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Review

Identification and analysis of phosphopeptides

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Abstract

Reversible phosphorylation of serine, threonine and tyrosine residues in proteins is one of the key events in signal transduction. To understand the process of signal transduction on a molecular level, it is imperative to identify phosphorylation sites in proteins. In this review, we offer an overview of the different methods/technologies currently available to identify protein phosphorylation sites.

Keywords: Reviews; Phosphorylation; Post-translational modification; Phosphopeptides

Contents

1. Introduction

Reversible phosphorylation of serine, threonine and tyrosine residues in proteins is one of the key events in signal transduction. To understand the process of signal transduction on a molecular level, it is imperative to identify phosphorylation sites in proteins. In this review, we offer an overview of the different methods/technologies currently available to identify protein phosphorylation sites. In the last chapter, we present data on exciting new developments in the field of Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry. Due to the wealth of information in this field, the review cannot entirely be comprehensive and we apologize for the many publications that could not be cited due to limited space.

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2. Two-dimensional phosphopeptide maps

A first step toward examination of phosphorylation sites within proteins was the use of proteolytic cleavage and the subsequent analysis of the resulting peptides by two-dimensional phosphopeptide maps of phosphorylated proteins. This technology, described in great detail by Boyle et al. [1], was used to visualize radiolabeled phosphopeptides. The peptides are spotted onto a thin layer plate and electrophoretically separated in the first dimension according to their net charge. Thin layer chromatography is used for separation in the second dimension according to hydrophobicity. Upon exposure to X-ray film, this approach results in the appearance of several spots representing the phosphopeptides. A typical example of such an analysis is shown in Fig. 1 (reproduced from Dhillon et al. [2]). Although this technology is well suited to visualize even minute amounts of radiolabeled phosphopeptides, it is evidently hampered by the inability to actually identify the phosphopeptide and pinpoint the phosphorylation site.

To circumvent these problems, phosphopeptides were eluted from the TLC plates and subjected to Edman degradation [3]. The sensitivity of this approach, however, was poor. In addition, other unphosphorylated peptides migrating at the same position greatly hampered the identification of the phosphopeptides. Further improvement of this technique could be gained by counting the radioactivity in the effluent after each single Edman sequencing step, thereby establishing the position of the phosphorylated amino acid [4]. As a general rule, identification of protein phosphorylation sites utilizing this technology is extremely sensitive, but tedious, time-consuming and often ambiguous.

From predicted migration patterns and the use of additional proteolytic enzymes, the phosphopeptide as well as the phosphorylation site could be narrowed down. A software program for the prediction of phosphopeptide migration patterns can be downloaded from http://www.pingu.salk.edu/~sefton/Hyper_protocols/pepsort1.html. It can be useful to select candidate phosphorylation sites from a limited number of options.

An elegant combination of Edman sequencing, mass spectrometry and ^{32}P labeling has been developed by the group of Nick Morrice in Dundee, UK [5]. Fig. 2 outlines their experimental scheme. The protein of interest is labeled with ^{32}P, digested with trypsin, and the tryptic peptides are separated by reversed-phase HPLC. Phosphopeptides labeled to >5000 cpm can be detected directly by an on-line radiodetector. Lower activity peptides are detected by spotting onto a PVDF membrane by use of a microblotter and subsequent autoradiography or phosphoimaging. Phosphopeptide-containing fractions are split and analyzed in parallel by matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and Edman degradation. MALDI-TOF provides a sensitive method for detection of phosphopeptides, typically in the femtomolar range. However, the sequencing capability of the commonly used MALDI-TOF instruments is very limited and usually does not allow for identification of the site of phosphorylation, in particular if multiple sites are phosphorylated. Phosphorylation site(s) may be found by Edman

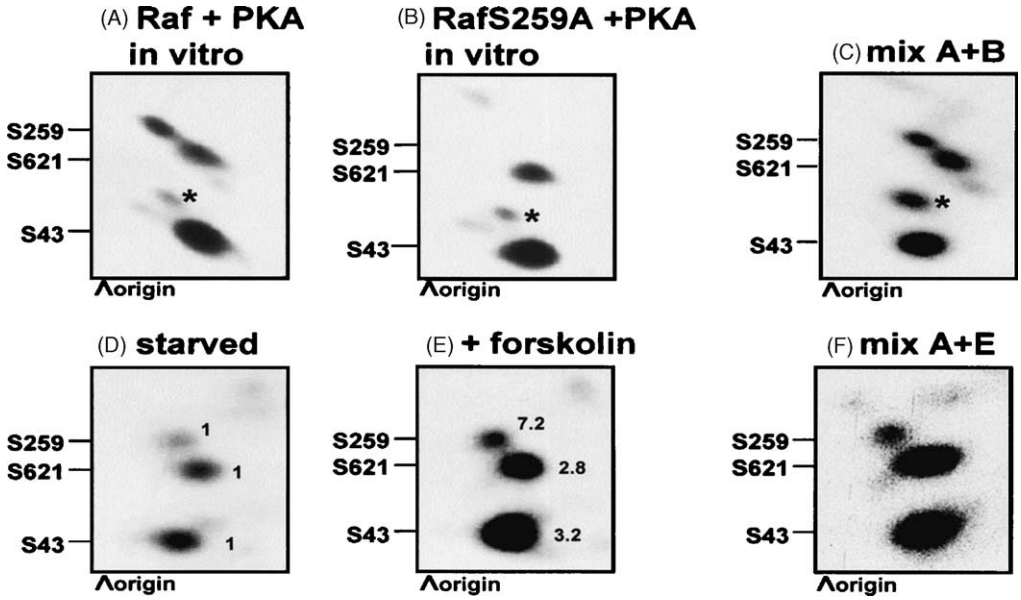

Fig. 1. Tryptic phosphopeptide maps of recombinant Raf-1 phosphorylated by PKA (Raf + PKA) in vitro (A), recombinant RafS259A phosphorylated by PKA (RafS259A + PKA) in vitro (B), a mixture of Raf + PKA and RafS259A + PKA (C), endogenous Raf-1 immunoprecipitated from serum-starved NIH 3T3 cells labeled with [^{32}P]orthophosphoric acid (D), endogenous Raf-1 from forskolin-treated cells (40 μM, 30 min) (E), and a mixture of panels A and E (F). Phosphorylation sites are indicated. Asterisks indicate spurious in vitro phosphorylation sites that do not occur in cells. In panels D and E, the spots were quantified with a phosphorimager and the relative changes are indicated. Equal amounts of Raf-1 protein were loaded. Figure reproduced from [2].

