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Structural identification and quantification of protein phosphorylations after gel electrophoretic separation using Fourier transform ion cyclotron resonance mass spectrometry and laser ablation inductively coupled plasma mass spectrometry

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Dedicated to Prof. Dr. Helmut Schwarz on the occasion of his 60th birthday.

Abstract

In the present work mass spectrometric approaches are described for the identification of phosphorylated protein structures, and the direct quantification of protein–phosphorus contents, using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). An ultrahigh resolution FT-ICR-MS method was developed and applied for the structural identification of phosphorylations in proteins, using direct peptide mapping analysis with high mass accuracy of tryptic phosphorylated fragments. The application of this method to human tau proteins, one of the key proteins for the formation of neurofibrillary tangles in Alzheimer's disease, provided the identification of 17 phosphorylation sites. A high-sensitivity inorganic mass spectrometric technique has been developed for the direct determination of phosphor and sulfur concentrations in proteins separated by two-dimensional (2D) gel electrophoresis. Quantitative P and S determination in protein gel spots was performed with an optimized method using a double-focusing sector field ICP mass spectrometer coupled to a laser ablation chamber (LA-ICP-MS). Two different quantification strategies were applied: (i) determination of P and S in gel spots by LA-ICP-MS, following the determination of these elements in blank gel after trypsin and HNO_3 digestion using ICP-SFMS; (ii) a new quantification procedure by LA-ICP-MS was developed for the direct microlocal analysis in small protein spots from 2D gels. A solution-based calibration strategy in LA-ICP-MS was proposed for the quantification procedure using an ultrasonic nebulizer for introduction of calibration standard solutions coupled to the laser ablation chamber. Cobalt was used as an internal standard element, and was added to the gel at a defined concentration. The quality of phosphor determination by LA-ICP-MS was ascertained with β-casein as reference material. In a first application to the multi-phosphorylated tau protein, an average phosphorus content of ca. 20% was determined. The present results demonstrate the analytical merit of the combination of high resolution FT-ICR-MS and LA-ICP-MS for the molecular characterization of phosphorylated protein structures and determination of phosphorus and sulfur from 2D gels.

Keywords: FT-ICR-MS; LA-ICP-MS; Phosphorus; Proteins; 2D gels

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1. Introduction

Recently, John B. Fenn and Kuichi Tanaka were honored with the 2002 Nobel Prize in Chemistry for inventing techniques used to identify and analyze proteins, appreciated as "revolutionary analytical methods for biomolecules" [1]. This is the first Nobel Prize in mass spectrometry for the discovery and invention of soft mass spectrometric ionization techniques (electrospray-ionization and laser desorption) which permit the identification of large biomolecules such as proteins [2–4].

The identification and structure determination of proteins, including the determination of post-translational modifications such as glycosylation, fatty acylation and phosphorylation, is a challenging task in analytical chemistry. Besides the structure analysis of proteins by MALDI and ESI mass spectrometry, the quantitative determination of phosphor and metal concentration such as iron in biological tissues and fluids is required, because, e.g., phosphorylation and oxidation (induced by Fe^{2+}) are the two most important modifications of proteins, and are of crucial relevance for many physiological as well as pathophysiological processes such as in carcinogenesis and neurodegenerative diseases [5,6]. For example, the hyperphosphorylation of the microtubule-associated tau protein has been recognized as a key process leading to protein aggregation and resulting in neurofibrillary degeneration [7]. While MALDI and ESI mass spectrometry can be used for the identification of phosphorylation structures in proteins [8,9], these techniques cannot provide direct quantitative determinations of phosphorus and metals in biological samples. In recent papers [9,10], inductively coupled plasma mass spectrometry (ICP-MS) was used for phosphor determination in intact protein samples, or tryptic protein digests after HNO_3 treatment. A serious disadvantage and limiting factor of ICP-MS is the multitude of isobaric interferences of atomic ions with molecular ions such as $^{15}N^{16}O^+$, $^{14}N^{17}O^+$ and $^{14}N^{16}O^1H^+$ for the determination of monoisotope phosphor at m/z 31. For the separation of isobaric interferences of $^{31}P^+$, a mass resolution ($m/\Delta m$) of $\geq$1500 is required. Therefore, a double-focusing sector field ICP-MS (ICP-SFMS) with the required mass resolution has proven advantageous for phosphor determination in small proteins samples. In a previous study a significantly lower detection limit for phosphor has been found by ICP-SFMS ($20\,pg\,g^{-1}$) in comparison to quadrupole-based ICP-MS with collision cell ($1.3\,ng\,g^{-1}$) [11], in which $^{31}P^{16}O^+$ molecular ions are formed by collision-induced reaction with oxygen. The potential of ICP-MS with a dynamic reaction cell for phosphor determination in protein and the determination of the state of phosphorylation was recently studied by Baranov et al. [12].

Interest in the analysis of phosphor in protein samples has recently focused on the development of methods for determining total phosphorus contents [13,14] and in different species [12,15]. Wind et al. [9] have coupled a capillary liquid chromatography to a sector field ICP-MS and applied this method to α- and β-casein. LA-ICP-MS is one of the most important techniques for direct microlocal analysis of solid samples [16,17]; it has been used to an increasing extent in the analysis of biological and medical samples [18,19]. The direct determination of P and S in proteins separated by 2D gel electrophoresis has been recently reported [19–21]. Marshall et al. [20] have developed a quantification strategy for phosphor in protein gel spots which showed good detection sensitivity for β-casein.

In the present study we have developed a direct microlocal technique for the determination of phosphor concentrations in small protein spots in LA-ICP-MS. The combination of this technique with high resolution FT-ICR-MS is shown to be a powerful tool for the molecular identification and quantification of protein phosphorylation.

