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Detection of phosphorylated subunits by combined LA–ICP–MS and MALDI–FTICR–MS analysis in yeast mitochondrial membrane complexes separated by blue native/SDS-PAGE

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Abstract

We report on the identification of phosphorylated subunits of yeast mitochondrial ATPase using a novel screening technique in combination with BN/SDS-PAGE. Protein complexes present in yeast mitochondrial membranes were separated in their native state in the first dimension and their subunit composition was resolved by SDS-PAGE in the second dimension. Laser ablation inductively coupled plasma mass spectrometry (LA–ICP–MS) was used to rapidly screen for the presence of phosphorus in the subunits. The detection limits of elements investigated in selected protein spots are in the low $\mu g\,g^{-1}$ concentration range. Sulfur was used as the internal standard element for quantification. Phosphorus was detected in two of the proteins, that were identified by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry (MALDI–FTICR–MS) as subunits Atp1p and Atp2p of the ATPase. These results were confirmed by Western blot analysis using antibodies directed against phosphorylated amino acids. The combination of LA–ICP–MS and MALDI–FTICR–MS with BN/SDS-PAGE provides a fast and sensitive tool for structure analysis of phosphorus and metal-containing subunits of membrane protein complexes.

Keywords: SDS-PAGE; LA–ICP–MS; MALDI–FTICR–MS; Mitochondria; Yeast; Phosphorylated proteins; BN-PAGE

1. Introduction

Many proteomic studies are currently in progress to unravel networks of cellular proteins. The protein mixtures from whole cells, organelles or purified protein complexes are usually separated by 2D (IEF/SDS) gel-electrophoresis prior to the identification of single proteins by matrix-assisted laser desorption/ionization mass spectrometry (MALDI–MS). While this protocol has been proven to be very successful to analyze soluble proteins [1], analysis of membrane proteins often encounters problems, and proteins may escape from detection. Blue native (BN)-PAGE is a powerful method to separate high molecular mass membrane complexes in their native state. SDS-PAGE as the second dimension allows the identification of complex subunits [2–5]. BN/SDS-PAGE is widely applied in the field of mitochondrial research as it allows separation of the abundant high molecular mass enzyme complexes of the inner membrane.

In addition to the identification of proteins, analysis of post-translational modifications like phosphorylation or metal binding is required to understand the physiological functions of proteins [6–8]. Reversible protein phosphorylation is a key determinant in many fundamental cellular functions, such as survival, differentiation, structural organization, stress responses and it is relevant for many pathophysiological processes in carcinogenesis or neurodegenerative diseases [9]. Similarly, metals as cofactors of proteins play important roles for a variety of cellular processes. The determination of phosphorus and metal concentration in very small amounts of protein samples as is the case of protein spots of analytical gels is a challenging task of analytical chemistry [10–12]. We use yeast mitochondria as a model system to employ LA–ICP–MS and MALDI–FTICR–MS as a tool for micro-local analysis of physiological relevant elements

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in mitochondrial enzyme complexes. It is well known that mitochondria as the site of aerobic energy metabolism in eukaryotic cells harbour a number of proteins, which carry metal ions (e.g., copper, iron and zinc ions) as essential cofactors. By contrast, the physiological role of protein phosphorylation in yeast mitochondria is largely unknown and remains to be elucidated. At present only few phosphorylated proteins in yeast mitochondria are known. Information on kinases and phosphatases, the enzymes that catalyze phosphorylation and dephosphorylation of mitochondrial proteins, respectively, are very limited.

The aim of our investigations is to use a screening technique for the determination of (e.g., P, Fe, Zn, Cu) in protein spots from two-dimensional polyacrylamide gels obtained after BN/SDS-PAGE by laser ablation inductively coupled plasma mass spectrometry (LA–ICP–SFMS) and to combine LA–ICP–MS with MALDI–FTICR–MS for structure analysis of phosphorus and metal containing proteins.

We combined BN/SDS-PAGE with LA–ICP–MS and MALDI–FTICR–MS in order to analyze yeast mitochondrial ATPase sub-units for the presence of physiologically relevant elements. This novel screening technique led to the identification of phosphorylated ATPase subunits, a so far unknown modification of this enzyme in yeast.

2. Experimental

2.1. 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, Omaha, NE, USA) was used for the micro-local analysis of phosphorus and sulfur, silicon and selected metals in protein spots on polyacrylamide gels. 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. To separate interfering molecular ions from atomic ions $^{31}P^+$, $^{32}S^+$, $^{28}Si^+$, $^{27}Al^+$, $^{63}Cu^+$ and $^{64}Zn^+$ all LA–ICP–SFMS measurements were performed at a mass resolution $m/\Delta m$ of 4000. Selected protein spots marked by silver staining were analyzed by an optimized micro-local analysis. The quantification of analytical data was performed using sulfur as internal standard element.

