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In-gel screening of phosphorus and copper, zinc and iron in proteins of yeast mitochondria by LA-ICP-MS and identification of phosphorylated protein structures by MALDI-FT-ICR-MS after separation with two-dimensional gel electrophoresis†

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A new screening technique using two-dimensional gels was developed in order to rapidly identify various elements in well-separated protein spots. Yeast mitochondrial proteins were separated using two-dimensional gel electrophoresis (blue native/SDS 2D-PAGE) and marked by silver staining. The 2D gels were systematically analyzed by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) using a double-focusing sector field instrument. From more than 60 mitochondrial protein spots in two-dimensional gels, phosphorus, sulfur and selected metals (Cu, Zn and Fe) were detected in a short analysis time by screening 2D gel with LA-ICP-MS using a focused laser beam. In selected protein spots a quantitative element determination was performed. Ion intensities of phosphorus and metals in single protein spots in the gels were measured at medium mass resolution using an optimized microanalytical method by LA-ICP-MS and in a solution of the gel (blank) after HNO_3 digestion by ICP-MS. For quantification purposes sulfur was used as the internal standard element. The detection limits for phosphorus, sulfur, copper, zinc and iron in protein spots, determined in the gel blank (Coomassie staining), were 0.18 μg g^{-1}, 1.3 mg g^{-1}, 6.4 μg g^{-1}, 17.6 μg g^{-1} and 9.5 μg g^{-1}, respectively. In silver staining gel a detection limit for sulfur of 137 μg g^{-1} was measured. Matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI-FTICR-MS) was applied for structure analysis and determination of phosphorylation sites of phosphorylated proteins. Results of the structure analysis of separated mitochondrial proteins obtained by MALDI-FTICR-MS were combined with those of the direct determination of phosphorus, sulfur and metal concentrations in protein spots in two-dimensional gels with LA-ICP-MS.

The proteomics area requires versatile tools allowing the detection of a particular feature within a large pool of proteins, *e.g.*, after electrophoretic separation in two-dimensional (2D) gels. The identification of singular protein spots in 2D gels is usually performed by organic mass spectrometry (*e.g.*, MALDI-MS) following the excision of the protein-containing gel area. Subsequent analysis of the derived peptide mixture upon tryptic digestion allows the determination of the post-translational modifications of proteins, *e.g.*, phosphorylation.[1–4] No screening method is yet available to detect post-translational modifications or bound metal ions (*e.g.*, Fe, Zn, Cu) directly from 2D gel separated proteins. Reversible protein phosphorylation is a key determinant in many fundamental cellular functions, such as survival, differentiation, structural organization, stress responses, and is relevant for many pathophysiological processes in carcinogenesis or neurodegenerative diseases, such as Alzheimer's and Parkinson's disease or Hallervorden–Spatz syndrome.[5]

In particular, the determination of phosphorus and metal concentration in very small amounts of protein samples, as is the case with protein spots of analytical gels, is of increasing interest.[1,4,6–8] We focused in our study on protein phosphorylation in yeast mitochondria. At present only a few phosphorylated mitochondrial proteins are known. Furthermore, only a few enzymes for phosphorylation (kinases) or dephosphorylation (phosphatases) of mitochondrial proteins have been identified up to now. The role of protein phosphorylation in yeast mitochondria is still unknown and remains to be elucidated. A motivation for this work is the study of the metal-binding proteins of mitochondria with a special focus on copper, iron and zinc ions. These elements play an important role as co-factors of proteins and are essential for their function. The only known mitochondrial enzyme complex that requires copper in order to function is the cytochrome oxidase (COX) of the respiratory chain. The delivery of copper from the cytoplasm to COX involves copper-binding proteins like Sco1p and Cox17p, but is still not fully understood.

Determination of P, Cu, Fe, Zn and other elements in very small protein volumes is a challenging task for analytical chemistry. Whereas for the element analysis of small protein solutions inductively coupled plasma mass spectrometry (ICP-MS) is the method of choice, laser ablation ICP-MS (LA-ICP-MS) is being used more and more frequently as a powerful microlocal analytical technique for fast element determination

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in solid biological and medical materials (plants or tissues), and nowadays also in protein research.[6–10] Special attention has recently been focused on the determination of phosphorus content in proteins selected from human brain (Alzheimer's disease).[10] In these studies LA-ICP-MS was applied for the microlocal analysis of selected protein spots in gels after two-dimensional (2D) gel electrophoresis. Due to the lack of suitable standard reference material, new strategies for direct microlocal analysis of phosphorus in protein spots using LA-ICP-MS were developed for the quantification of analytical data.[7,10] Recently, in our laboratory the application of a solution-based calibration strategy was proposed for quantitative phosphorus determination by LA-ICP-MS[6] and the simultaneous determination of P, S, Si, Al, Cu and Zn concentrations in human brain proteins (Alzheimer's disease).[10]

