Phosphorylation of B14.5a Subunit from Bovine Heart Complex I Identified by Titanium Dioxide Selective Enrichment and Shotgun Proteomics*[S]

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Shotgun proteomics was used to study the steady phosphorylation state of NADH:ubiquinone oxidoreductase (complex I) subunits from bovine heart mitochondria. A total tryptic digestion of enzymatically active complex I was performed, and the resulting peptide mixture was subjected to phosphopeptide enrichment by the use of titanium dioxide (TiO_2). The phosphopeptide-enriched fraction was separated and analyzed with nanoscale reverse-phase HPLC-ESI-MS/MS in single information-dependent acquisition. Hence two phosphorylated complex I subunits were detected: 42 kDa and B14.5a. Phosphorylation of 42-kDa subunit at Ser-59 has already been determined with fluorescent phosphoprotein-specific gel staining and mass spectrometry (Schilling, B., Aggeler, R., Schulenberg, B., Murray, J., Row, R. H., Capaldi, R. A., and Gibson, B. W. (2005) Mass spectrometric identification of novel phosphorylation site in subunit NDUFA10 of bovine mitochondrial complex I. *FEBS Lett.* 579, 2485–2490). In our work, this finding was confirmed using a non-gel-based approach. In addition, we report novel phosphorylation on B14.5a nuclear encoded subunit. We demonstrated evidence of the phosphorylation site at Ser-95 residue by collision-induced dissociation experiments on three different molecular ions of two tryptic phosphopeptides of B14.5a. ***Molecular & Cellular Proteomics 6: 231–237, 2007.***

NADH:ubiquinone oxidoreductase (complex I,[1] EC 1.6.5.3) has the most complex structure and complicated mechanism of action of all the five complexes of mitochondrial electron transport/oxidative phosphorylation system (OXPHOS). Complex I from bovine heart is known to contain 46 subunits, seven of which are encoded by the mitochondrial DNA, and the remaining are nuclear gene products that are imported into the mitochondria. This highly hydrophobic, multisubunit assembly is partly immersed into the mitochondrial inner membrane and partly extends into the mitochondrial matrix. Traditional gel-based proteomics is extensively used for the characterization of the subunit composition of OXPHOS complexes (1–3). We have recently suggested the use of an alternative, non-gel-based proteomics approach ("shotgun" proteomics) for analyzing composition and post-translational modifications of complex I carried out in a single experiment (4).

Reversible protein phosphorylation/dephosphorylation mediated by protein kinases and phosphatases is a dynamic post-translational process regulating virtually every cellular event including cell signaling, apoptosis, oncogenesis, and immune disorders (5). During phosphorylation/dephosphorylation a phosphate group is covalently attached to/removed from the hydroxyl residues of serine, threonine, and tyrosine. By reversible phosphorylation enzymatic activity, binding properties, stability, hydrophobicity, and localization of a protein can be regulated. Due to the high biological importance of protein phosphorylation, different methods have been developed and used both for the detection of the phosphorylation status and the determination of phosphorylation sites. Traditional methods include ^{32}P radioactive protein labeling followed by autoradiography and Edman sequencing and/or mass spectrometric analysis or alternatively immunochemical detection using antibodies against phosphoserine, phosphothreonine, and phosphotyrosine. *In vitro* phosphorylation of complex I with added ATP has been performed in a number of works to find potential substrates of mitochondrial kinases in particular protein kinase A (PKA) (6–10) and pyruvate dehydrogenase kinase (11, 12). In human (13) and mammalian cell cultures (7, 14) cAMP-dependent *in vitro* phosphorylation of complex I subunits has been demonstrated and was associated with stimulation of the NADH:ubiquinone oxidoreductase activity of the complex. cAMP has also been found to reduce

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[1] The abbreviations used are: complex I, NADH:ubiquinone oxidoreductase; OXPHOS, oxidative phosphorylation system; TiO_2, titanium dioxide; PKA, protein kinase A; ROS, reactive oxygen species.