2.2. MALDI–FTICR–MS instrumentation

Proteins of interest are identified by MALDI–FTICR–MS. 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 [13]. 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 20 laser shots were accumulated in the hexapole at 15 V and extracted at 7 V into the analyzer cell. A 100 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. A volume of 0.5 μl of matrix solution and 0.5 μl of sample solution was mixed on the stainless-steel MALDI sample target and allowed to dry.

2.3. Protein separation by two-dimensional gel electrophoresis

The proposed combination of atomic mass spectrometry (LA–ICP–MS) together with molecular mass spectrometry (MALDI–FTICR–MS) was tested on yeast mitochondrial protein complexes, which were separated by blue native gel electrophoresis (BN-PAGE [2]), followed by an electrophoresis under denaturing conditions (SDS-PAGE) in the second dimension. The gels were stained with silver or Coomassie blue. For preparative BN-PAGE, 10 gel slots were loaded with 200 μg mitochondria lysed by digitonin. After electrophoresis protein bands corresponding to the ATPase mono- or dimer were sliced, pooled and subjected to SDS-PAGE for separation of its subunits.

The combination of LA–ICP–SFMS with high-resolution MALDI–FTICR–MS was used as a suitable tool for protein iden-

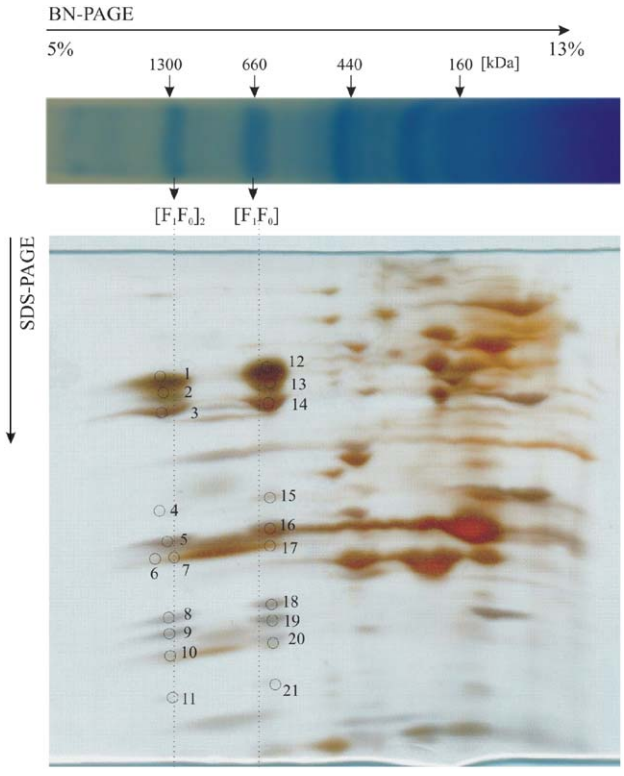

Fig. 1. Yeast mitochondrial enzyme complexes were isolated by BN-PAGE in a 5–13% polyacrylamide gel. Subsequently, subunits were separated in the second dimension under denaturing conditions (SDS-PAGE), and proteins were visualized by silver-staining. The positions of the monomeric ($[F_1F_0]$) and dimeric ($[F_1F_0]_2$) form of ATPase are marked. Numbers indicate spots selected for analysis by LA–ICP–MS.

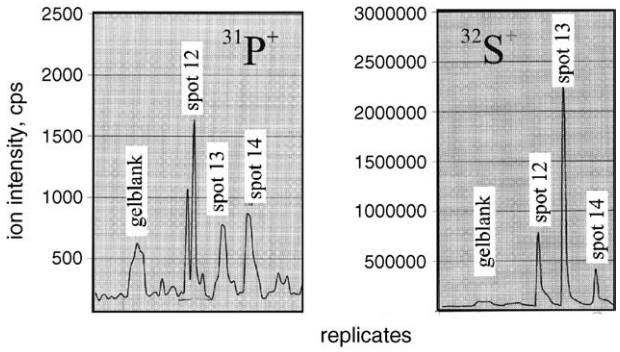

Fig. 2. Ion intensities of P^+ and S^+ of selected protein spots measured by LA–ICP–MS. Indicated spot numbers refer to Fig. 1.

tification, quantification of protein phosphorylation and determination of selected metal concentrations.

