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Review

Structural composition of alternative complex III: Variations on the same theme ☆

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ABSTRACT

Alternative complex III forms a recently identified family of enzymes with quinol:electron acceptor oxidoreductase activity. First biochemical and genomic analyses showed that ACIII is composed of six to eight subunits, most of which homologous to different proteins or domains already observed in other known enzymatic complexes. The increasing number of completely sequenced genomes led us to perform a new search for the genes coding for the different ACIII subunits. We have identified a larger number of gene clusters coding for ACIII, still confined to the bacterial domain, but extended to classes in which it was not observed before. We also found an unanticipated diversity in gene clusters, both in terms of its constitution and organization. The several unexpected gene arrangements brought new perspectives to the role of the different subunits of ACIII, namely in quinone binding and in proton translocation. This article is part of a Special Issue entitled: Respiratory complex III and related bc complexes.

1. Introduction

Quinol:electron acceptor oxidoreductase (namely quinol:cytochrome *c* oxidoreductase) is a central activity in the majority of the respiratory and photosynthetic electron transfer chains. This activity was, until recently, exclusively attributed to the cytochrome bc_1/b_6f complex family. This paradigm was changed by the identification and characterization of the so-called *A*lternative *C*omplex III (ACIII), particularly in organisms that lack bc_1/b_6f complexes, but ought to have quinol:electron acceptor oxidoreductase activity due to the presence of (periplasmatic electron carrier):oxygen oxidoreductases.

ACIII is structurally unrelated to the bc_1/b_6f complex family, but some of its subunits are observed in other complexes, namely in members of the *C*omplex *I*ron–*S*ulfur *M*olybdoenzyme (CISM) family [1,2].

Only two representatives of ACIII, those from the aerobic bacterium *Rhodothermus marinus* and the photosynthetic bacterium *Chloroflexus aurantiacus*, have been purified and biochemically and spectroscopically characterized [1–7]. ACIII isolated from both organisms is composed of seven subunits encoded by the gene cluster *ActABCDEFG* (Figs. 1 and 2A). Subunits ActB, ActC and ActF are homologous to subunits present in several members of the CISM family. The members of this family generally have three subunits: the catalytic subunit (with a molybdenum containing cofactor and, in most cases, also harboring one $[4Fe–4S]^{2+/1+}$ cluster), the iron sulfur protein (containing four $[4Fe–4S]^{2+/1+}$, or three $[4Fe–4S]^{2+/1+}$ and one $[3Fe–4S]^{1+/0}$ clusters) and a transmembrane α helical protein. Up to now two different types of membrane proteins have been identified in the members of the family [8]. One is a protein with 8 transmembrane helices, typified by subunit C (PsrC) of polysulfide reductase, whose crystal structure has already been obtained [9]. The second is a 5–6 transmembrane helix protein, which contains two B-type hemes, each one close to the opposite sides of the membrane [8]. ActC and ActF are membrane proteins homologous to each other and to PsrC. ActB is composed of two different domains designated as B1 and B2, homologous to the catalytic subunit and to the iron sulfur protein of the members of the CISM family, respectively. In spite of the predicted presence of a similar fold, the B1 domain of ActB does not contain molybdopterin nor any other molybdenum cofactor, in contrast to what is observed in the members of the CISM family [3,6]. A similar situation occurs in respiratory complex I, in which the C-terminal domain of the NuoG subunit is homologous to the molybdopterin containing subunit of formate dehydrogenase, but the cofactor also is absent. The role of these domains that lost the binding motifs for the catalytic centers is not known, but a regulatory function is a possibility. ActA and ActE are cytochromes, with five (or six) and one C-type hemes, respectively. ActD and ActG are predicted to be transmembrane proteins and seem to be present exclusively in ACIII. A review on the properties of each subunit of ACIII has been published recently [10].

A gene cluster related to that encoding ACIII was also identified before [1,2]. This cluster codes for a complex composed of only three of the subunits of ACIII, but in which the two domains of ActB are separated proteins (ActAB1B2C) [1] (Fig. 1). Later on, an example of such type of complexes was purified from *Desulfovibrio vulgaris* Hildenborough having cytochrome *c*:quinone oxidoreductase activity, and therefore was named QRC (*Q*uinone *R*educing *C*omplex) [11].