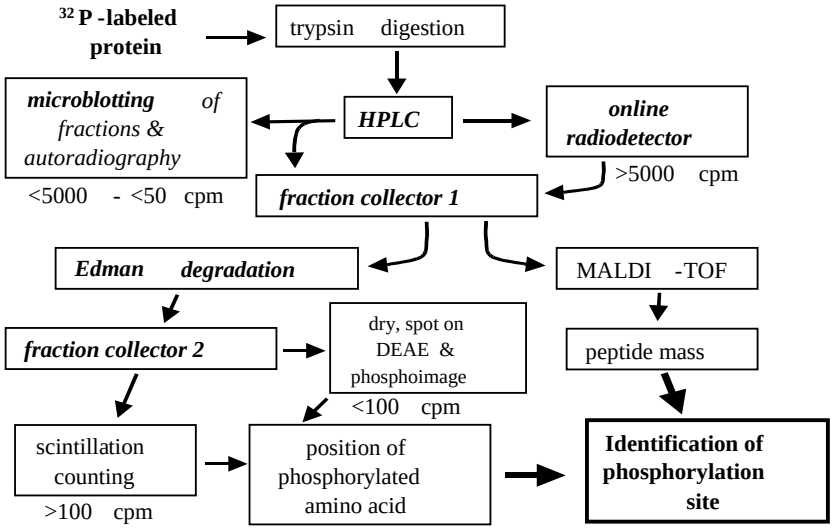

Fig. 2. A high throughput, multi-technique approach to the identification of sites of protein phosphorylation [5].

degradation. Peptide sequencing by Edman degradation is rather insensitive. However, if the cleavage products are not sequenced, but one determines only the cycle at which the phosphate elutes, sensitivity is more than 10,000-fold higher. By combining these methods, one may identify the phosphopeptide by the mass information provided by MALDI-TOF, and the site of phosphorylation from the Edman degradation. Thus, the sensitivity of phosphorylation site identification can be pushed to femtomolar levels. Typically, 500 fmol of phosphopeptide suffices to reliably identify the phosphorylation sites, even for multiply phosphorylated peptides.

3. Mass spectrometric characterization of protein phosphorylation

With the development of mass spectrometers and their application to peptide analysis, a new era in the analysis of protein phosphorylation appeared. The underlying idea was quite simple: phosphorylation of a polypeptide results in an easily detectable 80 Da increase of the molecular weight of the target peptide. However, phosphorylation of a specific site in vivo is seldom stoichiometric. Hence, most of the peptides are unphosphorylated, leading to low relative quantities of phosphopeptides (frequently far less than 5%) in a typical tryptic digest of an in vivo phosphorylated protein. As a result, phosphopeptides often have to be purified or enriched prior to analysis.

Two complementary ionization techniques, electrospray ionization (ESI) and MALDI are currently available to generate ionized polypeptides that can subsequently be analyzed with by a variety of different mass spectrometers (see below). The detection of elemental phosphorus by inductively coupled plasma (ICP) mass spectrometry offers an alternative approach. When coupled with liquid chromatography, ICP MS can establish the elution times of phosphorylated peptides, and quantify the amount of phosphorus in each peptide. A separate LC ESI MS analysis can then determine the mass of the peptide(s) detected at the appropriate elution time(s) [6]. Because ICP MS is able to quantify sulfur as well as phosphorus, the stoichiometry of phosphorylation may be determined in a single experiment. The relative molar quantities of the phosphorylated peptide and a non-phosphoryalted cysteine or methionine containing peptide yield the relative extent of phosphorylation [7].

3.1. Electrospray ionization (ESI)

Electrospray ionisation (ESI) [8–10] is an attractive ionization method for the characterization of phosphopeptides for several reaons. ESI is directly compatible with liquid phase separation, as well as concentration techniques, such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). In addition, multiply protonated peptide ions fragment efficiently during collision-induced dissociation (CID) mass spectrometry/mass spectrometry (MS/MS), a primary tool for gas-phase sequencing of peptides. Finally, multiply-charged ions are required for dissociation by electron capture MS/MS, a promising new method for identifying phosphorylation site(s) by mass analysis (see below).

Mass spectrometric detection of phosphopeptides is frequently carried out with ^{32}P radiolabeled samples. Following enzymatic hydrolysis, peptides are analyzed by LC/MS incorporating a radioactivity detector [11]. The radioactive trace, plus MS and MS/MS serves to identify unphosphorylated peptides that co-elute with the ^{32}P labeled peptides. A typical example is given in Fig. 3 (from Janosch et al. [11]). This method, however, requires the use of radioactivity and, a subsequent proof that the phosphopeptide is indeed correctly identified (e.g. by comparison to mutant proteins).

