The alternative complex III: A different architecture using known building modules

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Abstract

Until recently cytochrome bc1 complexes were the only enzymes known to be able to transfer electrons from reduced quinones to cytochrome c. However, a complex with the same activity and with a unique subunit composition was purified from the membranes of *Rhodothermus marinus*. This complex, named alternative complex III (ACIII), was then biochemical, spectroscopic and genetically characterized. Later it was observed that the presence of ACIII was not exclusive of *R. marinus* being the genes coding for ACIII widespread, at least in the Bacteria domain. In this work, a comprehensive description of the current knowledge on ACIII is presented. The relation of ACIII with members of the complex iron-sulfur molybdoenzyme family is investigated by analyzing all the available completely sequenced genomes. It is concluded that ACIII is a new complex composed by a novel combination of modules already identified in other respiratory complexes.

1. Introduction

In electron transfer respiratory chains the exergonic transfer of electrons through membrane bound complexes is coupled to the translocation of protons across the membrane leading to the formation of a transmembrane electrochemical potential. The dissipation of the membrane potential energy can be used by ATP synthase to produce ATP.

Cytochrome bc1 complexes are part of the respiratory chains and have quinol: cytochrome c oxidoreductase activity. They are widespread enzymes composed of a dihemic cytochrome b, a cytochrome c1 and a Rieske iron-sulfur subunit [1,2]. The family also comprises cytochrome bc1f complexes, present in chloroplasts and cyanobacteria [3].

Besides the bc1 complex family, several other enzymes are able to oxidize quinols, namely quinol oxidases from the heme-copper oxygen reductases superfamily [4], and DMSO reductase, nitrite and nitrate reductases from the complex iron-sulfur molybdoenzyme (CISM) family [5]. However, and until recently, the cytochrome bc1 complex family was the only one described to receive electrons from quinols and transfer them to cytochrome c. The alternative complex III (ACIII), purified for the first time from *Rhodothermus (R.) marinus* membranes and structurally and functionally characterized [6–8], was the first example of a complex performing the same function as the bc1 complex but not belonging to its family.

2. The alternative complex III is a widespread quinol: electron acceptor oxidoreductase

The *R. marinus* respiratory chain has been extensively studied [9–12] and the presence of three different oxygen reductases, a caa3 [13,14], a ba3 [15] and a cbb3 [16] was observed. These enzymes are unable to receive electrons from reduced quinones and therefore a complex which transfers electrons from quinols to periplasmatic electron carriers is required. A cytochrome bc1 complex was never observed at the protein level and its absence has now been corroborated by the analysis of the recently sequenced *R. marinus* genome [17], in which genes coding for such a complex are not present. Instead, a seven subunits complex [18] with quinol: HiPIP oxidoreductase activity was isolated from the membranes of *R. marinus* [6–8]. This complex is structurally different from the cytochrome bc1 complexes, even though it performs the same function. By this reason the complex was named alternative complex III (ACIII) [6,19].

The presence of ACIII is not exclusive of *R.marinus*. A homologous complex was also isolated from the membranes of the green nonsulfur proteobacterium *Chloroflexus (C.) aurantiacus* [19–21] and was recently shown to have menaquinone: aurocyanin oxidoreductase activity [19]. As in the case of the *R. marinus* enzyme [7], it was not inhibited by the typical inhibitors of the cytochrome bc1 complex, such as antimycin A. The genes coding for ACIII were also identified in the *δ-Proteobacterium Myxococcus xanthus* and the same function for the complex was proposed [22]. Furthermore, Yanyushin and coworkers observed that the new complex was widespread and related to the complex iron-sulfur molybdoenzyme (CISM) family (see below) [21]. Due to the similarity with the members of that family, the name MFcc (molybdopterin, FeS, integral membrane subunits, with two c-
type heme subunits) has been proposed. This designation may be misleading since the complex does not contain molybdenum [6,21], and thus the name alternative complex III was adopted [6,19].

In this work, we performed an exhaustive search for ACIII coding genes in organisms with a completely sequenced genome, by September 2009. We confirmed that ACIII is a widespread enzyme in the Bacteria domain (Fig. 1). The genes coding for the complex may be present in genomes that do not contain coding genes for bc1 complex subunits and in which genes coding for a quinol:cytochrome c oxidoreductase should exist, but there are also examples of genomes where ACIII genes coexist with those coding for the classical complexes III (Fig. 1).

