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Biochimica et Biophysica Acta 1757 (2006) 1438–1450
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Shotgun proteomics for the characterization of subunit composition of mitochondrial complex I

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Received 13 February 2006; received in revised form 13 April 2006; accepted 24 May 2006
Available online 3 June 2006

Abstract

Here we propose shotgun proteomics as an alternative method to gel-based bottom-up proteomic platform for the structural characterization of mitochondrial NADH:ubiquinone oxidoreductase (complex I). The approach is based on simultaneous identification of subunits after global digestion of the intact complex. Resulting mixture of tryptic peptides is purified, concentrated, separated and online analyzed using nano-scale reverse-phase nano-ESI-MS/MS in a single information dependent acquisition mode. The usefulness of the method is demonstrated in our work on the well described model system of complex I from bovine heart mitochondria. The shotgun method led to the identification and partial sequence characterization of 42 subunits representing more than 95% coverage of the complex. In particular, almost all nuclear (except MLRQ) and 5 mitochondria DNA encoded subunits (except ND4L and ND6) were identified. Furthermore, it was possible to identify 30 co-purified proteins of the inner mitochondrial membrane structurally not belonging to complex I. The method's efficiency is shown by comparing it to two classical 1 D gel-based strategies. Shotgun proteomics is less laborious, significantly faster and requires less sample material with minimal treatment, facilitating the screening for post-translational modifications and quantitative and qualitative differences of complex I subunits in genetic disorders.

Keywords: Complex I; Bovine heart mitochondria; Subunit composition; Shotgun proteomics; Mass spectrometry

1. Introduction

NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) is the largest in the number of subunits and molecular mass in the family of protonmotive enzyme-complexes of the respiratory chain of mitochondria. This membrane-bound complex has the most complicated structure and mechanism of action among the mitochondrial electron transport/oxidative phosphorylation system (OXPHOS) complexes [1]. Complex I from bovine heart mitochondria is an excellent model system for both the characterization of the corresponding human enzyme [2] and the study of hydrophobic multisubunit membrane-bound protein complexes in general. Proteins of complex I assemble into an L-shaped structure with the hydrophobic moiety spanning the inner mitochondrial membrane and the catalytic sector extending into the matrix [3]. Complex I of bovine heart mitochondria consists of at least 46 subunits (Table 1), 7 of which (ND1–ND6, and ND4L) are encoded by the mitochondrial DNA (mtDNA) [4–6].

Complex I deficiency is the most common and most severe of oxidative phosphorylation defects. It can be caused by alterations in both nuclear and mitochondrial encoded genes [7]. Impairment of complex I activity can be found in various pathologies including neurodegenerative diseases like Huntington's [8], Parkinson's [9,10] and Alzheimer's diseases [11]

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doi:10.1016/j.bbabio.2006.05.037

Table 1
Mitochondrial NADH:ubiquinone oxidoreductase (complex I) subunits from bovine heart

Swiss-Prot access number	Name of the gene	Short name of the subunit	Mw [a]	Gravy score [b]	Known PTMs [c]	Number of cysteines
P15690	NUAM_BOVIN	75 kDa	77.0 kDa	−0.124	–	17
P25708	NUBM_BOVIN	51 kDa	48.5 kDa	−0.296	–	12
P17694	NUCM_BOVIN	49 kDa	49.2 kDa	−0.337	–	6
P23709	NUGM_BOVIN	30 kDa	26.4 kDa	−0.456	–	2
P04394	NUHM_BOVIN	24 kDa	23.8 kDa	−0.294	–	5
P42026	NUKM_BOVIN	20 kDa	20.1 kDa	−0.152	–	5
P42028	NUIM_BOVIN	23 kDa	20.2 kDa	−0.487	–	8
P34942	NUDM_BOVIN	42 kDa	36.7 kDa	−0.480	P-Ser59 [d]	5
P34943	NUEM_BOVIN	39 kDa	39.1 kDa	−0.189	–	1
Q02375	NUYM_BOVIN	18 kDa	15.3 kDa	−0.928	–	0
Q02379	NIPM_BOVIN	15 kDa	12.5 kDa	−1.015	–	4
P23934	NUMM_BOVIN	13 kDa-A	10.5 kDa	−0.671	–	3
P25712	NUOM_BOVIN	9 kDa	8.4 kDa	−1.346	–	0
Q02374	NIGM_BOVIN	AGGG	8.5 kDa	−0.602	–	0
Q02372	NIAM_BOVIN	ASHI	18.7 kDa	−0.958	–	1
Q8HXG5	NESM_BOVIN	ESSS	14.5 kDa	−0.679	P-Ser20 [e]	1
Q02376	NIKM_BOVIN	KFYI	5.8 kDa	−0.465	–	0
Q01321	NUML_BOVIN	MLRQ	9.3 kDa	−0.433	–	0
Q02378	NINM_BOVIN	MNLL	7.0 kDa	−0.490	–	0
Q02377	NIMM_BOVIN	MWFE	8.1 kDa	−0.211	P-Ser55 [e]	1
Q02373	NIDM_BOVIN	PDSW	20.8 kDa	−0.989	–	5
P42029	NUPM_BOVIN	19k Da	20.0 kDa	−0.717	–	8
P52505	ACPM_BOVIN	9.6 kDa	10.7 kDa	−0.225	PPant-Ser44	1
Q02380	NISM_BOVIN	SGDH	16.7 kDa	−0.331		0
Q02369	NI2M_BOVIN	B22	21.7 kDa	−1.078	N-Ac [f]	4
Q02368	NB8M_BOVIN	B18	16.5 kDa	−1.003	N-Myr [f]	4
O97725	N7BM_BOVIN	B17.2	17.1 kDa	−0.804	N-Ac [f]	1
Q02367	NB7M_BOVIN	B17	15.4 kDa	−0.613	N-Ac [f]	0
Q95KV7	NB6M_BOVIN	B16.6	16.6 kDa	−0.322	N-Ac [f]	0
P48305	NB5M_BOVIN	B15	15.1 kDa	−0.688	N-Ac [f]	0
Q8HXG6	N5BM_BOVIN	B14.7	14.7 kDa	0.245	N-Ac [f]	4
Q05752	N4AM_BOVIN	B14.5a	12.6 kDa	−0.700	N-Ac [f]	1
Q02827	N4BM_BOVIN	B14.5b	14.1 kDa	−0.350	N-Ac [f]	1
Q02366	NB4M_BOVIN	B14	15.0 kDa	−0.591	N-Ac [f]	0
P23935	NUFM_BOVIN	13 kDa-B	13.2 kDa	−0.424	N-Ac [f]	1
Q02365	NB2M_BOVIN	B12	11.1 kDa	−0.692	N-Ac [f]	0
Q02371	NI9M_BOVIN	B9	9.3 kDa	−0.093	N-Ac [f]	0
Q02370	NI8M_BOVIN	B8	11.0 kDa	−0.372	N-Ac [f]	2
P03887	NU1M_BOVIN	ND1	35.7 kDa	0.798	–	1
P03892	NU2M_BOVIN	ND2	39.3 kDa	0.785	–	0
P03898	NU3M_BOVIN	ND3	13.1 kDa	0.863	–	1
P03910	NU4M_BOVIN	ND4	52.1 kDa	0.826	–	3
P03920	NU5M_BOVIN	ND5	68.3 kDa	0.637	–	4
P03924	NU6M_BOVIN	ND6	19.1 kDa	1.031	–	1
P03902	NU4LM_BOVIN	ND4L	10.8 kDa	1.259	–	3