We have performed an exhaustive search for the genes coding for ACIII subunits, which revealed the presence of these complexes in 66

Abbreviations: ACIII, alternative complex III; CISM, complex iron sulfur molybdoenzyme

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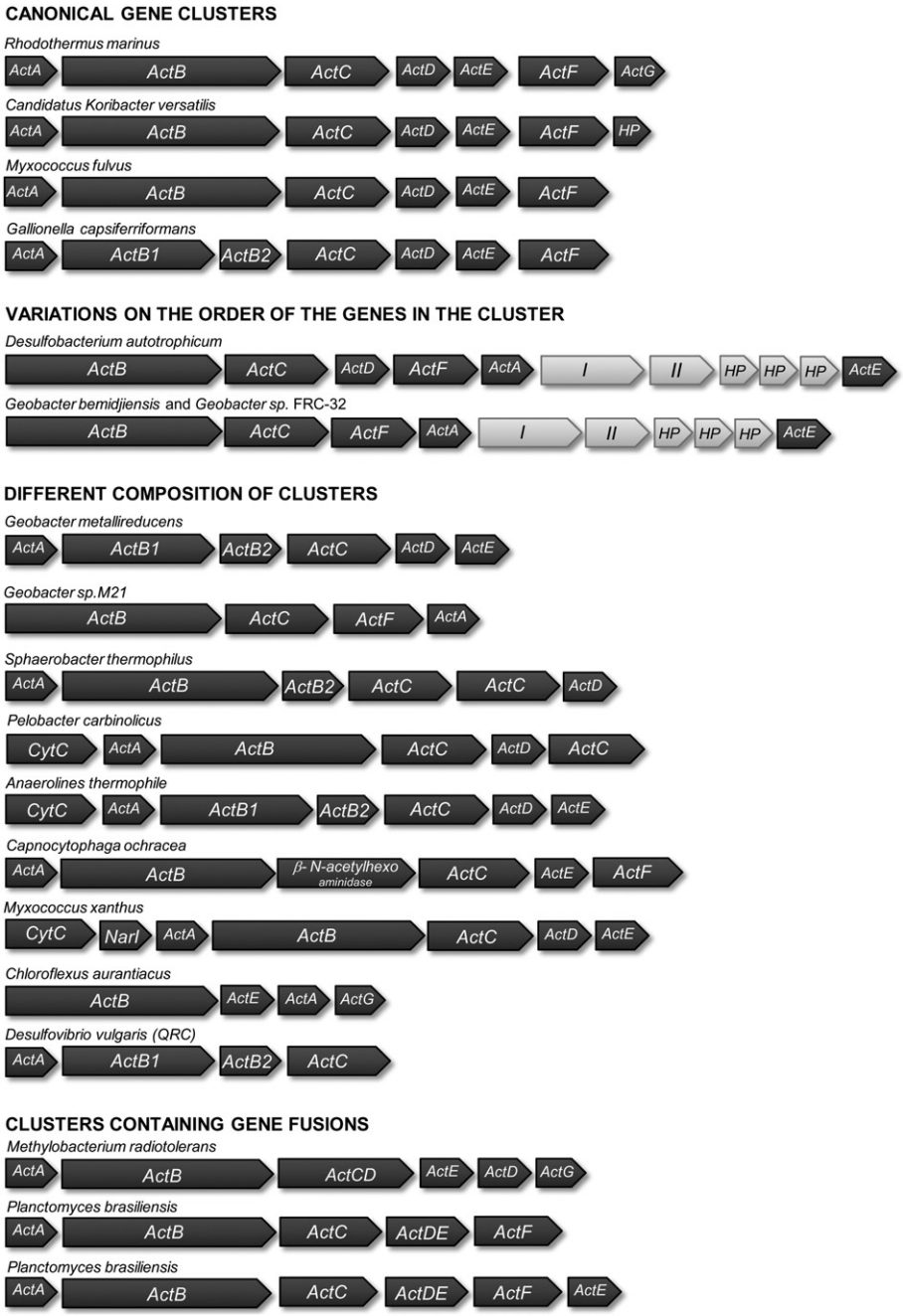

Fig. 1. Different organizations for the gene clusters coding for alternative complexes III. I and II represent the two subunits of the heme-copper oxygen reductase. HP means hypothetical protein.

organisms, all belonging to the domain of the Bacteria [2]. Genes coding for ACIII are mainly present in organisms in which the genes coding for a bc_1/b_6f complex are absent, but gene clusters coding for both complexes can also coexist in some genomes [1,7]. Furthermore, it has been reported previously that the gene clusters encoding ACIII may have three different compositions: *ActABCDEFG*, *ActABCDEF* or *ActAB1B2CDEF*, which in several cases are followed by gene clusters coding for heme-copper oxygen reductases (Fig. 1) [1,2].