2. Experimental

2.1. FT-ICR-MS instrumentation and measurements

MALDI-FT-ICR-MS and ESI-FT-ICR-MS measurements on protein samples after separation by

2D gel electrophoresis and subsequent tryptic *in gel* digestion were performed with a Bruker Apex II FT-ICR instrument equipped with an actively shielded 7T superconducting magnet, a cylindrical infinity ICR analyzer cell, and external MALDI and ESI ion sources. A detailed description of instrumentations has been given elsewhere [7]. The MALDI source with pulsed nitrogen laser is operated at 337 nm, and ions are directly desorbed into a hexapole ion guide while being cooled during formation using Ar as the collision gas. Ions generated by five laser shots were accumulated in the hexapole at 15 V and extracted at 7 V into the analyzer cell. A 20 mg mL^{-1} solution of 2,5-dihydroxybenzoic acid (DHB; Aldrich, Germany) in acetonitrile, 0.1% trifluoroacetic acid in water (2:1) was used as the matrix. One microliter of matrix solution and 1 μL of sample solution were mixed on the stainless-steel MALDI sample target and allowed to dry. The ESI-FT-ICR spectra were obtained with an APOLLO (Bruker Daltonics) electrospray/nano-ESI source was coupled to the FT-ICR-spectrometer; a microchip ESI-FT-ICR system employed for the measurements of tau protein has been recently described [7]. Sample solutions of 0.5–5 μL (0.0005–0.01 μg μL^{-1}) were applied to the chip reservoir, and the chip fixed into the sample holder. The spray voltage increased slowly from 0 V to the working voltage of 1000–2300 V, and the onset of the spray was inspected with a microscope. Mass spectra were obtained by the acquisition of four 32 single scans at 45 70 V capillary exit voltage.

2.2. LA-ICP-MS instrumentation

A double-focusing sector field ICP-MS (ICP-SFMS, Element, Finnigan MAT, Bremen, Germany) coupled with a commercial laser ablation system LSX 200 (CETAC LSX 200, CETAC Technologies, Inc., Omaha, NE, USA) was used for the microlocal analysis of phosphor and sulfur in protein gel spots. The experimental arrangement is shown in Fig. 1. The ablated material is transported by argon as a carrier gas into the inductively coupled plasma (ICP). The ions formed in the ICP were extracted in the sector field mass spectrometer and separated according to their mass-to-charge (m/z) ratios. In order to separate interfering molecular ions from atomic ions P^+ and S^+, all LA-ICP-SFMS measurements were performed at a mass resolution $m/\Delta m$ of 4000. The $^{31}P^+$ ions are clearly separated from $^{15}N^{16}O^+$ and $^{14}N^{16}O^1H^+$ providing accurate phosphor determinations with only blank correction. Fig. 2 shows a mass spectrum at m/z 31 using a sector field ICP-MS at medium resolution.

The ICP torch was shielded with a grounded platinum electrode (GuardElectrode™, Finnigan MAT). For calibration a single gas flow solution-based procedure was developed using an ultrasonic nebulizer (USN; Aridus, CETAC Technologies Inc.). The main advantage of this arrangement is the possibility of simultaneously optimizing the nebulizer gas flow rate for USN and the carrier gas flow rate for the transport of laser-ablated material in ICP. The experimental parameters of LA-ICP-MS were optimized with respect

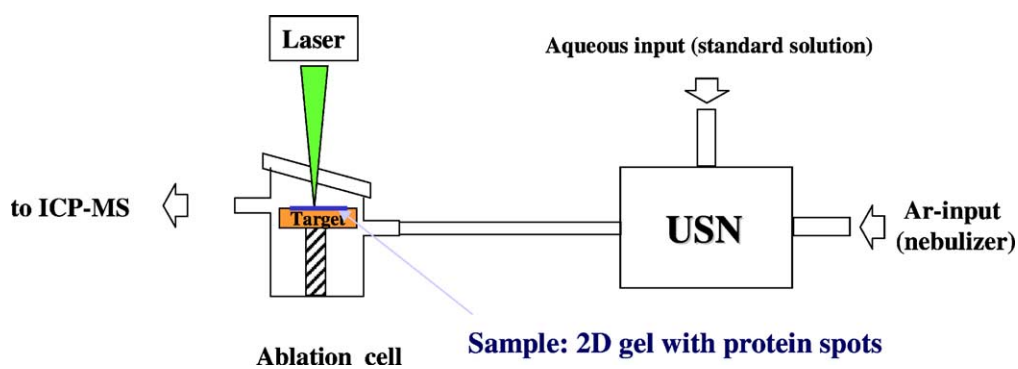

Fig. 1. Experimental set-up of solution calibration for P and S determination in gel spots by LA-ICP-MS.