[a] Average molecular masses were calculated based on the amino acid sequences considering the known post-translational modifications (PTMs) using GPMAW 5.11 software (Lighthouse Data, Odense, Denmark).
[b] GRAVY scores were calculated using Protalizer Version 0.1 software (http://microbio1.biologie.uni-greifswald.de/emaupt).
[c] Post-translational modifications: N-Ac = N-terminal acetylated, N-Myr = N-terminal myristylated, P = phosphorylation, PPant = modified by pantetheine 4′ phosphate.
[d] Schulenberg et al,. J. Biol. Chem. (2003) 278, 2725.
[e] Chen et al., J. Biol. Chem. (2004) 279, 26036.
[f] Carroll et al., Mol. Cell. Prot. (2005) 4, 693.

as well as in HIV infection [12], diabetes [13], Down-syndrome [11] and cancer [14–17]. To date, diagnosis of OXPHOS dysfunction is made by enzymatic and immunochemical analysis of individual multiprotein complexes. Such analyses require significant amounts of biopsy tissue or cell culture material. To be able to associate specific gene alterations (mutation, deletion, amplification) with structural modifications at the protein level and/or impairment in the activity of complex I would be highly important in understanding the functional role of complex I subunits. Furthermore, elucidation of the genotype–phenotype correlation would help in clarifying the pathogenic mechanism of a disorder long before symptoms appear, with the possibility of effective timely therapy.

Improvements in mass spectrometry (MS) instrumentation and methodology and the rapid growth of genomic databases have allowed the development of high-throughput proteomic

approaches used in identifying and quantifying a large number of different proteins in cell organelles such as mitochondria. Mitochondrial proteome maps of five different organisms have been published [17–20] and are continuously updated. The most widely applied proteomic strategy in the field of mitochondria and in the studies of the OXPHOS complexes is bottom-up proteomics [21–24]. There are two basic strategies in bottom-up proteomics. In gel-based methods the protein mixture is separated prior to enzymatic cleavage. Protein identification is based on the peptide mass fingerprint of the protein obtained by accurate mass measurement of the enzymatically cleaved peptide fragments using MALDI-TOFMS or ESI-MS. Alternatively, peptide sequencing can be carried out by means of tandem MS/MS [24]. In the second approach (named 'shotgun approach') global digestion of the pool of proteins is performed to produce a mixture of peptides which is then separated and analyzed usually by multidimensional liquid chromatography (LC) coupled to a tandem ESI mass spectrometer. For the structural analysis of complex I gel-based methodologies have been applied so far. Combination of rigorous purification steps, different fractionation of the intact complex into subcomplexes (ion-exchange, ammonium sulfate precipitation and gel filtration), separation of subunits (one-dimensional SDS-PAGE, two-dimensional isoelectrofocusing/SDS-PAGE, and reverse-phase HPLC) and two different MS-based protein identification methods (Peptide mass fingerprinting (PMF) and MS/MS based sequencing) allowed the identification of the known 46 subunits of complex I [4] (Table 1). Blue-native polyacrylamide gel electrophoresis (BN-PAGE) separation of the intact multienzyme complexes is also extensively applied for the characterization of mitochondrial OXPHOS complexes [25]. BN-PAGE combined with a second SDS-PAGE separation is used to generate two-dimensional map of the individual subunits which subsequently can be identified by MALDI-TOF PMF [25,26]. Recently, combination of BN-PAGE with LC nano-ESI-MS/MS led to the identification of 32 complex I subunits out of 46 including some hydrophobic components, except for the most problematic ND4L and ND6 subunits [27].

As a part of an effort to find more efficient analytical methods, here we propose a novel shotgun proteomic platform based on the simultaneous identification of subunits after global digestion of the intact mitochondrial complex as an alternative, and more efficient analytical method for the characterization of membrane-bound complexes. The scope of this work was to analyze the protein composition of a fully active preparation of isolated complex I from bovine heart mitochondria using the shotgun proteomic approach and to compare its performance to the traditional gel-based techniques. Different digestion protocols were tested in order to optimize experimental conditions for the total digestion of the protein mixture using low amount of starting material. Peptides were separated with reverse-phase nano-HPLC and on-line analyzed by nano-ESI-MS/MS using information dependent analysis (IDA) on a quadrupole time-of-flight instrument (QTOF). The same sample was also analyzed by traditional gel-based proteomic approaches combined by MALDI-TOFMS and HPLC-ESI-MS/MS analysis of the tryptic peptides (Fig. 1).

2. Materials and methods

Protein molecular weight markers for SDS-PAGE analyses were from GE Healthcare (Amersham Biosciences AB, SE-751 84 Uppsala, Sweden); 30% Acrylamide/bis solution (37.5:1, 2.6%C), Coomassie Brilliant Blue G-250 were from Bio-Rad (Hercules, CA, USA); sequencing grade, modified trypsin (porcine) was from Promega (Madison, WI, USA). HPLC gradient grade solvents (LichroSolv) were from Merck (Whitehouse Station, NJ, USA). α-cyano-4-hydroxycinnamic acid was from Bruker Daltonics (Billerica, MA, USA). RapiGest was from Waters (Milford, MA USA). ICAT kit was from Applied Biosystems (Foster City, CA, USA).

Mitochondria were prepared from bovine heart as in [28]. Complex I was isolated and purified by cholate solubilization and salts fractionation using the standard procedure of Hatefi [29]. Concentration of flavin mononucleotide (FMN) determined according to Ragan et al. [30] amounted to 0.8 nmol/mg. Protein concentration of the sample was 47 mg/mL determined by Biuret assay [31].

2.1. SDS-PAGE based proteomic identification of proteins

Complex I sample (2.2 μL,100 μg) was diluted in 5 μL buffer (0.125 M Tris (hydroxymethyl) methylamine hydrochloride (Tris–HCl) pH 6.8, 4%, sodium dodecyl sulfate (SDS), 20% Glycerol, 0.2 M dithiothreitol (DTT), 0.02% bromophenol blue) containing 4 M Urea and 0.1% dodecylmaltoside solution and boiled at 100 °C for 6 min. The sample was loaded on 12–22% polyacrylamide gradient gel (16 cm × 20 cm) according to the method of Laemmli [32]. Electrophoresis was carried out using the Tris–glycine–SDS buffer system (25 mM Tris, 192 mM glycine and 0.1% SDS) on a Protean II xi cell apparatus (BioRad, Hercules, CA, USA) at 20 mA/gel until the dye front reached the bottom edge of the gel. Low molecular weight standards were used in the gel. Gel was fixed and stained with Coomassie Brilliant Blue G-250. Selected gel bands were cut manually and placed into eppendorf tubes. Reduction, alkylation and hydrolysis of proteins from gel bands were performed according to Shevchenco [33] using 30 μL 10 mM DTT in 100 mM Ammonium Bicarbonate (NH_4HCO_3), 30 μL 55 mM Iodoacetammide (IAA), in the same buffer and 30 μL trypsin at 6 ng/μL concentration in 25 mM NH_4HCO_3.