A problem with LA-ICP-MS and ICP-MS is the appearance of isobaric interferences, *e.g.*, the determination of phosphorus, iron and copper, is disturbed by the formation of molecular ions such as $^{15}N^{16}O^+$, $^{14}N^{17}O^+$ and $^{14}N^{16}O^1H^+$ at mass 31 u, $^{40}Ar^{16}O^+$ and $^{32}S_2{}^+$ at $^{56}Fe^+$ and $^{64}Zn^+$, respectively. Therefore, double-focusing sector field ICP-MS (ICP-SFMS) at a mass resolution ($m/\Delta m \sim 4400$) has been applied for the separation of these isobaric interferences by molecular ions for P, Fe and Zn determination in small protein samples. Another possibility for solving interference problems is the use of ICP-MS with a dynamic collision/reaction cell, *e.g.*, for P determination *via* the measurement of interference-free $^{31}P^{16}O^+$ molecular ions.[11,12] This technique is helpful for phosphorus determination if no sector field ICP-MS is available, but under optimized experimental conditions (using a quadrupole-based ICP-CC-QMS with hexapole collision cell from Micromass) significantly higher detection limits for phosphorus determination in aqueous solution were observed. By using ICP-CC-QMS the detection limit for phosphorus in aqueous solution was found in our laboratory to be 1.3 ng g^{-1} compared with ICP-SFMS with 20 pg g^{-1}.[6,11] With decreasing amount of protein the danger of contamination during sample preparation increases, therefore a direct analytical method for the determination of phosphorus and metals in protein spots in gels such as LA-ICP-MS is helpful. Phosphorus determination in protein samples in a one-dimensional gel[13] or two dimensional gels by LA-ICP-MS has been reported in different papers.[7,8,10] Recently, different analytical ways in phosphoproteomics and metallomics as a new frontier in analytical chemistry and the combination of atomic and molecular (organic) mass spectrometry were reported by Szpunar[14] and Wind and Lehmann.[15]

Phosphorylation of proteins and metal concentrations can be determined *via* the quantitative analysis of phosphorus and metals.[7,10] In addition to atomic mass spectrometric determination of element concentration, the identification and structure determination of investigated proteins is required by organic mass spectrometry, including the determination of post-translational modifications such as glycosylation, fatty acylation and phosphorylation. Mass spectrometers that employ soft ionization techniques (electrospray ionization–ESI and matrix-assisted laser desorption ionization–MALDI) permit the identification of large biomolecules such as proteins.[16–18] While MALDI and ESI mass spectrometry can be used for the identification of phosphorylation sites in proteins, these techniques cannot provide direct quantitative determinations of phosphorus and metals in biological samples.

In the present study, a direct microlocal analytical technique was developed for the simultaneous multielement determination of phosphorus, sulfur and metal concentrations *via* in-gel screening in small protein spots in two-dimensional gels by LA-ICP-MS. The combination of LA-ICP-MS with high-resolution MALDI-FTICR-MS is used as a powerful tool for the molecular identification and quantification of protein phosphorylation as well as other element concentrations.

Experimental section

LA-ICP-MS instrumentation

A double-focusing sector-field ICP-MS (ICP-SFMS, ELEMENT, Thermo Electron, Bremen, Germany) coupled with a commercial laser ablation system LSX 200 (CETAC LSX 200, Cetac Technologies, Omaha, NE, USA) was used for the microlocal analysis of phosphorus, metals and sulfur in protein gel spots. The ablated material was 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 ratios. In order to separate interfering molecular ions from atomic ions P^+, S^+, Cu^+, Zn^+ and Fe^+, all LA-ICP-SFMS measurements were performed at a mass resolution $m/\Delta m$ of 4400. As demonstrated in a previous paper,[19] all analyte ions were measured without interference problems, *e.g.*, the $^{31}P^+$ ions are clearly separated from $^{15}N^{16}O^+$ and $^{14}N^{16}O^1H^+$ molecular ions. The ICP torch was shielded with a grounded platinum electrode (GuardElectrode™, Thermo Electron). For optimization of experimental parameters an ultrasonic nebulizer (USN, CETAC Technologies Inc., Omaha, Nebraska, USA), which is coupled on-line to the laser ablation chamber, was used.[7] The background intensity of P, S, Cu, Fe and Zn was determined after microwave digestion of a small cut of the blank gel and measurement using ICP-SFMS. The optimized experimental parameters of LA-ICP-SFMS and ICP-SFMS measurements are summarized in Table 1.

Table 1 Experimental parameters for LA-ICP-SFMS and ICP-SFMS (ELEMENT, Thermo Electron) for determination of P, S, Fe, Cu and Zn in protein spots and blank gel

	LA-ICP-SFMS	ICP-SFMS
Laser ablation system	LSX 200 (CETAC)	
Wavelength of Nd:YAG laser/nm	266	
Laser energy per pulse/mJ	5	
Laser power density/W cm^{-2}	5×10^8	
Nebulizer type	USN (for calibration)	Microconcentric
Spray chamber	With desolvator	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}	1.32	0.7
Solution uptake rate/mL min^{-1}	2	0.05
Ion extraction lens voltage/V	2000	2000
Mass resolution, ($m/\Delta m$)	4400	4400
Analysis time/min	5	5
Number of runs	6	20
Number of blocks of runs	5	6

