Biochimica et Biophysica Acta 1797 (2010) 1477–1482

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

# The alternative complex III of *Rhodothermus marinus* and its structural and functional association with $caa_3$ oxygen reductase

Patrícia N. Refojo, Miguel Teixeira, Manuela M. Pereira *

*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. da República, EAN, 2780-157 Oeiras, Portugal*

ARTICLE INFO

*Article history:*
Received 8 January 2010
Received in revised form 22 February 2010
Accepted 24 February 2010
Available online 4 March 2010

*Keywords:*
Alternative complex III
$bc_1$ Complex
$caa_3$ Oxygen reductase
Quinol
HQNO
Complex iron–sulfur molybdoenzyme family

ABSTRACT

An alternative complex III (ACIII) is a respiratory complex with quinol:electron acceptor oxidoreductase activity. It is the only example of an enzyme performing complex III function that does not belong to $bc_1$ complex family. ACIII from *Rhodothermus (R.) marinus* was the first enzyme of this type to be isolated and characterized, and in this work we deepen its characterization. We addressed its interaction with quinol substrate and with the $caa_3$ oxygen reductase, whose coding gene cluster follows that of the ACIII. There is at least, one quinone binding site present in *R. marinus* ACIII as observed by fluorescence quenching titration of HQNO, a quinone analogue inhibitor. Furthermore, electrophoretic and spectroscopic evidences, taken together with mass spectrometry revealed a structural association between ACIII and $caa_3$ oxygen reductase. The association was also shown to be functional, since quinol:oxygen oxidoreductase activity was observed when the two isolated complexes were put together. This work is thus a step forward in the recognition of the structural and functional diversities of prokaryotic respiratory chains.



## 1. Introduction

In aerobic organisms most of energy is obtained by oxidative phosphorylation. The energy released by the transfer of electrons through membrane bound complexes is used to translocate protons and other ions across the membrane. This process, consequently creates an electrochemical potential difference that can be used by ATP synthase to produce ATP. The mitochondrial electron transfer respiratory chain is composed by four different membrane complexes (I–IV). Complexes I and II are the electron entry points while complex IV reduces the final electron acceptor, dioxygen. Complex III, also called cytochrome $bc_1$, mediates electron transfer between complex I or II and complex IV by accepting electrons from reduced quinones and reducing cytochrome *c*. Prokaryotic respiratory chains are quite diverse, having several alternative enzymatic complexes, as well as distinct types of electron carriers. However, until recently no alternative complex to the cytochrome $bc_1$ was known.

The complexes of the respiratory chain of the bacterium *Rhodothermus (R.) marinus* have been widely studied. *R. marinus* is a Gram negative bacterium belonging to the Bacteroidetes phylum [1]. It is a strict aerobe and moderate halophile that grows optimally at 65 °C with 1–2% of NaCl [2,3].

Like in mitochondria, complex I [4,5] and complex II [6,7] are the electrons entry points. Oxygen can be reduced by three different oxygen reductases: $caa_3$ [9,10], $cbb_3$ [11] and $ba_3$ [12] which belong to the A2, C and B families of heme–copper oxygen reductases, respectively [10]. One of the unique features of the respiratory chain of this bacterium is the presence of a different complex III [8]. This was the first recognized example of a complex alternative to the canonical cytochrome $bc_1$ complex, that possess a completely different structural composition, while performing the same catalytic reaction: namely linking quinol oxidation to the reduction of electron carriers, in this case a HiPIP. The presence of this type of complex is not exclusive of *R. marinus*. In fact, it was recently shown to be a widespread enzyme at least in the Bacteria domain, given that the genes coding for its subunits are present in several organisms [13,14] in which the genes coding for the canonical $bc_1$ complex are absent [13–16]. Alternative complex III is composed by peripheral and transmembrane proteins. One of the latter is predicted to have quinone binding sites, while the largest peripheral subunit has one binding motif for a $[3Fe–4S]^{1+/0}$ cluster and three binding motifs for $[4Fe–4S]^{2+/1+}$ clusters. Two other subunits, one with five and another with one *c*-type heme binding motifs are also present in the complex [8,16]. The genes coding for the subunits of ACIII are clustered together [13,14,16]. Moreover, in several organisms the gene cluster of ACIII is followed by that coding for oxygen reductases [13,14]. The $caa_3$ oxygen reductase of *R. marinus* is composed by four subunits and has five redox centres. A low-spin heme *a* and a binuclear centre (high-spin heme $a_3$ and a $Cu_B$ centre) are present in subunit I, while a $Cu_A$ and a low-spin heme *c* are present in subunit II. This enzyme reduces oxygen to water in a reaction coupled to proton pumping [17]. The genes coding for the $caa_3$

*Abbreviations:* ACIII, alternative complex III; DDM, n-dodecyl β-D-Maltoside; HQNO, 2-heptyl-4-hydroquinoline-N-oxide

* Corresponding author. Tel.: +351 214469321; fax: +351 2144269314.
*E-mail address:* mpereira@itqb.unl.pt (M.M. Pereira).