accumulation of oxygen free radicals from human and murine cells in culture (15, 16). Changes in the reactive oxygen species (ROS) level appeared to be inversely related to cAMP-dependent activation of complex I (17). In purified bovine complex I, two serine-phosphorylated subunits with molecular masses of 18 and 10 kDa were detected in the presence of cAMP, [^{32}P]ATP, and PKA and identified, by the use of mass spectrometry, as ESSS (Ser-20) and MWFE (Ser-55) (10). *In vitro* study of mitochondrial tyrosine-phosphorylated proteins has identified the 39-kDa subunit of complex I from rat brain mitochondria (11). Although *in vitro* studies provide valuable information on phosphorylable proteins, these phosphorylation sites are not necessarily phosphorylated in living systems. Therefore, direct analysis of naturally occurring phosphorylation of proteins is particularly important, but at the same time it is extremely difficult due to the low level of phosphorylated proteins in a given time and the transient nature of many phosphorylation events. In fact, no phosphorylated subunits have been revealed by traditional gel- and non-gel-based proteomics in native complex I probably due to their low degree of modification. There are two strategies to overcome these difficulties: (*a*) highly specific detection of phosphoproteins and (*b*) selective enrichment of phosphorylated proteins or their phosphopeptides. Recently a novel method was applied for the characterization of dynamic and steady-state phosphorylation of complex I subunits from bovine heart using novel phosphoprotein-specific gel staining. With this method, only the 42-kDa subunit was shown to be phosphorylated in both states (18, 19). Based on comparative MS/MS analysis between synthesized phosphopeptides and the tryptic phosphopeptide derived from the 42-kDa protein, serine 59 was identified as the phosphorylation site (20).

Selective enrichment of phosphoproteins or their phosphopeptide fraction can also facilitate the study of steady-state phosphorylation status of low abundance proteins in complex mixtures. Different enrichment methods have been developed for phosphopeptides, *i.e.* IMAC, immunoprecipitation using specific anti-phospho antibodies, and selective chemical modifications. Recently Pinkse *et al.* (21) have reported a highly selective and sensitive method of isolation of phosphopeptides from complex cell lysate using a TiO_2 column directly coupled to an ESI mass spectrometer. The method has been improved by Larsen *et al.* (22) and most recently by Chen and Chen (23). In the present work, we developed a modified TiO_2 affinity approach in which the affinity probe is used in a single tube experiment. The method was combined with shotgun proteomics to study the steady phosphorylation state of complex I subunits. The intact complex is enzymatically digested, and the resulting peptide mixture is separated by HPLC and analyzed by nano-ESI-MS/MS (4). Here we show that besides the 42-kDa protein there is another phosphorylated complex I subunit. This protein is the nuclear encoded B14.5a subunit. The phosphorylation site was determined by CID tandem mass spectrometry.

EXPERIMENTAL PROCEDURES

Isolation of Complex I—Bovine heart mitochondria was prepared as described previously (24). Complex I was isolated and purified by cholate solubilization and salt fractionation using the standard procedure of Hatefi (25) in the presence of a mixture of phosphatase (Mixture Set II) and protease (Mixture Set III) inhibitors (Calbiochem, Merck KGaA). The protein concentration of the sample was 47 mg/ml as determined by biuret assay. 10 μl (470 μg) of complex I sample was denatured, reduced, and digested as described previously (4). The sample was diluted to a final volume of 100 μl containing 0.1% TFA.

Phosphopeptide Enrichment Using TiO_2—Tryptic peptides were selectively enriched in phosphopeptides using a modified procedure of Larsen *et al.* (22). Briefly 1 mg of TiO_2 particles (Reanal, Budapest, Hungary) was placed in an Eppendorf tube, washed three times with 100 μl of 80% acetonitrile in 0.1% TFA, and conditioned by 100 μl of 0.1% TFA. The slurry was centrifuged on a bench-top centrifuge, and solvent was removed. The complex I digest (100 μl containing 470 μg of tryptic digest) was mixed with TiO_2 particles and incubated at room temperature under shaking for 30 min. Particles were centrifuged, and the supernatant was removed. Non-phosphorylated peptides were eluted by 10 μl of 80% acetonitrile in 0.1% TFA. Particles were washed five times by 200 μl (80% acetonitrile in 0.1% TFA). Phosphopeptides were eluted by adding 20 μl of 50 mM ammonium hydrogen carbonate at pH 11.5. After centrifugation, the supernatant (containing the phosphopeptide fraction) was removed and acidified to pH 7.8 using formic acid.

