Analytical Biochemistry 407 (2010) 104–110

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Subunit composition of *Rhodothermus marinus* respiratory complex I

Ana P. Batista, Catarina Franco, Marta Mendes, Ana V. Coelho, Manuela M. Pereira *

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, 2780-157 Oeiras, Portugal

ARTICLE INFO

Article history:
Received 25 May 2010
Received in revised form 30 July 2010
Accepted 30 July 2010
Available online 6 August 2010

Keywords:
Mass spectrometry
NADH:quinone oxidoreductase
NADH dehydrogenase
Respiratory chain

ABSTRACT

The basic structural characterization of complex I is still not trivial due to its complexity, not only in the number of its protein constituents but especially because of the different properties of the several subunits. Bacterial complex I generally contains 14 subunits: 7 hydrophilic proteins located in the peripheral arm and 7 hydrophobic proteins present in the membrane arm. It is the identification of the hydrophobic proteins that makes the characterization of complex I, and of membrane proteins in general, very difficult. In this article, we report the identification of the subunits of complex I from *Rhodothermus marinus*. The original approach, presented here, combined several protein and peptides separation strategies (different reversed phase materials, high-performance liquid chromatography, and gel electrophoresis) with different identification methods (electrospray ionization–tandem mass spectrometry, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry, and Edman degradation analysis) and represents a step forward in the characterization of membrane proteins that studies are still technically highly challenging. The combination of the different methodologies allowed the identification of complex I canonical subunits and also a possible novel subunit, namely a pterin-4α-carbinolamine dehydratase (PCD). This was the first time that a PCD was suggested to be part of complex I, and its possible regulatory role is discussed.

Respiratory complex I couples electron transfer from nicotinamide adenine dinucleotide (reduced form, NADH)[1] to quinone with charge translocation across the membrane, thereby contributing to the transmembrane difference of electrochemical potential. The mitochondrial enzyme contains more than 40 subunits, whereas the bacterial complex is composed of the minimal functional unit, generally with 14 subunits named Nqo1 to Nqo14. Complex I is L-shaped, having 7 subunits located in the peripheral part [1]. The crystallographic structure of this hydrophilic part was already determined, showing the localization of the prosthetic groups, a series of iron–sulfur centers (binuclear and tetranuclear), and one non-covalently bound flavin mononucleotide (FMN) [2]. The other 7 subunits are hydrophobic, constitute the membrane part of the complex, and most likely are involved in quinone reduction and charge translocation.

Complex I from *Rhodothermus marinus* was isolated with one FMN and six to eight iron–sulfur centers of the $[2Fe\text{-}2S]^{2+/1+}$ and $[4Fe\text{-}4S]^{2+/1+}$ types [3,4]. It is sensitive to the complex I inhibitor rotenone, and its reconstitution in liposomes showed proton translocation coupled to NADH:quinone oxidoreductase activity. The genes coding for the Nqo subunits are clustered in two operons, *nqo*1–7 (nqoA) and *nqo*10–14 (nqoB), and two independent genes, *nqo*8 and *nqo*9. Among complex I genes, and cotranscribed with them, two additional genes encoding a pterin-4α-carbinolamine dehydratase (PCD) and a putative Nha-type sodium/proton antiporter were identified [5].

The polypeptide composition of complex I and the specific assembly of its subunits into a large complex are fundamental aspects to understand its function. Because complex I is composed of proteins with very different hydrophobic properties, the identification of all the subunits was revealed to be highly challenging. There are several methods described in the literature for membrane protein identification, including the subunits of complex I [6–13]. However, the identification process is still not straightforward.

To identify the subunits of *R. marinus* complex I, different methods, such as mass spectrometry (MS), Edman degradation, and reversed phase (RP) high-performance liquid chromatography (HPLC), were used. The originality of our approach was based on the combination of a sequential elution of the intact and digested complex in four differently packed RP microcolumns (POROS R1, R2, R3, and graphite) with MS analysis. The identification of the

* Corresponding author. Fax: +351 214469314.
E-mail address: mpereira@itqb.unl.pt (M.M. Pereira).

[1] *Abbreviations used:* NADH, nicotinamide adenine dinucleotide (reduced form); FMN, flavin mononucleotide; PCD, pterin-4α-carbinolamine dehydratase; RP, reversed phase; HPLC, high-performance liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; DDM, *n*-dodecyl-β-D-maltoside; DMN, 2,3-dimethyl-1,4-naphthoquinone; BN, blue native; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; ACN, acetonitrile; MALDI–TOF MS, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry; NH_4HCO_3, ammonium bicarbonate; R, arginine; K, lysine; MS/MS, tandem mass spectrometry; PMF, peptide mass fingerprinting; ESI, electrospray ionization.

0003-2697/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2010.07.038

canonical subunits, as well as the PCD, was possible only by integrating the different methodologies.

Materials and methods

General procedures

Cell growth and protein purification

Bacterial growth, membrane preparation, and solubilization were done as described previously [14] except that the growth medium contained 100 mM glutamate. Complex I was purified according to an established procedure [4], optimized by introducing a further chromatographic step, a Mono Q column. Briefly, the sample was submitted to two successive High Performance Q-Sepharose columns, using as buffer 20 mM Tris–HCl (pH 8.0), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.1% *n*-dodecyl-β-D-maltoside (DDM), and was eluted in a linear gradient, 0 to 1 M NaCl. The fraction containing complex I was then applied to a gel filtration S200 column, eluted with 20 mM Tris–HCl (pH 8.0), 1 mM PMSF, 0.1% DDM, and 150 mM NaCl, and finally applied on a Mono Q column using 20 mM Tris–HCl (pH 8.0), 1 mM PMSF, and 0.1% DDM as buffer. The complex was eluted in a linear gradient, 0 to 1 M NaCl.