2.1.1. MALDI-TOFMS analysis of gel separated proteins

0.5 μL of the tryptic peptide mixture was mixed with 0.5 μL of a saturated solution of α-cyano-4-hydroxycinnaminic acid matrix (10 mg/mL in 50% acetonitrile containing 25 fmol/μL angiotensin and 125 fmol/μL Adrenocorticotropic Hormone fragment 18–39 (ACTH) as internal standards). The mixture was spotted on a MALDI target plate and dried under ambient condition. Mass spectra were generated on a MALDI-TOF mass spectrometer Voyager DE™ PRO (Applied Biosystems, Foster City, CA, USA), operating in positive-ion reflectron mode. The laser intensity (N_2, 337 nm) was set just above the ion generation threshold and pulsed every 10 ns. Mass spectra were acquired from each sample in the 400–3500 *m/z* range, by accumulating 100 laser shots and were calibrated using the monoisotopic peaks of angiotensin (*m/z* 931.5154) and ACTH (*m/z* 2465.1989) as internal standards. All mass values are reported as monoisotopic masses. Protein identification was achieved by using the MALDI mass spectral data for database search against the SwissProt database using the MASCOT search algorithm (http://www.matrixscience.com). Parameters for database search were as follows: mammalian as taxonomic category, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues and one missing cleavage and 30 ppm as mass tolerance for the monoisotopic peptide masses.

2.1.2. Nano-HPLC ESI-MS and MS/MS analysis

Analysis of tryptic digest was performed on a QTOF Micro instrument (Waters, Milford, MA, USA) equipped with a nanoflow electrospray ion source employing coated silica capillaries (360 OD/100 ID, Tip 30 ID, New Objective, Woburn, MA, USA) as nanoflow tip. Samples were loaded, purified and concentrated on a pre-column PepMap, C18, 5 mm length, 300 Å, (LCPackings, Sunnyvale, CA, USA) at 20 μL/min flow rate. Peptides were separated on a

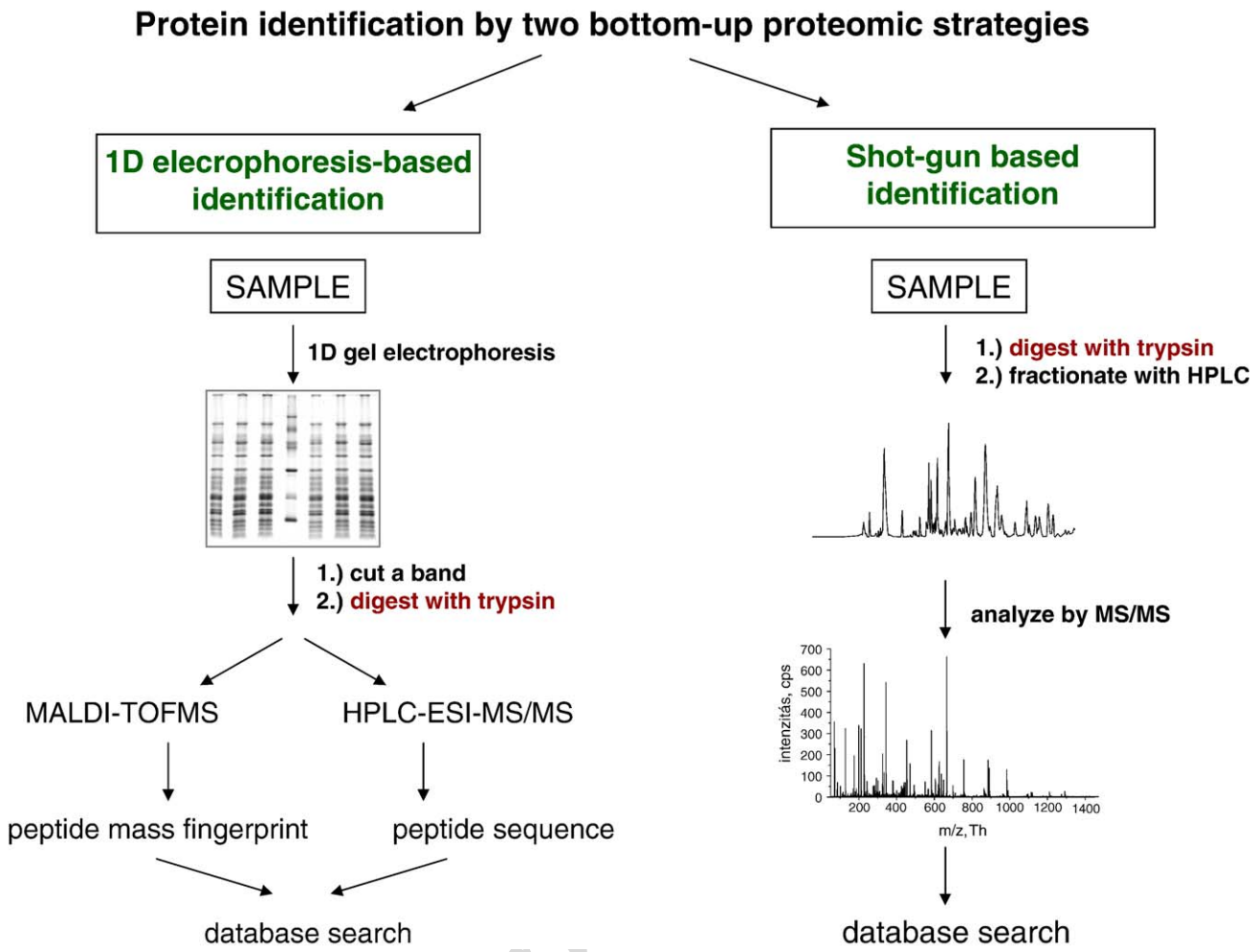

Fig. 1. Flow chart of different mass spectrometry based proteomic approaches used for the identification of complex I subunits in this work.

capillary column Pepmap, C18, 15 cm length, 75 μm ID, 300 Å, (LCPackings, Sunnyvale, CA, USA), using a CapLC micro HPLC (Waters, Milford, MA, USA). Flow rate was split from 5 μL/min to approximately 200 nL/min. Eluents: (A) 2% acetonitrile in 0.1% formic acid (HCOOH) and B) 95% acetonitrile in 0.1% HCOOH were used. Separation was performed by a linear gradient: 2–60% B in 30 min, 60–95% B in 2 min. Collision induced dissociation (CID) was used to generate peptide fragments from multiply charged molecular ions. Argon was used as collision gas. Peak lists of survey experiments were generated by *ProteinLynx Global Server 2.1* (Waters, Milford, MA, USA) and used in Mascot program (http://www.matrixscience.com) for database search. Search criteria were as follows: species: mammals, type of search: MS/MS Ion Search, enzyme: Trypsin, fixed modifications: Carbamidomethyl (C), variable modifications: Acetyl (N-term), Oxidation (M), Phosphorylation (S,T,Y); mass values: monoisotopic, peptide mass tolerance: 100 ppm, fragment mass tolerance: 0.1 Da and number of maximum missed cleavages: 2.