Nano-HPLC-ESI-MS/MS Analysis—The total complex I digest and the phosphopeptide-enriched fraction were analyzed using a hybrid Q-TOF instrument, QTOF Micro (Waters) equipped with a nanoflow electrospray ion source. A metal-coated silica tip (360-μm outer diameter/20-μm inner diameter; tip, 10-μm inner diameter; New Objective, Woburn, MA) was used as a nanospray emitter. Samples (1 μg in the case of total protein digest and ⅓ of the phosphopeptide fraction) were loaded, purified, and concentrated on a precolumn (PepMap, C_{18}, 5-mm length, 300 Å; LCPackings, Sunnyvale, CA) using a CapLC micro-HPLC system (Waters) at 20 μl/min flow rate. A capillary column (PepMap, C_{18}, 15-cm length, 75-μm inner diameter, 300 Å; LCPackings) was used for peptide separation with a flow rate split to ~200 nl/min using solvents A (2% acetonitrile in 0.1% formic acid (HCOOH)) and B (95% acetonitrile in 0.1% HCOOH). Separation was performed using the following linear gradients: 1) 2–60% B in 120 min and 60–95% B in 2 min for the total protein digest and 2) 2–60% B in 60 min and 60–95% B in 2 min for the phosphopeptide-enriched fraction. CID experiments were performed in data-directed analysis (parent survey) mode using the MassLynx 4.0 software. Argon was used as collision gas. A peak list was generated by ProteinLynx Global Server 2.1 (Waters). Database searching was performed by Phenyx program (26) using the following criteria: database, UniProt Swiss-Prot (release 48.8 of January 10, 2006); taxonomy, *Bos taurus*; type of search, MS/MS ion search; enzyme, trypsin; fixed modifications, carbamidomethyl; variable modifications, acetyl (N-terminal), oxidation on methionine, and phosphorylation on serine, threonine, and tyrosine; mass values, monoisotopic; parent tolerance, 0.15 Da, *z* score, ≥3; *p* value, ≤1.0E−3; Access score = 5; and number of maximum missed cleavages, 2. Enzymatic digestion, phosphopeptide enrichment, and nano-ESI-MS/MS experiments were repeated at least three times using both the same complex I preparation and a complex I sample isolated from a different biological source.

RESULTS

Complex I samples were prepared from bovine heart mitochondria according to Hatefi (25). In this preparation complex