Analytical procedures

Protein concentration was determined by the bicinchoninic acid method [15]. Flavin was extracted with trichloroacetic acid [16], and its content was determined by fluorescence spectroscopy. The iron content was determined by the 2,4,6-tripyridyl-1,3,5-triazine method [17].

Catalytic activity assays

NADH:quinone oxidoreductase activity was monitored at 55 °C following the NADH oxidation at 330 nm ($\varepsilon = 5930\ M^{-1}\ cm^{-1}$). The reaction mixture contained 100 mM potassium phosphate (pH 7.5), 0.1% (w/v) DDM, 20 μM 2,3-dimethyl-1,4-naphthoquinone (DMN), and 50 μM NADH. The reaction was started by the addition of complex I. In the inhibition assays, the complex was preincubated with rotenone (50 μM) or piericidin A (100 μM) for 5 min at room temperature.

Blue native electrophoresis

Molecular mass of the native complex was determined by blue native (BN)–polyacrylamide gel electrophoresis (PAGE) using a gradient gel (5–13%) [18].

Protein separation

Electrophoresis

Separation of the subunits was performed by tricine–sodium dodecyl sulfate (SDS)–PAGE (12.5%) or gradient gel (10–20%) [19,20].

HPLC

The separation of complex I (3.5 mg) subunits by RP–HPLC was performed using a C4 column (150 × 3.9 mm, 300 Å, 5 μm, Delta-Pak, Waters), equilibrated with 0.1% trifluoroacetic acid (TFA) and eluting with 20 to 100% (v/v) acetonitrile (ACN), at 1 ml/min (System Gold Beckman HPLC).

Mass spectrometry analysis

Molecular mass of complex I subunits

Purified complex I was washed (see "in-solution digestion" section below) to remove the detergent, concentrated (15 mg/ml), and acidified with 5% (v/v) formic acid. Prior to matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI–TOF MS) analysis, the sample was desalted and concentrated using a GELoader tip packed with POROS R1 (Applied Biosystems). Elution of the retained proteins was done either with the matrix solution (0.5 μl sinapinic acid in 70% [v/v] ACN and 0.1% [v/v] TFA) or by 0.5 μl of 10% stepwise elution using 20 to 100% (v/v) ACN and 0.1% (v/v) TFA and applied directly onto a MALDI plate using the dry droplet method. External mass calibration was performed with ProMix3 (LaserBioLabs) and CalMix3 (Applied Biosystems).

In-gel digestion

Excised gel bands from tricine–SDS–PAGE were washed with 50% (v/v) ACN to remove the Coomassie dye. Gel pieces were then dehydrated by incubation with ACN and evaporated by centrifugation under vacuum. For the reduction and alkylation of cysteines, gel pieces containing the proteins were successively exposed to 10 mM dithiotreitol/100 mM ammonium bicarbonate (NH_4HCO_3) and 55 mM iodoacetamide/100 mM NH_4HCO_3. Modified trypsin (6.7 ng/μl in 50 mM NH_4HCO_3, Promega) was added to the dried gel pieces, which were incubated at 37 °C overnight. Supernatant was recovered, dried by centrifugation under vacuum, and dissolved in 5% (v/v) formic acid prior to MS analysis [21].

In-solution digestion

Complex was first washed to remove the detergent and PMSF (protease inhibitor) by cycles of concentration/dilution steps with a DDM- and PMSF-free buffer. DDM content was decreased to a final concentration of 0.025%. The detergent- and PMSF-free complex (200 fmol) was dried in a vacuum concentrator and dissolved in 400 mM NH_4HCO_3 and 8 M urea. In-solution digestion of the R1 microcolumn fractions was performed as described elsewhere [22]. Briefly, reduction was performed by the addition of 5 μl of 45 mM dithiothreitol and incubation at 50 °C for 15 min. Subsequent alkylation with 5 μl of 100 mM iodoacetamide was performed for 15 min in the dark. The solution was diluted to 2 M urea using water, and after the addition of trypsin (40 pmol), the sample was incubated at 37 °C for 16 h.

Purification and concentration of the peptides using RP microcolumns

Desalting and concentration of the acidified supernatants (5% [v/v] formic acid) containing the tryptic digested peptides was carried out with custom-made chromatographic microcolumns using GELoader tips packed with material of increasing hydrophobicity with POROS R2 (20 μm bead size), POROS R3 (20 μm bead size), and graphite (Sigma) materials [23,24]. Peptides were directly eluted from the microcolumns with the matrix solution (0.5 μl α-cyano-4-hydroxy-*trans*-cinnamic acid in 70% [v/v] ACN and 5% [v/v] formic acid) or by 10% stepwise elution using 0.5 μl of 20 to 100% (v/v) ACN and 5% (v/v) formic acid.

Peptide mass fingerprinting

Monoisotopic peptide masses were determined using MALDI–TOF MS equipment, and external mass calibration was performed with PepMix1 (LaserBioLabs). Protein identification was performed using MASCOT software (online available version 2.2.07, Matrix Science) [25]. Searches were done on the MSDB nonredundant protein sequence database (version 20050929) having the following conditions: a minimum mass accuracy of 100 ppm, one missed cleavage in peptide masses, and carbamidomethylation of cysteines and oxidation of methionines as fixed and variable amino acid modifications, respectively. To accept the identification, the considered criteria were significant homology scores achieved in MASCOT, significant sequence coverage values (>15%), and distribution of the identified peptides over the sequence. An additional criterion, the arginine (R)/lysine (K) terminated peptides ratio, was

used in the case of proteins identified via manual inspection of the mass spectra. Proteins were considered if the R/K ratio was greater than 1 [26].