LA-ICP-MS measurements and quantification procedure

The separated protein spots in 2D gels (Fig. 1) were screened using LA-ICP-MS at medium mass resolution at a mass-to-charge ratio of 31, 32, 56, 63 and 64 with respect to the occurrence of P, S, Fe, Cu and Zn. Several protein spots containing P, S, Cu, Zn and Fe were selected for further studies. The quantification procedure was performed by measuring ion intensities for $^{31}P^+$, $^{63}Cu^+$, $^{64}Zn^+$ and $^{56}Fe^+$ in the protein spots as described in literature.[10] The concentrations of these elements in the blank gel marked in Fig. 1 were determined after microwave digestion by ICP-SFMS. The element concentration $^{sp}C_X$ (with X = P, Cu, Zn and Fe) in protein spots was determined with respect to the known sulfur concentration (if sulfur-containing protein was identified by MALDI-FTICR-MS, and the number of S atoms was determined) using the formula described in literature.[10]

MALDI-FT-ICR-MS instrumentation and measurements

MALDI-FTICR-MS measurements on protein samples after separation by 2D gel electrophoresis and subsequent tryptic in-gel-digestion were performed with a Bruker Apex II FTICR instrument equipped with an actively shielded 7 T superconducting magnet, a cylindrical infinity ICR analyzer cell, and an external MALDI ion source. A detailed description of this instrumentation has been given elsewhere.[20] 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 10 laser shots were accumulated in the hexapole at 15 V and extracted at 7 V into the analyzer cell. A 100 μg 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. A volume of 1 μL of matrix solution and 1 μL of sample solution were mixed on the stainless-steel MALDI sample target and allowed to dry.

Standards and reagents

Concentrated nitric acid of supragrade purity from Merck (Darmstadt, Germany) was used for sample digestion. Phosphorus, sulfur, iron, copper and zinc standard stock solutions for the calibration procedures were obtained from Merck (Darmstadt, Germany) and from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). For all dilutions, deionized Milli-Q water (18 MΩ) was obtained from a Millipore Milli-Q-Plus water purifier.

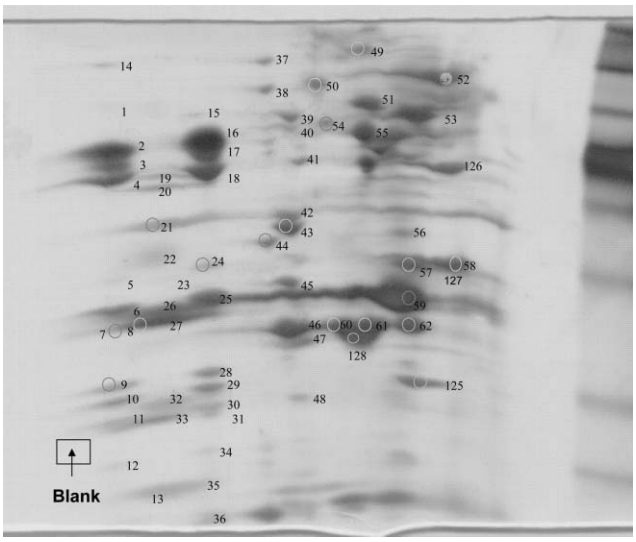

Fig. 1 2D gel electrophoretical separation of proteins from yeast mitochondria; staining was performed with silver.

Samples and sample preparation

Protein mixtures from yeast mitochondria were analyzed directly with respect to phosphorus, sulfur, copper, zinc and iron content by LA-ICP-MS after 2D gel electrophoresis and

Table 2 Qualitative results of analysis of protein spots obtained by screening of 2D gel by LA-ICP-MS[b]

Number of spot	P	S	Cu	Zn	Fe
1	−	−	−	−	−
2	+	−	−	−	−
3	−	−	−	−	−
4	−	+	−	−	−
5	−	+	−	−	−
6	+	+	−	−	−
7	++	++	+	++	++
8	++	++	−	++	+
9	++	+	−	−	−
10	+	−	−	−	−
11	−	++	−	−	−
12	−	++	−	−	−
13	++	−	−	−	−
14	−	−	(+)	+	−
15	−	−	−	−	+
16	+	+	−	−	+
17	−	++	−	−	−
18	−	−	−	−	−
19	−	++	−	−	−
20	−	++	−	−	−
21	++	+	+	+	−
22	−	−	−	−	−
23	−	−	−	−	−
24	−	+	++	++	++
25	+	++	−	−	−
26	−	+	−	−	−
27	−	−	−	−	−
28	−	++	−	+	+
29	−	++	−	+	−
30	+	−	+	+	−
31	+	−	+	+	−
32	+	−	+	+	−
33	+	−	+	+	−
34	−	−	+	+	−
35	+	++	+	+	−
36	+	−	+	+	−
37	−	−	−	−	−
38	−	+	−	++	−
39	−	+	−	−	++
40	−	+	−	−	−
41	−	+	−	+	−
42	−	+	−	−	−
43	++	−	−	−	−
44	+	−	++	−	+
45	−	−	−	+	−
46	−	+	+		−
47	−	+	+	−	−
48	−	−	+	−	−
49	++	+	++	++	n.d.[a]
50	+	−	++	+	n.d.
51	+	−	−	−	n.d.
52	+	−	++	+	n.d.
53	−	−	−	−	n.d.
54	+	+	−	−	n.d.
55	−	−	−	−	n.d.
56	−	−	−	−	n.d.
57	+	−	−	−	n.d.
58	−	−	−	−	n.d.
59	++	+	++	+	+
60	++	+	+	+	+
61	+	+	+	+	++
62		+		++	+
125	−	+	+	+	+
126	−	−	−	−	−
127	+	+	+	++	++
128	+	+	+	+	++

[a] n.d.: not determined. [b] ++ ion intensity was 10× higher than the background signal.