The genes encoding ACIII form a cluster composed by six to eight genes (ActABCDEFG); in some cases ActG is absent, while in others ActB is split into two different genes ActB1 and ActB2, which correspond to the two domains of the gene ActB product (see below). A gene cluster with a similar organization but in which the ActD, ActE and ActF genes are absent was also earlier identified and its product named MFIc complex [21]; in this gene cluster ActB is also split in two genes. The presence of MFIc complexes is only predicted for δ-Proteobacteria (Fig. 1), and their function has not been established yet.

The analysis of the gene clusters coding for ACIII in the sequenced genomes, in relation to their neighboring genes revealed that they may be isolated, ie, without any obvious functional relationship with preceding and following genes or gene clusters; or they may be followed by a gene cluster coding for a heme-copper oxidoreductase. This latter situation presents four possibilities; illustrative examples of each one are represented in Fig. 2. R. marinus is one example of the most observed organization in which the following gene cluster codes for subunits of the caa3 oxygen reductase. Salinibacter ruber represents a similar example but in this case the SCO1 gene (whose product is involved in copper incorporation [131]) is absent. The presence of a following gene cluster coding for subunits of cbb3 oxygen reductases can also be observed (Opinitutu terreae), as well as genes coding only for subunits I and II of other oxygen reductases (Flavobacterium psychrophilum). Thermus thermophilus exemplifies a situation in which the gene cluster coding for ACIII is isolated. In Fig. 1, the type of oxygen reductase (A1, A2, B and C-type, [23]) encoded by the gene cluster that follows the genes coding for ACIII is indicated.

### 3. Structural characterization of the alternative complex III

The first gene of the cluster (ActA) codes for a 27 kDa protein with five c-type heme binding motifs (CXXCH); the fifth motif is one amino acid residue apart from the C-terminus. Three methionine and seven other histidine residues are present in the sequence and are thus candidates for the sixth ligand of the hemes. However, the alignment of the amino acid sequence of the subunits A of all ACIII showed that four histidine (H54, H57, H129, H132, R. marinus enzyme numbering) and one methionine (M160) residues are strictly conserved and are thus the most probable sixth heme ligands. A possible signal peptide and thus the name alternative complex III was adopted [6,19]. Despite the unique gene organization and subunit composition of the ACIII, the different subunits have homology with subunits of enzymatic complexes already characterized. In order to determine the most related proteins, the output number of sequences obtained by blast searches was enlarged. The sequences with the lower E-values obtained, excluding those of ACIII, were the subunits of the MFIc complex, followed by the sequences of subunits of complexes belonging to the complex iron-sulfur molybdoenzyme family (CISM family) [5].

A relation between subunits B and C of ACIII and three subunits of those complexes had already been observed [6,21]. The CISM family is characterized by the presence of three subunits [5]. A catalytic subunit which has a molybdo-bis(pyranopterin guanine dinucleotide) (Mo-bisPGD) cofactor and in some cases an iron-sulfur center (named F50), a protein with four iron-sulfur clusters (FS1-FS4) named four cluster protein (FCP), and a membrane anchor protein (MAP), this family includes complexes such as DMSO reductase (DmsABC), polysulfide reductase (DmsABC) and cytochrome bc1 complex family (bc1/bc). The type of heme-copper oxidoreductases (HCO) coded by the gene cluster following the ACIII gene cluster is also indicated [23]. The black dots represent the presence of the corresponding gene clusters. For the construction of this phylogenetic profile only aerobic organisms were considered.

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**Fig. 1.** Phylogenetic profile of the presence of alternative complex III (ACIII), MFIc complex and cytochrome bc1 complex family (bc1/bc). The type of heme-copper oxidoreductases (HCO) coded by the gene cluster following the ACIII gene cluster is also indicated [23]. The black dots represent the presence of the corresponding gene clusters. For the construction of this phylogenetic profile only aerobic organisms were considered.
Archaea

Thaumarchaeota
Thermoprotei
Euryarchaeota

Acidobacteria
Bacteroidetes/Chlorobi

Deinococci
Chlamydiae/Verrucomicrobia
Verrucomicrobia

Chloroflexi
Actinobacteria
Firmicutes
Cyanobacteria

Bacteria

Proteobacteria

Gamma proteobacteria
Beta proteobacteria
Alpha proteobacteria
Epsilon proteobacteria
Delta proteobacteria

