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Invited review

Bioenergetics at extreme temperature: *Thermus thermophilus* ba_3- and caa_3-type cytochrome *c* oxidases☆

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ABSTRACT

Seven years into the completion of the genome sequencing projects of the thermophilic bacterium *Thermus thermophilus* strains HB8 and HB27, many questions remain on its bioenergetic mechanisms. A key fact that is occasionally overlooked is that oxygen has a very limited solubility in water at high temperatures. The HB8 strain is a facultative anaerobe whereas its relative HB27 is strictly aerobic. This has been attributed to the absence of nitrate respiration genes from the HB27 genome that are carried on a mobilizable but highly-unstable plasmid. In *T. thermophilus*, the nitrate respiration complements the primary aerobic respiration. It is widely known that many organisms encode multiple biochemically-redundant components of the respiratory complexes. In this minireview, the presence of the two cytochrome *c* oxidases (C*c*O) in *T. thermophilus*, the ba_3- and caa_3-types, is outlined along with functional considerations. We argue for the distinct evolutionary histories of these two C*c*O including their respective genetic and molecular organizations, with the caa_3-oxidase subunits having been initially 'fused'. Coupled with sequence analysis, the ba_3-oxidase crystal structure has provided evolutionary and functional information; for example, its subunit I is more closely related to archaeal sequences than bacterial and the substrate–enzyme interaction is hydrophobic as the elevated growth temperature weakens the electrostatic interactions common in mesophiles. Discussion on the role of cofactors in intra- and intermolecular electron transfer and proton pumping mechanism is also included. This article is part of a Special Issue entitled: Respiratory Oxidases.

1. Introduction

Thermus thermophilus is a Gram-negative and an extremely-thermophilic bacterium. The genomes of two very closely-related strains, HB8 and HB27, have been sequenced (NCBI GPID: 13202 and 10617) [1]. HB8 is a facultative anaerobe, with a nitrate reductase gene cluster (GenBank ID: Y10124) being responsible for growth on nitrate under anaerobic conditions [2]. In the sequenced genome, the genes were not detected and it has been suggested that the conjugative plasmid carrying the genes is not stable [3]. The strictly-aerobic HB27 strain can be engineered to grow on nitrate anaerobically by the gene transfer through conjugation [4]. This has been exploited to develop the *T. thermophilus* HB27::*nar* strain for anoxia-inducible protein expression system [5].

However, sequence analysis alone is insufficient to provide an explanation of its overall bioenergetic mechanisms, including the existence of two terminal oxidases. At 70 °C, the solubility of O_2 in water is only ~60% compared to at 25 °C [6]. *T. thermophilus* has been revealed to encode two cytochrome *c* oxidases (C*c*O). The low O_2-affinity caa_3-oxidase is constitutively expressed whereas the ba_3-oxidase possesses high affinity and is coexpressed with the caa_3-oxidase under low oxygen tension.

The intriguing facts surrounding the C*c*O of *T. thermophilus* are not limited to their structure–function relationships. Apart from being able to reduce nitric oxide (NO) [7], their general features including the hydrophobic substrate–enzyme interaction and subunit compositions do not conform to the canonical features of bacterial and mitochondrial complexes. Within three decades, old data have been revised and new data are being constantly obtained. The ba_3-oxidase originally described as comprising only one subunit [8] was later found to contain three subunits [9], the c_1-cytochrome of caa_3-oxidase [10–12] is actually a covalently-bound cytochrome *c* [13,14] and the two-polypeptide, three-subunit caa_3-oxidase [15] is now known as a three-polypeptide, four-subunit complex (T. Soulimane, unpublished data).

2. Terminal oxidases of *T. thermophilus*

The transfer of electrons to the terminal acceptor O_2 is catalyzed by a group of proteins in the heme-copper oxidases (HCO) superfamily.

Abbreviations: HEF, hydroxyethylfarnesyl; HEGG, hydroxyethylgeranylgeranyl; SD, Shine–Dalgarno sequence; TMH, transmembrane helices

☆ This article is part of a Special Issue entitled: Respiratory Oxidases.

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This superfamily does not encompass all terminal oxidases, as evidenced by the *bd*-type quinol oxidases with no copper redox center [16,17]. It is divided into two distinct families — the C*c*O and quinol oxidase, with the latter lacking the mixed-valence homodinuclear copper center Cu_A [18]. The sequence motif of the Cu_A binding region (His-Xaa$_{35}$-Cys-Xaa-Glu-Xaa-Cys-Xaa$_3$-His-Xaa$_2$-Met) allows for the classification of an oxidase as either a C*c*O or quinol oxidase [19]. Surprisingly, the distribution of these HCO does not correlate with growth conditions [20] and the heme type is not related to the phylogeny of the organism, the electron donor or sequence similarities between the enzymes [18]. Thus, HCO are best classified according to their proton pumping-related sequence conservation where three types (A, B and C) have been proposed [21]. The A-type is split into the A1 and A2 subfamilies; both are similar in that the oxidases have the D- and K-pathways for proton pumping except for sequence conservation. For instance, the *T. thermophilus* caa_3-oxidase is of the A2-type because the GluI-278^P, [1] residue at the hydrophilic end of the D-pathway, which is otherwise highly conserved in members of the A1, is absent. The GluI-278^P residue may be functionally replaced by the conserved Tyr-Ser residues in the caa_3-oxidase (YS motif; Section 2.2.1) [20]. Accordingly, the ba_3-type oxidase belongs to the B-type HCO as it does not have the classical D- and K-pathways for proton pumping but instead has a pathway analogous to the K-pathway (K-analogue) [22]. Finally, the C-type consists of cbb_3-oxidases which do not show significant sequence similarities among themselves or to the other types. A classification tool and database for HCO (http://www.evocell.org/hco) has been developed recently [23]. This tool does not include the splitting of the B-type oxidases into several other HCO types as proposed by Hemp and Gennis [24]. These new types are designated D, E, F, G and H, of which the D–F types are found exclusively within the *Sulfolobales* class of the *Crenarchaeota* phylum. While the number of prokaryotic genome sequences is increasing, detailed biochemical and biophysical studies into these unique HCO lags far behind those from the 'model' organisms.