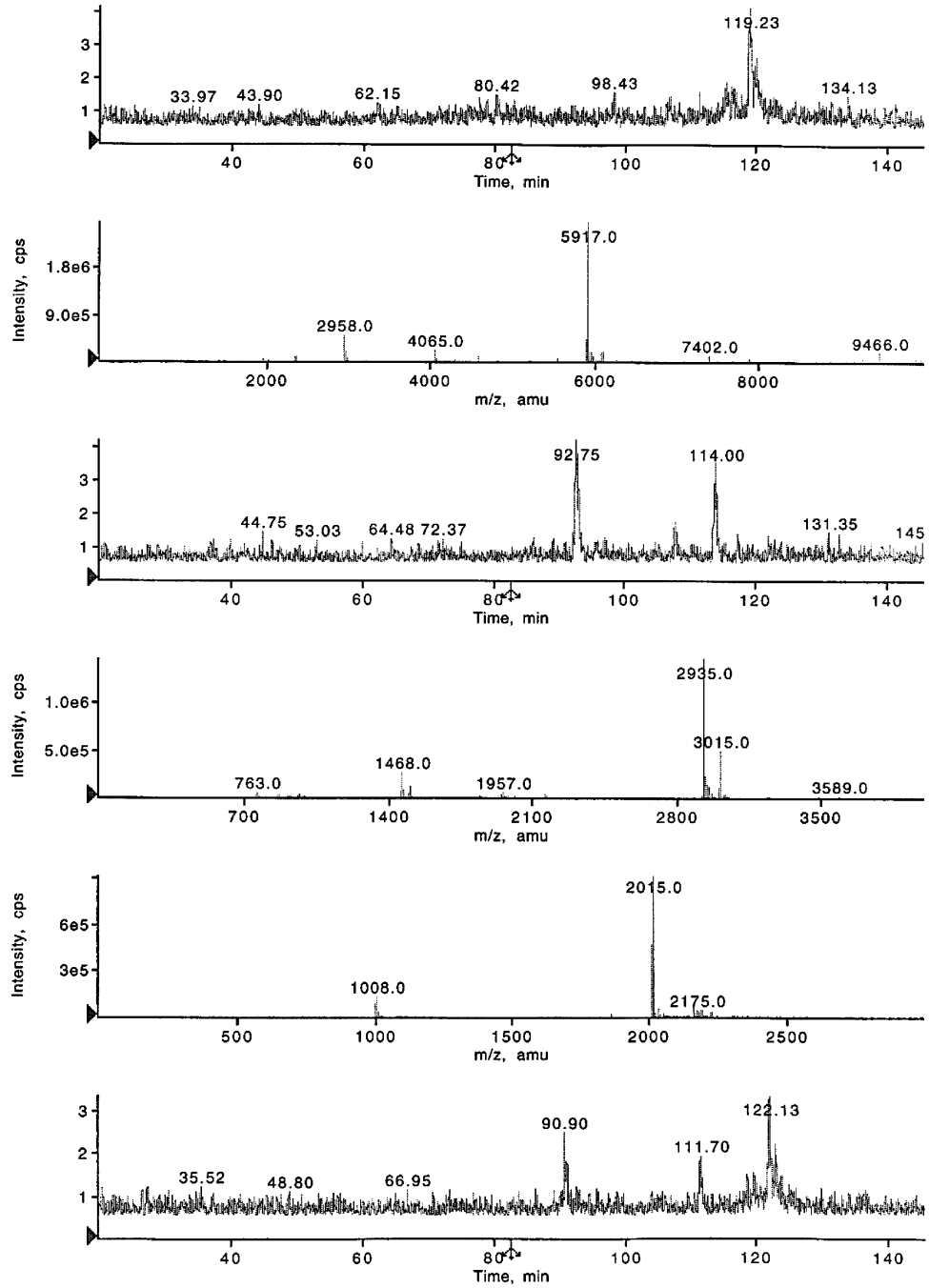

Fig. 3. HPLC-mass spectroscopy analysis of IKB phosphorylation sites. Recombinant IKB was phosphorylated and subsequently digested with trypsin. The top panel shows the radioactivity in the HPLC run. The second panel is the reconstructed mass spectrum of the radioactive peak. The third panel shows the radioactivity in the HPLC run of a trypsin and Asp-N digest of IKB. The fourth and fifth panels are the reconstructed mass spectra of the first and second radioactive peaks, respectively. The bottom panel shows the radioactivity in the HPLC run of a mixture of the tryptic digest with the trypsin and Asp-N digest of phosphorylated IKB. Figure reproduced from [11].

Early attempts to specifically detect phosphorylated peptide ions involved the identification of PO_3^- (79 Da) formed by nozzlel-skimmer CID of the corresponding phosphopeptide during on-line HPLC MS [12]. Although the detection of the PO_3^- ion indicates the point at which point the phosphopeptides elute, there is no connectivity between the PO_3^- ion and its ionic precursor(s). Scanning in the negative ion mode for precursors of PO_3^- with a tandem-in-space instrument provides that connectivity [13–15]. Detection is thus more specific, and "chemical noise" is reduced because the only species detected are those that lose PO_3^- from a given precursor. The detection limit for this technology applied to synthetic phosphopeptides is 0.5–5 fmol for both tandem quadrupole and quadrupole ToF instruments [16]. For a

tryptic digest, however, the minimum amount of protein required will be slightly greater. The ability to detect sub-picomolar amounts of serine, threonine and tyrosine-phosphorylated peptide ions (independent of ion charge state) from a single experiment has made this approach one of the most successful and widely applied methods [17–21].

Tyrosine-phosphorylated peptides may be detected by positive ion mode precursor ion scanning with a Q-ToF instrument. The resolving power of the reflectron ToF analyzer (∼10,000) can distinguish pY immonium ions (216.04 Da) from other isobaric ions of the same nominal mass (∼216 Da) [22]. The tyrosine phosphopeptides may then be sequenced with a product ion analysis, without recourse to negative ion analysis. For example, six novel (and three known) sites of tyrosine phosphorylation were recently identified by this method for the p185 form of Bcr/Abl [23].

Neutral loss analysis by tandem quadrupole MS/MS can screen for phosphopeptide ions that lose H_3PO_4 (98 Da) upon CID [24]. However, because the instrument scans for the loss of 98/*z*, each experiment detects only phosphopeptide ions of a pre-determined charge state, *z*. In our experience the selectivity of this approach is significantly poorer than scanning for precursor ions that lose PO_3^-, and often results in multiple false positives. In those instances, unrelated product ions appear at *m*/*z* 98/*z* below the precursor ion *m*/*z* value. In a relatively new and more effective approach based upon the detection of the neutral loss of 98/*z* by Q-ToF MS/MS [25], false positives are reduced by virtue of the higher resolution of the Q-ToF relative to the unit mass resolution of the sequential quadrupole mass analyzer. Once the phosphopeptide has been identified, the instrument automatically acquires a product ion spectrum from which the location of the modification may be assigned. The sensitivity of nanoscale HPLC ESI MS over direct infusion ESI offers an additional benefit.

The so-called shotgun approach to the identification of phosphorylated peptides [26] also benefits from the sensitivity of nanoscale HPLC MS. In that approach, proteins are digested with three different enzymes (one specific, and two non-specific) and three separate LC/MS/MS experiments are performed. Because each site of phosphorylation appears in a different peptide for each of the enzymatic digests, the probability of detecting each site of phosphorylation increases relative to a single enzymatic digestion LC/MS/MS experiment.

3.2. Matrix assisted laser desorption ionisation (MALDI)

MALDI MS [27,28] is widely applied to the characterization of protein phosphorylation, typically based on the 80 Da mass decrease in a phosphopeptide mass after alkaline phosphatase digestion [29–31]. Although phosphopeptides exhibit low ionization efficiency in positive ion mode [32,33], they have higher relative abundance in negative ion mode [34]. Ma et al. have exploited that behavior to differentiate phosphorylated peptides from (excess) unphosphorylated polypeptides. The phosphopeptides are subsequently sequenced, and hence positively identified, with product ion analyses performed by ESI Q-ToF MS. Interfacing a MALDI source with a ToF–ToF [35] or Q-ToF tandem mass spectrometer, rather than the more common ToF analyzer, allows for direct sequencing of MALDI-generated phosphopeptide ions [36].

4. Enrichment of phosphoproteins

4.1. Immobilized metal ion affinity chromatography

In immobilized metal ion affinity chromatography (IMAC) phosphopeptides bind selectively to immobilized metal ions via their phosphate moiety and may thus be enriched or even purified, and then subsequently eluted and examined by mass spectrometry as in an early application of IMAC by Neville et al. [37]. Later, it was shown that Ga^{3+} and Al^{3+} offered higher selectivity than Fe^{3+} [38]. Although effective for synthetic phosphopeptides as well as model phosphoproteins such as casein, the method was less reliable for "real" proteins. Nevertheless, a number of successful studies have been reported [29,37,39,40]. Our own laboratories reveal that certain phosphopeptides bound with rather low affinity under the conditions described by Posewitz and Tempst [38], and hence tend to bleed from the IMAC column. Based on the radioactivity profile from peptides labeled with γ-^{32}P-ATP, up to 90% of the phopshopeptide was found in the flow-through and in the wash fraction. In contrast, acidic unphosphorylated peptides bound with rather high affinity. Thus, the selective enrichment of phosphopeptides frequently was in fact due to selective enrichment of acidic peptides. Ficarro et al. [41] sought to circumvent this problem by converting the acidic peptides into methyl esters by use of methanolic HCl, so as to reduce their binding to the IMAC column. Methylation resulted in a much higher selectivity of the IMAC column and the isolation of more than 1000 phosphopeptides from *Saccharomyces cerevisiae*.