2.3.1. Western blot analysis

Proteins were separated by SDS-PAGE [14] and blotted onto PVDF-membranes (Millipore) by semi-dry blotting for 1 h at 25 V and 60 mA using transfer buffer (192 mM glycine, 25 mM Tris, 20% (v/v) methanol, 0.05% (w/v) SDS). Immunological detection was performed with antibodies directed against the phosphorylated moieties of serine, threonine and tyrosine (all from Sigma), and against Atp2p (MolecularProbes) according to manufacturer's instructions.

For phosphatase treatment, membranes were incubated for 2 h at 30 °C in buffer I (50 mM Tris/HCl, pH 7.5; 5 mM $MgCl_2$) in presence of 50 U CIAP (Invitrogen) and 50 U SAP (MBI Fermentas) alkaline phosphatase.

3. Results and discussion

3.1. Identification of mitochondrial phosphorus or metal ion containing proteins using MALDI–FTICR–MS

Two-dimensional gel electrophoresis is one of the most powerful analytical procedures in proteomics. By combining 2D gel electrophoresis with LA–ICP–MS the fast detection of physiologically relevant elements (P, Cu, Zn and Fe) in protein spots is possible. Recently, we reported on a combined LA–ICP–MS and MALDI–FTICR–MS analysis of selected silver-stained protein spots that were obtained by 2D separation of yeast mitochondrial proteins (Fig. 1), with respect to P^+, Fe^+, Cu^+ and Zn^+ ion intensities [11]. The detection limits for phosphorus, copper, zinc and iron – determined in the Coomassie-stained gel blank – were 0.2, 6, 18 and 10 $\mu g\,g^{-1}$, respectively. The study revealed that some of the known mitochondrial proteins possess so far unknown metal binding properties or contain bound phosphorus [11]. For example, the spots representing the major mitochondrial ADP/ATP translocator (Aac2p) and the acetyl-CoA hydrolase (Ach1p) contained all analytes tested with relatively high intensities [11]. In case of the mitochondrial aconitase Aco1p, an iron–sulfur cluster bearing protein, we were able to detect Cu, Zn and at the same time phosphorus. This is in line with the previous finding of phosphorylation of mitochondrial aconitase in potato [15]. The accumulation of various metals in this spot may hint at the metal-binding properties of Aco1p.

In the present study, we focused on yeast mitochondrial ATPase, a complex of the oxidative phosphorylation system composed of at least 15 polypeptides [16]. Inspection of the LA–ICP–MS data indicates that some proteins that possibly represent subunits of the ATPase complex, may be phosphorylated (Fig. 2). To verify phosphorylation of ATPase subunits, the monomeric and dimeric form of the enzyme was isolated by preparative BN-PAGE. The subunit composition was revealed by a subsequent SDS-PAGE and Coomassie blue staining (Fig. 3a). Two of the bands that showed the most intensive staining, were sliced from the gel and identified by MALDI–FTICR–MS as subunit Atp1p (58.6 kDa) and Atp2p (54.8 kDa).

The MALDI–FTICR–MS spectrum of Atp1p (Fig. 4) revealed 3 phosphorylated peptides (Table 1; peptide ^{411}V–^{428}K with 2 phosphorylations on ^{413}S und ^{426}S, peptide ^{265}Y–^{295}R with a single phosphorylation on either ^{265}Y or ^{281}Y, and peptide ^{422}Q–^{452}K with a phosphorylation on ^{434}Y) that could be iden-