Tandem mass spectrometry experiments: Micro-RP HPLC–linear ion trap MS

Peptides were concentrated and desalted on an RP precolumn (0.18 × 30 mm, BioBasic 18, Thermo Electron) and online eluted on an analytical RP column (0.18 × 150 mm, BioBasic 18) at 2 μl/min. Peptides were eluted using 33-min gradients: 5 to 60% solvent B (solvent A: 0.1% [v/v] formic acid and 5% [v/v] ACN; solvent B: 0.1% [v/v] formic acid and 80% [v/v] ACN). Linear ion trap was operated in data-dependent ZoomScan and tandem mass spectrometry (MS/MS) switching mode using the three most intense precursors detected in a survey scan from m/z 450 to 1600 (three microscans). Singly charged ions were excluded for MS/MS analysis. ZoomScan settings were as follows: maximum injection time, 200 ms; zoom target parameter, 3000 ions; and the number of microscans, 3. Normalized collision energy was set to 35%, and dynamic exclusion was applied during 10-s periods to avoid fragmenting each ion more than twice from each chromatographic peak.

Peptide MS/MS data were evaluated using Bioworks 3.3.1 software. Searches were performed against an indexed UniRef100 database (5.888.655 entries when downloaded in April 2008) and a specific database containing the sequences of the proteins from *R. marinus* complex I that contains protein sequences not included in the UniRef100 database. The following constraints were used for the searches: tryptic cleavage after arginine and lysine, up to two missed cleavage sites, and tolerances of 1 Da for precursor ions and 0.5 Da for MS/MS fragment ions. Modifications allowed were carbamidomethylation of cysteines and oxidation of methionines as fixed and variable amino acid modifications, respectively. Only protein identifications with two or more distinct peptides, $P < 0.01$, and *Xcorr* thresholds of at least 2.0/2.5 for doubly/triply charged peptides were accepted. Protein identification was further validated by manual inspections of MS/MS spectra.

Instrumentation

Intact masses and peptide mass fingerprinting (PMF) experiments were performed using MALDI–TOF MS Voyager DE-STR (PerSeptive Biosystems). Tandem mass experiments were performed using microLC–electrospray ionization (ESI) linear ion trap mass spectrometer model LTQ (Thermo Finnigan). The raw data are available at the PRIDE database (http://www.ebi.ac.uk/pride).

N-terminal sequence analysis

For N-terminal amino acid sequence analysis, proteins separated by tricine–SDS–PAGE were transferred to a poly(vinylidene difluoride) membrane and stained with Ponceau S dye. Sequencing was performed by automated Edman degradation in an Applied Biosystems model 491HT sequencer [27].

Results and discussion

Complex I integrity and purity

The integrity of *R. marinus* complex I was investigated by NADH:DMN oxidoreductase activity. The value of 0.4 μmol NADH min^{-1} mg^{-1}, in the range of other isolated bacterial enzymes [28], was obtained. Decreases of 50 to 70% in the specific activity were observed in the presence of complex I inhibitors (rotenone and piericidin A). A flavin/complex ratio of 1 and the iron content of the complex (~27 iron/flavin) were in agreement with the presence of one flavin, six $[4Fe\text{-}4S]^{2+/1+}$, and two $[2Fe\text{-}2S]^{2+/1+}$ centers. These quantifications indicated the presence of all the expected prosthetic groups in the purified complex. BN–PAGE revealed that the complex has an apparent molecular mass of 450 ± 50 kDa, in agreement with the sum of the predicted molecular mass of the all *R. marinus* complex I subunits (data not shown).

Identification of complex I subunits

To be identified, the subunits of complex I needed to be separated and isolated. The process of isolating at least 14 subunits with very different hydrophobic properties is not trivial. It required the optimization of several technical procedures such as SDS–PAGE and chromatographic fractioning (HPLC and POROS R1). To facilitate the reading of the description of complex I subunit separation and identification, a graphical representation of the strategy used is included as Fig. 1.

Determination of the intact masses of complex I subunits

Mass spectrum of complex I in solution is shown in Fig. 2. Nearly all relevant m/z peaks observed in the spectrum could be assigned to the expected canonical subunits (Nqo1–Nqo14) considering charges +1 (Table 1). The exceptions are subunits Nqo8, Nqo10, Nqo12, and Nqo14, whose predicted molecular mass could not be observed. It was shown previously that among complex I genes, and cotranscribed with them, two additional genes encoding for a PCD and an Nha-like antiporter were present [5]. The expected molecular masses for these two proteins are approximately 11.3 and 58.4 kDa, respectively. Peak m/z values corresponding to proteins with compatible masses were observed in the mass spectrum. However, in the case of the antiporter, its assignment is questionable because Nqo13 has a close predicted molecular mass (58,308 Da vs. 58,413 Da for the antiporter). The obtained identifications were further corroborated by elution of complex I subunits through a POROS R1 microcolumn using a stepwise ACN gradient (see Supplementary Fig. 1 in supplementary data).

Complex I subunits identified by PMF (in-gel digestion)

Subunits Nqo1 to Nqo6 and Nqo9 were also identified by PMF after SDS–PAGE separation (Supplementary Fig. 2 and Supplementary Table 1). Interestingly, Nqo3, Nqo4, Nqo5, and Nqo9 subunits were identified at different positions, corresponding to the predicted molecular mass and also to higher or lower molecular mass. For Nqo3, the occurrence of proteolysis was excluded because the N- and C-terminal sequences were identified in all of the gel bands.