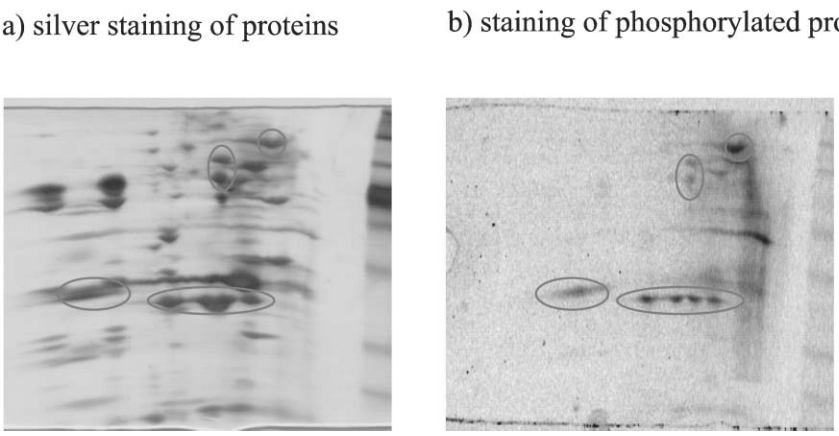

Fig. 2 Comparison of mitochondrial yeast proteins from in silver-stained 2D gel (a) and phosphorus-stained 2D gel (b): phosphorus-containing proteins are marked.

by ICP-MS after microwave digestion. Proteins were digested with trypsin (Progema, Mannheim, Germany) and analyzed by MALDI-FTICR-MS as previously described.[20]

Protein separation by two-dimensional gel electrophoresis and *in-gel* staining of phosphorylated proteins

Mitochondria of lactate-grown yeast cells of wild-type strain BY4741 (EUROSCARF acc.no Y10000) were isolated and lysed by the mild detergent digitonin (detergent : protein ratio = 4 : 1) in the presence of phosphatase inhibitor mix I and II (Sigma, Germany) and proteinase inhibitor mix (Roche, Mannheim, Germany). 200 μg of mitochondrial proteins was separated in a 5–13% blue native gel according to Schägger.[26] Strips from the first dimension BN-PAGE were excised, incubated in 1% SDS solution with 1% mercaptoethanol for 2 h and used for a second dimension SDS-PAGE. Proteins were stained using ProteoSilver™ Silver Stain Kit (Sigma) for LA-ICP-MS or by Coomassie G250 (Serva, Heidelberg, Germany) for MALDI-FTICR-MS or LA-ICP-MS. All protein separations were performed in duplicate by using the selected separated proteins in parallel from one gel spot for analysis by MALDI-FTICR-MS, and the second gel for P, S, Cu, Zn and Fe determinations directly by LA-ICP-MS.

Staining of phosphorylated proteins in gels was performed with the Pro-Q Diamond phosphoprotein gel stain (Molecular Probes).

Results and discussion

Separation of mitochondrial proteins and screening of protein spots in two-dimensional gels by LA-ICP-MS

Yeast mitochondrial protein complexes were separated by BN-PAGE as described in the Experimental part. Separation of the complex constituents was performed by a second dimension under denaturing conditions (SDS-PAGE). Protein spots were stained with silver (Fig. 1) or Coomassie and subjected to a systematic screen for various elements (P, S, Cu, Zn and Fe) by the new screening analytical technique using LA-ICP-SFMS. This technique enables the fast detection of protein spots within the gel matrix with accumulations of various elements (P, Cu, Zn and Fe). Table 2 summarizes the results of the qualitative analysis of more than 60 protein spots in 2D gels of yeast mitochondria. Whereas in some protein spots (spot numbers 1, 3, 18, 22, 23, 27, 37, 55, 56 and 58) none of the elements of interest were found, in some protein spots (*e.g.*, 7, 49, 59, 60 and 61) in part high ion intensities of the analytes were measured. In several proteins only selected elements are present (*e.g.*, high P content in spot 13 or 43). Abundant phosphorylated proteins can also be detected by a special fluorescent staining technique.[23] Compared to this staining technique the LA-ICP-SFMS has a significantly higher sensitivity. Spots of phosphorylated proteins, *e.g.*, 59, 60, 61, 62, 125 and 128, marked by the phosphorus staining technique were investigated by LA-ICP-MS and MALDI-FTICR-MS. In Fig. 2(b) phosphorylated protein spots are marked and compared with the silver-stained 2D gel of yeast mitochondrial proteins (see Fig. 2(a)). 29 proteins containing phosphorus were found by the microlocal analysis of protein spots in 2D gel. A further feature of LA-ICP-SFMS is that the screening of separated protein spots in a two-dimensional gel and the qualitative measurements of a multitude of protein spots was performed in just a few hours. This is the main advantage of this analytical screening procedure, because the time required for structure analysis can be reduced significantly by a preselection of protein spots containing P, S and metals of interest using LA-ICP-SFMS. After a qualitative survey analysis several protein spots from yeast mitochondria containing P, S, Cu, Zn and Fe were analyzed in more detail with respect to their structure and sequence by MALDI-FTICR-MS. Although MALDI-FTICR-MS is an excellent tool for quickly identifying proteins and determining the structure and mass of proteins with high mass accuracy, one should bear in mind that this does not necessarily reflect their physiological state. The denaturing and reducing conditions during the separation of proteins in the second dimension may lead to a partial release of the naturally bound metal ions. However, we cannot exclude the possibility that contamination by metal ions may be bound after removal of the denaturing and reducing conditions during washing and staining steps. This may explaine the