Nitrospumilales
Crenarchaeotes
Desulfurococcales
Sulfobacterales
Thermoproteales
Thermoplasmales
Halobacteriales
Gemmatales
Sphingobacteriales
Flavobacteriales
Chloroibiales
Deinococcales
Thermales
Chiromycales
Methylacidiphilum
Optitales
Verrucomicrobiales
Aquificales
Thermotogales
Herpetosiphonales
Chloroflexales
Acidimicrobiales
Actinomycetaciales
Clostridiales
Bacillales
Gloeobacteriales
Acidobacteria
Prochlorales
Nostocales
Oscillatoriales
Chroococcales
Spirochaetales
Planctomycetaceae
Maripondales
Methylococcus
Acidithiobacillales
Pastuniales
Aeromonadales
Vibrionales
Alteromonadales
Oceanospirillales
Methylcoccales
Xanthomonadales
Chromatiales
Legionellales
Enterobacteriales
Pseudomonadales
Thiocyanates
Rhodocyclales
Netesiniales
Methylphilales
Hydrogenophiles
Burkholderiales
Nitrosomonadales
Parvulariales
Caulobacteriales
Sphingomonadales
Rhodobacteriales
Rhodospirillales
Rickettsiales
Rizobiales
Campylobacteriales
Bdellovibrioales
Syntrophobacteriales
Desulfofthetas
Desulforomonadales
Mycococcales
Fig. 2. Organization of the gene clusters coding for alternative complex III subunits (dark grey) and those coding for the different heme-copper oxygen reductases (light grey). SCO1 gene product is involved in the incorporation of copper. I, II, III and IV represent the different subunits of oxygen reductases while FixN, FixP and cyt c are the genes coding for subunits of cbb3 oxygen reductase. An example of an organism for each organization is indicated. In eight of the sixteen cases exemplified by Thermus thermophilus, ActG is absent.

Fig. 3. Schematic representation of the alternative complex III. The subunits (modules) of the complex may be separated according to their proposed function. The membrane attachment and quinone interacting modules correspond to subunits C, D, F and G, while subunits A, B and E are electron transfer modules. The spheres represent c-type hemes; cubes and pyramids represent [4Fe-4S]^{2+/1+} and [3Fe-4S]^{1+/0} clusters, respectively.
reductase (PsrABC), formate dehydrogenase (FdnGHI), and nitrate reductase (NarGHI). Also related to this family are the complexes nitrite reductase (NrfABCD), arsenite oxidase (AoxAB), TMAO reductase (TorCA), formate dehydrogenase (FdhAB), nitrate reductase (NapAB), ethylbenzene dehydrogenase (EbdABC), and selenate reductase (YnfEFG).

Schematic representations of some of these enzymes are presented in Fig. 4 and the respective gene cluster organization is shown in Fig. 5.

To obtain the amino acid sequence of each subunit of the complexes of this family, a new blast search against all genomes deposited at Kegg server (http://www.genome.jp/kegg/) [27–29] was performed using as initial query a sequence from a model organism, generally Escherichia coli. Also, when orthology information was available, all genes annotated as coding for proteins of the CISM family or for related proteins were retrieved. The gene organization of each complex within an organism was automatically inspected, and dubious gene organizations were manually inspected. All retrieved sequences were then mapped on NCBI Taxonomy using the BioSQL package from April 2009 available to download at ftp://ftp.ncbi.nih.gov/.

4.1. The iron-sulfur protein - Subunit B

Domain 1 of subunit B showed similarity with the catalytic subunit of the members of the CISM family, while domain 2 presented similarity with the four cluster protein. Since the two domains observed in subunit B are homologous to different proteins, the sequence was divided (800 amino acid residues from the N-terminal-Domain 1 and 240 amino acid residues from the C-terminal-Domain 2) and the two parts analyzed separately.
For the analysis of subunit B, the subunit NuoG (or Nqo3) of the complex I (NADH: quinone oxidoreductase) was included, since this subunit is also related to the CISM family. Furthermore, this subunit is another example of a protein that has a molybdopterin-like domain, but lacks any molybdenum cofactor, and has an iron-sulfur domain [30]. As in the case of subunit B of ACIII, the NuoG amino acid sequence was also separated in two parts (NuoG_1, N-terminal and NuoG_2, C-terminal). Interestingly, subunit NuoG has the domains in a reverse order: the molybdopterin domain is located in the C-terminus while the iron-sulfur centers binding motifs are at the N-terminus, thus suggesting independent fusion processes. NuoG_1 and NuoG_2 were analyzed with B2 and B1 domains of subunit B, respectively.