4.2. Phospho-specific antibodies

As outlined above, the identification of protein phosphorylation sites is hampered by the frequently low stochiometry of phosphorylation in vivo. One is thus led to consider enrichment for phosphoproteins by use of antibodies specific for phosphoamino acids. For tyrosine phosphorylation, several excellent antibodies suitable for selective immunoprecipitation of tyrosine-phosphorylated proteins facilitate identification of tyrosine phosphorylation sites. Given the relative abundances, phosphotyrosine:phosphothreonine:phosphoserine ≈ 1:200:1800 [42], tyrosine-phosphorylated proteins are clearly over-represented in the literature, due to their greater accessibility to analysis.

Specific phosphoserine and phosphothreonine antibodies have recently been applied to phosphoprotein analysis [43]. For example, Gronborg et al. selectively immunoprecipitated proteins that become phosphorylated on serine and/or threonine residues in vivo upon treatment with calyculin-A. Isolation of the immunoprecipitated proteins from SDS gels and subsequent analysis by MS or MS/MS led to the identification of novel phosphoproteins and phosphorylation sites. Given that the resolution of fractionation in the SDS gel could be improved greatly by use of 2D-electrophoresis, that approach holds great promise for identification of the in vivo phosphoproteome.

If the phosphoproteins are known, capillary electrophoresis with in-column immunoaffinity concentration of (phospho)proteins of interest can be employed. The combination of the selectivity of affinity-CE with the sensitivity of laser-induced fluorescence detection allows for protein quantification. This approach was employed by Phillips and Smith, who quantified the phosphorylated and non-phosphorylated forms of the regulatory STAT-1 and STAT-3 proteins [44]. The review of Guzman and Stubbs contains a more detailed discussion of the benefits of affinity capillary electrophoresis [45].

4.3. Chemical modification of phosphopeptides

Recently, several alternative ways to identify and, in some case, purify phosphopeptides have been based on selective chemical modification of the phosphoamino acids prior to analysis, with a number of advantages over characterization of the an unmodified phosphopeptide. It should, however, be noted that high-yield conversion is required at each step of the reaction to maintain the sensitivity of these methods.

Jaffe et al. has described beta-elimination of the phosphate, followed by addition of ethanthiol [46]. Upon completion of the reaction, the mass of the previously phosphorylated residue increases by 44 Da compared to that of serine or threonine. LC/MS/MS of the tryptic digest was then used to identify the sites of modification, and by inference, the sites of phosphorylation. Advantages of this method are: (1) The removal of the acidic phosphate group improves the retention of the peptides on reversed-phase HPLC stationary phases; (2) ionization efficiency may also increase for positive ions; (3) CID of ethanthiol modified peptide ions often yields more complete sequence coverage relative to phosphopeptide ions, for which losses of H_3PO_4 often dominate CID product ion spectrum. However, tyrosine phosphopeptides do not undergo beta-elimination of H_3PO_4, and therefore cannot be detected with this method.

In a modified beta-elimination method later reported by Oda et al. [47], phosphorylated serine or threonine residues are converted to a biotinylated residue, allowing for specific isolation of the previously phosphorylated peptides by avidin/biotin affinity chromatography.

Zhou et al. [48] introduced a powerful technology that resulted in the identification of several phosphoproteins, as well as phosphorylation sites in *S. cerevisiae*. Shortly after amino protection, the phosphopeptides are derivatized to phosphoramidate. After regeneration of the phosphate and reduction, free SH-groups are generated. Phosphopeptides are captured by their SH-groups on immobilized iodoacetyl groups. In the last step, the purified phosphopeptides are regenerated with trifluoroacetic acid and characterized by LC ESI MS/MS and database searching.

Modification of these methods to incorporate stable isotope labels allows for the *relative* quantification of protein phosphorylation [49]. Oda et al. have also described a method to characterize the relative quantification of protein phosphorylation, in which the stable isotope label (^{14}N or ^{15}N) is incorporated into all proteins during cell culcture [50]. The important problem of the *absolute* quantification of phosphoproteins is also beginning to be addressed [51].

5. Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry

Recent advances in FT-ICR MS and its associated ion activation techniques are beginning to produce a variety of powerful new approaches to the characterization of post-translationally modified proteins and peptides. High-field FT-ICR mass spectrometers provide 10–100 times higher mass resolution, resolving power, and mass accuracy than any other mass analyzer [52,53]. The FT-ICR experiment is based on measurement of the cyclotron frequencies of ions trapped in a spatially uniform magnetic field [54,55]. It is the measurement of frequency which provides the high performance of FT-ICR MS (frequency can be measured more accurately than any other physical property). A number of key FT-ICR parameters scale linearly (resolving power, data acquisition speed (for real-time LC/MS and LC/MS/MS), upper mass limit for peak coalescence (the ultimate limit to FT-ICR mass resolution)), or quadratically (maximum number of trapped ions (which in turn maximizes the dynamic range of highest to lowest magnitude signal), upper mass limit due to trapping potential, maximum ion kinetic energy (for more efficient CID), maximum trapping period (for ion cooling and reactivity experiments)) with increasing magnetic field strength [56]. The performance of FT-ICR MS therefore continues to improve as higher field strength magnets become available.

Both ESI [55] and MALDI have been interfaced with FT-ICR mass spectrometers. Although MALDI offers high degrees of automation and sample throughput [57,58], ESI provides a number of alternative advantages. Whereas MALDI generates predominantly singly charged ions, ESI produces multiply-charged ions, which are detected at lower m/z values. The mass resolving power and mass accuracy of FT-ICR MS improve at lower m/z ratios, and detection efficiency increases with charge (z). The use of ESI therefore maximizes the performance of the FT-ICR MS experiment.

Accurate mass measurement can determine unique *elemental* composition only for ions up to ~300 Da at a mass accuracy of 1 ppm (easily attained at 9.4 T) [53]. Although proteolytic peptides typically have higher masses, their *amino acid* compositions can nevertheless be determined by mass alone by virtue of particular constraints. For example, reduced protein databases may be created from a knowledge of those proteins likely to be expressed in a specific system, for example, cerebrospinal fluids [59]. If limiting the protein database is not practical, cysteine residues may be modified with alkylating agents containing chlorine (which has a naturally abundant ^{37}Cl isotopic component). The presence (or absence) of cysteine within any peptide may then be determined from altered (or normal) isotopic distributions. That information may then be combined with accurate mass measurement prior to database searching [60]. Because most post-translational modifications involve a change in mass (e.g. 79.9663 Da for HPO_3), identification of modified proteins (or proteolytic peptides) is also possible from upon highly accurate mass measurements, however, the database search must again be constrained.

5.1. FT-ICR MS/MS and MS^3

Although mass data serves to identify the amino acid composition and post-translational modifications of proteolytic peptides, the primary amino acid sequence (including the location of any modification) cannot be determined by mass alone. Tandem mass spectrometry can solve that problem. FT-ICR mass spectrometers function as tandem-in-time instruments, and are therefore able to perform MS^n experiments (MS, MS/MS, MS/MS/MS, etc.). For precursor ion selection, all other ions can be removed from the ICR cell by stored waveform inverse Fourier transform (SWIFT) ion ejection [61,62]. Alternatively precursor ions may be isolated and accumulated outside the magnet prior to transfer into the ICR cell [63–66].