2. Methods

Gene clusters coding for ACIII subunits were analyzed by Blast searches against all genomes deposited at the Kegg server (http://www.genome.jp/kegg/) [12–14], using the amino acid sequence of the B1 domain of ActB from *R. marinus* as query. All the sequences that showed an E value lower than one were kept for further inspection.

In order to exclude amino acid sequences belonging to the members of the CISM family, manual inspections for the presence of molybdenum cofactor binding motifs were performed based on the patterns deposited on Prosite server (www.prosite.expasy.org) as "Prokaryotic molybdopterin oxidoreductases signatures": Pattern 1 — [STAN]x Xx(x)C[STAG][GSTVMF]xCx[LIVMFYSW]x[LIVMA]xxx (x)[DENQKHT]; Pattern 2 — [STA]x[STAC][STAC]xx[STA]D[LIVMY] [LIVMY]LPx[STAC][STAC]xxE. Genomes of the organisms containing homologues of ActB or ActB1 were manually inspected in order to identify the gene clusters coding for ACIII and neighboring genes.

The type of heme-copper oxygen reductase encoded downstream of the ACIII gene cluster was identified using the database classifier available online (http://www.evocell.org/hco/search/blast/) [15].

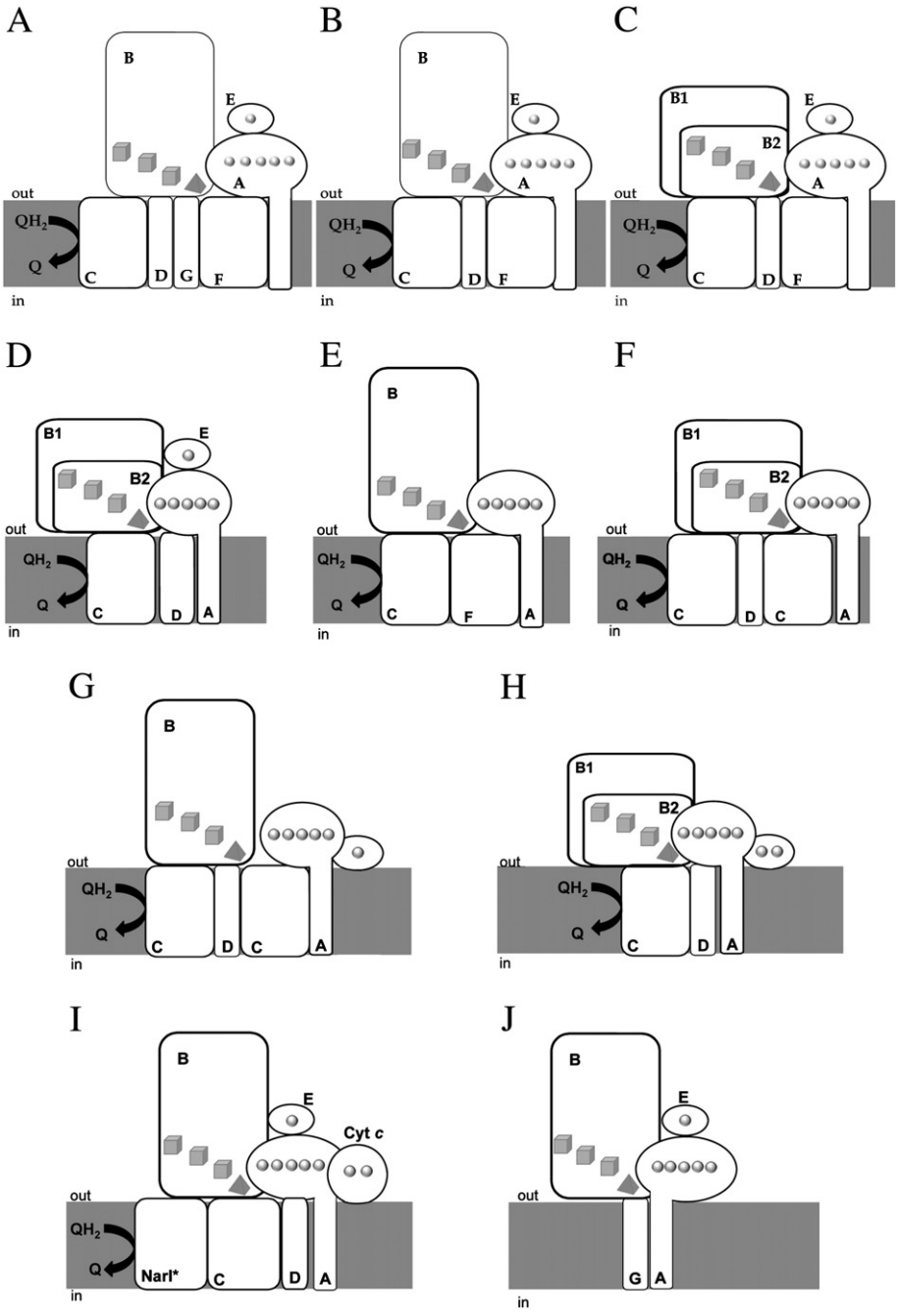

Fig. 2. Schematic representation of the subunit constitution of the different alternative complexes III encoded by the gene clusters: *ActABCDEFG* (A), *ActABCDEF* (B), *ActAB1B2CDEF* (C), *ActAB1B2CDE* (D), *ActBCFA* (E), *ActAB1B2CCD* (F), *ActCytcABCD* (G), ActCytc1AB1B2CDE (H), *ActCytc2NarIABCDE* (I) and *ActBEAG* (J). The gray spheres represent the C-type hemes and cubes and pyramids represent $[4Fe–4S]^{2+/1+}$ and $[3Fe–4S]^{1+/0}$ centers, respectively. The orientation of subunit ActB (or ActB1 and ActB2) in relation to the membrane is uncertain, as discussed recently [10]. For simplicity only the orientation towards the periplasm is represented.

3. Results

3.1. Search for ACIII clusters

In the last years the number of organisms with their genomes completely sequenced and deposited in data bases has increased exponentially. Aiming at getting all available ACIII gene clusters, searches in databases were preformed with Blast algorithm using as query the amino acid sequence of domain 1 of ActB from *R. marinus*. All the sequences retrieved were manually analyzed for the presence of the molybdopterin binding motifs (based on the available data from Prosite). From the 192 amino acid sequences obtained, 47 