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The alternative complex III from *Rhodothermus marinus* – A prototype of a new family of quinol:electron acceptor oxidoreductases

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Abstract **The biochemical and genetic search for a bc_1 complex in *Rhodothermus marinus* was always fruitless; however, a functional equivalent, i.e. having quinol:cytochrome *c* oxidoreductase activity was characterized. Now, with the sequencing of *R. marinus* genome, it was possible to assign the N-terminal sequences of several proteins of this complex to its coding genes. The alternative complex III from *R. marinus* has the same genomic organization of the so-called MFIcc complexes, proposed to be oxidoreductases of the respiratory and photosynthetic electron transfer chains. In this report, we establish undoubtedly the existence of an alternative complex III, a functional substitute of the bc_1 complex, by its identification at both the biochemical and genomic level.**

Keywords: MFIcc; Complex III; bc_1 Complex; Oxygen reductase

1. Introduction

Rhodothermus marinus is a strict aerobic, moderate halophilic and non-phototrophic Gram negative bacterium, with an optimum growth temperature of 65 °C and salt concentration of 1–2% [1,2]. It belongs to the group of *Flexibacter*, *Bacteroids* and *Cytophaga* species (FBC-group) [3]. Its respiratory chain has been extensively studied. Besides a complex I NADH:quinone oxidoreductase [4,5] and a succinate:quinone oxidoreductase [6,7] it possesses three oxygen reductases, a caa_3 [8–10], a cbb_3 [11] and a ba_3 [12] members of type A2, C and B families of heme–copper oxygen reductases [13], respectively. All these oxygen reductases receive electrons from periplasmatic proteins, namely a High Potential iron–sulfur Protein (HiPIP) and a cytochrome *c*. Thus, a complex III in *R. marinus* membranes had to be present to link quinol oxidation to HiPIP and cytochrome *c* reduction. In fact, a functional equivalent protein complex, i.e., a complex having quinol:HiPIP/cytochrome *c* oxidoreductase activity was isolated from the membranes of *R. marinus* and biochemical and spectroscopically characterized [14]. It was proposed to be a multihemic cytochrome complex containing at least five low-spin heme centers. Spectroscopic data strongly suggested that two of the hemes are in van der Waals contact, yielding a split Soret band. EPR spectra of the oxidized complex showed resonances of five low-spin ferric heme centers and of a $[3Fe–4S]^{1+/0}$ centre, which has a high reduction potential of +140 mV. The hemes have reduction potentials in the range of −45 to +235 mV [14].

With the increasing number of prokaryotic genome sequences, it is now possible to identify in silico so far unknown respiratory complexes, namely when accompanied with a thorough biochemical characterization at the protein level, which results from the still largely unexplored enormous biodiversity of the microbial world. Based on sequence analysis of the genomes so far sequenced, Yanyushin and co-workers anticipated the presence of a protein complex, proposing to be an alternative complex III, involved in the respiratory and in the photosynthetic electron transfer chains [15]. Furthermore, it was observed the presence of this complex in several genomes in which the genes coding for the bc_1 complex are absent and in which it is expected to exist a quinol:electron acceptor oxidoreductase, since genes coding for oxygen reductases, oxidizing periplasmatic electron donors are present. The gene cluster identified by those authors is constituted by six genes. Two of those are homologous to genes coding for the three subunits of molybdopterin containing oxidoreductases of the DMSO reductase family and other two code for *c* type cytochromes [15]. In this report we show that *R. marinus* complex III [14] is a MFIcc complex like the one proposed by Yanyushin and co-workers [14], and establish undoubtedly the existence of a different complex III by its identification at the biochemical and genomic levels.

2. Materials and methods

2.1. Bacterial growth and protein purification

Rhodothermus marinus strain PRQ62b growth and protein purification were performed as described in [14].

2.2. Electrophoresis

Tricine–SDS/PAGE was carried out as described by Schägger and von Jagow [16] with 10%T, 3%C, and heme staining followed Goodhew et al. [17].

2.3. Protein, heme and metal determination

Protein concentrations were determined using the bicinchoninic acid (BCA) method [18] and an apparent molecular mass of 247 kDa,

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determined by tricine–SDS/PAGE (considering a 1:1 stoichiometry for all subunits) was considered to define metal and heme content. Heme content was determined by pyridine hemochrome [19], and HPLC analysis after heme extraction as described in [14]. Iron and molybdenum were analyzed by atomic absorption on a graphite chamber, at the Laboratório de Análises, Instituto Superior Técnico, Lisbon and Faculdade de Ciencias e Tecnologia, Universidade Nova de Lisboa.

2.4. N-terminal determination

The enzyme subunits were transferred to a polyvinylidene difluoride (PVDF) membrane. Each transferred sample was submitted to N-terminal protein sequence analysis by automated Edman degradation [20] using an Applied Biosystems Procise 491 HT protein sequencer.

2.5. Sequence identification

The gene cluster of complex III from *R. marinus* was identified in silico from a local Prokaria genome database of *R. marinus* ITI378 by using the N-terminal sequences obtained for the several proteins. The gene library was prepared as follows. DNA was fragmented by nebulization and cloned into pTrueBlue (Stratagene). Plasmids were isolated by high-throughput minipreparation, and sequencing was performed. Contigs were assembled with the Phred–Phrap package [21], and putative open reading frames (ORFs) were identified with the GetORF program from the EMBOSS package [22], followed by BLASTP searches [23] against protein sequence databases. Gaps were closed by PCR amplification using sequences from flanking contigs.