Table 3 Identified proteins using MALDI-FTICR-MS

Spot	Protein	M.W./kDa (exp.)	M.W./kDa	S atoms
49	Ach1p (acetyl-CoA hydrolase)	~100–120	58.68[a]	17
50	Sdh1p (yeast succinate dehydrogenase)	~70–80	70.19	26
52	Aco1p (aconitase, mitochondrial)	~80–90	85.31	23
59	Aac2p (major mitochondrialATP/ADP translocator)	~35–40	34.40	14

[a] monomeric or mannose-glycosylated state

detection of protein spots in which more than one metal ion was detected, although there are a number of examples of mitochondrial proteins, *e.g.*, Cox1p, which bind several metal ions simultaneously.

In contrast, phosphorus is covalently attached to the protein and will not be removed by the electrophoretic procedure. To avoid dephosphorylation of the proteins by phosphatases a mixture of phosphatase inhibitors was added throughout the isolation and the lysis of mitochondria.

Identification of proteins by high-resolution MALDI-FTICR-MS

The methods for the identification and characterization of proteins by mass spectrometry generally include the following analytical procedure: (i), degradation of the protein into small peptides by enzymatic treatment; and (ii), differential peptide mapping before and after alkaline phosphatase treatment. The development of FTICR mass spectrometry has recently enabled a breakthrough in the high-resolution mass spectrometric structure analysis of proteins using MALDI ionization,[3,20,24] in combination with 2D gel electrophoresis,[21] the high (sub-ppm) mass determination accuracy and isotopic fine structure of FT-ICR-MS providing particular advantages for the identification of proteins of medium and low abundance. The complete primary structure of selected proteins could be directly identified[22] after the tryptic digestion by MALDI-FTICR-MS.

Results of the mass spectrometric analysis of protein spots in 2D gels are summarized in Table 3. For example, spot 49 was identified as Ach1p (acetyl CoA hydrolase) with a molecular weight of 58.68 kDa. In the 2D gel the protein was located in a mass range between 100 and 120 kDa, possibly reflecting either its dimeric state or its mannose-glycosylated state. (The proteins identified by MALDI-FTICR-MS, which have molecular weights between 34–85 kDa, contain 14–26 sulfur atoms.) Spot 59 represents Aac2p, the major mitochondrial ADP/ATP translocator (Fig. 3(a)). Sdh1p, a subunit of complex II of the respiratory chain, was detected in spot 50.