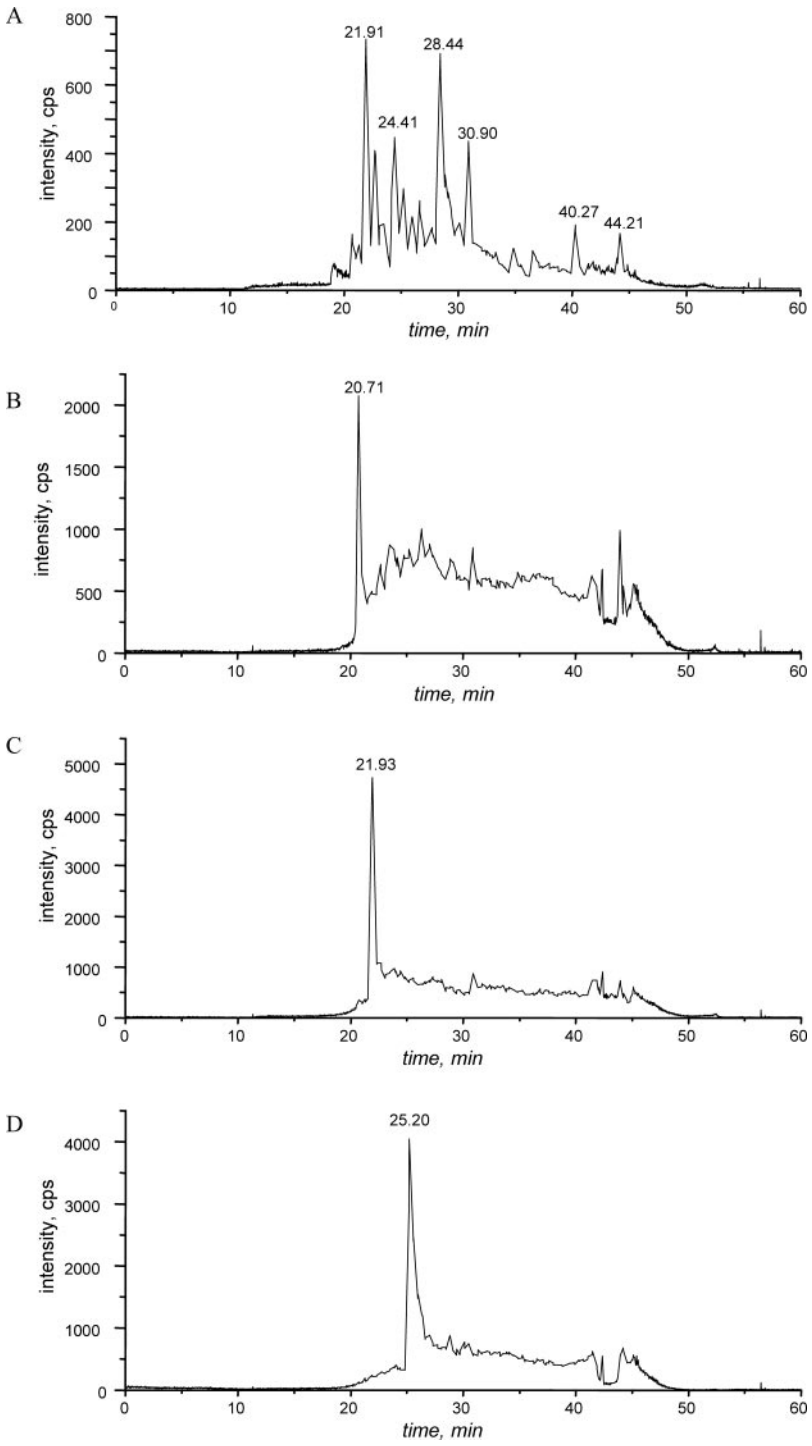

FIG. 1. **Shown are the base peak ion chromatogram (*A*) of nano-HPLC-ESI-MS experiment performed on the phosphopeptide-enriched fraction of complex I and selected ion chromatograms due to *m/z* 415.21 triply and *m/z* 622.3 doubly charged molecular ions (*B*), *m/z* 558.27 doubly charged molecular ion (*C*), and *m/z* 786.34 doubly charged molecular ion (*D*) of phosphopeptides.** *cps*, counts per second.

I retains its inhibitor sensitivity and enzymatic activity. The preparation is known to have a relatively high lipid content and contains significant amounts of transhydrogenase and other proteins related to the OXPHOS system that may have a role in the function, assembly, and stability of the complex (27). In our previous work (4) we showed that shotgun proteomics can deal with this not highly purified, but biologically active, complex I. Here a modified TiO_2-based phosphopeptide enrichment in combination with shotgun proteomics was applied to study the steady-state phosphorylation status of enzymatically active bovine complex I. The solid phase affinity purification is based on the method published earlier by Larsen *et al.* (22) and was modified for application in a simple five-step single tube experiment using centrifugal force for separating liquid and solid phases. The method was optimized using a peptide mixture containing phosphopeptides