Tandem FT-ICR experiments have been performed with a number of ion activation techniques. Sustained off-resonance irradiation (SORI) CID allows for the fragmentation of ions trapped within the ICR cell [67]. However, the presence of collision gas into the ICR cell limits mass resolution, and requires the inclusion of long “pump down” events into the experimental sequence (often as high as 20–30 s). Infrared multiphoton dissociation (IRMPD) provides a powerful alternative [68,69]. Ion activation by irradiation with IR photons induces peptide fragmentation similar to that from CID (i.e. *b* and *y* type ions from cleavage of the backbone peptide linkage, see Fig. 4), but without the need to compromise the high vacuum within the ICR cell. The short duration of the IRMPD event (typically 50–500 ms) is also compatible with the timescale of CE or HPLC ESI experiments.

Electron capture dissociation (ECD) offers two primary benefits for the characterization of protein phosphorylation, and is at present unique to FT-ICR instruments [70,71]. First,

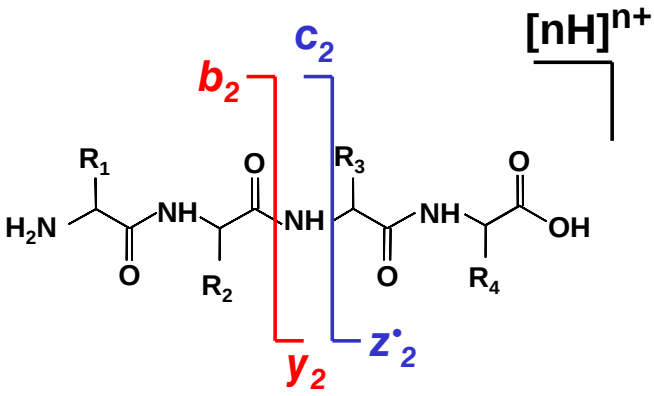

Fig. 4. Location of peptide backbone cleavages to form *b* and *y* (CID) and *c* and $z^\bullet$ (ECD) ions.

ECD typically produces *c* and $z^\bullet$ type ions (see Fig. 4) and thus also cleaves the peptide backbone to yield sequence information, but provides more extensive sequence coverage than CID with little or no loss of labile post-translational modifications [72,73]. The extended sequence information obtained from activated ion (AI) ECD has allowed the sequencing of proteins with molecular weights of up to 42 kDa [74]. Second, in contrast to CID and IRMPD, which typically cleave phosphate and sugar linkages (thereby precluding determination of their attachment to a phospho- or glycopeptide), ECD tends to leave the phosphate attached while breaking the backbone peptide linkages (see below).

5.2. Characterization of protein phosphorylation with FT-ICR MS and tandem MS

The commonly observed neutral loss of H_3PO_4 from phosphopeptide ions following CID is also observed upon IRMPD. Flora and Muddiman exploited that diagnostic property in negative ion mode ESI FT-ICR experiments, in which phosphopepitde ions were identified through the observation of the loss of H_3PO_4 following irradiation with an IR laser for 4 s (11.1 W) [75].

The value of mass-based methods for the identification of post-translational modifications was recently demonstrated in a study characterizing the phosphorylation of protein kinase C (PKC) [76]. The use of capillary liquid chromatography provided increased sensitivity and dynamic range relative to direct infusion methods. The mass-based approach allowed the identification of multiple co-eluting peptides, largely independent of the observed signal magnitude, thereby increasing the experimental duty cycle (i.e. the fraction of time during which ions are actually collected for subsequent observation) and dynamic range relative to LC/MS/MS experiments, which select only the few most abundant ions within the survey mass spectrum for product ion analysis. Fig. 5 (top and middle) shows two mass spectra obtained from the LC ESI FT-ICR analysis of a PKCα tryptic hydrolysate. Two previously unknown

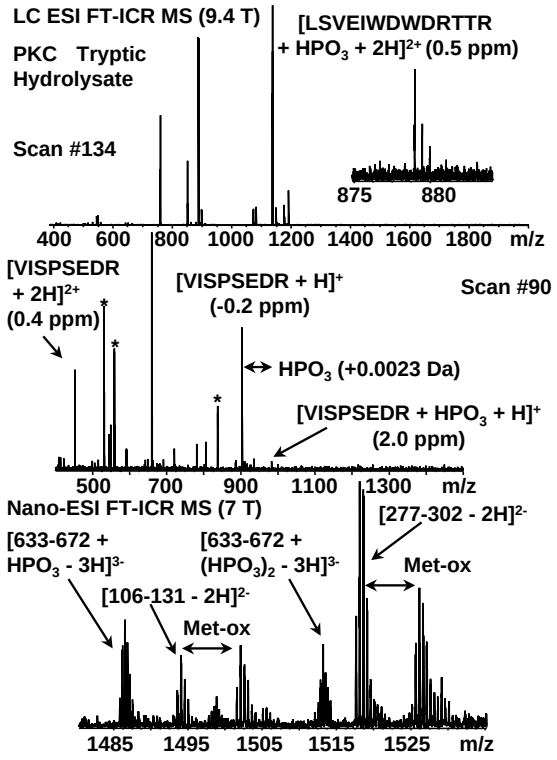

Fig. 5. Top: mass spectrum obtained from scan 134 of LC ESI FT-ICR MS (9.4 T) analysis of PKCα tryptic hydrolysate. Following internal calibration, the $[L_{240}SVEIWDWDRTTR_{252} + HPO_3 + 2H]^{2+}$ phosphopeptide ion was mass measured with an accuracy of +0.5 ppm (+0.0004 Th). Middle: mass spectrum obtained from scan 90 of the LC ESI FT-ICR MS (9.4 T) analysis of PKCα tryptic hydrolysate. ∗: calibrant ions. Both the phosphorylated and non-phosphorylated analogs of the $V_{317}ISPSEDR_{324}$ peptide are seen. The experimental mass difference between the phosphorylated and non-phosphorylated forms ($[M+H]^+$ ions) was 79.9686 + 0.0023 Da (HPO_3 = 79.9663 Da). Bottom: expanded segment of a negative ion direct infusion nano-ESI FT-ICR (7 T) mass spectrum of a PKCα tryptic hydrolysate. Both the singly and doubly phosphorylated forms of the T87 peptide (residues 633–672) are observed. Observation of those peptides confirms two of the three known sites of PKCα phosphorylation. Figure and caption reproduced from [76].

phosphopeptides were identified. A subsequent negative ion nanoelectrospray FT-ICR MS experiment (bottom) identified the singly and doubly phosphorylated forms of the T87 peptide (residues 633–672). Detection of those peptides confirmed two of the three known sites of PKCα phosphorylation.