The amino acid sequence of domain II of subunit B was compared with sequences of catalytic subunits of complexes of the CISM family. From the dendogram obtained (Fig. 6A) eleven different clades are observed, each clade being composed by subunits of the same enzymatic complex. The similarity between the subunits of the members of the CISM family is in agreement with previous analyses [5,31]. Domain I of subunit B of ACIII, subunit B1 of MFCi and NuoG are clearly related to those members. The subunits of ACIII and of MFCi are clustered together and seem to have a common origin. Besides the absence of molybdenum, the domain B1 of subunit B of ACIII, the NuoG amino acid sequence was also compared with sequences of other multi-hemes subunits of the CISM family [32,33]. The analysis of subunit B, the subunit NuoG (or Nqo3) of the complex I (NADH: quinone oxidoreductase) was included, since this subunit is also related to the CISM family. Furthermore, this subunit is another example of a protein that has a molybdopterin-like domain, but lacks any molybdenum cofactor, and has an iron-sulfur domain [30].

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4.3. c-type heme containing subunits- A and E

4.3.1. Subunit A

Although the typical CISM family members do not have c-type heme cytochrome subunits, variations of the subunit composition of complexes related to the family have been identified and subunits containing c-type hemes have been observed [5,32,33]. Therefore, the sequence of the pentaheme cytochrome c of the alternative complex was compared with that of other multi-hemes subunits of the CISM family and related complexes such as NrfA, NrfB, NrfH, NapC and TorC. All these proteins belong to the Napc/NrfH family of cytochromes [34] with the exception of NrfA and NrfB. That family plays an important role in the electron transfer between the quinone/quinol pool and oxidoreductases located outside the cytoplasmatic membrane [33,34]. NapC and NrfH are typical examples of the family, which have four c-type hemes and a membrane helix at the N-terminus; both are involved in the transfer of electrons to other cytochrome domains. NapA transfers electrons to the di-heme protein NapB from the NapAB complex [34], while NrfH transfers electrons to NrfA [36]. NrfA and NrfB are also pentaheme c-type cytochromes but the transmembrane anchor is absent [32,37]. TorC belongs also to the NapC/NrfH family [38,39]; it contains at the C-terminus an additional domain with a c-type heme described to be responsible for the interaction with the molybdenum-containing TMAO reductase [38]. Three different clades can be considered in the dendogram represented in Fig. 6D: one formed by NapC, TorC, NrfH and NrfB, a second one formed only by NrfA and a last one formed by subunits A of the alternative complexes III and of the MFCi complexes. Within their clade, NapC and TorC are clustered together as expected, since both proteins belong to the same family; NrfH formed a sub-group inside of this clade, as previously observed [40]. NrfA is the only one of the analyzed proteins with an intrinsic catalytic activity; its catalytic heme is bound through an unconventional binding motif where a lysine replaces the typical histidine residue (CXCK) [41,42]. The specific properties of NrfA are in agreement with its place as an individual clade. The subunits A of the ACIII and of the MFCi complex were found to be part of the same clade, being closely related, and appear to have had the same evolutionary origin.

4.3.2. Subunit E

There are multiple examples of monoheme c-type cytochromes. The amino acid sequence of subunit E of ACIII was aligned with sequences from diverse c-type cytochromes, cytochrome c1 from cytochrome bc1 complex and also with monohemicyanides of the oxygen reductases (c-domain of subunit II of cbb3 oxygen reductase and c domain of FixP subunit of cbb3 oxygen reductase). A dendogram was constructed (data not shown); however, it was not possible to determine any closer protein since the bootstrap values obtained for the different branches of the dendogram were extremely low. Yet, it was possible to conclude that
the subunit E of ACIII formed an independent clade. These observations suggest that the monoheme c-type subunit of ACIII is another example of a subfamily of c-type cytochromes.

4.4. The other membrane proteins- Subunits D and G

We were unable to identify any protein homologous of these two proteins; their presence seems to be restricted to the ACIII.

5. The alternative complex III is a different complex composed by "old" modules

The ACIII has a unique subunit composition. However, the different constituting subunits show similarities with subunits of complexes already known, namely those of the CISM family. The subunits of the different complexes can be divided into several modules according to their function: 1-electron transfer, 2-catalytic and 3-membrane attachment and quinone interacting modules. These modules can be...
observation of complexes combined in multiple ways. The type of function performed could have influenced the different combinations of those subunits. This idea of modularity in the construction of respiratory complexes has been proposed before, and respiratory complexes have been constructed by in vitro reconstitution of the respiratory chain. Characterization of its flavocytochrome c oxidase and phylogeny of hydrogenases, FEBS Lett. 512 (2002) 125–128.