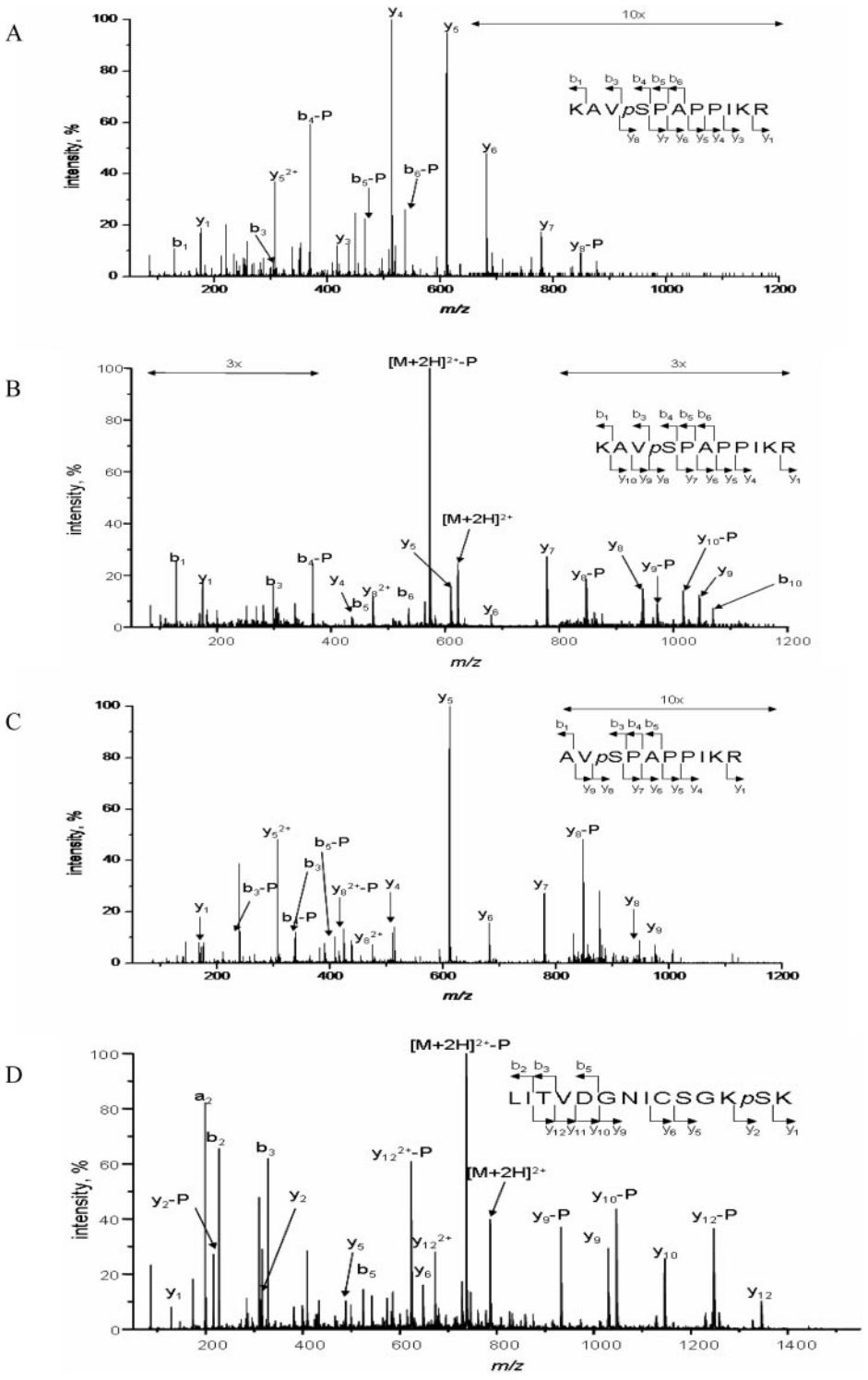

FIG. 2. **CID tandem mass spectra of phosphopeptides detected after TiO_2 affinity enrichment.** *A*, $[M + 3H]^{3+}$ triply charged molecular ion at *m/z* 415.21 eluted between 20.5 and 21.0 min (Fig. 1*B*). *B*, $[M + 2H]^{2+}$, doubly charged molecular ions at *m/z* 622.32 eluted between 20.5 and 21.0 min (Fig. 1*B*). *C*, $[M + 2H]^{2+}$, doubly charged molecular ion at *m/z* 558.27 eluted between 21.6 and 22.1 min (Fig. 1*C*). *D*, $[M + 2H]^{2+}$, doubly charged molecular ion at *m/z* 786.34 of phosphopeptide eluted between 25.0 and 25.7 min (Fig. 1*D*). Neutral loss of phosphoric acid (H_3PO_4) is indicated as "*-P*."

from precipitated milk caseins (data not shown). Nano-HPLC-ESI-MS/MS in data-directed analysis mode was performed to compare complex I sample before and after solid phase affinity enrichment. Analysis of starting sample yielded a rich ion chromatogram with a high number of peptides that were eluted and sequenced, resulting in the identification of 54 proteins including 37 complex I subunits, nine proteins from other OXPHOS complexes, and eight other mitochondria-related proteins (Supplemental Table 1). According to our previous observations, there was no phosphorylated peptide with statistically significant score detected in complex I without phosphopeptide enrichment (4).

TABLE I
Results obtained on B14.5a subunit by ESI-MS/MS data-dependent analysis of the total tryptic digest and the phosphopeptide-enriched fraction of complex I

Modified residues are indicated in bold.

