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.
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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
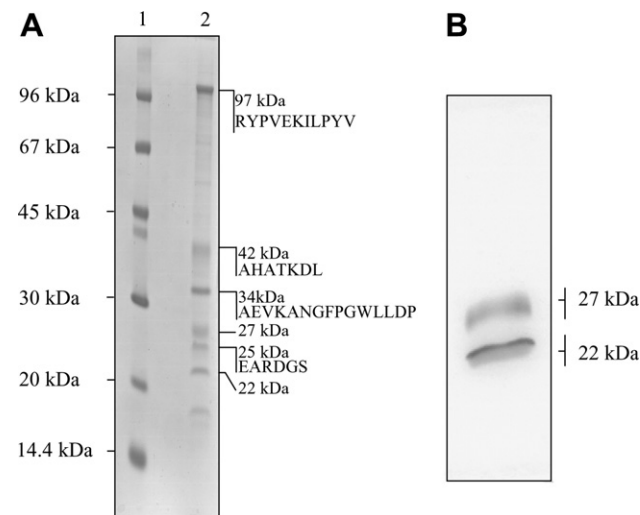

Fig. 1. SDS–PAGE of the complex III from *Rhodothermus marinus*. Coomassie (A) and heme staining (B). The N-terminal sequences obtained by Edman degradation are indicated for the respective band from which they were sequenced.

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.

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
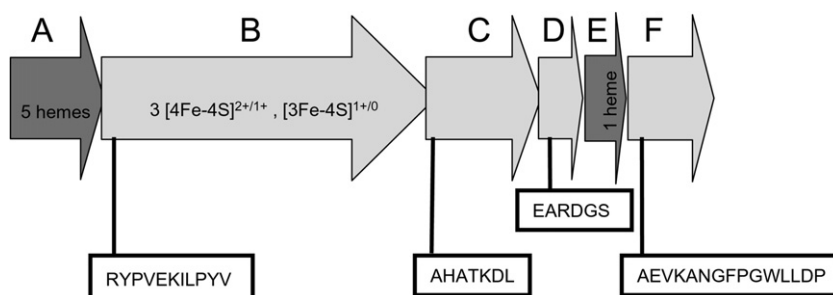
Fig. 2. Gene cluster organization of the complex III from *Rhodothermus marinus*. The gene cluster is composed of six genes (A–F). Gene A codes for a protein containing five heme C binding motifs. Gene B is the fusion of two genes, a putative molybdopterin containing protein (N-terminal) and an iron–sulfur protein (C-terminal), containing three binding sites for $[4Fe–4S]^{2+/1+}$ clusters and one for a $[3Fe–4S]^{1+/0}$ cluster. Genes C and F code for transmembrane proteins with 10 TM helices each and are both homologs of the membrane proteins of some members of the DMSO reductase family responsible for the interaction with quinones. Gene D codes for a hypothetical protein predicted to have two transmembrane helices and gene E codes for a monohemic cytochrome *c*.

family responsible for the interaction with quinones. Topology prediction reveals the possibility of ten transmembrane helices. Two possible quinone binding sites, as proposed by Fisher and Rich [27], are present in the transmembranes helices. Gene D codes for a hypothetical protein predicted to have two transmembrane helices. The protein coded by gene E contains one CXXCH motif, and several histidine and methionine residues that can be the heme sixth ligand. However, this is most probably a methionine residue in the conserved motive MPA present in other cytochromes (e.g. [28]). No transmembrane helices were predicted to be present. Finally, gene F codes for a protein homologous to the one coded by gene C.

The genomic organization shown in Fig. 2 is the same observed by Yanyushin and co-workers [15], which was proposed to codify for a new class of bacterial membrane bound oxidoreductases involved in the respiratory and in the photosynthetic electron transfer chains. Searching in all genomes so far available the authors observed that these complexes are distributed in almost all bacterial phyla, with special relevance for those where genes coding for a bc_1 complex are not present, but quinol:electron acceptor activity has to be present.

3.4. Protein complex composition

Based on the genomic organization, on our previous biochemical and functional studies and on what is known for members of the DMSO reductase family, the *R. marinus* complex III may look like as schematized in Fig. 3. The order of prosthetic groups is not known; here it is only intended to show that subunits containing the prosthetic groups should be peripheral and facing the periplasm, based on the observation of the signal peptides present in the genes coding for these proteins. The presence of *c* type cytochromes and of a $[3Fe–4S]^{1+/0}$ centre has already been established in the previous characterization of *R. marinus* complex III. The presence of three $[4Fe–4S]^{2+/1+}$ centers, besides the similarity with the members of DMSO reductase family, from which several structures are known, is highly supported by the iron content of the sample, considering the presence of five hemes, three $[4Fe–4S]^{2+/1+}$ centers and a $[3Fe–4S]^{1+/0}$.