2.2. Analysis based on Shotgun proteomics

Three different protocols were used parallely for the enzymatic digestion of complex I.

2.2.1. Protocol A

Sample (200 μg) was denatured using 36 μL of 50 mM Tris–HCl in 0.1% SDS and reduced in boiling water for 10 min with 1 μL of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP). Alkylation was performed using 5 μL of 55 mM IAA in 50 mM NH_4HCO_3 for 30 min in dark at room temperature. The reduced and alkylated sample was digested by trypsin (40 μL, 6 ng/μL in 25 mM NH_4HCO_3). After overnight incubation at 37 °C, the resulting peptide mixture was acidified with 40 μL 0.1% TFA.

2.2.2. Protocol B

Complex I sample (200 μg) was dissolved in 20 μL 0.2% (w/v) RapiGest SF solution. 5 μL 30 mM DTT in 50 mM NH_4HCO_3 was added and the solution was incubated at 60 °C for 30 min. After boiling the sample for 5 min, 11 μL of 55 mM IAA in 50 mM NH_4HCO_3 was added and the sample was placed in the dark for 30 min. Digestion and acidification were performed according to Protocol A.

2.2.3. Isotope-coded affinity tag (ICAT) labeling experiment

In another experiment isotope labeling was used in order to reduce sample complexity by separating the mixture into two fractions: cysteine containing peptides and cysteine non containing peptides, using ICAT labeling, cation exchange and affinity chromatography [34]. 200 μg of sample was denatured and reduced according to Protocol A. Alkylation was performed using light chain ICAT reagent ($^{12}C_{43}H_{70}N_7O_{12}Si$) of the ICAT kit (Applied Biosystems, Foster City, CA, USA). After the alkylation step, digestion was performed according to protocol A. Peptides were purified by cation exchange chromatography and labeled peptides were separated using avidin affinity column. Fractions were collected and analyzed directly by nano-HPLC-ESI-MS/MS.

2.2.4. Nano-HPLC- ESI-MS/MS analysis of enzymatically digested complex I sample

Nano-HPLC separation of peptide mixtures were performed using an Integral 100Q HPLC system (PerSeptive Biosystems, Framingham, MA USA). Flow rate was split from 400 μL/min to 200 nL/min using a flow splitter

(Accurate, LCPackings, Sunnyvale, CA USA). Eluents: (A) 5% acetonitrile in 0.08% HCOOH and 0.01% TFA and (B) 95% acetonitrile in 0.08% HCOOH and 0.01% TFA. Gradient: 5–50% B in 90 min 50–100% B in 5 min. 1 μg of digested sample (about 1 pmol calculated for the pure complex I) was loaded, purified and concentrated on a pre-column, PepMap, C18, 5 mm length, 300 Å (LCPackings, Sunnyvale, CA, USA) using an external Phoenix HPLC pump (Fisons-Instruments, Manchester, UK) at 20 μL/min flow rate. Peptides were separated on a capillary column, C18 PepMap, 15 cm length, 75 μm ID, 300 Å (LCPackings, Sunnyvale, CA, USA). For ESI-MS and MS/MS analysis a QTOF type mass spectrometer (QStar-Pulsar, Applied Biosystems, Foster City, CA, USA) equipped with a nanoflow electrospray ion source fitted with pulled uncoated silica capillary (150 OD/50 ID) as nanoflow tip was employed. Experiments were performed in IDA mode. Precursor ions were selected for fragmentation using the following MS to MS/MS switch criteria: ions greater than *m/z* 300.0, charge state 2 to 4, intensity exceeds 15 counts, former target ions were excluded for 60 s, ion tolerance 50.0 mmu. CID was used to fragment multiply charged ions. Nitrogen was used as collision gas. Automatically generated peak lists were used for MS/MS ion search using the Mascot program against the SwissProt database. The criteria for database searches were the ones already used in the nano-HPLC-ESI-MS/MS experiments performed on the QTOF-Micro instrument.

3. Results

We performed analysis using traditional 1D gel-based (MALDI PMF and ESI-MS/MS peptide sequencing) and shotgun proteomic approaches (Fig. 1) to compare their performances for the characterization of subunit composition of a highly hydrophobic, multi-subunit protein complex. The main difference between shotgun and gel-based approaches is that in the first case subunits are not separated prior analysis but directly digested by a proteolytic enzyme. This results in a very complex mixture of peptides which is than separated and online analyzed by ESI mass spectrometry using information dependent MS and MS/MS analysis in an automated fashion.

Complex I was isolated from bovine heart mitochondria using the standard procedure of Hatefi [29]. In this preparation complex I retains its inhibitor sensitivity and enzymatic activity [35]. The preparation is also known to have a relatively high lipid content and can contain significant amounts of transhydrogenase and other proteins related to the OXPHOS system.

3.1. SDS-PAGE based proteomic method

The pattern of protein bands separated on SDS-polyacrylamide gradient gel (Fig. 2) shows similarities to those obtained on a highly purified complex I sample by Carroll et al. [4]. Sample purity and differences in experimental conditions (such as gel gradient), however, can influence the 1D gel pattern. Thirty-one bands detected by Coomassie staining were excised from the gel (Fig. 2) and the proteins present in each band were digested with trypsin analyzed both by MALDI-TOFMS and by nano-HPLC-ESI-MS/MS (Fig. 1). All bands except one (band 1) contained more than one identified protein (Table 2 and Supplementary Table 1).

3.1.1. Proteins identified by PMF

MALDI-TOFMS analysis of 31 gel bands led to the identification of 37 mitochondrial proteins: twenty nine

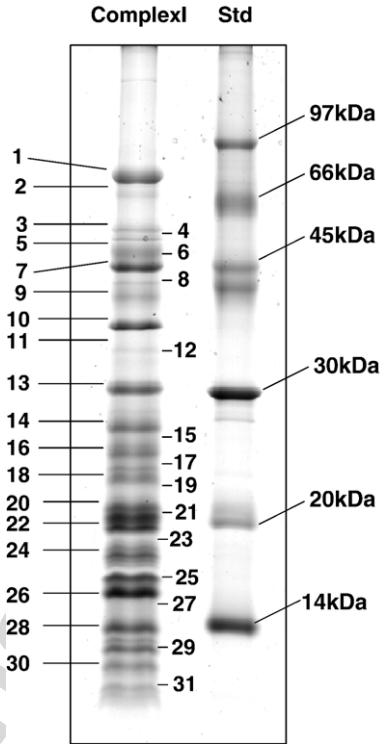

Fig. 2. SDS-PAGE image of mitochondrial complex I from bovine heart. Gel bands 1–31 visualized by Coomassie staining were excised and analyzed by MALDI-TOFMS and nano-HPLC-ESI-MS/MS. Lane on the right shows the molecular weight standards.