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.02.029


**Fig. 1.** *Rhodothermus marinus* genomic organization of the genes coding for the alternative complex III (black boxes) and those coding for the $caa_3$ oxygen reductase (gray boxes).

oxygen reductase from *R. marinus* are organized in a five gene cluster in which the first one, SCOI, codes for a protein involved in the incorporation of copper [10].

Functional associations involving the complexes from mitochondrial respiratory chains have been already described, namely, involving complex III and oxygen reductases. These associations were also found in some bacteria like *Mycobacterium smegmatis* [18], *Corynebacterium glutamicum* [19], *Thermophilic bacterium PS3* [20] and *Paracoccus denitrificans* [21].

Herein, a deeper characterization of the alternative complex III is presented, namely its interaction with menadiol, a quinol analogue, and with the $caa_3$ oxygen reductase.

## 2. Experimental procedures

### 2.1. Bacterial growth and protein purification

Growth of *R. marinus* strain PRQ62b was performed as described before [8]. Solubilised membranes (prepared according to [8]) in 20 mM Tris–HCl, 1 mM PMSF, and 0.05% n-dodecyl β-D-maltoside (DDM) pH 8 were applied into a Q-Sepharose High Performance column. The sample was eluted applying a gradient from 0 to 0.5 M of NaCl in the same buffer. A fraction eluted with approximately 0.35 M of NaCl, called D5, and containing both the ACIII and the $caa_3$ oxygen reductase was obtained. This fraction was used for further studies, including a Blue Native (BN)-PAGE and a Tricine-SDS-PAGE. The same fraction was also submitted to further chromatographic procedures in order to isolate the ACIII and the $caa_3$ oxygen reductase as described before [8,17].

### 2.2. DNA techniques

*R. marinus* genomic DNA was extracted from a liquid grown culture using a GenElute Bacterial Genomic DNA kit (Sigma). In order to confirm that the gene clusters coding for the ACIII and for the $caa_3$ oxygen reductase were consecutive, appropriate primers were designed. The forward primer (5′-ATG GCC GAA G TG AAA GCG AA-3′) was designed to hybridize with the last gene of the cluster coding for ACIII while the reverse primer (5′-CCT TTA CCC CAC CAC CGC AT-3′) was designed to hybridize with the first gene of the cluster coding for the $caa_3$ oxygen reductase. The sequence of the PCR product obtained was translated using an expasy tool (http://www.expasy.org/tools/dna.html).

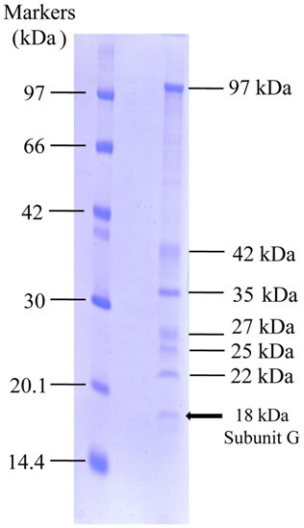


**Fig. 2.** Coomassie stained Tricine-SDS-PAGE of the alternative complex III from *Rhodothermus marinus* highlighting the subunit G with an apparent molecular mass of 18 kDa.

### 2.3. Prediction of transmembrane topology

Transmembrane topology was predicted using ConpredII at http://bioinfo.si.hirosaki-u.ac.jp/~Conpred2/ [22].

### 2.4. Fluorescence spectroscopy

The binding of HQNO (2-heptyl-4-hydroxyquinoline-N-oxide) to the ACIII was measured on a Cary Varian Eclipse fluorescence spectrophotometer. The excitation wavelength was set at 341 nm and the emission spectra were recorded between 370 and 600 nm. These measurements were performed considering the quenching of the HQNO fluorescence by the addition of ACIII. The complex was added to a 6 μM HQNO solution in small aliquots to a maximum concentration value of 4.4 μM. The fluorescence of the ACIII at the mentioned wavelengths was also measured and subtracted. The number of binding sites ($n$) and the binding constant ($K$) were determined from the following equation [23],

$$\log\ (F_0 - F)/F\ =\ \log K\ +\ n \log\ [Q] \qquad (1)$$

where the values $F$ and $F_0$ are the fluorescence intensities of HQNO in the presence and absence of the ACIII (quencher), respectively.

### 2.5. Electrophoresis

The BN-PAGE was performed as in [24] and the Tricine-SDS-PAGE was carried out as in [25] with 10% T, 3% C. Heme staining was done as in [26] to identify the protein bands of the complex having covalently bound hemes. The bands of BN-PAGE were also stained for cytochrome *c* oxidase activity according to [27].