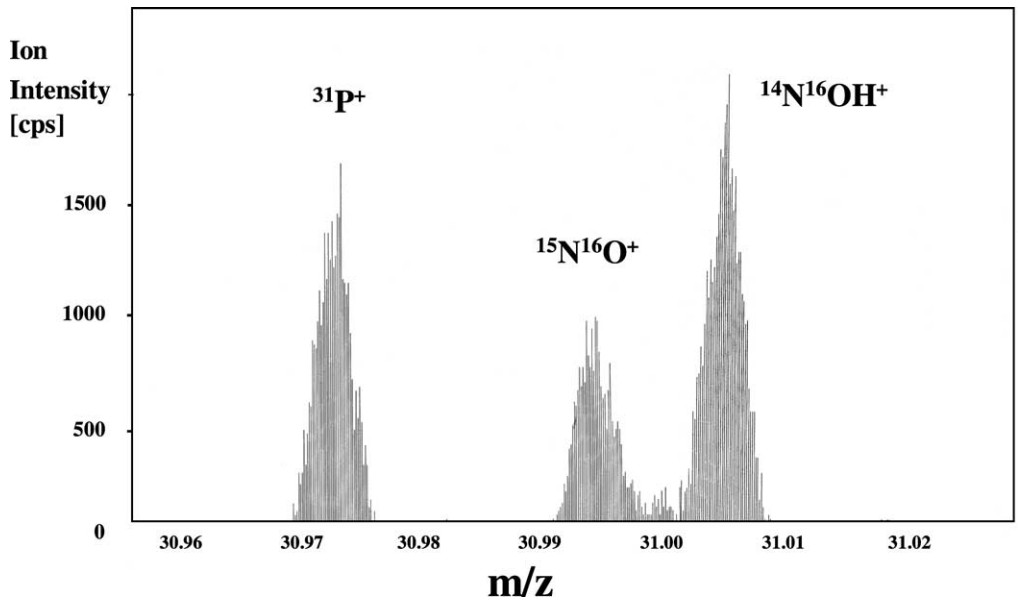

Fig. 2. Mass spectrum at m/z 31 measured in a digested protein sample by ICP-SFMS; mass resolution: $m/\Delta m \sim 4000$.

to the maximal ion intensity of $^{31}P^+$ using a 1 μg L^{-1} phosphorus solution introduced by the USN, which is coupled on-line to the laser ablation chamber. Maximal ion intensity was observed at a carrier gas flow rate of 1 L min^{-1} for the transport of ablated material to ICP-MS and an optimal mixing of nebulized standard solutions and laser-ablated solid sample directly in the ablation chamber is possible.

2.3. LA-ICP-MS measurement procedure

For phosphor determination in gel protein spots two different quantification strategies in LA-ICP-MS were applied: (i) the response for P and S in protein spots and in gel (blank) was determined by LA-ICP-MS, following the concentration of these elements in gel (blank) after trypsin and HNO_3 digestion had been measured using ICP-SFMS. Then the P (or S) concentration in protein spots can be calculated from the known response ratio P_{spot}/P_{gel} and P concentration in gel (or sulfur response ratio S_{spot}/S_{gel} and S concentration in gel); (ii) a new quantification procedure for direct microlocal analysis using a solution-based calibration, where an USN for the nebulization of calibration standard solutions of phosphor and sulfur was coupled to the laser ablation chamber. Cobalt was added as an internal standard element to the gel after separation of the protein mixture for correction of possible variations of gel ablation rate from point to point. Homogeneous distribution of Co was achieved by placing gel fragments into aqueous Co solution with 1 μg mL^{-1} Co concentration. In this work an external calibration procedure was applied by the nebulization of standard solutions in LA-ICP-MS, which had been previously developed for geological samples [22] and for high-purity graphite [23]. In order to achieve matrix matching phosphorus and sulfur standard solutions with increasing concentration (from 1 to 20 ng g^{-1}) are nebulized successively with the USN during solution calibration and the gel blank is simultaneously ablated with the focused laser beam. In this way a calibration curve and an optimal matrix matching is achieved. The protein spots in gel were measured using the same experimental arrangement where 1% nitride acid is simultaneously nebulized with the USN. Analyte concentrations (P and S) in the protein spots were calculated taking account of relative sensitivity coefficients (RSC) for P and S measured in advance

and expressed as the ratio of analyte responses in solution and in gel normalized to the concentration unit. The solution-based calibration can be used for simultaneous multi-element determination in gels. A certified standard (CRM) BCR-273 (single-cell proteins with a P concentration of $26.8 \pm 0.4\,mg\,g^{-1}$) was from IRMM (Geel, Belgium). BCR-273 represents a dried mixture of different proteins, in which only the average phosphor concentration is certified. The separation of this mixture by electrophoresis is not possible because the proteins are destroyed. Furthermore, BCR-273 is not suited for microlocal analysis because the inhomogeneity of the CRM might influence the measurement accuracy [10]. Therefore, this CRM was only used for testing the accuracy of determination of phosphorus in protein matrix by ICP-SFMS with liquid sample introduction. β-Casein is better suited for quality control in LA-ICP-SFMS since it has fewer modifications (genetic variants) and the phosphorylation states of this protein are known. The optimized experimental parameters of LA-ICP-SFMS and ICP-SFMS measurements are summarized in Table 1.

2.4. Standards and reagents

Concentrated nitric acid of supragrade purity from Merck (Darmstadt, Germany) was used for sample digestion. Phosphor and sulfur standard stock solution for the calibration procedures was obtained from Merck and from the National Institute of Standards and Technology (NIST). For all dilutions deionized Milli-Q water (18 MΩ) was obtained from a Millipore Milli-Q-Plus water purifier. Certified reference material (CRM) BCR-273 (single-cell proteins with the certified P concentration of $26.8 \pm 0.4\,mg\,g^{-1}$) was obtained from IRMM.

2.5. Samples and sample preparation

β-Casein (with unknown P concentration) for phosphorus and sulfur determination was obtained from Sigma (Deisenhofen, Germany) and was analyzed by LA-ICP-MS after 2D gel electrophoresis directly and by ICP-MS after digestion in solution. Phosphorus determination in protein spots from 2D gels was performed using the analytical methods for human serum and human tau protein (*E. coli*; Sigma). Proteins were digested with trypsin (Progema, Mannheim, Germany) and analyzed by MALDI- and ESI-FT-ICR-MS as previously described [7]. Other protein samples were directly analyzed by LA-ICP-MS following separation by 2D gel electrophoresis.