contained the conserved cysteine residues for molybdopterin binding included in the motif described as Pattern 1 (see Methods), while the motif designated as Pattern 2 was not detected. However, only six of those 47 sequences contained all amino acid residues constituent of Pattern 1, i.e. the full Pattern. Subsequently, neighboring genes were manually analyzed in order to assign the encoded complex as a member of the CISM family or as ACIII. We observed that 14 sequences out of the remaining 41 without the full Pattern 1, were encoded by genes composing ACIII gene clusters (either *ActB* or *ActB1*) while the rest of the sequences were encoded by genes included in typical gene clusters of the members of the CISM family (catalytic subunit, iron–sulfur protein and membrane protein). Unexpectedly, two of the six sequences that have the entire Pattern 1 were part of gene clusters having the same organization as those coding for ACIII (*Leptospira biflexa* and *Melioribacter roseus*).

It remains to be established if the complexes assigned as members of the CISM family are indeed molybdopterin containing enzymes, or if they are some intermediary version of the CISM or ACIII families. A similar question may be raised for complexes having the same subunit composition as ACIII, but which apparently have the sequence binding motifs for the molybdopterin. Only future biochemical characterization of these complexes may answer those questions.

3.2. Taxonomic distribution of ACIII clusters

We have previously identified (using all the available sequenced genomes at Kegg database by September 2009), 66 organisms containing gene clusters encoding ACIII [2]. The present reanalysis shows an increase in that number of almost 2.5 times. The presence of ACIII gene clusters is still confined to the domain of the Bacteria, with a high predominance in the Bacteroidetes phylum. ACIII encoding genes were now also identified in the Deinococcales class, from the Thermus–Deinococcales phylum. Interestingly and in contrast to the observation for ACIII gene clusters, the number of identified clusters encoding QRCs did not change and as before they appear to be limited to the Deltaproteobacteria class.

3.3. A new perspective on the gene composition of ACIII clusters

An unexpected diversity of ACIII gene clusters was found, in terms of the number of genes, their relative order and the type of proteins they code for (Figs. 1 and 2).