Complex I proteins identified by LC–MS/MS (in-gel digestion)

Subunits separated by electrophoresis and subjected to trypsin digestion were also analyzed by LC–ESI–MS/MS. In this way, the identification of the peripheral subunits (Nqo1–Nqo6 and Nqo9) was corroborated by sequence determination (MS/MS) (Supplementary Table 2). Furthermore, this procedure also allowed to correct the sequence of Nqo6 deposited at the UniRef database. As shown in Fig. 3, two of the fragmented peptides with monoisotopic masses 2763.2 and 1099.6 helped to reassign the N-term region of Nqo6.

Complex I proteins identified by LC–MS/MS (in-solution digestion)

There are several methods described in the literature for membrane protein identification [6–13], and most of these couple electrophoresis (native or denaturant gels) techniques to MS analyses. In the current study, this type of approach was undertaken, with success in the identification of the peripheral subunits but ineffi-

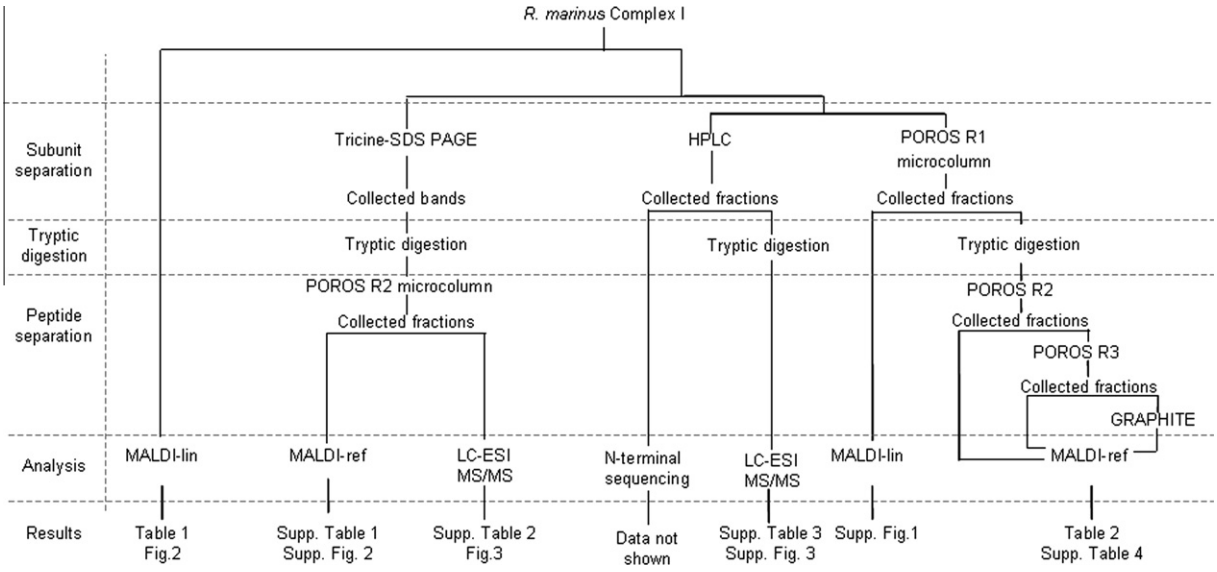

Fig. 1. Graphical representation of the strategy used for the separation and identification of complex I subunits. MALDI-lin, MS spectra collected using MALDI–TOF in linear mode; MALDI-ref, MS spectra collected using MALDI–TOF in reflector mode; Supp, Supplementary. POROS R1, R2, R3, and GRAPHITE correspond to the different RP materials used.

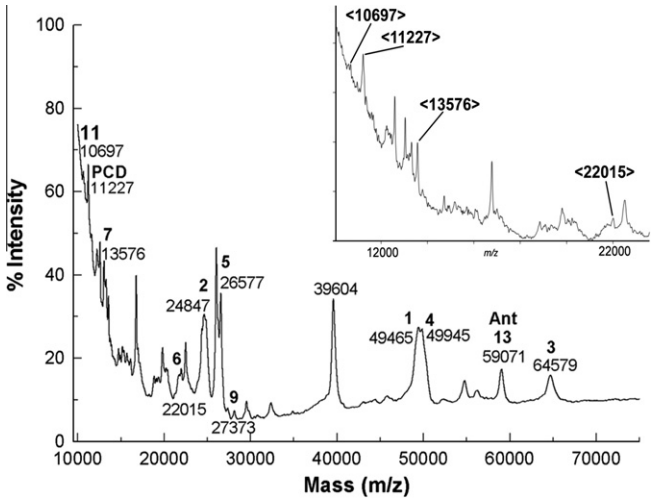

Fig. 2. MALDI mass spectrum of *R. marinus* complex I in solution (1:10 in H_2O). Matrix: sinapinic acid. Calibration mixture: ProMix3. The numbers refer to the respective subunits (e.g., Nqo1 is represented by 1) with charge of +1. Ant, antiporter.

ciency in the identification of the membrane subunits. The unsuccessful identification of the membrane subunits could be due to an incomplete extraction of peptides from the gel after digestion; thus, a non-gel approach was undertaken. Each fraction obtained by HPLC (Supplementary Fig. 3) was digested and analyzed (Supplementary Table 3). Identifications of the peptides eluted between 20% and 44% ACN were successful and in agreement with the results obtained by Edman degradation (see below). Identification of the subunits present in the HPLC fractions eluted with higher ACN concentrations was not possible due to protein precipitation. All of the subunits of the peripheral part were again identified.