Once the phosphorylated peptides are identified, tandem FT-ICR MS^n experiments serve to locate the modified residue. It is the ability of FT-ICR mass spectrometers to perform both IRMPD (or SORI CID) and ECD that allows for a more complete characterization of modified peptides than is possible with other types of tandem mass spectrometers. Both Stensballe et al. [77] and Shi et al. [78] have demonstrated that ECD provides more sequence information from phosphopeptides than does CID. Importantly, no significant losses of H_3PO_4, HPO_3 or H_2O were observed from either the intact precursor ions, or the phosphorylated product ions. The study by Stensablle et al. combined IMAC isolation of the phosphopeptides prior to nanoelectrospray ECD FT-ICR MS/MS. The fragmentation of the phsophorylated peptides was compared following both ECD and SORI CID. For the serine phosphorylated β-casein peptide, FQpSEEQQQTEDELQDK, SORI CID of the $[M + 3H]^{3+}$ ion cleaved 9 of 15 peptide backbone bonds, whereas ECD cleaved 15 out of 15. The study also demonstrated that extensive sequence information could be obtained from large, highly serine-phosphorylated peptides. Twenty-three out of 27 peptide bonds were cleaved following the ECD of the 3476 Da β-casein LEELNVPGEIVEpSLpSpSpSEESITRINK peptide $[M + 4H]^{4+}$ ion. All four sites of phosphorylation were located from the ECD data.

The work by Shi et al. also demonstrates that phosphopeptides can be sequenced by ECD. Increased sequence coverage with ECD, relative to SORI CID, was demonstrated for a 28 residue peptide. SORI CID cleaved 20 out of 27 backbone bonds with multiple losses of H_3PO_4. In contrast, the ECD data provided 100% sequence coverage from 81 fragment ions, with little loss of H_3PO_4. The study then went on to show that activated ion ECD [74] was able to provide extensive sequence information from intact phosphoproteins (87 out of 208 backbone bonds were cleaved). Importantly, that work demonstrated that the top–down [79,80] approach to protein sequencing is feasible for phosphoproteins.

The preceding studies indicated very early on in the development of ECD that the technique would prove to be an important new tool for phosphopeptide characterization. Since that time a number of improvements have been made to the ECD experiment. High sensitivity ECD (10 fmol μl^{-1}) has been demonstrated [81]. In addition, the long irradiation period of these early ECD experiments (up to 8 s [77]) has since been reduced to a few milliseconds. Fig. 6 shows a comparison between the IRMPD (top) and ECD (bottom) of a serine-phosphorylated peptide. To generate the fragments shown in Fig. 6 (bottom), the peptide was irradiated with electrons for only 20 ms [82], clearly speeding up the ECD experiment to a period short enough for coupling with low flow rate on-line liquid chromatography. LC ESI ECD FT-ICR MS/MS experiments have since been published, albeit with large amounts of sample and no precursor ion selection [83]. The introduction of mass-selective external ion accumulation has allowed for a increase in the dynamic range and sensitivity of FT-ICR MS^n experiments [63–66], allowing for performance of ECD or IRMPD for low-abundance ions in complex mixtures. The benefits of an instrumental geometry that allows simultaneous and optimized IRMPD and ECD has recently been described. Combined ECD/IRMPD MS^3 experiments (similar in concept to AI ECD) can provide increased peptide sequence information relative to ECD alone [82]. The application of that approach to the characterization of protein phosphorylation shows that the ECD/IRMPD MS^3 experiment provides increased sequence information for two (out of the three) PKA phosphopeptides studied [84]. Although the use of FT-ICR

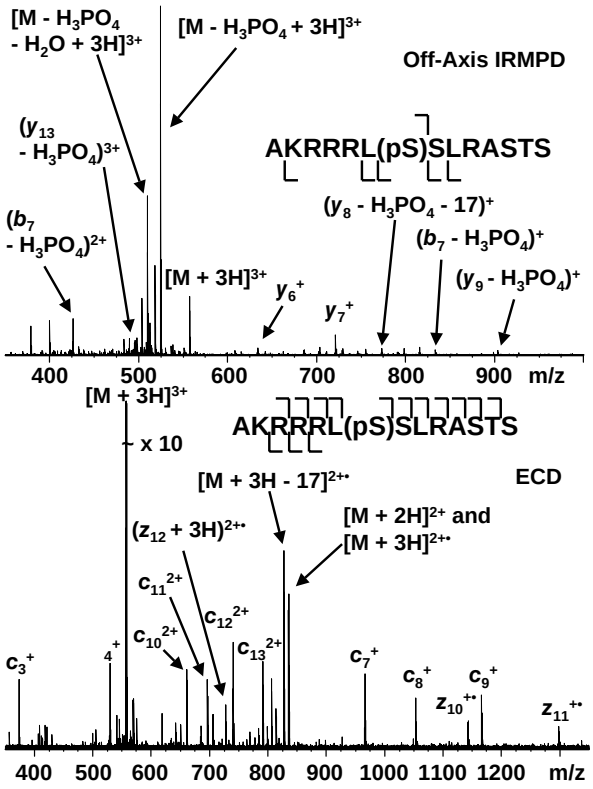

Fig. 6. Top: product ion spectrum obtained from off-axis IRMPD FT-ICR MS/MS of a population of quadrupole- and SWIFT-isolated [AKRRRL(pS)SLRASTS + 3H]$^{3+}$ phosphopeptide ions. The spectrum is dominated by ions resulting from neutral losses of H_3PO_4, NH_3 and H_2O. Five (out of 13) peptide backbone bonds are broken, and the location of the phosphorylation site is identified only by observation of the (y_8–H_3PO_4–NH_3) ion (present at very low abundance) and the singly and doubly charged (b_7–H_3PO_4) ions. Irradiation was for 500 ms at ~36 W laser power, and the data represent a sum of 10 scans. Bottom: product ion spectrum obtained from ECD (20 ms irradiation) FT-ICR MS/MS of the same quadrupole- and SWIFT-isolated phosphopeptide as in Fig. 6, top. Twelve out of 13 peptide backbone bonds are cleaved, and the location of the phosphate is readily assigned by observation of the abundant c_7 ions. Figure and caption adapted from [82].

MS and MS/MS for the characterization of protein phosphorylation has been somewhat limited to date, the role of the technique can be expected to expand in the future.

6. Conclusions

Examination of protein phosphorylation and identification of phosphorylation sites can successfully be performed on a routine basis when larger amounts of purified proteins are employed. In vivo, unfortunately, most proteins involved in signal transduction are certainly not abundant. Limiting amounts of protein, low stoichiometry of phosphorylation, as well as the technical problem that they frequently cannot easily be purified (e.g. cut out as single band from SDS gels) certainly limits the use of mass spectrometry to determine in vivo phosphorylation sites. It is, however, expected that mass spectrometric techniques and enrichment procedures will continue to improve.

For proteins of low abundance, the method of choice for the analysis of in vivo phosphorylation is still the 2D phosphopeptide map performed on in vivo ^{32}P labeled proteins. Comparison of the in vivo data with the in vitro labeled ^{32}P proteins, and characterization of the in vitro phosphorylation sites with mass spectrometry appears to offer the best means for identification of the in vivo phosphorylation site(s).