At a first glance it may appear unexpected that having a protein similar to a molybdopterin reductase this complex does not contain molybdenum. This situation is not unique. The structure of the C-terminal part of Nqo3/NuoG, the largest subunit of respiratory chain complex I, is also similar to the
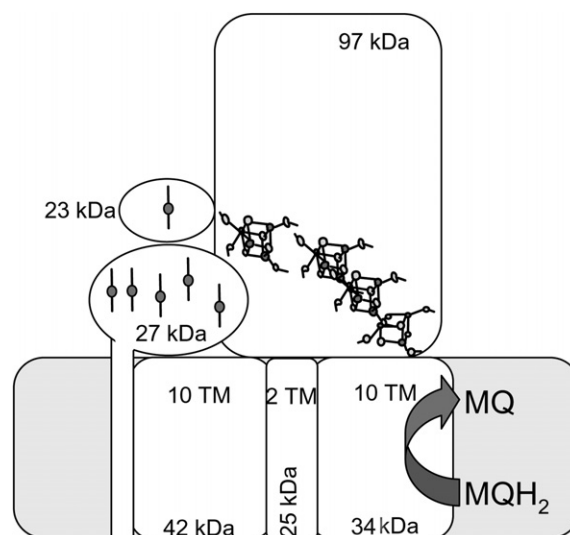

Fig. 3. Schematic representation of the alternative complex III from *Rhodothermus marinus*. Based on the genomic organization, on our previous biochemical and functional studies and on what is known for members of the DMSO reductase family.

family of molybdopterin reductases [29]. In fact, it presents the same four structural domains, where domains II and III create a cavity, which in the other members of the family is occupied by the molybdopterin guanine dinucleotide (MGD) prosthetic group. In the case of Nqo3, the cavity is occupied by domain IV. The function of the C-terminal of Nqo3 is not known, being a flexible area and suggested to have a regulatory role [29].

Similarly to what is observed for the nitrate reductases that are members of the DSMO reductase family, coded by the NarGHI operons, for the succinate:quinone oxidoreductase [30,31] and for the DMSO reductase of *Escherichia coli* engineered with a $[3Fe–4S]^{1+/0}$ cluster [32], this centre should be the one that interacts with the quinone/quinol. In this case, the subunit of *R. marinus* complex III containing the iron–sulfur centers should be the one receiving the electrons from the membrane subunits and transferring these to the *c* type cytochromes. Also taking into account the structure of the formate dehydrogenase from *E. coli*, the iron–sulfur centers should form a wire for electron transfer [33].

The two transmembrane subunits with the higher molecular mass are homologous to each other and homologous to several membrane proteins of the DMSO reductase family. Interaction studies of 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) with DMSO reductase from *E. coli* showed a binding stoichiometry of 1:1, thus indicating the presence of one quinone binding site [34]. As mentioned above, inspection of the amino acid sequences of these subunits reveals the presence of at least two possible quinone binding sites, as proposed by Fisher and Rich [27], and so the presence of more than one quinone binding site cannot be excluded. The reason that the complex III from *R. marinus* contains two of such membrane subunits is not known, but again taking the example of complex I this is not unique since in this type of complex it is observed the presence of homologous subunits within the complex, such as Nqo 12, Nqo13 and Nqo14 [35,36].

The small transmembrane subunit seems to be unique for these alternative complexes and its function is unknown and quinone binding sites seem not to be present.

Comparing the composition of the alternative complex III here described with the one of the *bc*$_1$ it can be observed that macroscopically they are not much different. Both have transmembrane subunits, which interact with quinone/quinols and transfer electrons to an iron–sulfur containing protein, which in its turn promotes the electron transfer to cytochromes.

The existence of two complexes performing the same reaction, i.e. two different complexes III, in this case, is not unique in respiratory chains. For all the other respiratory complexes there are alternatives. In the case of complex I, Type II (NDH-II) and Na$^+$-pumping NADH:quinone oxidoreductases are alternatives. Different types of oxygen reductases of the heme–copper family and of the *bd* family are observed as well as succinate:quinone oxidoreductases with different types of membrane attachments. In this report, we have established undoubtedly the existence of an alternative complex III by its identification at the biochemical and genomic levels.

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