Complex I subunits identified by PMF (in-solution digestion)

A new approach was undertaken to identify the membrane subunits. The R1 microcolumn fractions were tryptic digested, and the obtained peptides were loaded sequentially in the RP microcolumns POROS R2 (similar to C18), POROS R3 (similar to C8), and graphite and eluted with the matrix solution (0.5 μl α-cyano-4-hydroxy-*trans*-cinnamic acid in 70% [v/v] ACN and 5% [v/v] formic acid) to be analyzed by PMF. The sequential elution increased the number of peptides to be analyzed, minimizing a possible ion suppression effect, by which the best ionizable peptides mask the signal of the worst ionizable ones. The use of these three resins with different hydrophobic proprieties allowed different peptides to be obtained, enhancing sequence coverage of the identified proteins. Subunit Nqo3 was identified in the first fractions (20–40% ACN) with a sequence coverage of 60.9% (Table 2). Manual inspection of the spectra allowed the identification of other peptides with compatible masses to other *R. marinus* complex I subunits such as Nqo1, Nqo2, Nqo5, Nqo6, and Nqo9 (Table 2 and Supplementary Table 4). The criteria used to validate the identification of a protein were amino acid sequence coverage higher than 15% and the distribution of identified peptides over the sequence. Based on these criteria, the membrane subunits Nqo7, Nqo12, Nqo13, and Nqo14 were identified for the first time. In addition, the PCD was identified as a possible novel subunit with amino acid sequence coverage of 53%. The identification of Nqo8 or Nha-like antiporter was not considered because the obtained amino acid coverage was lower than 15% and/or the identified peptides were not well distributed in the sequence. Not surprisingly, the membrane subunits were observed in fractions that eluted at an ACN percentage higher than 40% (see also Supplementary Fig. 1).

Trypsin cleaves peptide bonds specifically after the carboxyl group of arginine and lysine amino acid residues. It is known that the peptides that end with R are better ionized on MALDI than those that end with K [26]. This observation was used as an additional criterion to validate the results (see Materials and methods). The determined R/K ratio (Table 2) further supported the identification of the subunits Nqo7, Nqo12, Nqo13, Nqo14, and PCD.

Identification of the canonical subunits was expected because these proteins are necessary for a correct assembly of functional complex I. However, the identification of PCD as a possible complex I subunit was a new observation. This protein has been considered as rare in prokaryotes. In fact, PCD is well characterized at the molecular genetics level in mammals, but only one bacterial

Table 1
Molecular masses of *R. marinus* complex I subunits: Theoretical and determined by MALDI–TOF MS

Subunit	Theoretical molecular mass (Da)[a]	Experimental molecular mass (Da)/MS resolution
Nqo1	49,299	49,464
Nqo2	25,042	24,846
Nqo3	64,724	64,578
Nqo4	50,186	49,944
Nqo5	26,622	26,576
Nqo6	18,676/21,593[b]	22,014
Nqo7	13,765	13,575
Nqo8	37,374	–
Nqo9	27,120	27,372
Nqo10	18,337	–
Nqo11	10,904	10,696
Nqo12	72,339	–
Nqo13	58,308	59,070
Nqo14	53,230	–
Antiporter	58,413	59,070
PCD	11,277	11,226

[a] Considering the gene sequences.
[b] Correct molecular mass (determined after the reassignment of N-terminal sequence).

Table 2
Identification of *R. marinus* complex I subunits by PMF after sequential elution of the complex subunits with R1 and of their tryptic peptides by R2, R3, and graphite microcolumns

Percentage ACN for R1 elution	Subunit identified	Sequence coverage (%)	Number of identified peptides[a]	R/K[b]
20–40	Nqo1	66.14	32	3.4
20–30	Nqo2	47.32	10	1.0
20–30	Nqo3	60.94	38	2.4
40–50	Nqo4	47.17	24	2.4
20–30	Nqo5	76.96	16	4.0
20–60	Nqo6	53.09	12	2.0
70–80	Nqo7	17.50	3	2.0
20	Nqo8	14.37	5	0.7
20–60	Nqo9	66.09	18	1.1
–	Nqo10	–	–	–
–	Nqo11	–	–	–
60	Nqo12	25.13	16	6.5
40–80	Nqo13	19.66	9	3.0
40–80	Nqo14	22.60	7	2.5
20–40	Antiporter	17.27	7	0.8
60–70	PCD	53.54	4	4.0

[a] Considering one possible missed cleavage and a peptide mass range between 720 and 5000.
[b] Ratio between sequences that end with arginine (R) or lysine (K).