The presence of the six genes, organized as *ActABCDEF*, was thought to be the canonical composition of ACIII clusters and in fact it is the most common one (~40% occurrence) (Figs. 1 and 2). 18% of the clusters also contain *ActG*, as in the cases of *R. marinus* and *C. aurantiacus* (Figs. 1 and 2), while in 12%, a gene encoding a hypothetic protein was identified as a putative 7th gene (e.g. *Candidatus Koribacter versatilis* cluster, Fig. 1). This protein is different from ActG, but is also predicted to have only a single transmembrane helix. The cluster *ActAB1B2CDE*, which encodes the two domains of ActB as separated proteins, is the least frequent (~5%).

In the Deltaproteobacterium *Geobacter metallireducens*, *ActF* (gene coding for a membrane protein) is absent (*ActAB1B2CDE*) (Figs. 1 and 2D), while *ActE* (gene coding for the monohemic cytochrome) is not detected in *Geobacter* sp. M21, *Sphaerobacter thermophilus* and *Pelobacter carbinolicus*. In the case of *Geobacter* sp. M21, besides the absence of *ActE*, the gene cluster encoding ACIII is only composed of four genes, organized as *ActBCFA*, which is followed by a cluster encoding a type C heme copper oxygen reductase (Figs. 1 and 2E). In the gene cluster from *S. thermophilus* the domains of ActB are coded by separate genes and two copies of *ActC* are present (*ActAB1B2CCD*) (Figs. 1 and 2F). *ActE* may be substituted by a 45 kDa protein, having a monoheme cytochrome *c* domain at the C-terminus, whose coding gene ($cytc_a$) is located upstream of the ActA gene (*ActCytc$_a$ABCDC*) (Figs. 1 and 2G). This is the case of *P. carbinolicus*, which also has two copies of *ActC*. The green non sulfur bacterium *Anaerolinea thermophile*, lacks *ActF* and contains a gene ($cytc_b$) coding for a diheme cytochrome *c* with a predicted molecular mass of ~35 kDa, which follows the gene cluster coding for ACIII (*ActCytc$_b$AB1B2CDE*) (Figs. 1 and 2H). Upstream of one of the three gene clusters coding for ACIII in *Myxococcus xanthus*, two additional genes encoding another diheme cytochrome *c* ($cytc_c$) and a protein homologous to NarI were observed (Figs. 1 and 2I). NarI is a transmembrane diheme cytochrome *b* subunit responsible for the interaction with quinol in the nitrate reductase [8], which is similar to the second type of membrane proteins observed in the members of the CISM family. Interestingly, no *ActF* is present in this cluster. The three types of cytochrome c mentioned above, do not show any amino acid sequence similarity to those from ACIII, neither between themselves, nor to any other known family of c-type cytochromes. In *Capnocytophaga ochracea*, the cluster has an unexpected gene annotated as a beta-N-acetylhexosaminidase, between *ActB* and *ActC* (Fig. 1).

In *C. aurantiacus*, *Chloroflexus aggregans* and *Chloroflexus* sp. Y-400-fl, in addition to the gene cluster coding for ACIII, which, at least in the *C. aurantiacus* case, is expressed in photosynthetic conditions, a gene cluster formed by *ActBEAG* followed by another one coding for a type A2 heme-copper oxygen reductase was identified (Fig. 2J).

We also observed that the order of the genes within the cluster may vary. In *Desulfobacterium autotrophicum*, the order is *ActBCDFA*, while in two *Geobacter* species (*Geobacter bemidjiensis* and *Geobacter* sp. FRC-32) is *ActBCFA*. Interestingly, in the former example the gene coding for the ActE subunit is not part of the cluster, being observed downstream of the following gene cluster, which encodes a type C heme-copper oxygen reductase (Fig. 1).

Fusion of some of the genes may also occur (apart from the already mentioned ActB1 and ActB2 versus ActB). This is the case of *Methylobacterium radiotolerans*, whose genes coding for ActC and ActD are fused giving origin to a protein with twelve predicted transmembrane helices (ActAB(CD)EFG) (Fig. 1). The fusion *ActDE* was also found, as for example in *Planctomyces brasiliensis*, *Planctomyces limnophilus* and *Rhodopirellula baltica*, *ActABC(DE)F*. The product of this fusion should result in the attachment of the soluble monoheme cytochrome *c* to the membrane via the transmembrane helices. A membrane association was previously proposed for ActE from *R. marinus* to occur via a lipid moiety due to the presence of a lipobox motif at the N-terminus, immediately after the translocation signal peptide [10]. Accordingly, that sequence is not present in the product of the *ActDE* fusion. *Ignavibacterium album* and *M. roseus* have also an extra copy of *ActE* (*ActABC(DE)FE*), besides the fused *ActDE*. Interestingly, it was observed that clusters containing only one copy of the gene encoding the monoheme subunit, are followed by gene clusters encoding type A2 heme-copper oxygen reductases, whose subunit II has a C-type heme in the C-terminus. The reciprocal situation is observed, i.e. when two copies of the monoheme cytochrome are present, the subunit II of the heme-copper oxygen reductase does not contain the C-terminal extension corresponding to the cytochrome domain (Fig. 3).