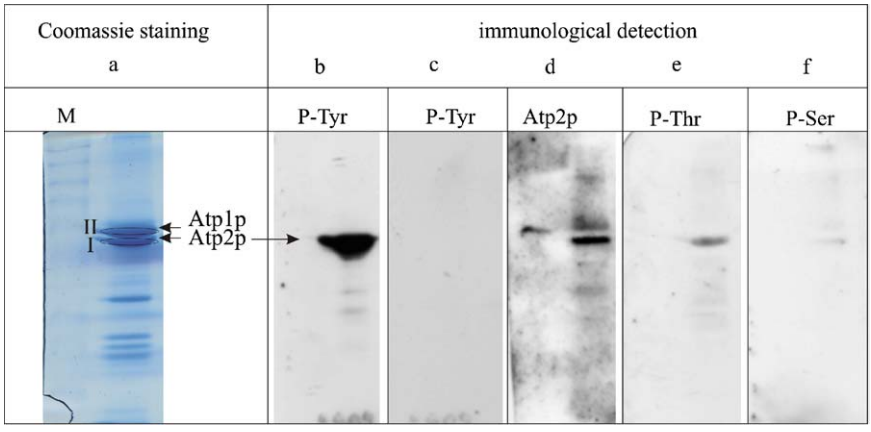

Fig. 3. Subunits of the ATPase complex were separated by preparative BN- and subsequent SDS-PAGE, and either stained by Coomassie blue (a) or transferred onto a PVDF-membrane for immunological detection with antibodies directed against phosphorylated amino acids as indicated or Atp2p (b–f). As a control, the membrane from (b) was treated with phosphatase (c) and subsequently again detected with Atp2p antibodies (d). Protein bands indicated (I, II) in (a) were subjected to MALDI–FTICR–MS.

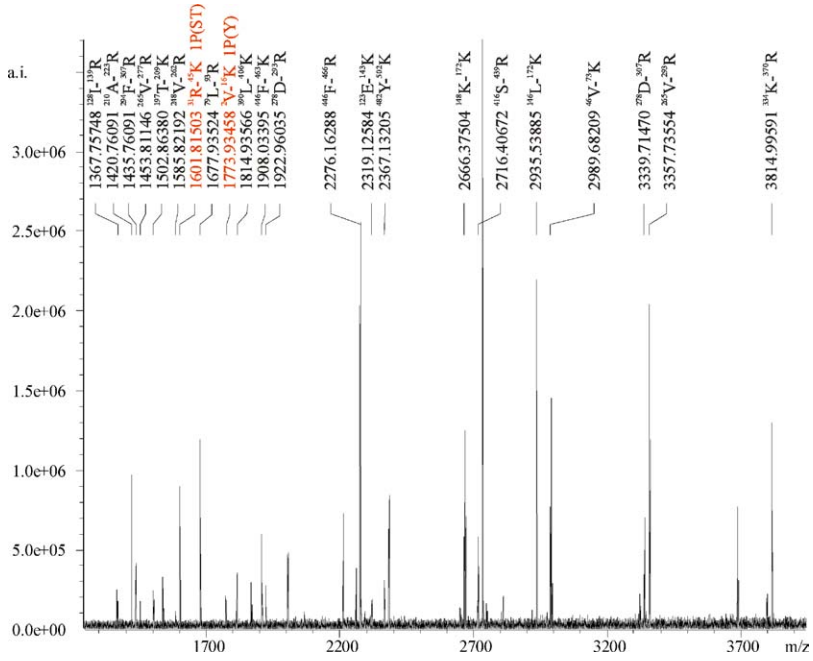

Fig. 4. MALDI–FTICR–MS of Atp1p (band II in Fig. 3a). Identified peptides and phosphorylated peptides are indicated.

tified by database search (www.matrixscience.com). For Atp2p two phosphorylated peptides (Table 1; peptide ^{31}R–^{45}K with the phosphorylation on a serine or threonine residue, and peptide ^{2}V–^{16}K with a tyrosine phosphorylation on the ^{7}Y residue) were identified as seen in the MALDI–FTICR–MS spectrum (Fig. 5).

To confirm the results of the LA–ICP–MS-derived data other detection techniques specific for phosphoproteins were applied. To this end ATPase subunits were separated by a preparative BN/SDS-PAGE as described above, transfered onto a PVDF-membrane and immunologically analyzed with antibodies directed against phosphoserine (P-Ser), phosphothreonine (P-Thr), and phosphotyrosine (P-Tyr) (Fig. 3 b, c, e and f). Comparison of the band corresponding to Atp2p revealed the most intensive signal in case of P-Tyr antibodies (Fig. 3b), indicating preferential phosphorylation of Atp2p at tyrosine residues.

Table 1
Identified protein spots from SDS-PAGE gel (Fig. 3a) with the identified phosphorylated peptides

Spot	Protein	Phosphopeptide
I	Atp2p	1P (^{31}R–^{45}K) RCMA*S*AAQ*ST*PI*T*GK (Phospho S or PhosphoT) 1P (^{2}V–^{16}K) VLPRL*Y*TATSRAAFK (Phospho Y)
II	Atp1p	2P (^{411}V–^{428}K) VG*S*AAQVKALKQVAG*S*LK (Phospho S) 1P (^{265}Y–^{295}R) *Y*SIIVAATASEAAPLQ*Y*LAPFTAASIGEWFR (Phospho Y) 1P (^{422}Q–^{452}K) QVAGSLKLFLAQ*Y*REVAAFAQFGSDLDASTK (Phospho Y)

Specificity of the detection was shown by removal of phosphate groups by incubation of the blot membrane with phosphatase-containing buffer. As expected, no signal was obtained for phosphorylated Tyr (Fig. 3c). Subsequent immunological detection of Atp2p excluded that the negative result was due to release of proteins from the membrane. In addition to P-Tyr, weak signals were obtained for P-Ser and P-Thr (Fig. 3 e and f), suggesting that Atp2p may also phosphorylated at those amino acids. Taken together, these data confirm the results obtained by MALDI–FTICR–MS. In case of Atp1p, no phosphorylated amino acid were immunological detectable. Possibly, the antibodies were not able to recognize their epitops due to steric hindrance.