example (*Pseudomonas aeruginosa*) has been biochemically characterized [29–31]. PCD is described as a bifunctional protein with catalytic and regulatory properties [29,32]. It is involved in the regeneration of tetrahydrobiopterin, an essential cofactor of aromatic amino hydroxylase, and catalyzes the conversion of 4α-hydroxytetrahydrobiopterin to quinoid-dihydrobiopterin. In mammals, this protein has also been shown to stabilize the dimeric homodomain transcription factor HNF1 and to enhance its activity. The relation between the enzymatic activity of PCD and its regulatory function as a dimerization cofactor of HNF1 is not understood [32]. Crystal structures showed that mammalian PCDs are tetramers (dimers of dimer configuration), whereas bacterial PCDs are dimers [32]. It is believed that the regulatory function, as dimerization of transcription factor HNF1, is related to this tetramerization, with the bacterial PCD having no role in transcription [29,33]. Crystal structures, as well as site-directed mutagenesis studies and in vivo PCD activity assays, have shown that the motif [EDKH]-*x*(3)-H-[HN]-[PCS]-*x*(5,6)-[YWF]-*x*(9)-[HW]-*x*(8,15)-D is the signature for PCD activity [33]. This motif is present in the *R. marinus* PCD amino acid sequence (accession number: AAY42130.1); however no genes coding for a typical phenylalanine hydroxylase are present in the genome of this bacterium (www.genome.jp). This strongly suggests that PCD may have a different function. An auxiliary role in the metabolism of molybdenum cofactor or its precursors was proposed [33]. Although no molybdenum cofactor is present in complex I, it is believed that the C-terminal part of Nqo3 evolved from an ancestor protein containing a molybdopterin [34]. The structure of complex I revealed that the Nqo3 C terminal is one of the most flexible regions, and a possible regulatory site corresponding to the ancestor MGD-binding site was suggested [2]. The possible presence of PCD in *R. marinus* complex

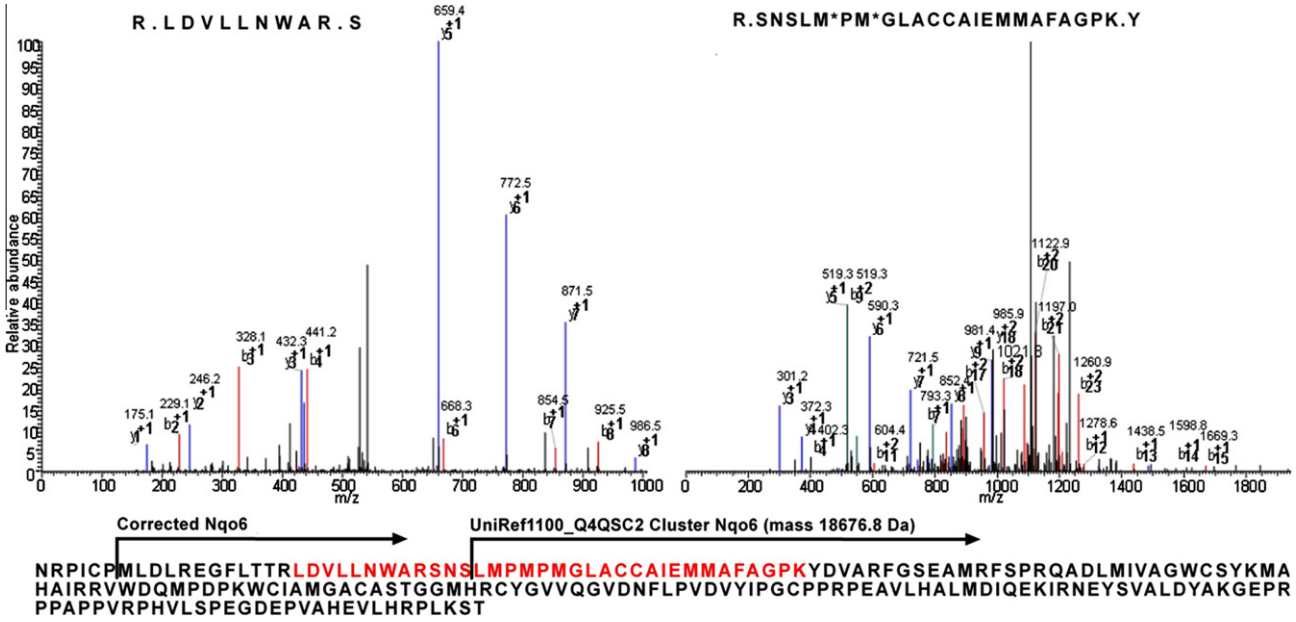

Fig. 3. MS/MS spectra of two Nqo6 peptides with monoisotopic masses 2763.2 and 1099.6. These two peptides (highlighted) allowed the N-terminal sequence of Nqo6 to be reassigned in the UniRef100 protein database.

Table 3
Summary of the identification of complex I subunits by the different methodologies employed in this study

Subunit	Theoretical molecular mass (Da)[a]	MALDI linear mode (whole complex)	LC–ESI–MS/MS (in-gel trypsin digestion)	MALDI–TOF PMF (subunit separated by sequential elution and trypsin digestion)	N-terminal sequencing (Edman degradation)
Nqo1	49,299	+	+	+	+
Nqo2	25,042	+	+	+	+
Nqo3	64,724	+	+	+	+
Nqo4	50,186	+	+	+	+
Nqo5	26,622	+	+	+	+
Nqo6	21,593	+	+	+	–
Nqo7	13,765	+	–	+	–
Nqo8	37,374	–	–	–	–
Nqo9	27,120	+	+	+	–
Nqo10	18,337	–	–	–	–
Nqo11	10,904	+	–	–	+
Nqo12	72,339	–	–	+	–
Nqo13	58,308	+	–	+	–
Nqo14	53,230	–	–	+	–
Antiporter	58,413	[b]	–	–	–
PCD	11,277	+	–	+	–

[a] Considering the gene sequences.
[b] The presence of the antiporter is questionable because its predicted molecular mass (58,413 Da) is very close to that of Nqo13 (58,308 Da).