2.6. Protein separation by 2D gel electrophoresis

The 2D gel electrophoresis separation of serum samples was performed as described by Tissot et al. [24]. Isoelectric focusing (IEF) in the first step was

Table 1
Optimized experimental parameters used LA-ICP-SFMS and ICP-SFMS (Element, Finnigan MAT) for determination of phosphorus and sulfur in protein

Technique	LA-ICP-SFMS	ICP-SFMS
Laser ablation system	LSX 200 (CETAC)	
Nebulizer type	Ultrasonic nebulizer (for solution calibration)	Microconcentric
Spray chamber		Minicyclonic
RF power (W)	1250	1200
Cooling gas flow rate ($L\,min^{-1}$)	18	14
Auxiliary gas flow rate ($L\,min^{-1}$)	1.1	1.4
Nebulizer (carrier) gas flow rate ($L\,min^{-1}$)	1.3	0.7
Solution uptake rate ($mL\,min^{-1}$)	2	0.05
Ion extraction lens (V)	8000	8000
Mass resolution ($m/\Delta m$)	4000	4000
Analysis time (min)	5	5
No. of runs	6	20
No. of blocks of runs	5	6

carried out on Immobiline DryStrip gels (Immobilized pH Gradient Strip) with pH 4–7 and 3–10. All protein separations were performed in duplicate by using the selected separated proteins in parallel from one gel spot for analysis by FT-ICR-MS, and the second gel for P and S determinations directly by LA-ICP-MS.

3. Results and discussion

3.1. Separation and isolation of proteins by 2D gel electrophoresis

Fig. 3 shows an example of a 2D gel separation of human serum proteins with different well-separated protein spots (e.g., albumin, immunoglobulins light chain and heavy chain, serrotransferrin, apolipoprotein, antitrypsin). At the standard conditions of isoelectric focusing and separation (see Section 2), the major and medium-abundant proteins are well resolved within the Immobiline pH gradients 4–7 and 3–10. For comparative characterization, the major protein spots were excised from the gel using standard techniques and the gel-immobilized protein digested with trypsin, followed by extraction and MALDI- and ESI-MS analysis of the resulting peptides. Mass spectrometric proteome analysis using either MALDI-TOF- or MALDI-FT-ICR-MS provided unequivocal identification of proteins from all of the peptide mixtures analyzed, in agreement with identifications of serum proteins previously reported in the literature by using Swiss-Prot database and/or related databases [25] (results not shown). Several areas from these 2D gels were employed as references and gel blanks for phosphorus determinations by LA-ICP-MS, as described below.

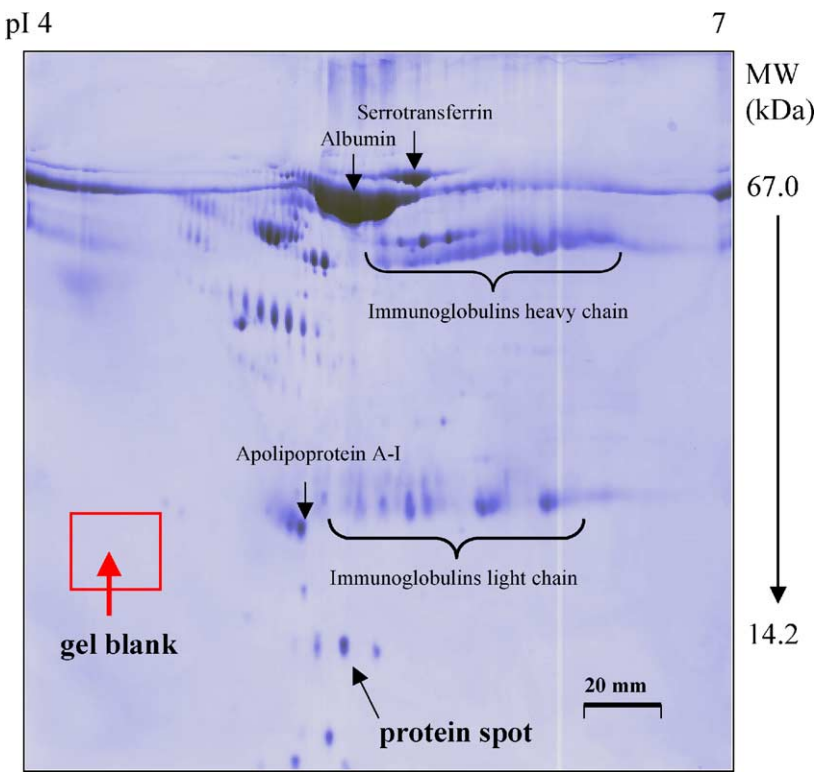

Fig. 3. 2D gel electrophoresis separation of human serum serum proteins within pH 4–7 using Immobiline gel strip; staining was performed with Coomassie Blue.