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References

[1] W.J. Boyle, P. Vandergeer, T. Hunter, Methods Enzymol. 201 (1991) 110.
[2] A.S. Dhillon, C. Pollock, H. Steen, P.E. Shaw, H. Mischak, W. Kolch, Mol. Cel. Biol. 22 (10) (2002) 3237.
[3] P. Vandergeer, T. Hunter, Electrophoresis 15 (3–4) (1994) 544.
[4] Y.H. Wang, C.J. Fiol, A.A. Depaoliroach, A.W. Bell, M.A. Hermodson, P.J. Roach, Anal. Biochem. 174 (2) (1988) 537.
[5] D.G. Cambell, N.A. Morris, J. Biomol. Tech. 13 (2002) 119.
[6] M. Wind, M. Edler, N. Jakubowski, M. Linscheid, H. Wesch, W.D. Lehmann, Anal. Chem. 73 (1) (2001) 29.
[7] M. Wind, H. Wesch, W.D. Lehmann, Anal. Chem. 73 (13) (2001) 3006.
[8] C.M. Whitehouse, R.N. Dreyer, M. Yamashita, J.B. Fenn, Anal. Chem. 57 (1985) 675.
[9] M. Wilm, M. Mann, Anal. Chem. 68 (1) (1996) 1.
[10] M.R. Emmett, R.M. Caprioli, J. Am. Soc. Mass Spectrom. 5 (7) (1994) 605.
[11] P. Janosch, M. Schellerer, T. Seitz, P. Reim, M. Eulitz, M. Brielmeier, W. Kolch, J.M. Sedivy, H. Mischak, J. Biol. Chem. 271 (23) (1996) 13868.
[12] M.J. Huddleston, R.S. Annan, M.F. Bean, S.A. Carr, J. Am. Soc. Mass Spectrom. 4 (9) (1993) 710.
[13] M. Wilm, G. Neubauer, M. Mann, Anal. Chem. 68 (3) (1996) 527.
[14] S.A. Carr, M.J. Huddleston, R.S. Annan, Anal. Biochem. 239 (2) (1996) 180.
[15] G. Neubauer, M. Mann, Anal. Chem. 71 (1) (1999) 235.
[16] H. Steen, B. Kuster, M. Mann, J. Mass Spectrom. 36 (7) (2001) 782.
[17] R.S. Annan, M.J. Huddleston, R. Verma, R.J. Deshaies, S.A. Carr, Anal. Chem. 73 (3) (2001) 393.
[18] F. Zappacosta, M.J. Huddleston, R.L. Karcher, V.I. Gelfand, S.A. Carr, R.S. Annan, Anal. Chem. 74 (13) (2002) 3221.
[19] T.A. Knotts, R.S. Orkiszewski, R.G. Cook, D.P. Edwards, N.L. Weigel, J. Biol. Chem. 276 (11) (2001) 8475.
[20] R. Verma, R.S. Annan, M.J. Huddleston, S.A. Carr, G. Reynard, R.J. Deshaies, Science 278 (5337) (1997) 455.
[21] A. Watty, G. Neubauer, M. Dreger, M. Zimmer, M. Wilm, S.J. Burden, PNAS 97 (9) (2000) 4585.