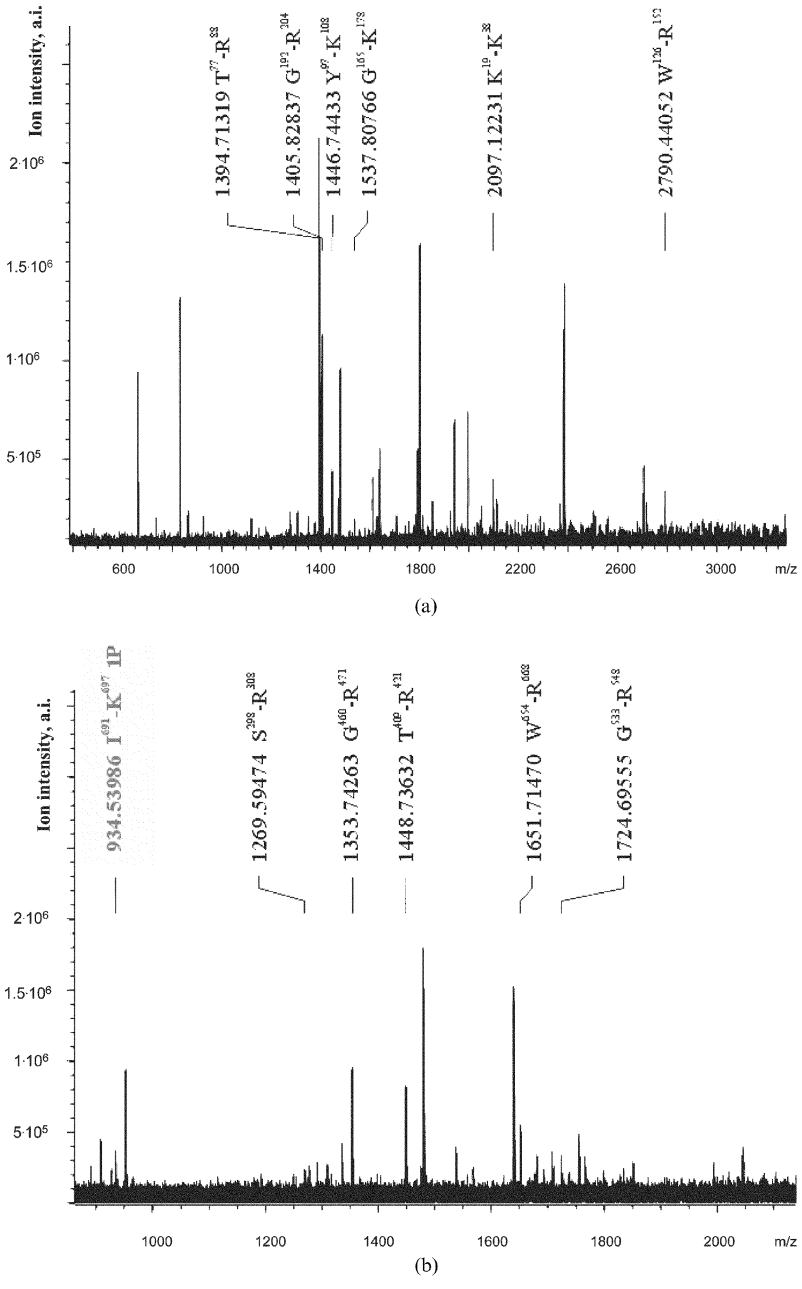

Fig. 3 (a) MALDI-FTICR mass spectrum of spot 59 (major mitochondrial ADP/ATP translocator) with the identified peptides; (b) MALDI-FTICR mass spectrum of spot 52 (Acotinase, mitochondrial) with the identified peptides and phosphopeptides.

Sdh1p binds FAD covalently (FAD contains two P-atoms) and was recently described as a phosphorylated protein in potato mitochondria.[28] Aco1p (spot 52), the mitochondrial aconitase, contains iron–sulfur cluster and is phosphorylated in potato mitochondria.[28] The observed molecular weight of proteins in spots 50, 52 and 59 in the 2D gel corresponds to the calculated value.

Determination of element concentrations in protein spots after separation with 2D gel electrophoresis by LA-ICP-SFMS

LA-ICP-MS is a promising and powerful surface analytical technique for the direct μ-local analysis of protein spots in a two-dimensional gel, by means of which the occurrence of P, S, Cu, Zn, Fe and other elements in proteins can be detected fast and simultaneously *via* a multielement survey analysis. Due to the relatively high background of some elements studied in gels (especially of sulfur in gels using Coomassie for staining) it is sometimes difficult to determine element concentration in protein spots.[6]

Calibration way of phosphorus-containing proteins. In order to quantify measured ion intensities by LA-ICP-SFMS and to determine the amount of phosphorylated proteins, one-dimensional calibration gels containing different amounts of ovalbumin (see Fig. 4) were analyzed. In Fig. 4a transient signals of phosphorus ion intensities of 500, 100, 10, 1 and 0.1 ng of ovalbumin are shown, and a corresponding calibration curve is given in Fig. 4b. Owing to inhomogeneous protein distribution (no, one or two sites of ovalbumin may be phosphorylated) in one-dimensional gel the correlation curve has a correlation coefficient of only 0.89. The unknown element concentration in proteins with similar matrix composition can be determined *via* external calibration.

Results of element determination in selected protein spots. Phosphorus was detected in proteins of spots 49 (Ach1p), 50 (Sdh1p), 52 (Aco1p) and 59 (Aac2p). So far there are no published data available concerning the phosphorylation of Ach1p and Aac2p. The results for Sdh1p and Aco1p are in agreement with those of previous phosphorylation analysis in plant mitochondria.[28]

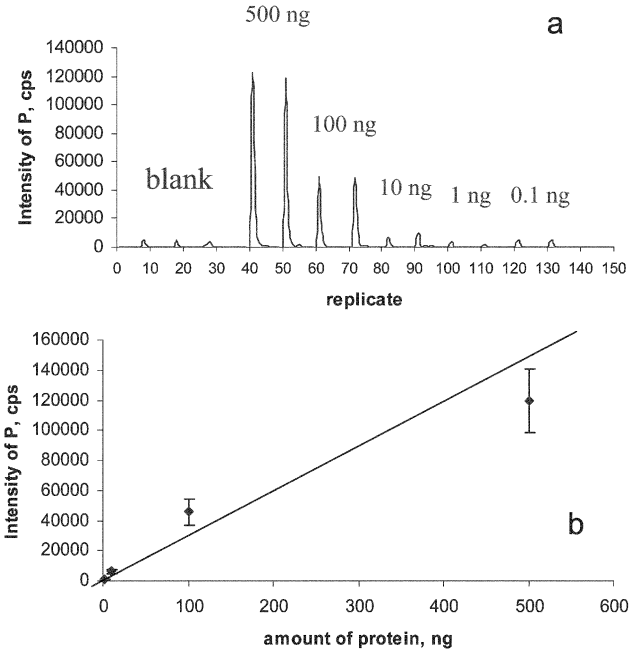

Fig. 4 a, Transient signals of P measured in protein spots of ovalbumin measured by LA-ICP-SFMS at mass resolution $m/\Delta m$ = 4400; b, calibration curves for P in protein spots for phosphorus in ovalbumin measured by LA-ICP-SFMS.