3.2. Identification of phosphorylation structure of tau protein by high resolution FT-ICR mass spectrometry

The methods for the identification and characterization of phosphorylated proteins by mass spectrometry generally include the following analytical procedure: (i) degradation of the phosphoprotein into small peptides by specific enzymatic treatment; (ii) separation of the phosphorylated peptides, with or without purification by metal–ion-affinity enrichment (IMAC); (iii) differential peptide mapping before and after alkaline phosphatase treatment; and (iv) mass spectrometric identification of phosphorylation sites using several fragmentation approaches (including MS/MS). The development of FT-ICR mass spectrometry has recently enabled a breakthrough for the high resolution mass spectrometric structure analysis of phosphorylated proteins using both ESI and MALDI ionization [26–29]; in combination with 2D gel electrophoresis the high (sub-ppm) mass determination accuracy and isotopic fine structure by FT-ICR-MS provide particular advantages for the identification of phosphorylated proteins with medium and low abundance. Fig. 4 shows the ESI-FT-ICR mass spectra of the trypsin-digested peptide mixture of human neurofibrillary tau protein within the *m*/*z* ranges 200–2200 and 1615–1630 (inset), respectively; identical results were obtained by conventional ESI-FT-ICR-MS and with a recently developed thin-film microchip [7]. The complete primary structure could be directly identified from the tryptic peptides, in part with very low abundances. In the peptide mixture a total of 18 serine and threonine phosphorylations were identified with their specific modification sites which clearly demonstrate the high analytical performance of the precise mass determinations by ESI-FT-ICR-MS. For example, the mass determinations of tryptic peptides provided the direct identification of 7 and 5 phosphorylation sites in the multi-phosphorylation domains (512–538) and (382–398) (T386, S388, T395, T396, S397); these phosphorylation “clusters” are assumed to play a particular functional role, e.g., for the supramolecular association of tau, which needs to be clarified in future biochemical studies.

The MALDI-FT-ICR mass spectra of tryptic peptides of tau protein provided complementary results corroborating the phosphorylation structure (data not shown). The complete primary structure and multi-phosphorylation pattern identified in tau is summarized in Fig. 5.

3.3. Phosphorus determinations in protein 2D gel electrophoresis bands by LA-ICP-SFMS

The direct microlocal analysis of proteins by LA-ICP-MS in gel bands is feasible within a short analysis time, in contrast to ICP-MS analysis of trypsin-digested protein spots excised from gels. At the initial stage of the present study the gel after 2D separation was dried for several days between two glass plates and then subjected to LA-ICP-SFMS analysis. With this procedure major problems in the phosphorus quantification were encountered without using an internal standard added to the gel before separation. Since LA-ICP-MS provides accurate analyte ratio measurements (e.g., P/S) S was suitable as an internal standard element. The following quantification procedure for the determination of P and S in protein spots was performed:

(i) Ion intensities for P^+ and S^+ in blank gel in the protein spots and near the spots of interest were determined using LA-ICP-MS (when ablating gel with 200 laser shots using single point analysis); as shown in Fig. 6, S intensities in gel blank and albumin spots were well reproducible (R.S.D. 1.9–3.6%).
(ii) Blank gel near the protein spots were excised and digested using HNO_3 as described in ref. [26].
(iii) P and S concentrations in the digested and diluted blank gel were determined by external calibration or standard addition.
(iv) Phosphorus and sulfur concentrations are then calculated from the ion intensity ratios of P^+/S^+ in gel and spots, measured via ICP-MS.