### 2.6. Mass spectrometry assays

The protein bands present in the Tricine-SDS-PAGE having as sample the D5 lane of the BN-PAGE were excised from the gel and submitted to proteolytic digestion with Trypsin or Chymotrypsin. The mass spectra of the peptides were acquired by MALDI-TOF in the positive reflection mode in the Mass Spectrometry Laboratory, Analytical Services Unit of ITQB/IBET. The identification of the peptides was performed either by searching in the databases with Mascot software (http://www.matrixscience.com) or by direct comparison of the molecular masses predicted for the peptides with those experimentally obtained. The molecular masses of the peptides were predicted using PeptideMass at http://expasy.org/cgi-bin/peptide-mass.pl.

The protein band observed in the Tricine-SDS-PAGE with an apparent molecular mass of 18 kDa was also analysed by MS/MS after *in-gel* digestion with Trypsin.

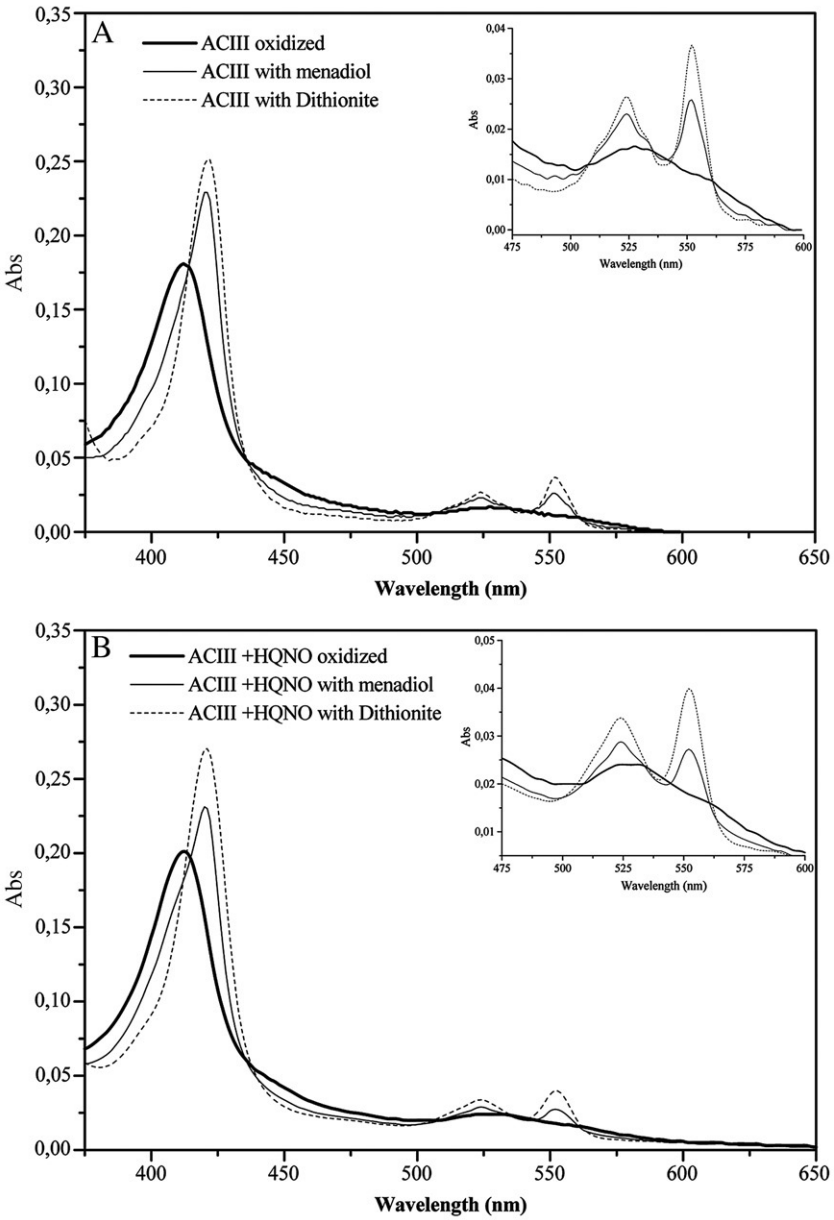


**Fig. 3.** UV–Visible absorption spectra of the alternative complex III in the oxidized state (**━**) and reduced by menadiol (—) or by sodium dithionite (- - -). In the absence (A) and presence (B) of HQNO.

### *2.7. UV–Visible absorption spectroscopy*

UV–Visible absorption spectroscopy was performed using a Shimadzu UV-1603 spectrophotometer. The ACIII (0.28 μM) spectra were measured under anaerobic conditions using a mixture of glucose (3 μM), glucose oxidase (4 U/mL) and catalase (132 U/mL). HQNO (used three times more concentrated than ACIII) was used as inhibitor.