nuclear DNA encoded proteins from the complex I assembly and eight subunits from other OXPHOS complexes. None of the mtDNA encoded subunits nor subunits ESSS, B14.7, B12, B9, KFYI, MLRQ, AGGG, MWFE and 9.6 kDa were identified. It should be of note that all of the missing subunits are hydrophobic, characterized by positive Gravy scores (Table 1). Hydrophobic proteins entering into the gel can result in diffused gel bands and poor staining with Comassie blue dye. In addition their 1D gel-based analysis is usually hampered by their low solubility. Some of the subunits (such as 75 kDa, 51 kDa, 39 kDa, PDSW, B8) were identified in more than one gel band, thus making 1D gel-based image complicated. Co-migrating proteins due to the low resolving power of electrophoresis is another problem. For example, in band 19, four different subunits (19 kDa, B22, B18 and PDSW) were identified simultaneously based on a single MALDI mass spectrum (Table 2). Suppression effects, caused by co-migrated proteins, lower protein scores and therefore limit the PMF-based protein identification.

Eight proteins not directly belonging to complex I were also identified in the sample by MALDI PMF. These include succinate dehydrogenase flavoprotein (band 2) from complex II; complex III core protein I (band 6) and cytocrome c1 (band 12); three subunits of cytocrome-c oxidase (complex IV): IV (band 24), Va (band 27) and VIb (band 30); and two ATP synthase (complex V) subunits: alpha (band 3) and beta (bands 3, 4). Their presence can be explained by their co-

Table 2
Identified subunits and corresponding MASCOT scores obtained by 1D gel separation combined with MALDI-TOFMS PMF and nano-HPLC-ESI-MS/MS peptide sequencing

Band[a]	Subunit[b]	Swiss-Prot access number	MALDI-TOFMS PMF			ESI-MS/MS ion search		
			Score[c]	Match[d]	Coverage (%)[e]	Score[c]	Number of peptides sequenced	Coverage (%)[e]
1	75 kDa	P15690	331	32/52	50	1167	24	42
2	75 kDa	P15690	294	30/46	45	600	19	28
	49 kDa	P17694	–	–	–	40	2	5
3	75 kDa	P15690	107	18/96	28	278	6	12
	51 kDa	P25708	63	11/96	25	79	3	5
4	51 kDa	P25708	113	13/53	31	227	8	17
	75 kDa	P15690	–	–	–	101	3	5
5	51 kDa	P25708	159	12/20	30	545	13	28
6	51 kDa	P25708	192	20/66	43	487	11	25
	49 kDa	P17697	173	18/66	40	357	8	18
	75 kDa	P15690	–	–	–	29	1	1
7	49 kDa	P17694	174	14/33	41	595	12	31
	51 kDa	P25708	–	–	–	108	3	9
	42 kDa	P34942	–	–	–	32	2	7
	75 kDa	P15690	–	–	–	29	1	1
8	49 kDa	P17694	156	17/68	38	205	7	18
	42 kDa	P34943	71	10/68	21	192	4	12
	75 kDa	P15690	59	11/68	17	53	2	3
	51 kDa	P25708	60	7/32	16	–	–	–
9	42 kDa	P34942	156	14/42	44	399	8	26
	49 kDa	P17694	–	–	–	68	3	7
	75 kDa	P15690	–	–	–	53	2	6
10	39 kDa	P34943	217	19/45	51	497	10	31
	42 kDa	P34942	–	–	–	15	1	2
11	39 kDa	P34943	66	7/27	18	32	3	11
	75 kDa	P15690	64	8/27	10	–	–	–
12	39 kDa	P34943	–	–	–	11	1	2
13	30 kDa	P23709	219	17/40	58	307	7	28
14	24 kDa	P04394	109	8/23	37	305	6	25
	B22	Q02369	41	4/15	18	123	4	20
	PDSW	Q02373	–	–	–	51	3	20
	23 kDa	P42028	–	–	–	37	1	5
	39 kDa	P34943	–	–	–	18	2	5
15	24 kDa	P04394	–	–	–	215	5	24
	B22	Q02369	–	–	–	203	5	28
	23 kDa	P42028	–	–	–	202	4	22
	PDSW	Q02373	92	7/26	37	153	4	28
	30 kDa	P23709	–	–	–	64	2	9
	49 kDa	P17697	–	–	–	28	2	4
16	23 kDa	P42028	52	5/27	27	244	4	22
	PDSW	Q02373	80	7/34	45	207	5	34
	B22	Q02369	–	–	–	188	5	28
	30 kDa	P23709	–	–	–	117	3	13
	24 kDa	P04394	–	–	–	25	1	5

(continued on next page)

Table 2 (*continued*)

Band[a]	Subunit[b]	Swiss-Prot access number	MALDI-TOFMS PMF			ESI-MS/MS ion search		
			Score[c]	Match[d]	Coverage (%)[e]	Score[c]	Number of peptides sequenced	Coverage (%)[e]
17	PDSW	Q02373	97	8/26	45	301	8	50
	23 kDa	P42028	74	6/18	21	220	6	27
	B22	Q02369	–	–	–	227	5	28
	30 kDa	P23709	–	–	–	66	1	4
	19 kDa	P42029	–	–	–	62	2	14
	24 kDa	P04394	–	–	–	53	2	9
	49 kDa	P17697	–	–	–	42	2	4
	20 kDa	P42026	–	–	–	30	1	4
	51 kDa	P25708	–	–	–	20	1	2
18	PDSW	Q02373	166	16/53	71	264	6	34
	B22	Q02369	69	9/53	59	188	5	28
	23 kDa	P42028	68	7/28	28	113	3	14
	20 kDa	P42026	–			102	3	14
19	B22	Q02369	79	10/67	57	130	4	23
	20 kDa	P42026	–			121	3	14
	PDSW	Q02373	56	6/38	37	104	3	18
	19 kDa	P42029	80	12/67	52	49	1	6
	B18	Q02368	70	7/45	41	39	2	19
	23 kDa	P42028	–	–	–	26	1	5
	ASHI	Q02372	–	–	–	11	2	10
20	B18	Q02368	92	9/64	64	167	5	36
	19 kDa	P42029	100	10/64	64	117	5	38
	20 kDa	P42026	48	5/38	15	104	3	14
	ASHI	Q02372	62	7/64	41	98	4	25
	18 kDa	Q02375	–			22	2	10
21	B18	Q02368	71	7/36	41	363	8	58
	19 kDa	P42029	53	5/29	31	140	5	27
	18 kDa	Q02375	–	–	–	126	3	16
	20 kDa	P42026	–	–	–	71	3	14
	ASHI	Q02372	–	–	–	19	2	10
22	18 kDa	Q02372	75	9/62	41	225	4	22
	B17	Q02367	55	6/44	35	158	4	27
	B18	Q02368	57	6/38	34	136	3	28
	ESSS	Q8HXG5	–	–	–	54	2	14
	19 kDa	P42029	83	9/62	55	48	1	7
	75 kDa	P15690	–	–	–	28	1	1
	20 kDa	P42026	–	–	–	27	1	4
23	B17.2	O97725	82	6/24	46	239	8	55
	SGDH	Q02380	50	5/24	20	138	4	20
	B16.6	Q95KV7	–	–	–	56	3	21
	19 kDa	P42029	–	–	–	29	1	6