2.6. Prediction of transmembrane topology

Transmembrane topology was predicted using ConpredII at http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/ [24].

2.7. Nucleotide sequence accession number

The gene sequence coding for complex III gene cluster of *R. marinus* has been deposited in GenBank under accession number 924811.

3. Results and discussion

3.1. Subunit and prosthetic group compositions

The purified *R. marinus* complex III shows six bands in a tricine–SDS/PAGE, corresponding to subunits with apparent molecular masses of 97, 42, 34, 27, 25 and 22 kDa (Fig. 1A). The bands corresponding to subunits with apparent molecular masses of 27 and 22 have also colored with heme staining, indicating the presence of C-type hemes (Fig. 1B). The cytochromes of the complex III from *R. marinus* have been previously characterized by HPLC analysis and UV–visible and EPR spectroscopies and a total of five hemes were showed to be present in the complex [14]. Besides the presence of four C-type hemes, the presence of a B-type heme was reported based on the maximum absorption band at 557 nm for one of the hemes and on HPLC analysis. The heme content was here reanalyzed. The peak at 557 nm is still present, but no hemes were detected by HPLC analysis, meaning that only C-type hemes are present in the complex. The complex did not contain molybdenum and the iron content was now determined by atomic absorption and a value of 20 ± 0.5 Fe per protein molecule was obtained.

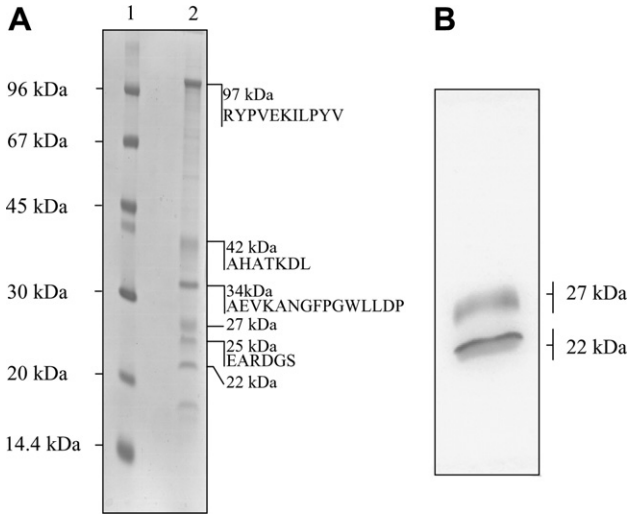

Fig. 1. SDS–PAGE of the complex III from *Rhodothermus marinus*. Coomassie (A) and heme staining (B). The N-terminal sequences obtained by Edman degradation are indicated for the respective band from which they were sequenced.

3.2. Sequence comparison

The N-terminal sequences for four of the bands have been determined: for the 97 kDa band, RYPVEKILPYV, for the 42 kDa band, AHATKDL, for the 34 kDa band, AEVKANGFPGWLLDP and for the 25 kDa band, EARDGS. Attempts to sequence the N-terminal of the two other bands were always fruitless. Search in *R. marinus* strain ITI378 gene database showed that the first sequence corresponds to the N-terminal sequence of a gene coding for a putative molybdopterin containing reductase fused to an iron–sulfur protein. The two sequences obtained for the 42 and 34 kDa bands correspond to two N-terminal sequences of different transmembrane proteins present in several complexes of the DMSO reductase family (e.g. DmsC; PsrC, NrfD). The last sequence is the N-terminal of a hypothetical protein present in several genomes.

3.3. Gene cluster organization and gene sequence analysis

The genes coding for these proteins seem to form a gene cluster with two other genes organized as shown in Fig. 2. This gene cluster (genes A–F) also contains two genes (A and E) coding for two type *c* cytochromes with predicted molecular masses of 27 and 23.5 kDa, which is in agreement with the results of the heme staining of the SDS–PAGE.

Gene A codes for a protein containing five heme C binding motifs, CXXCH, being the fifth motif one amino acid residue apart from the C-terminal. Seven other histidine and three methionine residues are present in the sequence being candidates for the sixth ligand of the heme irons. A possible signal peptide in the N-terminal region may be present, but the putative cleavage site is inside the predicted transmembrane helix and thus it is possible that this cytochrome is attached to the membrane by this transmembrane helix. Gene B is the fusion of two genes, a putative molybdopterin containing protein (N-terminal) and an iron–sulfur protein (C-terminal), whose genes cluster together in several genomes coding for complexes of the DMSO reductase family. Three binding sites for [4Fe–4S]$^{2+/1+}$ clusters and one for a [3Fe–4S]$^{1+/0}$ cluster are observed. In the previous characterization of *R. marinus* complex the [3Fe–4S]$^{1+/0}$ cluster was identified by EPR spectroscopy. This gene, like the ones coding for the molybdopterin containing oxidoreductase of the DMSO reductase family has a twin arginine translocase (Tat) signal peptide [25,26]. Gene C codes for a homolog of nrfD, which as mentioned above is a transmembrane protein of some members of the DMSO reductase