4. Discussion

The diversity of ACIII subunit composition described here brings new perspectives in the discussion of the role of each subunit, namely those involved in quinone or quinol interaction and possible proton translocation.

The number and localization of quinone/quinol binding sites in ACIII are still unknown. One quinone binding site has been determined for the *R. marinus* enzyme by fluorescence quenching assays [7]; however, the presence of additional quinone/quinol binding sites cannot be ruled out. By analogy to the members of the CISM family, namely to polysulfide reductase (Psr) [9], the quinone/quinol binding sites were suggested to be present in ActC and/or ActF. The homology of these two subunits could indicate the presence of two quinone/quinol binding sites in ACIII. The structure of PsrC also hypothesizes the presence of proton channels, which could be involved in proton translocation. The possible existence of an ACIII-like complex without ActC and ActF questions the role of these subunits in quinone/quinol interaction. The *ActBEAG* gene cluster, present in *Chloroflexus* species, is an example in which genes coding for the large membrane subunits, ActC and ActF are not present. If quinol: electron acceptor oxidoreductase activity is observed for such complex and knowing that no other large membrane protein typical of ACIII is present in the genome, the constitution of the cluster strongly suggests that quinols may interact at ActA, the multiheme cytochrome, which is predicted to contain a transmembrane helix. Such a situation would be similar to that of the multiheme cytochrome NrfH subunit from the nitrite reductase NrfAH [16].

If ActA is the local of quinone/quinol interaction, then ActC and ActF would be exclusively involved in proton translocation and in such case one coupling site could be present in each subunit. This situation would resemble that of respiratory complex I and group 4

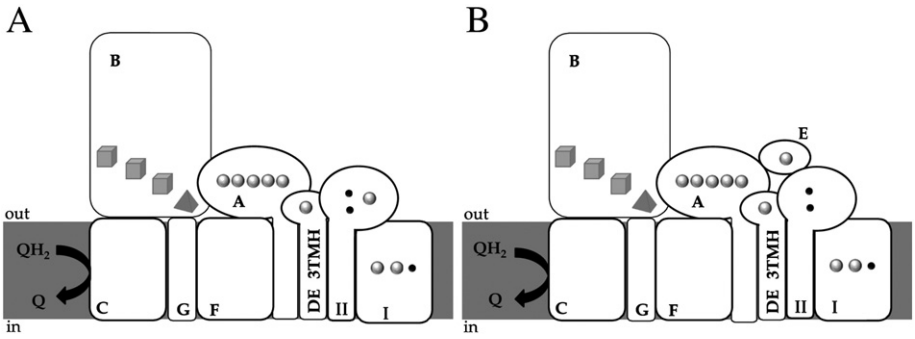

Fig. 3. Schematic representations of two alternative complex III in which ActD and ActE are fused. In (A) ACIII only contains one copy of ActE and a C-type heme in the subunit II of the heme-copper oxygen reductase. In (B) the ACIII is composed of two copies of ActE and no C-type heme is present in subunit II. I and II correspond to the two subunits of a heme-copper oxygen reductase. Gray spheres — C-type hemes; small black spheres — copper ions; cubes and pyramids — $[4Fe–4S]^{2+/1+}$ and $[3Fe–4S]^{1+/0}$ centers, respectively.

membrane bound [NiFe] hydrogenases. Complex I contains three homologous subunits thought to be responsible for proton translocation, while in those [NiFe] hydrogenases only one of such subunits is present [17,18]. The number of "repeated" subunits was suggested to correlate with an increase in energy conservation efficiency [18,19].

The presence of two monoheme cytochromes seems to be necessary to allow electron transfer between ACIII and a heme-copper oxygen reductase, irrespective of which complex these cytochromes are part of (Fig. 3). This observation together with the fact that in many cases the gene cluster coding for ACIII is followed by one encoding a heme-copper oxygen reductase supports the notion that the functional and structural association between the two encoded complexes, observed for the *R. marinus* complexes, [7] may be present in other organisms.

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