### *2.8. Activity assays*

The menaquinol:oxygen oxidoreductase activity was determined by the oxygen consumption measured polarographically with a Clark-type oxygen electrode, YS Model 5300, from Yellow Springs. The assays were carried out at 30 °C in 20 mM potassium phosphate pH 6.5 buffer. Menadiol, obtained by reducing menadione with sodium dithionite [28] was used as the electron donor for the menaquinol: oxygen oxidoreductase activity measurements. The ACIII and the $caa_3$ oxygen reductase were used in a 1:1 ratio. KCN (≈0.7 mM) and HQNO (the same ratio as before) were used as inhibitors of $caa_3$ oxygen reductase and ACIII, respectively. The activity values were calculated per milligram of $caa_3$ oxygen reductase.

## 3. Results

### *3.1. The genomic organization and the new subunit of the alternative complex III*

In the genome of several organisms the gene cluster coding for the ACIII precedes the gene cluster coding for oxygen reductases [13,14]. In most genomes in which this type of organization is observed the oxygen reductase genes code for a $caa_3$, nevertheless examples of genes coding for other oxygen reductases, such as $cbb_3$ oxygen reductases are also present [13].

In order to investigate whether *R. marinus* genome contains such a gene organization, suitable primers were designed to amplify the

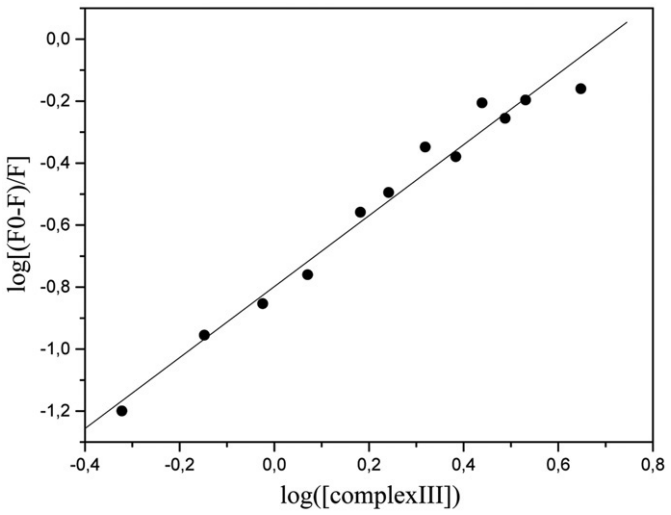


**Fig. 4.** Double-log plot of the quenching effect on HQNO fluorescence by the alternative complex III monitored at 479 nm. The data were fitted using Eq. (1).

region between the two clusters. A PCR product with ca 1800 bp was obtained and sequenced. The N-terminal and C-terminal parts of this sequence corresponded to those of the *ActF* and *SCOI* deduced sequences, showing that indeed in *R. marinus* the gene clusters coding for the ACIII and for the $caa_3$ oxygen reductase were consecutive (Fig. 1).

Furthermore, the PCR product encoded an ORF, named *ActG*, which did not correspond to any of the already known subunits of the two complexes. The protein encoded by this gene, named G, was predicted to have 14.5 kDa and one transmembrane helix and no binding motifs for redox cofactors. The subunit G was assigned to the protein band observed in the SDS-PAG of the ACIII with an apparent molecular mass of 18 kDa (Fig. 2). This assignment was confirmed by peptide mass fingerprint analysis (data not shown).

### 3.2. Interaction of alternative complex III with menadiol

The interaction of ACIII with menadiol, a menaquinol analogue, was investigated by UV–Visible absorption spectroscopy. It was observed that the oxidized ACIII was approximately 60% reduced by menadiol (Fig. 3 A). The fully reduced state of the complex was achieved by the addition of sodium dithionite. In order to check the specificity of menadiol reduction, HQNO, a menadiol structural analogue and an inhibitor of several quinone interacting enzymes, was used. In its presence the reduction by menadiol was 25% inhibited (Fig. 3 B).

Several studies showed that the fluorescence intensity of HQNO is quenched upon its binding to a protein (e.g. DMSO reductase [29] and nitrate reductase [30]). HQNO has a maximum fluorescence at 479 nm when excited at 341 nm. A decrease in its fluorescence intensity with the increasing concentration of the ACIII was observed (data not shown). The fluorescence quenching titration data were analysed considering the quenching of HQNO fluorescence intensity by ACIII as a static process [31]. A binding constant of 159 $nM^{-1}$ and the presence of 1 binding site ($n = 1.1$) were calculated from the fitting of Eq. (1) (Fig. 4).

The obtained results indicated that the ACIII of *R. marinus* has one binding site for HQNO. Nevertheless, the presence of more quinone binding sites, as observed for the $bc_1$ complex cannot be excluded.