I may reinforce that suggestion and extend it to the involvement of PCD in a possible regulatory mechanism. In *Salinibacter rubber* DSM 13855, a bacterium closely related to *R. marinus*, the gene coding for a PCD protein is also present among complex I genes (www.genome.jp), suggesting that such a protein could indeed be part of some complexes I, including that of *R. marinus*. The presence of extra subunits in bacterial complex I has also been observed in the case of *Thermus thermophilus*, where a frataxin-like protein was shown to be part of its peripheral arm [2].

Complex I proteins identified by N-terminal sequencing

In parallel with MS analyses, the proteins separated by electrophoresis and HPLC were subjected to Edman degradation, allowing the identification of the membrane subunit Nqo11 beside the peripheral proteins.

Conclusion

We have identified all of the canonical subunits of complex I with the exceptions of Nqo8 and Nqo10 (Table 3). Nqo8 subunit has a predicted molecular mass of 37.4 kDa that was not observed in the *R. marinus* complex I mass spectrum (Fig. 2). However, a peak *m*/*z* corresponding to a protein of 39.6 kDa was observed. This protein had eluted at an ACN percentage higher than 40% (Supplementary Fig. 1), as did all of the identified membrane subunits. Given that N- and C-terminal sequences of Nqo8 were not determined yet, a misassignment of any of these cannot be excluded, as observed for Nqo6, whose N-terminal sequence was reassigned in the current work (Fig. 3). A PCD that does not correspond to any previously known complex I subunit was also identified and suggested to be an additional protein of *R. marinus* complex I. To our knowledge, this study provides one of the few examples of detailed MS analysis of bacterial complex I [12,13]. Most of the reported studies have addressed the mitochondrial enzyme [6–8], and the identification of the membrane subunits is not always achieved [35]. In the current work, the identification of the subunits, especially the membrane ones, was possible only after establishing a new experimental protocol that involved preseparation of complex I subunits followed by their peptide digestion in solution. This approach is a step forward in the characterization of membrane proteins in general by MS, the studies of which are still highly challenging technically.

Acknowledgments

We thank Miguel Teixeira for critical reading of the manuscript, João Carita for cell growth, and Manuela Regalla (Analytical Services Unit, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa) for N-terminal sequencing and RP–HPLC. A.P.B. and C.F. are recipients of grants from Fundação para a Ciência e a Tecnologia (SFRH/BD/25288/2005 and SFRH/BD/29799/2006, respectively). This work was supported by project POCI/BIA-PRO/58374/2004 (to M.M.P.) and the National Mass Spectrometry Network (REDE/1504/REM/2005).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.07.038.

References

[1] U. Brandt, Energy converting NADH:quinone oxidoreductase (complex I), Annu. Rev. Biochem. 75 (2006) 69–92.
[2] L.A. Sazanov, P. Hinchliffe, Structure of the hydrophilic domain of respiratory complex I from *Thermus thermophilus*, Science 311 (2006) 1430–1436.
[3] A.S. Fernandes, M.M. Pereira, M. Teixeira, Purification and characterization of the complex I from the respiratory chain of *Rhodothermus marinus*, J. Bioenerg. Biomembr. 34 (2002) 413–421.
[4] A.S. Fernandes, F.L. Sousa, M. Teixeira, M.M. Pereira, Electron paramagnetic resonance studies of the iron–sulfur centers from complex I of *Rhodothermus marinus*, Biochemistry 45 (2006) 1002–1008.
[5] A.M. Melo, S.A. Lobo, F.L. Sousa, A.S. Fernandes, M.M. Pereira, G.O. Hreggvidsson, J.K. Kristjansson, L.M. Saraiva, M. Teixeira, A nhaD Na^+/H^+ antiporter and a pcd homologue are among the *Rhodothermus marinus* complex I genes, Biochim. Biophys. Acta 1709 (2005) 95–103.
[6] J. Carroll, M.C. Altman, I.M. Fearnley, J.E. Walker, Identification of membrane proteins by tandem mass spectrometry of protein ions, Proc. Natl. Acad. Sci. USA 104 (2007) 14330–14335.
[7] J. Carroll, I.M. Fearnley, J.M. Skehel, R.J. Shannon, J. Hirst, J.E. Walker, Bovine complex I is a complex of 45 different subunits, J. Biol. Chem. 281 (2006) 32724–32727.
[8] J. Carroll, I.M. Fearnley, J.E. Walker, Definition of the mitochondrial proteome by measurement of molecular masses of membrane proteins, Proc. Natl. Acad. Sci. USA 103 (2006) 16170–16175.
[9] C.C. Darie, M.L. Biniossek, V. Winter, B. Mutschler, W. Haehnel, Isolation and structural characterization of the Ndh complex from mesophyll and bundle sheath chloroplasts of *Zea mays*, FEBS J. 272 (2005) 2705–2716.
[10] A.S. Fandino, I. Rais, M. Vollmer, H. Elgass, H. Schagger, M. Karas, LC–nanospray–MS/MS analysis of hydrophobic proteins from membrane protein complexes isolated by blue-native electrophoresis, J. Mass Spectrom. 40 (2005) 1223–1231.
[11] I.M. Fearnley, J. Carroll, R.J. Shannon, M.J. Runswick, J.E. Walker, J. Hirst, GRIM-19, a cell death regulatory gene product is a subunit of bovine mitochondrial

