

Often times, ECD produces only charge reduction without actually fragmenting the molecule. It was perceived that these charge reduced ions were fragmented, but are held together by weaker bonds [33]. To address this question, an ECD charge reduced species of a tryptic digested glycoprotein from the lectin of the coral tree, *Erythrina corallodendron* was SWIFT isolated and irradiated with photons (Fig. 6). The IRMPD of the ECD charge reduced species produced an abundance of c and z ions. No subsequent loss of the glycosylation was noted.

4. Discussion

PTMs of proteins are essential in cell signaling and thus are equally essential to life and disease. The major thrust of proteomics in biomedical research is in the up or down regulation of specific proteins. Protein expression is important in many disease states, but modifications to proteins (such as phosphorylation, glycosylation, etc.) also play a major role in the biological system and in disease progression. The cascade effect of PTMs in intercellular signaling makes their identification and localization imperative in the study of normal vs. diseased states. The typical proteomics facility does not have the ability to monitor the extent of, location of, or structure of various PTMs. FT-ICR-MS is the perfect complement to an established proteomics facility because FT-ICR-MS has the unmatched ability to detect and identify these modifications. The unequalled resolution, mass accuracy, high sensitivity and MS^n permit the identification of biomolecules and their modifications at true biological levels. No other technique has the ability to determine the placement and structure of post-translational modifications at biological concentrations. To do this effectively, the FT-ICR-MS must have high sensitivity and the ability to perform both ECD and IRMPD in tandem. These two fragmentation techniques together are unique to FT-ICR-MS and are essential for the location (ECD) and structure determination (IRMPD) of post-translational modifications.

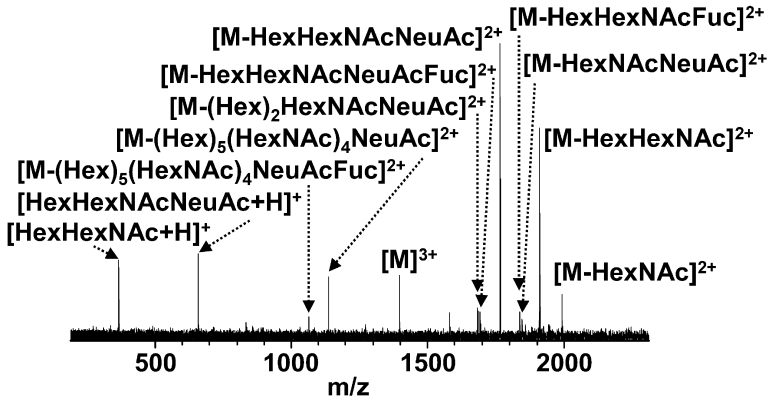

Fig. 4. IRMPD structure determination of human CSF glycoprotein. β-Trace glycoprotein was isolated from human CSF by 2D gel electrophoresis and targeted for analysis with a specific glycoprotein stain (PAS). After excising and in-gel digestion, the sample was analyzed by micro-ESI-FT-ICR-MS. IRMPD fragmentation was needed in addition to exact mass determination to confirm the carbohydrate that was attached to this peptide.

The biological examples presented here (Figs. 2–4 from human CSF samples) depict samples that were first separated on 2D gels and then specifically targeted for PTM analysis (in this case glycosylation) by specific staining procedures. No other separation technique permits specific targeting of PTM proteins prior to analysis. Comparison of normal vs. diseased samples with specific PTM stains permits targeted analysis of proteins with altered PTMs.

Once targeted, tryptic digested and excised from the gel, the sample is analyzed by micro-ESI-FT-ICR-MS. The unequalled mass accuracy permits the identification of many tryptic fragments based solely on mass alone. PTMs are mapped by ECD fragmentation (unique to FT-ICR), which rarely affects the labile PTM. Finally, IRMPD is used to gather structural information on complex PTMs such as glycosylation (i.e., structural information collected

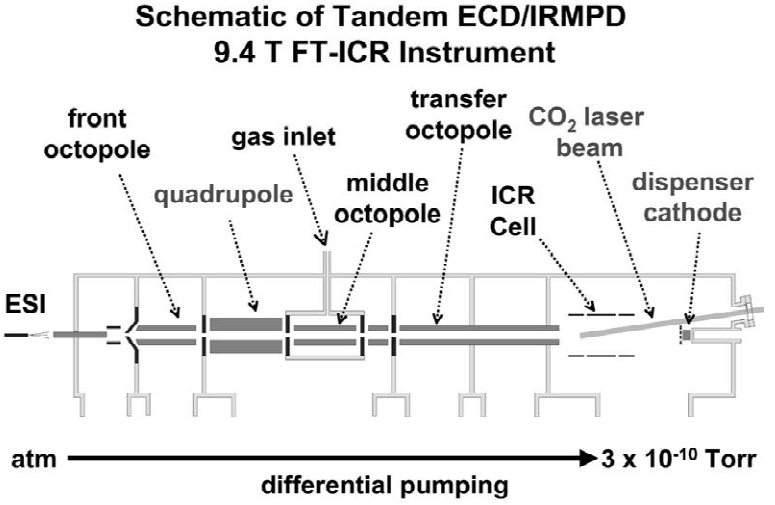

Fig. 5. Schematic of the tandem ECD–IRMPD-ESI-9.4 T FT-ICR system. The figure shows the modifications made to the 9.4 T FT-ICR permitting both rapid, on-axis ECD (with a cathode dispenser) and tandem off-axis IRMPD. This step permits rapid PTM localization and subsequent structural determination. 1 Torr = 133.322 Pa.