Clearly the combined LA–ICP–MS and MALDI–FTICR–MS analysis is superior compared to the immunological detection, both in sensitivity (detection limit at sub $\mu g\ g^{-1}$ range) and specificity. In addition this new screening technique allows the exact identification of phosphorylation sites.

In contrast to phosphorus that is covalently bound to specific amino acids of proteins, metals are usually coordinatively attached to amino acid residues (e.g., copper) or via prosthetic groups (e.g., heme-iron). Therefore, metal ions, but not phosphorus may be released by the denaturing and reducing conditions during separation of proteins in SDS gels. It cannot be excluded that metal ions present in buffers for subsequent treatment of the gel (washing and staining steps) bind unspecifically to the proteins in the gel. In this case the accumulation of metal ions in protein spots rather reflect the capability of a protein to bind metals. Recently, we were able to show by tracer experiments using enriched isotope spikes (^{65}Cu, ^{67}Zn and ^{54}Fe) that some proteins can bind metals after separation by 2D gel electrophoresis prior to silver staining, whereas other proteins retained their original metal complexes [17].

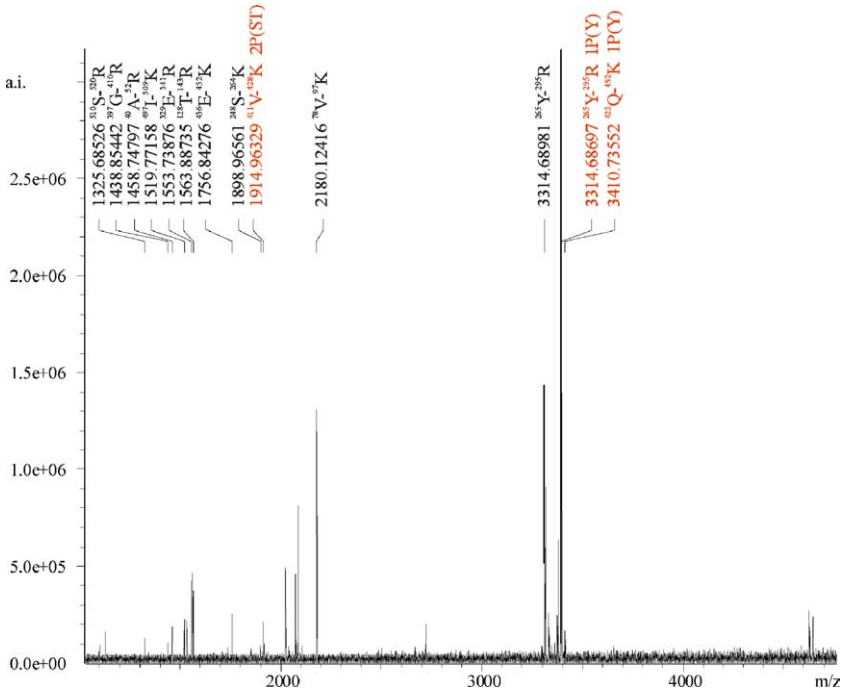

Fig. 5. MALDI–FTICR–MS of Atp2p (band I in Fig. 3a). Identified peptides and phosphorylated peptides are indicated.

4. Conclusion

The described method combines the fast simultaneous access to multi-element concentrations in gel-separated protein spots by LA–ICP–SFMS with the identification and structural information of the proteins derived from MALDI–FTICR–MS analysis. Phosphorus and metal concentration [11,18] in the $\mu g\,g^{-1}$ and sub-$\mu g\,g^{-1}$ range can be determined in protein spots. We were able to show that the method is suitable for detection of phosphorylated proteins in a highly sensitive and reliable manner. The most challenging problems will address the preservation of the naturally bound metal ions during gel separation and the avoidance of contaminations during sample preparation and staining. Future work will focus on improving the screening technique using a laser ablation system with a better lateral resolution and development of advanced methods for determination of metal concentrations, especially by on-line isotope dilution analysis in LA-ICP-MS.

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