The authors showed that both proteins are phosphorylated in plant mitochondria. In addition, Sdh1p binds FAD (contains 2 P) covalently, which may also be responsible for the detected signal. To confirm the phosphorylation of Aco1p MALDI-FTICR-MS was performed to identify the phosphopeptides (Fig. 3(b)). We could identify a phosphorylated peptide which comprises the aminoacid sequence from I691 to K697 (IHETNLK). The phosphorylated residue in the peptide might be threonine 694 or histidine 692. This finding shows that the applied method is suitable for detecting phosphorylated proteins in 2D gels.

The principle of this method for detection of metal containing proteins will be tested by analyzing the mitochondrial aconitase (spot 52) in a future work. This enzyme binds 1 4Fe-4S cluster per subunit. Fe was found by LA-ICP-MS and we detected also Cu and Zn in this spot, indicating a nonspecific accumulation of other metal ions. As mentioned above, the separation procedure of proteins interferes with the natural status of the proteins and therefore might change the authentic metal compositon of a protein.

In addition, we screened for further proteins, which contain metals. By investigating protein spots in the horizontal middle of the 2D gel (protein spots from numbers 37–48) with LA-ICP-SFMS mostly single elements (*e.g.*, in spot 38 Zn, spot 39 Fe; spot 43 P and spot 44 Cu) of higher concentrations were found. Most of the proteins contain Cu, Zn and Fe as well. Transient ion signals of $^{31}P^+$, $^{32}S^+$, $^{63}Cu^+$, $^{64}Zn^+$ and $^{56}Fe^+$ in different protein spots measured by LA-ICP-SFMS with 500 laser shots using single-point microlocal analysis are shown in Fig. 5. Each figure contains the 2D gel region of interest for better orientation. For example, protein spot 59 (Aac2p) shows relatively high P^+ and Cu^+ intensity; S, Zn and Fe were also detected.

The results of the measurement of element ratios in respect to sulfur (P/S, Cu/S, Zn/S and Fe/S) in selected protein spots determined in silver stained 2D gels are summarized in Table 4. The element ratios were always found to be lower than 1. Metal : sulfur ratios, *e.g.* Fe : S and Mn : S, were also applied to characterize metalloprotein samples by size exclusion chromatography hyphenated to inductively coupled plasma mass spectrometry with dynamic reaction cell technology (SEC-ICP-DRCMS) by Hann *et al.*[25]

The results of the quantification procedure using sulfur as an internal standard element for protein spot 59 are summarized in Table 5, which also contains the detection limits of elements measured by ICP-SFMS in the gel blank (Coomassie-stained) after digestion. Relatively high detection limits for S of 1.3 mg g^{-1} were found for the staining technique using Coomassie. Fig. 6 shows selected transient signals for $^{32}S^+$ using silver staining. Significantly lower ion intensities were measured at mass 32 u for blank gel, therefore the signal to noise ratios for the protein spot can be improved, as described recently.[10] The measurements by LA-ICP-MS result in lower detection limits, especially for S in the μg g^{-1} range.

Conclusions

In the present study, protein spots in two-dimensional gels were screened with respect to P, S, Fe, Cu and Zn content, and multielement determination was investigated in separated protein spots by LA-ICP-MS as the microlocal analytical technique. A combination of high-resolution MALDI-FTICR-MS and LA-ICP-SFMS represents a powerful tool for the identification of proteins from mitochondria and determination of element concentrations of P and selected metals in selected proteins. The complementary data from the two methods are particularly valuable in the case of multiple modifications where no other currently available technique will provide corresponding molecular information. The phosphorus