NADH:ubiquinone oxidoreductase (complex I), J. Biol. Chem. 276 (2001) 38345–38348.
[12] P. Prommeenate, A.M. Lennon, C. Markert, M. Hippler, P.J. Nixon, Subunit composition of NDH-1 complexes of *Synechocystis* sp. PCC 6803: Identification of two new ndh gene products with nuclear-encoded homologues in the chloroplast Ndh complex, J. Biol. Chem. 279 (2004) 28165–28173.
[13] P. Zhang, N. Battchikova, V. Paakkarinen, H. Katoh, M. Iwai, M. Ikeuchi, H.B. Pakrasi, T. Ogawa, E.M. Aro, Isolation, subunit composition, and interaction of the NDH-1 complexes from *Thermosynechococcus elongatus* BP-1, Biochem. J. 390 (2005) 513–520.
[14] M.M. Pereira, J.N. Carita, M. Teixeira, Membrane-bound electron transfer chain of the thermohalophilic bacterium *Rhodothermus marinus*: A novel multihemic cytochrome *bc*, a new complex III, Biochemistry 38 (1999) 1268–1275.
[15] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Measurement of protein using bicinchoninic acid, Anal. Biochem. 150 (1985) 76–85.
[16] S. Susin, J. Abian, F. Sanchez-Baeza, M.L. Peleato, A. Abadia, E. Gelpi, J. Abadia, Riboflavin 3′- and 5′-sulfate, two novel flavins accumulating in the roots of iron-deficient sugar beet (*Beta vulgaris*), J. Biol. Chem. 268 (1993) 20958–20965.
[17] D.S. Fischer, D.C. Price, A simple serum iron method using the new sensitive chromogen tripyridyl-*S*-triazine, Clin. Chem. 10 (1964) 21–31.
[18] H. Schagger, Membrane Protein Purification and Crystallization: A Practical Guide, 2nd ed., Elsevier Science, San Diego, 2003.
[19] H. Schagger, G. von Jagow, Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, Anal. Biochem. 166 (1987) 368–379.
[20] J.E. Walker, J.M. Skehel, S.K. Buchanan, Structural analysis of NADH:ubiquinone oxidoreductase from bovine heart mitochondria, Methods Enzymol. 260 (1995) 14–34.
[21] B. Spenglen, Post-source decay analysis in matrix-assisted laser desorption/ionization mass spectrometry of biomolecules, J. Mass Spectrom. 32 (1997) 1019–1036.
[22] S. Banuelos, M.J. Omaetxebarria, I. Ramos, M.R. Larsen, I. Arregi, O.N. Jensen, J.M. Arizmendi, A. Prado, A. Muga, Phosphorylation of both nucleoplasmin domains is required for activation of its chromatin decondensation activity, J. Biol. Chem. 282 (2007) 21213–21221.
[23] J. Gobom, E. Nordhoff, E. Mirgorodskaya, R. Ekman, P. Roepstorff, Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry, J. Mass Spectrom. 34 (1999) 105–116.
[24] M.R. Larsen, S.J. Cordwell, P. Roepstorff, Graphite powder as an alternative or supplement to reversed-phase material for desalting and concentration of peptide mixtures prior to matrix-assisted laser desorption/ionization–mass spectrometry, Proteomics 2 (2002) 1277–1287.
[25] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (1999) 3551–3567.
[26] E. Krause, H. Wenschuh, P.R. Jungblut, The dominance of arginine-containing peptides in MALDI-derived tryptic mass fingerprints of proteins, Anal. Chem. 71 (1999) 4160–4165.
[27] P. Edman, G. Begg, A protein sequenator, Eur. J. Biochem. 1 (1967) 80–91.
[28] S. Stolpe, T. Friedrich, The *Escherichia coli* NADH:ubiquinone oxidoreductase (complex I) is a primary proton pump but may be capable of secondary sodium antiport, J. Biol. Chem. 279 (2004) 18377–18383.
[29] J. Song, T. Xia, R.A. Jensen, PhhB, a *Pseudomonas aeruginosa* homolog of mammalian pterin-4α-carbinolamine dehydratase/DCoH does not regulate expression of phenylalanine hydroxylase at the transcriptional level, J. Bacteriol. 181 (1999) 2789–2796.
[30] G. Zhao, T. Xia, J. Song, R.A. Jensen, *Pseudomonas aeruginosa* possesses homologues of mammalian phenylalanine hydroxylase and 4α-carbinolamine dehydratase/DCoH as part of a three-component gene cluster, Proc. Natl. Acad. Sci. USA 91 (1994) 1366–1370.
[31] S. Koster, G. Stier, N. Kubasch, H.C. Curtius, S. Ghisla, Pterin-4α-carbinolamine dehydratase from *Pseudomonas aeruginosa*: Characterization, catalytic mechanism, and comparison to the human enzyme, Biol. Chem. 379 (1998) 1427–1432.
[32] D. Suck, R. Ficner, Structure and function of PCD/DCoH, an enzyme with regulatory properties, FEBS Lett. 389 (1996) 35–39.
[33] V. Naponelli, A. Noiriel, M.J. Ziemak, S.M. Beverley, L.F. Lye, A.M. Plume, J.R. Botella, K. Loizeau, S. Ravanel, F. Rebeille, V. de Crecy-Lagard, A.D. Hanson, Phylogenomic and functional analysis of pterin-4α-carbinolamine dehydratase family (COG2154) proteins in plants and microorganisms, Plant Physiol. 146 (2008) 1515–1527.
[34] M.M. Pereira, P.N. Refojo, G.O. Hreggvidsson, S. Hjorleifsdottir, M. Teixeira, The alternative complex III from *Rhodothermus marinus*: A prototype of a new family of quinol:electron acceptor oxidoreductases, FEBS Lett. 581 (2007) 4831–4835.
[35] H.R. Bridges, L. Grgic, M.E. Harbour, J. Hirst, The respiratory complexes I from the mitochondria of two *Pichia* species, Biochem. J. 422 (2009) 151–159.