24	B17.2	O97725	158	12/44	66	336	6	64
	B16.6	Q95KV7	52	5/26	25	275	6	48
	B14.5b	Q02827	–	–	–	41	1	9
	SGDH	Q02380	–	–	–	38	2	10
	19 kDa	P42029	–	–	–	32	1	6
25	B15	P48305	141	11/35	62	295	9	50
	B14.5a	Q05752	48	5/35	33	109	5	53
	B14.5b	Q02827	–	–	–	158	4	34
	15 kDa	Q02379	–	–	–	42	2	13
	51 kDa	P25708	–	–	–	32	3	6
	B14	Q02366	–	–	–	18	1	6
26	B14	Q02366	–	–	–	106	4	14
	B14.5b	Q02827	47	4/26	35	83	3	23
	B14.5a	Q05752	91	9/42	58	57	2	13
	15 kDa	Q02379	73	7/42	57	57	2	13
	B15	P48305	–	–	–	29	1	7
27	B12	Q02365	–	–	–	118	2	19
	B14.7	Q8HXG6	–	–	–	71	1	7
	B14	Q02366	52	6/43	35	45	2	18
	13 kDa-A	P23934	–	–	–	45	2	21
	B22	Q02369	–	–	–	35	1	7
	13 kDa-B	P23935	59	6/49	40	27	1	13
	B14.5b	Q02827	–	–	–	18	1	9
	23 kDa	P42028	–	–	–	17	1	5
28	13 kDa-A	P23934	74	6/36	54	275	6	41
	13 kDa-B	P23935	81	7/36	44	138	4	31
	B8	Q02370	–	–	–	112	3	33
	B12	Q02365	–	–	–	107	2	37
	B22	Q02369	–	–	–	26	1	7
	30 kDa	P23709	–	–	–	24	1	4
	B14.5b	Q02827	–	–	–	20	1	6
	B16.6	Q95KV7	–	–	–	20	1	6
29	B8	Q02370	138	11/38	75	222	4	37
	B14.7	Q8HXG6	–	–	–	29	1	7
	9 kDa	P25712	63	6/27	39	–	–	–
30	MNLL	Q02378	–	–		46	1	14
	B8	Q02370	58	5/25	45	–	–	–
31	MNLL	Q02378	45	3/16	33	–	–	–
	B9	Q02371	–	–	–	37	1	8

[a] Number of SDS-PAGE gel bands as shown in Fig. 2.

[b] Short name of the identified complex I subunits (as in Table 1). Other OXPHOS proteins identified by SDS-PAGE MALDI PMF and/or HPLC-ESI-MS/MS sequencing are listed in the text and in Supplementary Table 1.

[c] MASCOT score of PMF and MS/MS ion searches, respectively.

[d] Matched peaks/total number of peaks sent to MASCOT PMF search.

[e] Protein sequence coverage in percentage obtained by PMF and MS/MS identifications, respectively.

Table 3
Identified proteins and their respective MASCOT scores obtained by the shotgun approach using protocol A, protocol B and ICAT labeling experiment

Subunit	Swiss-Prot access number	Protocol A		Protocol B		ICAT labeling experiment	
		Score	Number of peptides sequenced	Score	Number of peptides sequenced	Score	Number of peptides sequenced
75 kDa	P15690	1412	31	859	24	992	24
51 kDa	P25708	1075	23	403	11	378	13
49 kDa	P17694	501	13	285	9	300	8
30 kDa	P23709	568	10	402	7	656	10
24 kDa	P04394	549	12	332	10	295	7
20 kDa	P42026	118	3	51	1	88	3
23 kDa	P42028	178	5	104	2	201	4
42 kDa	P34942	314	7	264	6	319	8
39 kDa	P34943	594	12	464	12	377	10
18 kDa	Q02375	270	7	183	5	108	3
15 kDa	Q02379	200	5	87	2	76	2
13 kDa-A	P23934	468	8	87	4	168	3
9 kDa	P25712	122	3	32	4	n.d.	–
AGGG	Q02374	26	1	28	1	n.d.	–
ASHI	Q02372	269	7	106	5	33	2
ESSS	Q8HXG5	178	5	61	2	77	4
KFYI	Q02376	n.d.	–	26	1	n.d.	–
MLRQ	Q01321	n.d.	–	n.d.	–	n.d.	–
MNLL	Q02378	42	1	13	2	9	1
MWFE	Q02377	21	1	7	2	25	1
PDSW	Q02373	499	11	350	11	229	6
19 kDa	P42029	387	9	248	6	280	9
9.6 kDa	P52505	330	8	206	6	263	8
SGDH	Q02380	164	4	26	2	90	2
B22	Q02369	329	8	160	5	146	4
B18	Q02368	236	6	127	4	360	8
B17.2	O97725	301	7	221	5	189	4
B17	Q02367	177	5	80	4	50	2
B16.6	Q95KV7	316	11	125	5	100	3
B15	P48305	234	5	126	5	70	3
B14.7	Q8HXG6	152	3	118	2	142	3
B14.5a	Q05752	355	9	155	4	81	2
B14.5b	Q02827	127	2	123	2	125	2
B14	Q02366	182	3	96	4	81	3
13 kDa-B	P23935	169	4	216	6	219	5
B12	Q02365	90	2	72	1	n.d.	–
B9	Q02371	42	2	35	1	n.d.	–
B8	Q02370	207	4	268	9	116	2
ND1	P03887	n.d.	–	109	3	113	2
ND2	P03892	n.d.	–	21	1	32	1
ND3	P03898	n.d.	–	n.d.	–	27	1
ND4	P03910	23	2	47	1	32	2
ND4L	P03902	n.d.	–	n.d.	–	n.d.	–
ND5	P03920	90	4	237	7	77	2
ND6	P03924	n.d.	–	n.d.	–	n.d.	–

purification during isolation protocol, however, it cannot be excluded that they play a role in the assembly or regulation of complex I.

3.1.2. Proteins identified by peptide sequencing

In this experiment peptide mixtures from in-gel tryptic digestion of the 31 SDS-PAGE bands (Fig. 2) were fractionated by reverse-phase nano-HPLC and the proteins were analyzed by nano-ESI-MS/MS in data dependent acquisition (DDA) mode (Fig. 1). In total, 50 proteins including thirty two complex I subunits out of 46 were identified (Table 2 and Supplementary Table 1). The presence of four subunits, ESSS (band 22), B14.7 (bands 27 and 29), B12 (bands 27 and 28) and B9 (band 31), not identified by MALDI PMF, was also confirmed. However, the seven mtDNA encoded subunits ND1–ND6 and ND4L, were not detected, nor were subunits 9 kDa (8437.4 Da), AGGG (8.493.4 Da), KFYI (5828.7 Da), MLRQ (9324.7 Da), MWFE (8105.4 Da), and 9.6 kDa (10109.7 Da). It should be noted that all of the missing nuclear encoded subunits have low-molecular masses and thus they migrate in a congested, high polyacrylamide percentage region of the gel (below 10 kDa) where their extraction and consequently their detection is difficult. In

addition, they generally produce only a few proteolytic fragments upon tryptic digestion, thus limiting protein identification by MS.