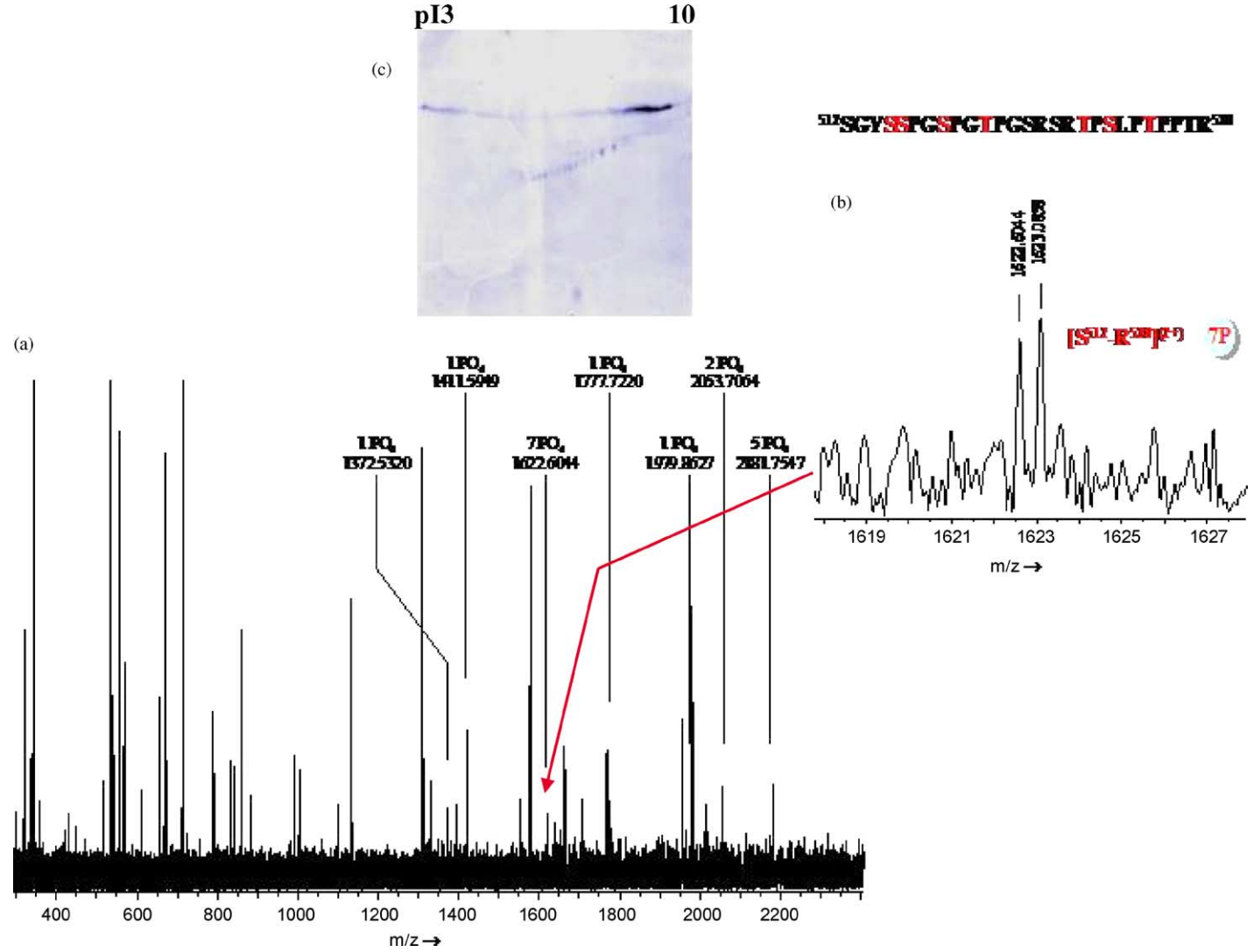

Fig. 4. ESI-FT-ICR mass spectrum of tryptic digest mixture of multi-phosphorylated human neurofibrillary tau protein. Mass assignments with *m*/*z* detection range, 200–2500, and partial sequences are shown for phosphorylated peptides. The inset shows the peptide fragment of the phosphorylation domain (512–538); the seven phosphorylation sites, identified using information of NiceProt View of Swiss-Prot database (primary accession number P10636), are indicated in red (C 113, H 191, N 36, O 61, P 7, doubly protonated; calc. 1622.55513, found 1622.60435, $\Delta = 30$ ppm).

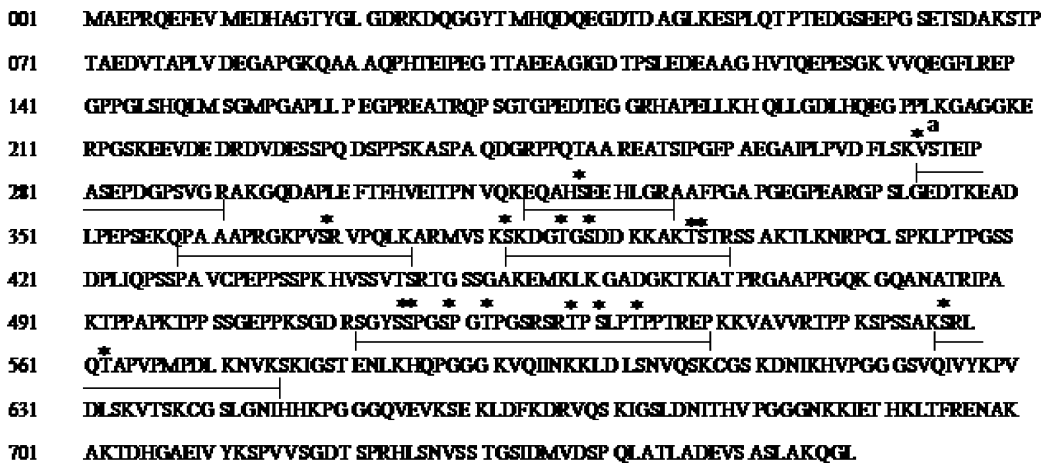

Fig. 5. Primary structure and phosphorylation sites of human tau identified by ESI-FT-ICR-MS. The 17 Ser- and Thr-phosphorylations are denoted by asterisks, underlined sequences denote phosphorylated tryptic peptides. (a) Phosphorylation position of tryptic peptide not assigned.

Phosphorus determinations in gel spots were limited by relatively high background concentrations of P and S as shown in Fig. 7 by transient signals at *m*/*z* 31 in gel blank and in the protein spot. It should be noted that impurities in the staining compounds might stick to the protein spots more extensively than to the blank gel. This is especially important for samples with high initial phosphorus concentration such as serum and cerebrospinal fluid, where free phosphate is present in cytoplasma as HPO_4^{2-} and in circulating fluids at millimolar concentrations. Even after separation of phosphate by gel electrophoresis-free phosphates and those neutralized by cations presented significant background intensity; therefore,

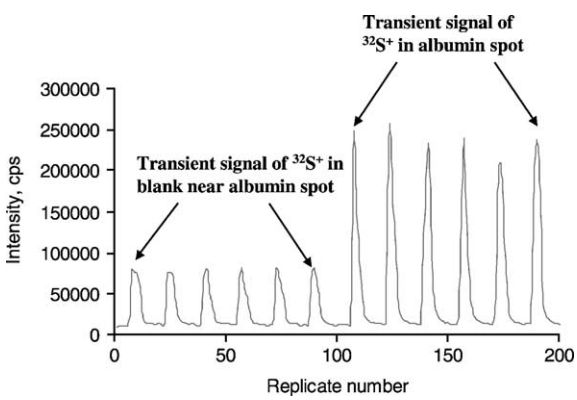

Fig. 6. Transient signals of $^{32}S^+$ intensity measured in gel blank and in albumin gel band (cf. Fig. 3).

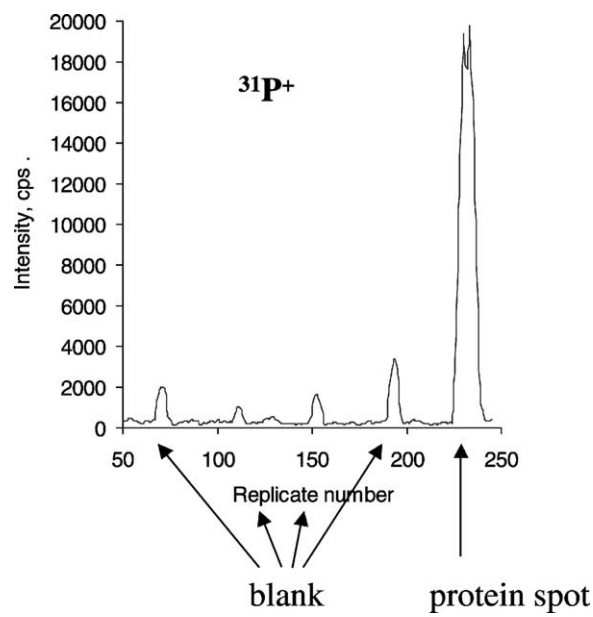

Fig. 7. Transient signals of $^{31}S^+$ in blank gel and protein spot measured by LA-ICP-SFMS.

phosphorus determination in protein spots is only possible if P^+ signals are at least 6σ over the mean background signal. In addition, relatively high concentrations of phosphor and sulfur originate from contamination by reagents used for sample and gel preparation. Hence, future investigations will focus on the development of "soft" non-destructive separation procedures with a high decontamination factor to allow full use of ICP-MS.