### 3.3. Interaction between alternative complex III and $caa_3$ oxygen reductase

#### 3.3.1. Structural association

The structural association of the ACIII and the $caa_3$ oxygen reductase was investigated by native gel electrophoresis (Fig. 5). For this process, i- the fraction D5, obtained after the first chromatographic step; ii- the isolated ACIII and iii- the $caa_3$ oxygen reductase were applied in independent lanes in a BN-PAG. The result of this electrophoresis showed that ACIII migrated with an apparent molecular mass of 361 kDa, while the $caa_3$ oxygen reductase showed an apparent molecular mass of 210 kDa. Bands corresponding to these masses could not be observed in the lane of the D5 fraction. Instead, a band with an apparent molecular mass of 550 kDa was detected, which suggested that D5 fraction contained an association of ACIII and $caa_3$ oxygen reductase. Replicates of the gel were submitted to other staining procedures. Heme staining (Fig. 5 B) revealed that the 550 kDa band was the only one which stained for covalently bound hemes in the lane of D5 (Fig. 5 B, lane 2). This result was consistent with the presence of an association of ACIII and $caa_3$ oxygen reductase. Lanes 3 and 4, containing ACIII and $caa_3$ oxygen reductase, respectively, as expected, stained both under this procedure. The *in-gel* cytochrome *c* oxidase activity assays showed a positive result for the $caa_3$ oxygen reductase and in the case of D5 fraction the activity marker was spread in the first half of the lane but it was absent in the 200 kDa region. The results from the two staining procedures corroborated the association of the ACIII and $caa_3$ oxygen reductase.

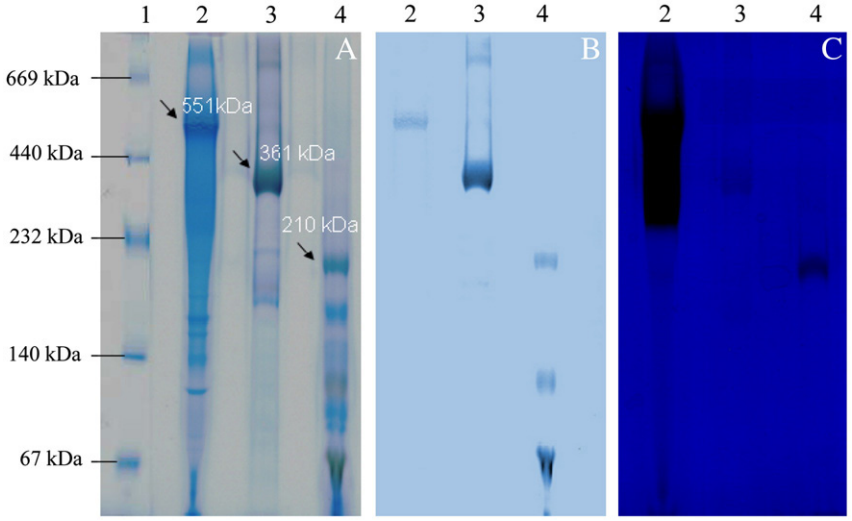


**Fig. 5.** Blue Native-PAGE of D5 fraction (lane 2), alternative complex III (lane 3) and $caa_3$ oxygen reductase (lane 4). Molecular mass protein markers are present in lane 1. The native gel was stained with Coomassie (A), with heme-staining procedures (B) and cytochrome *c* oxidase *in-gel* activity (C).

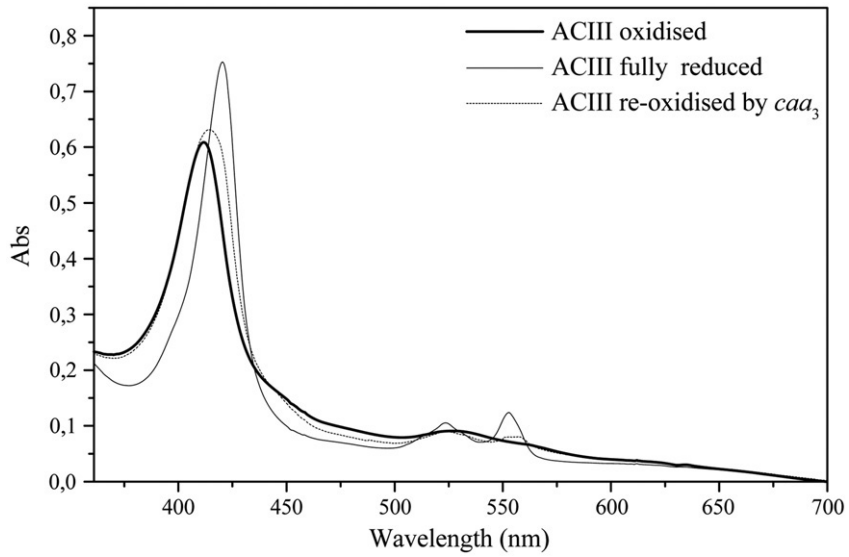


**Fig. 6.** UV–Visible absorption spectra of the alternative complex III in the oxidized and reduced state, and re-oxidized by the $caa_3$ oxygen reductase.