[22] H. Steen, B. Kuster, M. Fernandez, A. Pandey, M. Mann, Anal. Chem. 73 (7) (2001) 1440.
[23] H. Steen, M. Fernandez, S. Ghaffari, A. Pandey, M. Mann, Mol. Cell Proteomics 2 (2003) 138.
[24] A. Schlosser, R. Pipkorn, D. Bossemeyer, W.D. Lehmann, Anal. Chem. 73 (2) (2001) 170.
[25] R.H. Bateman, R. Carruthers, J.B. Hoyes, C. Jones, J.I. Langridge, A. Millar, J.P.C. Vissers, J. Am. Soc. Mass Spectrom. 13 (7) (2002) 792.
[26] M.J. MacCoss, W.H. McDonald, A. Saraf, R. Sadygov, J.M. Clark, J.J. Tasto, K.L. Gould, D. Wolters, M. Washburn, A. Weiss, J.I. Clark, J.R. Yates, Proc. National Academy Sci. U.S.A. 99 (12) (2002) 7900.
[27] M. Karas, F. Hillenkamp, Anal. Chem. 60 (20) (1988) 2299.
[28] K. Tanaka, H. Waki, Y. Ido, S. Akita, Y. Yoshida, T. Yoshida, Rapid Commun. Mass Spectrom. 2 (1988) 151.
[29] A. Stensballe, S. Andersen, O.N. Jensen, Proteomics 1 (2) (2001) 207.
[30] X.L. Zhang, C.J. Herring, P.R. Romano, J. Szczepanowska, H. Brzeska, A.G. Hinnebusch, J. Qin, Anal. Chem. 70 (10) (1998) 2050.
[31] M.R. Larsen, G.L. Sorensen, S.J. Fey, P.M. Larsen, P. Roepstorff, Proteomics 1 (2) (2001) 223.
[32] Y.L. Ma, Y. Lu, H.Q. Zeng, D. Ron, W.J. Mo, T.A. Neubert, Rapid Commun. Mass Spectrom. 15 (18) (2001) 1693.
[33] J.M. Asara, J. Allison, J. Am. Soc. Mass Spectrom. 10 (1) (1999) 35.
[34] J.L. Wolfender, F.X. Chu, H. Ball, F. Wolfender, M. Fainzilber, M.A. Baldwin, A.L. Burlingame, J. Mass Spectrom. 34 (4) (1999) 447.
[35] W.V. Bienvenut, C. Deon, C. Pasquarello, J.M. Campbell, J.C. Sanchez, M.L. Vestal, D.F. Hochstrasser, Proteomics 2 (7) (2002) 868.
[36] K.L. Bennett, A. Stensballe, A.V. Podtelejnikov, M. Moniatte, O.N. Jensen, Journal of Mass Spectrometry 37 (2) (2002) 179.
[37] D.C.A. Neville, C.R. Rozanas, E.M. Price, D.B. Gruis, A.S. Verkman, R.R. Townsend, Protein Science 6 (11) (1997) 2436.
[38] M.C. Posewitz, P. Tempst, Anal. Chem. 71 (14) (1999) 2883.
[39] K.E. Cleverley, J.C. Betts, W.P. Blackstock, J.M. Gallo, B.H. Anderton, Biochemistry 37 (11) (1998) 3917.
[40] P. Cao, J.T. Stults, Rapid Commun. Mass Spectrom. 14 (17) (2000) 1600.
[41] S.B. Ficarro, M.L. McCleland, P.T. Stukenberg, D.J. Burke, M.M. Ross, J. Shabanowitz, D.F. Hunt, F.M. White, Nat. Biotechnol. 20 (3) (2002) 301.
[42] T. Hunter, Philosophical Trans. Roy. Soc. London Ser. B-Biol. Sci. 353 (1368) (1998) 583.
[43] M. Gronborg, T.Z. Kristiansen, A. Stensballe, J.S. Andersen, O. Ohara, M. Mann, O.N. Jensen, A. Pandey, Mol. Cell Proteomics 1 (7) (2002) 517.
[44] T.M. Phillips, P. Smith, Biomed. Chromatogr. 17 (2–3) (2003) 182.
[45] N.A. Guzman, R.J. Stubbs, Electrophoresis 22 (17) (2001) 3602.
[46] Jaffe, H. Veeranna, H.C. Pant, Biochemistry, 37(46) (1998) 16211–16224.
[47] Y. Oda, T. Nagasu, B.T. Chait, Nat. Biotechnol. 19 (4) (2001) 379.
[48] H.L. Zhou, J.D. Watts, R. Aebersold, Nat. Biotechnol. 19 (4) (2001) 375.
[49] M.B. Goshe, T.D. Veenstra, E.A. Panisko, T.P. Conrads, N.H. Angell, R.D. Smith, Anal. Chem. 74 (3) (2002) 607.
[50] Y. Oda, K. Huang, F.R. Cross, D. Cowburn, B.T. Chait, Proc. National Academy Sci. U.S.A. 96 (12) (1999) 6591.
[51] S. Gerber, J. Rush, O. Stremmann, H. Steen, M. Kirschner, S.P. Gygi, Orlando, FL, 2002.
[52] A.G. Marshall, Int. J. Mass Spectrom. 200 (2000) 331.
[53] A.G. Marshall, C.L. Hendrickson, S.D.H. Shi, Anal. Chem. 74 (9) (2002) 253A.
[54] A.G. Marshall, C.L. Hendrickson, G.S. Jackson, Mass Spectrom. Rev. 17 (1998) 1.
[55] C.L. Hendrickson, M.R. Emmett, Annu. Rev. Phys. Chem. 50 (1999) 517.
[56] A.G. Marshall, S. Guan, Rapid Commun. Mass Spectrom. 10 (1996) 1819.
[57] E.C. Peters, A. Brock, D.M. Horn, Q.T. Phung, C. Ericson, A.R. Salomon, S.B. Ficarro, L.M. Brill, LC GC Europe 15 (7) (2002) 423.
[58] P.B. O'Connor, C.E. Costello, Anal. Chem. 72 (24) (2000) 5881.
[59] J. Bergquist, M. Palmblad, M. Wetterhall, P. Hakansson, K.E. Markides, Mass Spectrom. Rev. 21 (1) (2002) 2.
[60] D.R. Goodlett, J.E. Bruce, G.A. Anderson, B. Rist, L. Pasa-Tolic, O. Fiehn, R.D. Smith, R. Aebersold, Anal. Chem. 72 (6) (2000) 1112.
[61] A.G. Marshall, T.C.L. Wang, T.L. Ricca, J. Am. Chem. Soc. 107 (26) (1985) 7893.
[62] S.H. Guan, A.G. Marshall, Int. J. Mass Spectrom. Ion Processes 158 (1996) 5.
[63] C.L. Hendrickson, J.P. Quinn, M.R. Emmett, A.G. Marshall, Long Beach, CA, 2000.
[64] C.L. Hendrickson, J.P. Quinn, M.R. Emmett, A.G. Marshall, Chicago, IL, 2001.
[65] M.E. Belov, E.N. Nikolaev, G.A. Anderson, H.R. Udseth, T.P. Conrads, T.D. Veenstra, C.D. Masselon, M.V. Gorshkov, R.D. Smith, Anal. Chem. 73 (2001) 253.
[66] M.E. Belov, E.N. Nikolaev, G.A. Anderson, K.J. Auberry, R. Harkewicz, R.D. Smith, J. Am. Soc. Mass Spectrom. 12 (2001) 38.
[67] J.W. Gauthier, T.R. Trautman, D.B. Jacobson, Anal. Chim. Acta 246 (1) (1991) 211.
[68] R.L. Woodlin, D.S. Bomse, J.L. Beauchamp, J. Am. Chem. Soc. 100 (1978) 3248.
[69] D.P. Little, J.P. Speir, M.W. Senko, P.B. O'Connor, F.W. McLafferty, Anal. Chem. 66 (1994) 2809.
[70] F.W. McLafferty, D.M. Horn, K. Breuker, Y. Ge, M.A. Lewis, B. Cerda, R.A. Zubarev, B.K. Carpenter, J. Am. Soc. Mass Spectrom. 12 (3) (2001) 245.
[71] R.A. Zubarev, N.L. Kelleher, F.W. McLafferty, J. Am. Chem. Soc. 120 (1998) 3265.
[72] R.L. Kelleher, R.A. Zubarev, K. Bush, B. Furie, B.C. Furie, F.W. McLafferty, C.T. Walsh, Anal. Chem. 71 (19) (1999) 4250.
[73] K. Hakansson, H.J. Cooper, M.R. Emmett, C.E. Costello, A.G. Marshall, C.L. Nilsson, Anal. Chem. 73 (18) (2001) 4530.
[74] D.M. Horn, Y. Ge, F.W. McLafferty, Anal. Chem. 72 (20) (2000) 4778.
[75] J.W. Flora, D.C. Muddiman, Anal. Chem. 73 (14) (2001) 3305.
[76] M.J. Chalmers, J.P. Quinn, G.T. Blakney, M.R. Emmett, H. Mischak, S.J. Gaskell, A.G. Marshall, J. Prot. Res. 2 (2003) 373.
[77] A. Stensballe, O.N. Jensen, J.V. Olsen, K.F. Haselmann, R.A. Zubarev, Rapid Comm. Mass Spectrom. 14 (19) (2000) 1793.
[78] S.D.H. Shi, M.E. Hemling, S.A. Carr, D.M. Horn, I. Lindh, F.W. McLafferty, Anal. Chem. 73 (1) (2001) 19.
[79] N.L. Kelleher, H.Y. Lin, G.A. Valaskovic, D.J. Aaserud, E.K. Fridriksson, F.W. McLafferty, J. Am. Chem. Soc. 121 (4) (1999) 806.
[80] Y. Ge, B.G. Lawhorn, M. ElNaggar, E. Strauss, J.H. Park, T.P. Begley, F.W. McLafferty, J. Am. Chem. Soc. 124 (4) (2002) 672.
[81] K. Hakansson, M.R. Emmett, C.L. Hendrickson, A.G. Marshall, Anal. Chem. 73 (15) (2001) 3605.
[82] K. Håkansson, M.J. Chalmers, J.P. Quinn, M.A. McFarland, C.L. Hendrickson, A.G. Marshall, Anal. Chem. 75 (2003) 3256.
[83] M. Palmblad, Y.O. Tsybin, M. Ramstrom, J. Bergquist, P. Hakansson, Rapid Commun. Mass Spectrom. 16 (10) (2002) 988.
[84] M.J. Chalmers, K. Håkansson, J. Shen, R. Smith, R. Johnson, M.R. Emmett, A.G. Marshall, Proteomics (2003) in press.