With this approach generally more proteins were identified in a single gel band compared to the PMF-based method (Table 2) because the limited resolving power of SDS-PAGE was compensated by the HPLC separation of the peptides. HPLC-ESI-MS/MS experiments showed the presence of 18 proteins in the sample which do not belong to complex I (Supplementary Table 1): three subunits from Ubiquinol—cytochrome-c reductase complex (complex III): Core protein I (bands 5–6) and II (band 7) and iron–sulfur subunit (band 15); four subunits from Cytochrome c oxidase (complex IV) polypeptides: IV isoform 1 (band 24), Va (band 27), Vb (band 27) and VIb (band 29); and eight ATP synthase (complex V) chains: alpha (band 3), beta (bands 3–5) chain B (band 14), oligomycin sensitivity conferral protein (bands 16–17), chain D (bands 16–17), e chain (bands 27–28), f chain (bands 28–29) and delta chain (band 25) and three other mitochondrial proteins like Acetyl-CoA carboxylase 1 (band 7), NAD(P) transhydrogenase (band 17) and ADP, ATP carrier protein, heart isoform T1 (band 16) were identified.

3.2. Subunits identified by shotgun proteomic approach

Samples obtained by total tryptic digestion of complex I were analyzed by nano-HPLC-ESI-MS/MS in IDA mode. Two protocols (Protocols A and B) differing in denaturation and reducing conditions were tested to compare their efficiency in the identification of subunits, especially the most hydrophobic ones. These results are summarized in Table 3 (Supplementary Table 2 and 3). The results show generally higher MASCOT scores, reflecting a better sequence coverage per single subunit by protocol A. Based on the number of identified proteins, however, the performance of protocol B (RapiGest as denaturing buffer and DTT as reducing agent) (Supplementary Table 3) was slightly better than Protocol A (50 mM Tris–HCl in 0.1% SDS and TCEP) (Supplementary Table 2). Protocols A and B yielded the identification of 38 and 41 subunits, respectively. In the latest experiment, almost all nuclear encoded subunits (except MLRQ) plus four of the seven mitochondrial subunits (except ND3, ND4L and ND6) were detected.

In another experiment, after denaturation and reduction (which was performed according to protocol A) stabile isotope labeling of cysteine residues was carried out using acid-labile

Table 4
Proteins non belonging to complex I identified by the shotgun approach using Protocol A, Protocol B and ICAT labeling experiments, respectively

Name of the protein		Protocol A		Protocol B		ICAT labeling experiment	
		Score	Number of peptides sequenced	Score	Number of peptides sequenced	Score	Number of peptides sequenced
Complex V	ATP synthase alpha chain heart isoform	507	12	629	13	370	9
	ATP synthase beta chain	613	17	197	6	369	10
	ATP synthase gamma chain	27	1	27	1	n.d.	–
	ATP synthase delta chain	36	1	n.d.	–	n.d.	–
	ATP synthase B chain	27	1	27	1	n.d.	–
	ATP synthase D chain	26	1	71	2	87	3
	ATP synthase E chain	n.d.	–	31	1	n.d.	–
	ATP synthase F chain	37	1	39	1	63	1
	ATP synthase 8 (subunit 8)	n.d.	–	27	1	19	2
	ATP synthase oligomycin sensitivity conferral protein	n.d.	1	48	2	61	1
Complex III	Ubiquinol–cytochrome-c reductase complex core protein 1	87	2	48	1	142	4
	Ubiquinol–cytochrome-c reductase complex core protein 2	113	3	43	1	72	2
	Ubiquinol–cytochrome c reductase iron–sulfur subunit	13	1	n.d.	–	n.d.	–
	Ubiquinol–cytochrome c reductase complex 11 kDa protein	59	1	14	1	37	1
	Ubiquinol–cytochrome c reductase complex 14 kDa protein	n.d.	–	19	1	37	1
	Cytochrome c1, haem protein	17	1	36	1	55	2
Complex II	Succinate dehydrogenase [ubiquinone] flavoprotein subunit	8	1	71	1	52	1
Complex IV	Cytochrome c oxidase polypeptide II	14	1	35	1	n.d.	–
	Cytochrome c oxidase subunit IV isoform 1	93	4	41	2	11	1
	Cytochrome c oxidase polypeptide Va	101	2	46	1	37	1
	Cytochrome c oxidase polypeptide VIa-heart	34	2	20	1	n.d.	–
	Cytochrome c oxidase polypeptideVIIa-heart	38	1	25	2	42	1
	Cytochrome c oxidase polypeptide Vb	78	2	135	3	n.d.	–
	Cytochrome c oxidase polypeptide VIb	36	1	26	1	47	2
Other	Translation initiation factor IF-2	33	2	13	1	n.d.	–
	ADP,ATP carrier protein, heart isoform T1	121	5	48	1	51	2
	ADP, ATP carrier protein	n.d.	–	48	1	99	3
	NAD(P) transhydrogenase	909	24	426	15	313	16

ICAT light reagent with biotin tag (released by Applied Biosystems, Inc., www.appliedbiosystems.com) [34]. The scope of this analysis was to reduce sample complexity by separating the peptides into two fractions (peptides having cysteine residues and peptides without cysteines) (Table 1) in order to improve identification of subunits. After labeling, the peptides were separated by affinity capture of the biotin tag on immobilized avidin column. The flow-through and the binding fractions were analyzed by nano-HPLC-ESI-MS/MS in IDA mode. Scores of the individual subunits were calculated as a sum of the two analyses (Table 3 and Supplementary Table 4). The results show that ICAT labeling and separation was the most efficient in the identification of mitochondrial encoded subunits, identifying five of these (ND1–ND4 and ND5), however, some of the nuclear encoded subunits already identified by protocol B (10 kDa, AGGG, B9, KFYI and B12) (Table 3, Supplementary Table 3) could not be detected in this way. This shows that the complexity of the sample still hampers the successful identification of some of the highly hydrophobic nuclear encoded subunits.

It should be noted that identification of KFYI, MLRQ, ND4L and ND6 subunits is the most problematic. KFYI protein was identified only by protocol B (Table 3). MLRQ is 9 kDa protein whose presence in the complex is not certain. It was not detected in any of the reported experiments. The absence of ND4L and ND6 identification is due to the low number of theoretical cleavage sites: using tryptic digestion, only a few peptides can be generated in the high molecular mass region, making difficult subsequent MS analyses. The smallest peptides obtained from the theoretical enzymatic cleavage of ND6 and ND4L have, in fact, molecular masses of 2510.33 Da and 2700.30 Da, respectively. MS/MS sequencing of such high molecular weight peptides raise problems. ND4L also has the highest Gravy score (1.259) and the lowest molecular mass amongst the mitochondrial encoded highly hydrophobic proteins (Table 1).