The upper part (669–232 kDa) of the D5 lane of the BN gel lane was submitted to a Tricine-SDS-PAGE (2D) (Supplementary Fig. 1). Several bands were observed in this denaturant second dimension. Nevertheless, in the lane corresponding to the band with the apparent molecular mass of 550 kDa only subunits with apparent molecular masses compatible with subunits of the complexes III and IV were observed. It was not possible to assign the bands to each subunit just by analysing the migration profiles of the subunits because two of the subunits of ACIII [16] and two of the $caa_3$ oxygen reductase [17] show the same apparent molecular masses upon electrophoresis. The protein bands were further identified by MS analysis: bands 1, 3 and 4 were assigned to subunits B, F and E (monohemic cytochrome *c*), respectively, of the ACIII; band 5 was identified as subunit III of $caa_3$ oxygen reductase. These results unequivocally showed that both, ACIII and $caa_3$ oxygen reductase were present in the complex observed at 550 kDa in the BN gel.

### 3.3.2. Functional association

The functional association of the ACIII and the $caa_3$ oxygen reductase was addressed by investigating the direct oxidation of the former by the oxygen reductase. Fig. 6 shows the UV–Visible spectra of the ACIII in the oxidized state and reduced by sodium dithionite. In the presence of oxygen, sub-stoichiometric amounts of $caa_3$ oxygen reductase could reoxidise the ACIII.

Moreover, if the ACIII receives electrons from quinol and gives electrons to the $caa_3$ oxygen reductase, a complex formed by the two enzymes should have quinol:oxygen oxidoreductase activity. This activity was determined by measuring oxygen consumption by a 1:1 mixture of the two complexes upon addition of menadiol and a value of 77.3 μM $O_2\,min^{-1}\,mg^{-1}$ was obtained. Addition of KCN (the typical oxygen reductases inhibitor) completely abolished $O_2$ consumption, while the addition of HQNO inhibited this activity by 45% (34.8 μM $O_2\,min^{-1}\,mg^{-1}$).

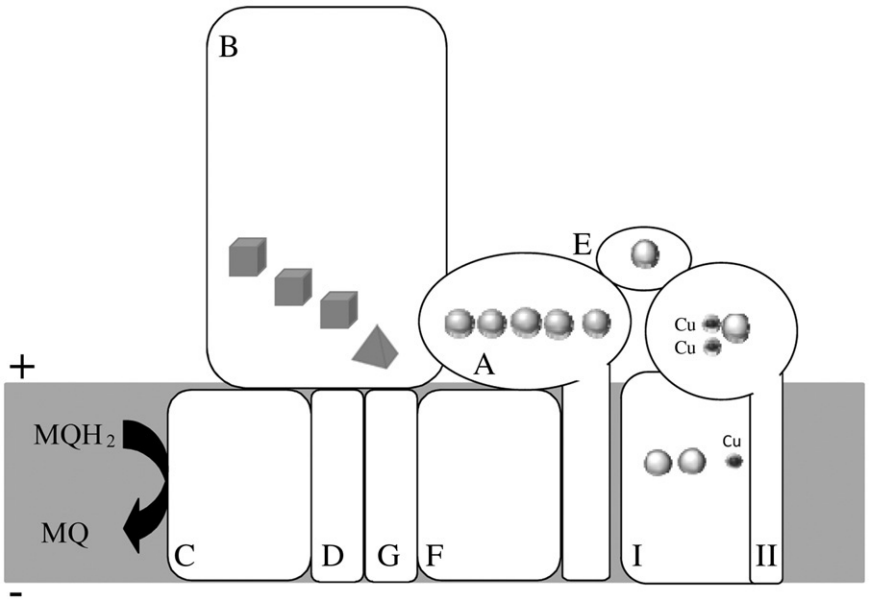


**Fig. 7.** Schematic representation of the structural and functional association between the alternative complex III (subunits A–G) and the $caa_3$ oxygen reductase (catalytic subunits I and II). The gray spheres represent *c*-type hemes, the smaller gray and black spheres represent copper ions while cubes and pyramids represent $[4Fe–4S]^{2+/1+}$ and $[3Fe–4S]^{1+/0}$ clusters, respectively.

## 4. Conclusion

In the electron transfer respiratory chain of the bacterium *R. marinus* the ACIII is the only enzyme which accepts electrons directly from reduced quinones. As previously shown, it is capable of performing the same function as the cytochrome $bc_1$ complex, although it does not belong to its family.