Fig. 8 shows an example of the transient ion signals of $^{31}P^+$ and $^{32}S^+$ measured by LA-ICP-SFMS with 200 laser shots using single point microlocal analysis. Concentrations of P and S in the blank gel were 0.11 and 0.39 mg g^{-1}, respectively, whereby the experiment was repeated three times (see signals 3, 4 and 5). Concentrations of P and S in a protein spot, calculated from the peak square ratio were 2.8 and 5.0 mg g^{-1}, respectively. Due to the high background of P and S in the gel the detection limit of LA-ICP-MS is relatively high so that in this investigation quantification in protein spots was not possible. Phosphorus concentrations in protein spots (assuming one phosphorylation site per protein molecule) were estimated to be detectable at $\geq 3 \times 10^6$ mol g^{-1}.

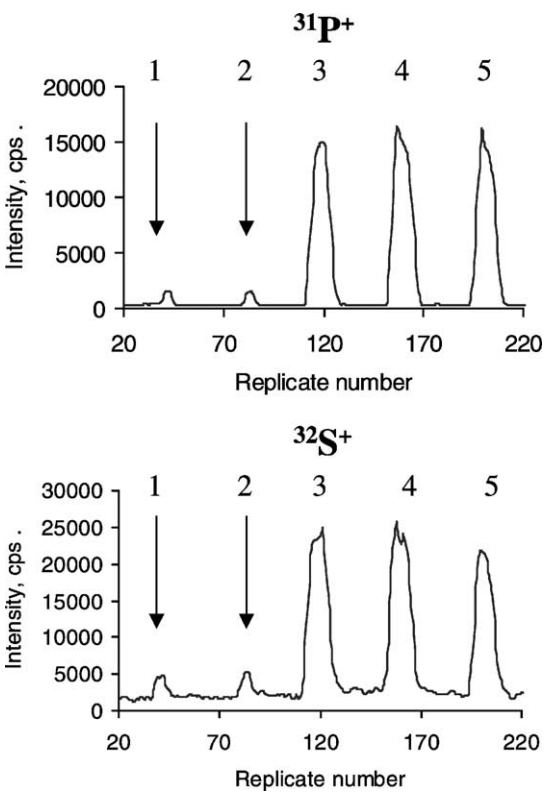

Fig. 8. Transient signals of $^{31}P^+$ and $^{32}S^+$ in blank gel and protein spots measured by LA-ICP-SFMS (1, 2: background signals in gel; 3, 4, 5: transient signals in protein spot).

A preliminary investigation via a depth profiling by microlocal LA-ICP-MS on protein spots showed that in most cases the maximum P and S concentrations were found on one of the surfaces of the gel. In general, concentrations of P and S changed with changing gel depth, but the ratio of P/S remained constant within measurement error. Location of proteins on the gel surface was also supported by microscopic investigation. Fig. 9 shows a magnification of a protein spot from human serum on the gel surface. Analysis of such objects in dried gel did not reveal any significant increase of phosphorus concentration above the gel background level. Therefore, wet gel was used for further analysis. Fig. 10 presents a fragment of gel surface after laser ablation (single craters with 0.3 mm diameter) together with a diagram of

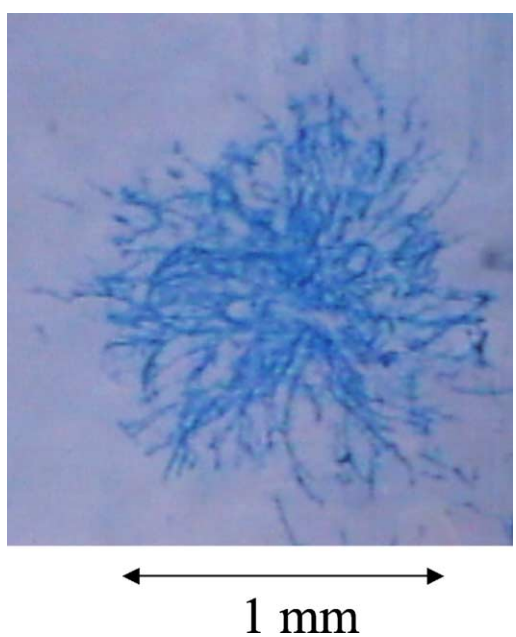

Fig. 9. Magnified surface of protein spot on 2D gel.