In several nuclear subunits of complex I the N-terminal methionine has been removed post-translationally; in other nuclear subunits the mitochondrial import sequence has been removed [36]. Fourteen of the nuclear encoded complex I subunits are known to have modified N-terminals (Table 1) [37]. We have found that five of these subunits (B22, B17.2, B8, 13 kDa-A and B16.6) are N-α-acetylated (Supplementary material, Table 2). Complex I from bovine heart has different potentially phosphorylated subunits including the 42 kDa (Ser-59) [38], ESSS (Ser-20) [39], MWFE (Ser-55) [39] and the 18 kDa (position 129–131) [40] subunits (Table 1). Our MS/MS experiments, however, did not confirm any steady-state phosphorylation of complex I subunits, probably due to their low degree of modification.

3.2.1. Other proteins identified by the shotgun proteomic approach

Using the shotgun approach, 30 co-purified proteins involved in the electron transport chain, but structurally not directly linked to complex I, were also identified (Table 4). The highest number of proteins were identified by Protocol B. Majority of these proteins belongs to the OXPHOS system: 11 proteins related to complex V subunit, 8 subunits of complex IV, 6 of complex III and 1 complex II subunit were identified. They can be co-purified proteins, or alternatively, proteins involved in the successful assembly and regulation of the complex.

4. Discussion

Given the importance of complex I impairment in a large number of pathologies, advanced genomic and proteomic tools have recently been employed to identify mutations and structural and functional alterations of constituent subunits and assembly factors of the complex specifically associated with the various diseases. Today, proteomic approaches based on gel electrophoresis combined with mass spectrometry are the preferred techniques for the characterization of the complex I [4,6,27]. In spite of the success of classical gel-based proteomics for determining the subunit composition of complex I, the approach has its limitations with regard to membrane proteins [41]. In addition these approaches require a relatively large quantity of samples (100 μg), laborious purification procedures and time-consuming analytical steps with the risk of

Table 5
Summary of the identified complex I subunits using different proteomic approaches

Strategy	Identification method	Nuclear encoded subunits	Mitochondrial encoded subunits	Number of identified subunits
1D gel-based	MALDI-TOFMS PMF	75 kDa, 51 kDa, 49 kDa, 30 kDa, 24 kDa, 20 kDa, 23 kDa, 42 kDa, 39 kDa, 18 kDa, 15 kDa, 13-AkDA, 9 kDa, ASHI, MNLL, PDSW, 19 kDa, SGDH, B22, B18, B17.2, B17, B16.6, B15, B14.5a, B14.5b, B14, 13 kDa-B, B8	None	29
	HPLC-ESI-MS/MS sequencing	75 kDa, 51 kDa, 49 kDa, 30 kDa, 24 kDa, 20 kDa, 23 kDa, 42 kDa, 39 kDa, 18 kDa, 15 kDa, 13 kDa-A, ASHI, ESSS, MNLL, PDSW, 19 kDa, SGDH, B22, B18, B17.2, B17, B16.6, B15, B14.7, B14.5a, B14.5b, B14, 13kDa-B, B12, B9, B8	None	32
Shotgun	HPLC-ESI-MS/MS sequencing	75 kDa, 51 kDa, 49 kDa, 30 kDa, 24 kDa, 20 kDa, 23 kDa, 42 kDa, 39 kDa, 18 kDa, 15 kDa, 13 kDa-A, 9 kDa, AGGG, ASHI, ESSS, KFYI, MNLL, MWFE, PDSW, 19 kDa, 9.6 kDa, SGDH, B22, B18, B17.2, B17, B16.6, B15, B14.7, B14.5a, B14.5b, B14, 13 kDa-B, B12, B9, B8	ND1 ND2 ND3 ND4 ND5	42

sample loss and sample alteration, therefore they cannot be applied in clinical screen testing.

In this work, we applied the shotgun proteomic approach for the identification of the proteins present in less than 1 μg enzymatically active complex I isolated from bovine heart. Table 5 shows the identified subunits obtained by three different proteomic strategies. The shotgun approach led to the identification of 42 subunits (corresponding to more than 95% of all complex I subunits) while 29 and 32 subunits were identified by the two SDS-PAGE based bottom-up strategies: MALDI-TOFMS PMF, and nano-HPLC-ESI-MS/MS sequencing, respectively. In particular, IDA experiments allowed the identification of five mtDNA (except ND4L and ND6) and almost all nuclear encoded subunits (except MLRQ), therefore demonstrating the superior performance of the shotgun approach compared to gel-based methods in the characterization of mitochondrial complexes.

Gathering all our data, we were able to identify 70 different mitochondrial proteins with the shotgun proteomic approach compared to 37 and 50 proteins identified with gel-based methods (MALDI-TOFMS and HPLC-ESI-MS/MS, respectively). This clearly shows that shotgun proteomics gave a better overall performance in the identification of the proteins present in a highly complex mixture. Some of these proteins structurally are not linked to complex I. These can be co-purified proteins, and their presence depend on the protocol used for the isolation of complex I. At the same time we cannot exclude the hypothesis that they have biochemical role in the assembly, stabilization, regulation and substrate channeling of complex I through the formation of supramolecular complexes. The association of the mitochondrial respiratory chain complexes into higher supramolecular structures called supercomplexes has been shown by isolation of structural associates using BN-PAGE [42]. Supercomplexes containing complexes I, III and IV have been isolated from bovine heart mitochondria. It has also been demonstrated that assembled complex III is required to stabilize the structure of complex I in mammalian cells [43] and the early stage of complex I assembly is linked to complex III and IV supercomplexes [44]. It should be noted that subunits from other OXPHOS complexes have been also found in a BN-PAGE separated complex I sample [27].

With the shotgun approach discussed herein, the time of the analysis was reduced considerably compared to gel based approaches. Nano-HPLC-ESI-MS/MS analysis of peptides generated from the digestion of the intact complex needs only a few hours, rendering it quite advantageous for clinical screening. The method can also be useful to probe post-translational modifications as it was proved by the identification of N-acetylation of some of the subunits. ICAT labeling also gives the opportunity to quantify changes in protein abundance [45]. In this manner, shotgun proteomics combined with isotope labeling can become a useful tool for relative quantification of samples, and also for the screening of post-translational modification. In addition, the shotgun approach could be useful for the analysis in human patients of defects in complex I assembly and characterization of sub-complexes [46,47]. Since increased complexity of the peptide mixture can be difficult to handle with one dimensional RP-HPLC separation, the method can be further improved by higher resolution chromatographic separation procedures like multidimensional HPLC.

Acknowledgements

Authors are grateful of the supports from “Rete di Spettrometria di Massa” (contract FERS n. 94.05.09.103, ARINCO N. 94.IT.16.028); Joint Projects between the National Research Council (CNR) of Italy and the Hungarian Academy of Sciences (MTA) 2004/2006; and the “Research Grant from Foundation Cassa di Risparmio di Puglia, 2004”.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2006.05.037.

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