Peptide sequence	Residues	Charge state	PTMs[a]	*m/z* observed	
				Total tryptic digest	Phosphopeptide fraction
VLVAGKPAESSAVAASEK	74–91	3+	ND[b]	572.02	ND
ASATRFIQWLRNWASGR	1–17	3+	ACET-Nterm	688.08	ND
LSNNYY**C**TR	48–56	2+	Cys_CAM	595.80	ND
AVSPAPPIK	93–101	2+	ND	440.30	ND
AV**S**PAPPIKR	93–102	2+	PHOS	ND	558.27
KAV**S**PAPPIKR	92–102	3+	PHOS	ND	415.21
KAV**S**PAPPIKR	92–102	2+	PHOS	ND	622.32

[a] Post-translational modifications: ACET-Nterm, N-terminal amino acid is acetylated; Cys_CAM, cysteine residue is carbamidomethylated; PHOS, serine residue is phosphorylated.
[b] ND, not detected.

On the other hand, the analysis of phosphopeptide-enriched fraction resulted in a relatively simple ion chromatogram (Fig. 1*A*) with only a few proteins identified (Supplemental Table 2). In this fraction, three phosphopeptides were identified with statistically significant scores based on the neutral loss of phosphoric acid (H_3PO_4) from the precursor as well fragment ions yielding characteristic 97.995-Da (nominal mass, 98 Da) differences in the CID mass spectra. Between 20.5 and 21.0 min (17.5% B) a peptide with molecular mass of 1242.63 Da was eluted giving *m/z* 415.21 triply and *m/z* 622.35 doubly charged molecular ions detected in the same scans (Fig. 1, *A* and *B*) and yielding very similar MS/MS spectra (Fig. 2, *A* and *B*). Based on a full set of y''_n and a partial set of b_1, b_3–b_6 fragment ions the sequence of this peptide is (K)^{92}KAV**pS**PAPPIKR^{102}(W) where pS is phosphoserine (residues 92–102, sequence numbering is based on the mature protein sequence after the removal of initial methionine) of B14.5a subunit of bovine mitochondrial complex I containing two missed cleavage sites. In this sequence the only amino acid subject to phosphorylation is Ser-95. Evidence of phosphorylation of Ser-95 was confirmed by the presence of b_4, $b_4 - 98$, and y''_8, $y''_8 - 98$ counter ion pairs, even if low in abundance, at *m/z* 466.19 and *m/z* 945.51, respectively (Fig. 2*B*). Phosphorylated Ser-95 was further confirmed by a second phosphopeptide eluted between 21.6 and 22.1 min (Fig. 1*C*) and leading an abundant doubly charged *m/z* 558.27 molecular ion ($M = 1114.59$ Da). Based on the CID spectrum of *m/z* 558.27 (Fig. 2*C*) and subsequent database search, this peptide was identified as (K)^{93}AV**pS**PAPPIKR^{102}(W), residues 93–102 of B14.5a (Supplemental Table 2). This second phosphopeptide is a single amino acid shorter than the first one and has only one miscleaved site. Miscleavage of B14.5a protein by trypsin could be explained by the presence of phosphate group that may prevent cleavage of the enzyme after Lys-92 and/or Lys-101. In fact, analysis performed on the total tryptic digest of complex I reveals the presence of a non-phosphorylated "regular" (without miscleaved site) (K)^{93}AVSPAPPIK^{101}(R) tryptic peptide based on the MS/MS analysis of *m/z* 440.3 $[M + H]^{2+}$ molecular ion (Table I and Supplemental Table 1). This indicates a partial phosphorylation of B14.5a subunit. The lack of detection of phosphorylated peptides in the starting sample is probably due to the complexity of the sample and the low degree of phosphorylation.

The third phosphopeptide eluted between 25 and 25.7 min gave an abundant doubly charged molecular ion at *m/z* 786.34 (Fig. 1*D*). Interpretation of the CID spectrum (Fig. 2*D*) combined with database search led to the identification of the 42-kDa subunit based on the (K)^{47}LITVDGNICSGK**pS**K^{60}(L) phosphopeptide sequence carbamidomethylated at Cys-55 and phosphorylated at Ser-59. We could confirm that phosphorylation occurred at Ser-59 by interpreting the low mass region of the spectrum. In agreement with the results published recently by Schilling *et al.* (20), we observed the presence of ion pair y_2, $y_2 - 98$ (*m/z* 314.09 and *m/z* 216.13, respectively) characteristic of Ser(P)-59 and the lack of ion at *m/z* 234.1, which would be the y_2 fragment ion in the case of phosphorylation at Ser-56.