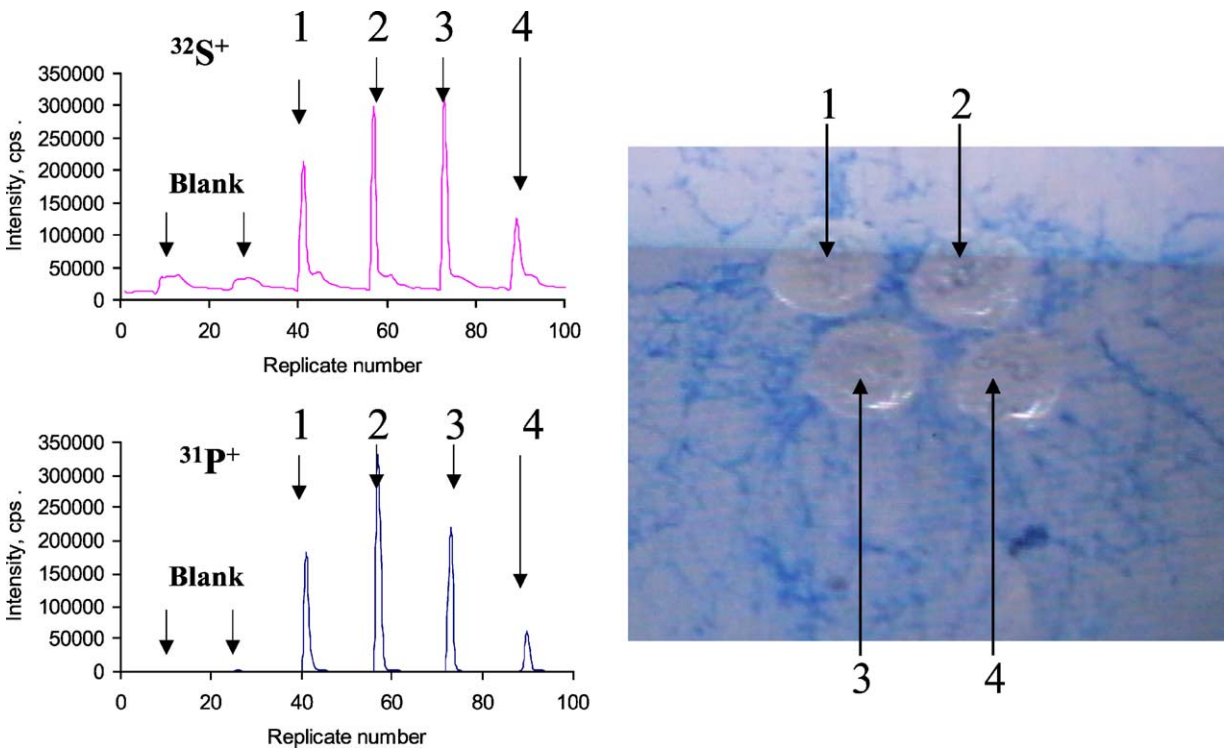

Fig. 10. Ablation craters on a protein spot (wet gel) and transient signals of $^{31}P^{+}$ and $^{32}S^{+}$ in blank gel and in the protein spots measured by LA-ICP-MS. Replicate numbers correspond to the numbers of craters.

the transient signal of $^{32}S^{+}$ and $^{31}P^{+}$ during ablation of blank gel and the protein spot, which was essentially background-free with sharp peaks of $^{32}S^{+}$ and $^{31}P^{+}$, suggesting that the signal originated from protein sputtered with a few laser shots. Thus, it was found that in the case of wet gel the proteins were effectively ablated from the gel surface, allowing the sensitive determination of P and S.

Application of this method to the phosphorylation analysis of β-casein provided S/P ratios in protein spots between 0.9 and 1.7. Bovine β-casein contains 5–8 methionine and cysteine residues, depending on genetic variation, and has 4–5 phosphorylation sites. Thus, S/P ratio is slightly higher than the value expected from the ratio of sulfur and phosphor atoms per molecule of β-casein (see Table 2) possibly due

Table 2
Phosphorus determinations and extent of phosphorylations in proteins by ICP-MS

Protein	Sample size (mg)	Molecular weight (Da)	P concentration ($mg\,g^{-1}$)	No. of phosphorylations	Phosphorylation concentration ($mol\,mol^{-1}$)	Phosphorylation extent (%)
β-Casein	0.001	25091	8.3 ± 0.8	4–5	6.7	∼25–50
Tau protein	0.001	78830	1.61 ± 0.12	17	∼4.0	∼23
Single-cell protein	1		27.5 ± 2.8			
CRM BCR-273			26.8 ± 0.4			

to excess of phosphate residues attached to the protein. When ablating proteins from the wet gel the analyzed material is evaporated rapidly from the gel surface. In the case of single ion detector ICP-MS the detector must switch from one isotope to another. In addition, the possible instability of the mass calibration at medium mass resolution in the ICP-SFMS applied required a relatively wide mass window to be scanned, which resulted in longer scanning time per one isotope and was disadvantageous when monitoring fast processes. In this case a multiple ion collector ICP-MS would be beneficial because it allows simultaneous measurement of both P and S.

4. Conclusions

In the present study we show that the combination of high resolution FT-ICR-MS and LA-ICP-MS is a powerful tool for the molecular characterization of phosphorylation structure, and quantitative phosphorylation state. The complementary data from both methods are particularly valuable in the case of multiple modifications where no other technique will provide corresponding molecular information. Thus, the example of human tau protein yields an average phosphorylation degree of ca. 20% based on the 17 phosphorylation sites identified (Table 2). By application of LA-ICP-MS as the microlocal analytical techniques together with a micronebulizer for solution calibration it is possible to quantify small protein spots in 2D gels; this application should be valuable for very low phosphorylation levels which require affinity-enrichment procedures. Furthermore, it was demonstrated that the application of sector field ICP-MS allows disturbing molecular ions $^{15}N^{16}O^{+}$, $^{14}N^{16}O^{1}H^{+}$ to be separated from analyte ions $^{31}P^{+}$, which is crucial for accurate phosphorus determinations in protein spots. The most important problems for the determination of phosphor in gel are possible contaminations during sample preparation. Future work will focus on minimizing the gel blank by improving the 2D gel electrophoretic separation of proteins from free phosphate in brain fluids.

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