In this work, a seventh gene (*ActG*) in the gene cluster coding for ACIII was revealed and the respective encoded protein was identified as a subunit of ACIII of *R. marinus*. This gene was observed to be also present in gene clusters coding for ACIII in other organisms [13].

Here we also addressed the interaction of ACIII with quinol and $caa_3$ oxygen reductase. We observed that the complex is reduced by menadiol, the analogue of *R. marinus* physiologic quinone, and that this reduction is inhibited by HQNO. The presence of at least one quinol binding site in the ACIII was determined by fluorescence quenching titration of HQNO. In the case of cytochrome $bc_1$ complexes the HQNO binds only to one (Qi) of the two quinone binding sites [32]; therefore, the presence of two or more quinol binding sites in the ACIII could not be excluded.

In several organisms, including *R. marinus*, ACIII coding genes are followed by those coding for $caa_3$ oxygen reductase. This observation led to the hypothesis of a direct interaction between the two complexes. The findings presented here showed that the ACIII and $caa_3$ oxygen reductase are structurally and functionally associated with a 550 kDa complex (Fig. 7).

The functional association of the ACIII and the $caa_3$ oxygen reductase was further demonstrated by the observation of menadiol:oxygen oxidoreductase activity, upon mixing the two purified complexes, which was KCN and HQNO inhibited. In cytochrome $bc_1$ complexes, and according to the Q-cycle mechanism, the cytochrome $c_1$ is the last electron acceptor within the complex, transferring electrons to the periplasmatic cytochrome *c* [33,34]. The monohemic subunit of the ACIII is proposed to perform an equivalent role. In the case of a direct interaction with $caa_3$ oxygen reductase this subunit is also proposed to replace the role of the periplasmatic electron carriers (such as cytochrome *c* and HiPIP).

## Acknowledgements

Patrícia N. Refojo is a recipient of a grant from Fundação para a Ciência e a Tecnologia (BD SFRH XXI/BD/24745/2005). João Carita is acknowledged for cell growth, Ana Coelho and Ana P. Batista for the Peptide Mass Fingerprint identifications and Eurico Melo for the helpful discussions on fluorescence measurements. This work was supported by Fundação para a Ciência e a Tecnologia (PTDC/BIA-PRO/66557/2006 and PTDC/QUI/66559/2006).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbabio.2010.02.029.

