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Subunit composition of *Rhodothermus marinus* respiratory complex I

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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

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[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.

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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