DISCUSSION

The combination of solid phase affinity method using TiO_2 particles in a simple one-tube experiment and shotgun proteomics can highly facilitate the investigation of phosphorylation status in the physiological state as demonstrated here in the study of steady-state phosphorylation of mitochondrial complex I from bovine heart. The enrichment method using TiO_2 is not highly specific: most of the peptides in the elution fraction are acidic tryptic peptides (Supplemental Table 2). However, after affinity separation we could detect three phosphopeptides identifying two proteins, 42 kDa and B14.5a. Steady-state phosphorylation of 42-kDa subunit was recently reported identifying the Ser-59 residue as phosphorylation site using a gel-based method and mass spectrometry (20). Here we confirmed this finding using a non-gel-based approach. We further show that B14.5a, encoded by the nuclear *NDUFA7* gene of complex I, is also phosphorylated in the

native complex. B14.5a subunit is a low molecular mass protein ($M_{average}$, 12,587.4 Da, calculated by considering the non-phosphorylated mature protein) mainly located in the extramembrane domain (subcomplex Iα) of the enzyme but also present in subcomplex Iλ (28). B14.5a has been proposed to be a ubiquinone-binding protein (28), but its precise role in the function of complex I enzyme is unknown. By analyzing tryptic digests of complex I before and after affinity enrichment, six peptides were detected from the B14.5a subunit (Table I). Three of these peptides (residues 1–17, 92–102, and 93–102) are post-translationally modified. Previously reported *N*-α-acetylation of B14.5a (19) was confirmed here by sequencing the N-terminal peptide 1–17 (Table I and Supplemental Table 1) present in the total tryptic digest. In addition, sequencing of two miscleaved tryptic peptides present in the phosphopeptide-enriched fraction and represented by three molecular ions demonstrates the phosphorylation of Ser-95 residue.

Differences in identified phosphoproteins between our studies and previous studies (10, 18–20) can be explained by the differences in the methods used for the preparation of the complex I sample, the systems studied (induced *versus* naturally occurring phosphorylation), and the techniques applied for detection. As for techniques, previous works applied gel electrophoresis using radiolabeling (10) and fluorescent phosphosensor dye (18, 19) combined with mass spectrometry-based identification. By using radiolabeling or the shotgun approach alone (without affinity enrichment) in contrast with fluorescent gel staining, there were no phosphorylated complex I proteins detected in the steady state. This demonstrates the superior sensitivity of fluorescent phosphosensor dye over traditional radiolabeling. On the other hand, the dynamic range and/or the sensitivity of shotgun proteomics alone has turned out to be insufficient for revealing phosphopeptides in the complex mixture deriving from enzymatic digestion of complex I (4). Applying combined TiO_2 enrichment and shotgun proteomics, however, seems to be more sensitive than the gel-based approaches because it detects the phosphorylation of both B14.5a and 42-kDa subunits.

Mitochondria play a central role in the regulation of apoptosis and are the major site of the ROS generation. Increased ROS production has been implicated in pathological disorders and in aging as well. Complex I is a main source of superoxide and thus possibly responsible for increased mitochondrial ROS production and redox signaling (17, 29). In this system, post-translational modifications, in particular oxidation (30), reversible protein glutathionylation (31), *S*-nitrosation (32), and phosphorylation (12), play important regulatory roles. Despite the presence of serine/threonine and tyrosine kinases in the mitochondrial membrane, there are surprisingly few mitochondrial proteins that have been found to be phosphorylated so far, and only one phosphoprotein (E1α subunit of pyruvate dehydrogenase complex) regulatory function has been determined (33). Regarding complex I from bovine heart mitochondria, cAMP-dependent PKA-driven phosphorylation of subunits ESSS, MWFE (10), and 42 kDa (20) have been described. In this context, B14.5a and 42-kDa subunits represent a step toward identification of proteins involved in reversible phosphorylation in the steady state of complex I. The regulatory function of these proteins remains to be explored.

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