## References

[1] S.H. Bjornsdottir, T. Blondal, G.O. Hreggvidsson, G. Eggertsson, S. Petursdottir, S. Hjorleifsdottir, S.H. Thorbjarnardottir, J.K. Kristjansson, Rhodothermus marinus: physiology and molecular biology, Extremophiles 10 (2006) 1–16.
[2] G.A. Alfredsson, J.K. Kristjansson, S. Hjorleifsdottir, K.O. Steiter, *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarin hot springs in Iceland, J. Gen. Microbiol. 134 (1988) 299–306.
[3] O.C. Nunes, M.M. Donato, M.S. da Costa, Isolation and characterization of *Rhodothermus* strains from S. Miguel, Azores, Syst. Appl. Microbiol. 15 (1992) 92–97.
[4] 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.
[5] 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.
[6] A.S. Fernandes, M.M. Pereira, M. Teixeira, The succinate dehydrogenase from the thermohalophilic bacterium *Rhodothermus marinus*: redox–Bohr effect on heme bL, J. Bioenerg. Biomembr. 33 (2001) 343–352.
[7] A.S. Fernandes, A.A. Konstantinov, M. Teixeira, M.M. Pereira, Quinone reduction by *Rhodothermus marinus* succinate:menaquinone oxidoreductase is not stimulated by the membrane potential, Biochem. Biophys. Res. Commun. 330 (2005) 565–570.
[8] 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.
[9] M.M. Pereira, F.L. Sousa, M. Teixeira, R.M. Nyquist, J. Heberle, A tyrosine residue deprotonates during oxygen reduction by the $caa_3$ reductase from *Rhodothermus marinus*, FEBS Lett. 580 (2006) 1350–1354.
[10] M.M. Pereira, M. Santana, M. Teixeira, A novel scenario for the evolution of haem–copper oxygen reductases, Biochim. Biophys. Acta 1505 (2001) 185–208.
[11] M.M. Pereira, J.N. Carita, R. Anglin, M. Saraste, M. Teixeira, Heme centers of *Rhodothermus marinus* respiratory chain. Characterization of its cbb3 oxidase, J. Bioenerg. Biomembr. 32 (2000) 143–152.
[12] A.F. Verissimo, M.M. Pereira, A.M. Melo, G.O. Hreggvidsson, J.K. Kristjansson, M. Teixeira, A ba3 oxygen reductase from the thermohalophilic bacterium *Rhodothermus marinus*, FEMS Microbiol. Lett. 269 (2007) 41–47.
[13] P.N. Refojo, F.L. Sousa, M. Teixeira, M.M. Pereira, The alternative complex III: a different architecture using known building modules, Bioenergetics (submitted for publication).
[14] M.F. Yanyushin, M.C. del Rosario, D.C. Brune, R.E. Blankenship, New class of bacterial membrane oxidoreductases, Biochemistry 44 (2005) 10037–10045.
[15] B. Goldman, S. Bhat, L.J. Shimkets, Genome evolution and the emergence of fruiting body development in *Myxococcus xanthus*, PLoS One 2 (2007) e1329.
[16] 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.
[17] M.M. Pereira, M. Santana, C.M. Soares, J. Mendes, J.N. Carita, A.S. Fernandes, M. Saraste, M.A. Carrondo, M. Teixeira, The caa3 terminal oxidase of the thermohalophilic bacterium *Rhodothermus marinus*: a HiPIP:oxygen oxidoreductase lacking the key glutamate of the D-channel, Biochim. Biophys. Acta 1413 (1999) 1–13.
[18] J.A. Megehee, J.P. Hosler, M.D. Lundrigan, Evidence for a cytochrome $bcc–aa_3$ interaction in the respiratory chain of *Mycobacterium smegmatis*, Microbiology 152 (2006) 823–829.
[19] A. Niebisch, M. Bott, Purification of a cytochrome $bc–aa_3$ supercomplex with quinol oxidase activity from *Corynebacterium glutamicum*. Identification of a fourth subunity of cytochrome $aa_3$ oxidase and mutational analysis of diheme cytochrome $c_1$, J. Biol. Chem. 278 (2003) 4339–4346.
[20] N. Sone, M. Sekimachi, E. Kutoh, Identification and properties of a quinol oxidase super-complex composed of a bc1 complex and cytochrome oxidase in the thermophilic bacterium PS3, J. Biol. Chem. 262 (1987) 15386–15391.
[21] E.A. Berry, B.L. Trumpower, Isolation of ubiquinol oxidase from *Paracoccus denitrificans* and resolution into cytochrome $bc_1$ and cytochrome $c–aa_3$ complexes, J. Biol. Chem. 260 (1985) 2458–2467.
[22] M. Arai, H. Mitsuke, M. Ikeda, J.X. Xia, T. Kikuchi, M. Satake, T. Shimizu, ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability, Nucleic Acids Res. 32 (2004) W390–W393.
[23] E. Gok, C. Ozturk, N. Akbay, Interaction of thyroxine with 7 hydroxycoumarin: a fluorescence quenching study, J. Fluoresc. 18 (2008) 781–785.
[24] H. Schagger, Membrane Protein Purification and Crystallization 2/e: A Practical Guide, Elsevier Science, New York, 2003.
[25] 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.
[26] C.F. Goodhew, K.R. Brown, G.W. Pettigrew, Haem staining in gels, a useful tool in the study of bacterial c-type cytochromes, Biochem. Biophys. Acta 852 (1986) 288–294.
[27] A.M. Molnar, A.A. Alves, L. Pereira-da-Silva, D.V. Macedo, F. Dabbeni-Sala, Evaluation by blue native polyacrylamide electrophoresis colorimetric staining of the effects of physical exercise on the activities of mitochondrial complexes in rat muscle, Braz. J. Med. Biol. Res. 37 (2004) 939–947.
[28] L.F. Fieser, Convinient procedures for the preparation of antihemorragic compounds, J. Biol. Chem. (1940) 391–396.
[29] V.W. Cheng, R.A. Rothery, M.G. Bertero, N.C. Strynadka, J.H. Weiner, Investigation of the environment surrounding iron–sulfur cluster 4 of *Escherichia coli* dimethylsulfoxide reductase, Biochemistry 44 (2005) 8068–8077.
[30] M.G. Bertero, R.A. Rothery, N. Boroumand, M. Palak, F. Blasco, N. Ginet, J.H. Weiner, N.C. Strynadka, Structural and biochemical characterization of a quinol binding site of *Escherichia coli* nitrate reductase A, J. Biol. Chem. 280 (2005) 14836–14843.
[31] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, second editionKluwer Academic/ Plenum Publishers, 1999.
[32] J.W. Cooley, T. Ohnishi, F. Daldal, Binding dynamics at the quinone reduction (Qi) site influence the equilibrium interactions of the iron sulfur protein and hydroquinone oxidation (Qo) site of the cytochrome $bc_1$ complex, Biochemistry 44 (2005) 10520–10532.
[33] P. Mitchell, Protonmotive redox mechanism of the cytochrome $b–c_1$ complex in the respiratory chain: protonmotive ubiquinone cycle, FEBS Lett. 56 (1975) 1–6.
[34] P. Mitchell, Possible molecular mechanisms of the protonmotive function of cytochrome systems, J. Theor. Biol. 62 (1976) 327–367.