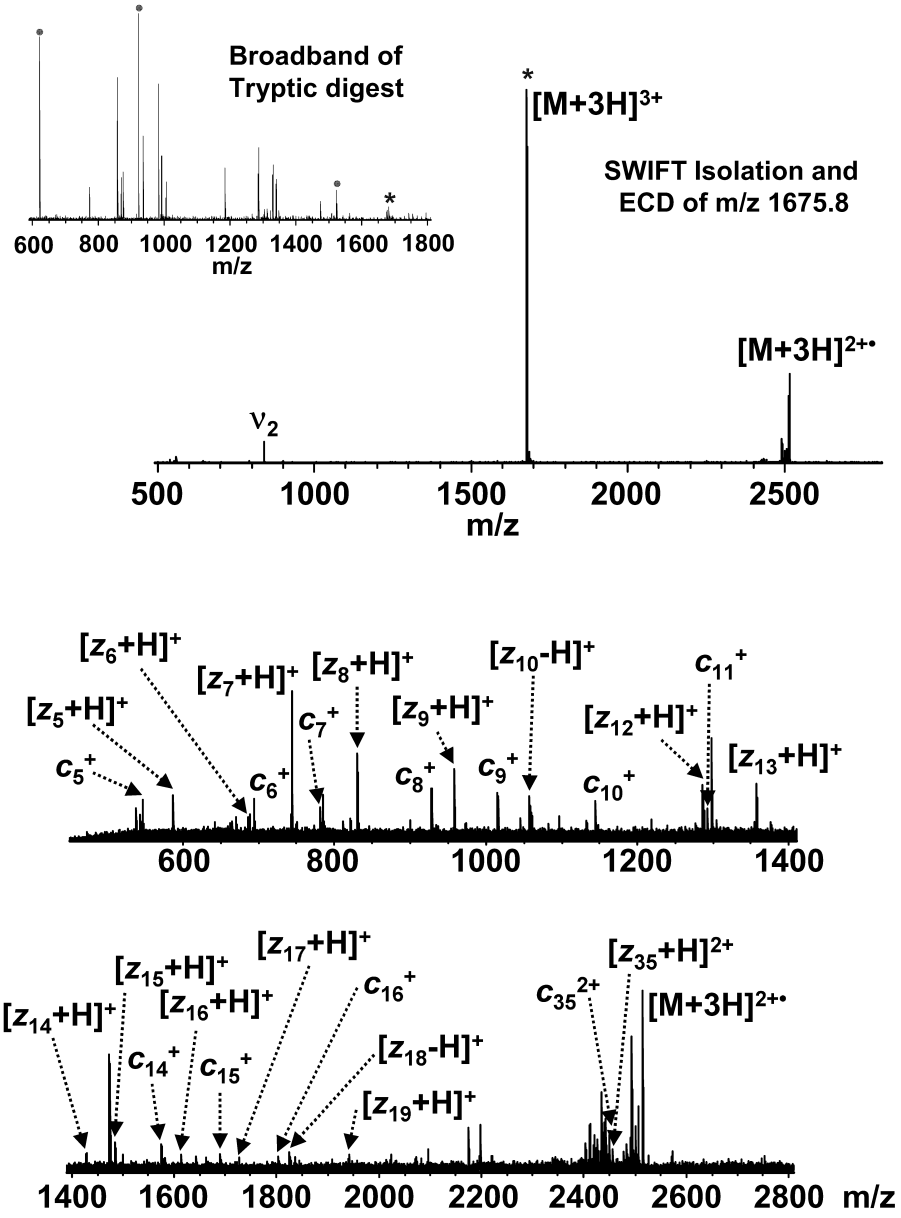

Fig. 6. IRMPD of ECD activated ions (tandem ECD–IRMPD). Top left figure depicts a typical broadband micro-ESI-FT-ICR-MS of a tryptic digest of a glycoprotein (starred peak represents glycosylated fragment). The spectrum to the left shows only charge reduction and no fragmentation after ECD. The two bottom spectra show the extensive fragmentation of the SWIFT isolated charge reduced species after IRMPD.

on released carbohydrates). All of these manipulations occur in the gas phase from a sample isolated from a single 2D gel spot, which was generated from a total of 300 μl of CSF sample applied to the first dimension of the gel. The data presented here demonstrate that FT-ICR-MS is the only analysis technique that can localize and identify PTMs at biological levels. The targeted 2D gel, FT-ICR-MS

technique is currently being applied to normal vs. Alzheimer's CSF samples [20] in search of specific glycosylation states for subsequent use in diagnosis of the disease.

In conclusion, high-sensitivity, low-flow electrospray FT-ICR-MS with tandem ECD–IRMPD capability is the method of choice for determination of the location, extent and structure of post-translational modifications. Tandem ECD–IRMPD techniques further permit ECD-type fragmentation of molecules that are only charge reduced with ECD alone (Fig. 6). The techniques demonstrated and discussed here are also applicable for other labile post-translational modifications as well. Coupled with a separation technique that permits specific targeting of PTM proteins (such as 2D gels), this system has the unequalled ability to determine changes in specific PTMs in normal vs. disease states from limited biological samples and at true biological concentrations.

Acknowledgements

Special thanks to staff at the NHMFL especially to TuKiet T. Lam, Michael J. Chalmers and Kristina Håkansson who collected the data presented. Thanks to John P. Quinn and Dan McIntosh for technical expertise, and useful discussions with Christopher L. Hendrickson, and Alan G. Marshall. Additional thanks goes to collaborators Carol L. Nilsson, Pia Davidsson and the Proteomics Facility Göteborg University, Göteborg, Sweden. Research funded by the NSF (CHE-99-09502), Florida State University, NHMFL-Tallahassee, FL, USA, Swedish National Research Council, Swedish Foundation for International Cooperation in Research and Higher Education (STINT).

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