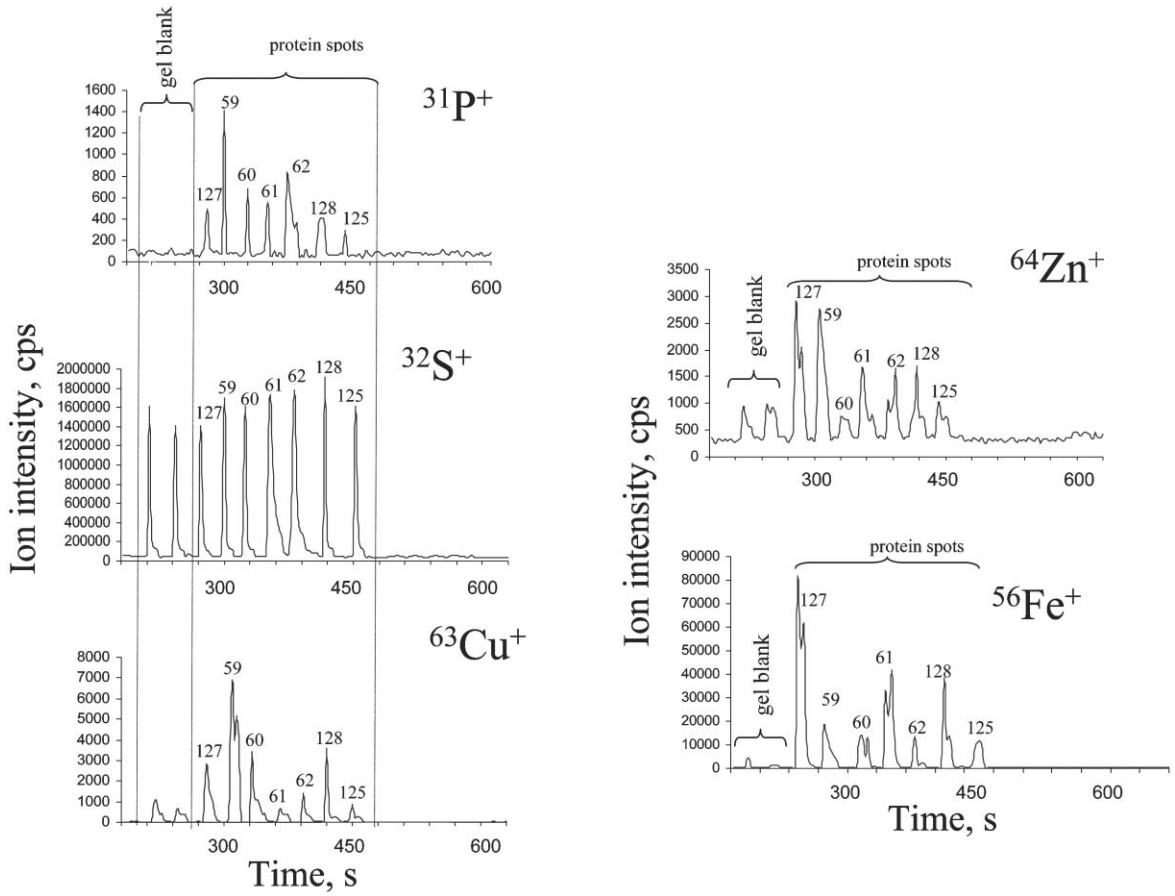

Fig. 5 Transient signals for $^{31}P^+$, $^{32}S^+$, $^{56}Fe^+$, $^{63}Cu^+$ and $^{64}Zn^+$ in protein spots of yeast mitochondria separated by 2-dimensional gel electrophoresis (after Coomassie staining) measured by LA-ICP-SFMS.

Table 4 Element ratios in protein spots measured by LA-ICP-MS[a]

Element ratios	Spot 59	Spot 60	Spot 61	Spot 62
P/S	0.005	0.003	0.001	0.002
Cu/S	0.021	0.013	—	0.002
Zn/S	0.006	—	0.002	0.002
Fe/S	0.03	0.044	0.117	0.024

[a] Relative standard deviation (RSD) of measured element ratios is about 30%

Table 5 Element concentration measured in protein spot 59 in 2D gel by LA-ICP-MS and limit of detection in blank gel (ICP-SFMS)

		Limit of detection/μg g^{-1}	
	Concentration[a]/mg g^{-1}	Coomassie staining	Silver staining
P	0.027	0.18	0.22
S	7.17	1327	137
Cu	0.94	6.4	8.2
Zn	3.4	17.6	20
Fe	0.87	9.5	n.d.[b]

[a] Relative standard deviation (RSD) of measured element concentration is about 30%. [b] n.d.: not determined.

and metal concentrations in the mg g^{-1} and sub-mg g^{-1} range were determined in selected protein spots. We could show that the method is suitable to detect phosphorylated proteins. The most important problems for the determination of phosphorus and other elements in the gel are the preservation of natural metal binding properties of the proteins and possible contaminations during sample preparation or by staining. Future work will focus on improving the screening technique using a laser ablation system with better lateral resolution and development of further quantification procedures, especially for proteins which do not contain sulfur.

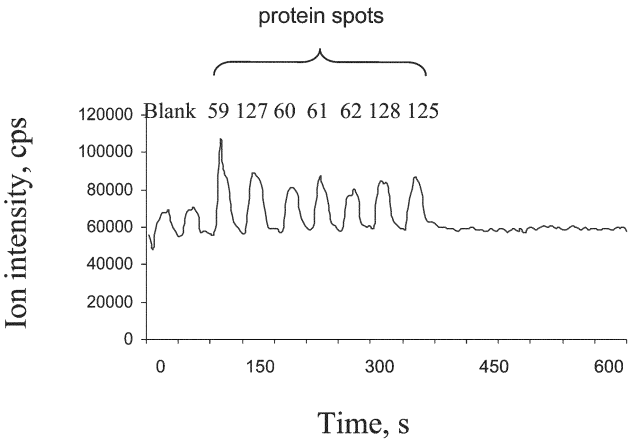

Fig. 6 Transient signals for $^{32}S^+$ in selected protein spots of yeast mitochondria separated by 2 D gel electrophoresis (after silver staining) measured by LA-ICP-SFMS.

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