2.1. ba_3-type C*c*O

Unlike the caa_3-oxidase subunits, the ba_3-type C*c*O subunits of both *T. thermophilus* strains are identical (Table 1). They are encoded by the loci TTHA1133–1135 on the chromosome of HB8 and TTC0768–0770 in HB27. This contiguous locus encodes all the three subunits — I, II and IIa.

2.1.1. Subunit I

The subunit I contains 13 transmembrane helices (TMH) and shows only a distant similarity to the other members of the HCO superfamily, indicating a possible early gene duplication event prior to the divergence of *Archaea* and *Bacteria*. Although Pereira *et al.* [18] suggested that the terminal oxidases in *Archaea* were acquired from *Bacteria*, more recent analyses indicate that B-type oxygen reductases originated from *Crenarhaeota* [25]. The presence of 13 TMH differentiates the *T. thermophilus* C*c*O from most of the members of HCO, as the canonical number of TMH is 12. The shortened loops, which are found connecting the TMH, have been generally postulated to play a role in enhancing the thermostability of proteins by decreasing the entropy of unfolding [26,27]. The crystal structure shows that the first nine residues (MAVRASEIS) predicted from the gene sequence are absent from the subunit I sequence (UniProt ID: Q5SJ79) [9]. Although it is likely the protein undergoes cleavage, prediction of cleavage sites using PeptideCutter (http://www.expasy.ch/tools/peptidecutter) indicates that there is no recognition site for a protease at the C-terminus of the last serine residue. Further, N-terminal sequencing of the crystallized protein shows that the residues are not retained. However, the recombinant ba_3-oxidase retains the residues SEIS at the N-terminus of subunit I (PDB ID: 1XME) [28,29]. As the only difference between the native and recombinant ba_3-oxidase is the presence of a hexahistidine tag between the Met and the first Ala, this could be the reason for the retention of the SEIS residues. Interestingly, the structures of recombinant ba_3-oxidase at 1.8 Å resolution (PDB ID: 3S8F and PDB ID: 3S8G) shows the retention of only the last Ser of the MAVRASEIS-like sequence [31].

Table 1
Genetic loci encoding cytochrome *c* oxidases in the two sequenced *Thermus thermophilus strains*. The caa_3-oxidase subunits of HB8 and HB27 strains differ to an extent while ba_3-oxidase subunits are completely identical.

Subunits	Genetic loci		Differing residues between HB8 and HB27
	HB8	HB27	
caa_3-oxidase			
I/III	TTHA0312	TTC1671	V175I
IIc	TTHA0311	TTC1672	F96L, M117L, A170S, A226Q, W269L, V318A
ba_3-oxidase			
I	TTHA1135	TTC0770	–
II	TTHA1134	TTC0769	–
IIa	TTHA1133	TTC0768	–

Three of the four redox centers – low-spin heme *b*, high-spin heme a_{s3} and a single Cu ion (Cu_B) – are located within the subunit I; the iron in heme a_{s3} together with Cu_B form a heterodinuclear center [9]. Based on the crystal structure, His72 and His386 act as the axial ligands of heme *b* whereas His233, His282, His283, His384 and Tyr237 form the coordination sphere of the heterodinuclear center. The metal centers are buried within the protein to avoid the release of reactive oxygen species during catalysis [18].

A formyl group at C8 and a hydrophobic hydroxyethylgeranylgeranyl (HEGG) moiety are present on the heme a_{s3}. This is in contrast with the typical heme *a* structure where hydroxyethylfarnesyl (HEF) is present on C2, as exemplified by the *Paracoccus denitrificans* aa_3-type C*c*O. HEGG is straight and reaches the cytoplasmic side without interfering with the proton pathways, and the increased hydrophobicity of HEGG compared to HEF might stabilize the heme at high growth temperatures [32,33]. This hypothesis fits with the shared phylogeny between the *T. thermophilus* ba_3-oxidase and the investigated archaeal C*c*O. Nevertheless, there are studies indicating otherwise — the $b(o/a)_3$-type[2] C*c*O of the thermophilic *Geobacillus stearothermophilus* (basonym *Bacillus stearothermophilus*) is related to archaea (B-family) but no heme A_s could be detected [34]; the C*c*O of the mesophilic *Corynebacterium glutamicum* is of aa_3-type with mass spectrometry analysis indicating presence of heme A_s [35]. Studies utilizing the aa_3 C*c*O-related *Escherichia coli* bo_3-type quinol oxidase has also shown that the long hydrocarbon side chain is crucial for the function of the high-spin heme iron center, but not for the low-spin one [36,37]. The structural analysis of the *Pseudomonas stutzeri* cbb_3-oxidase shows the absence of HEF in the native enzyme [38], raising questions about the requirements for the side chain in distant but related C*c*O types. Notably, the electron transfer mechanism in cbb_3-oxidase occurs without a Cu_A site but instead through a series of heme moieties before reaching the dinuclear center.

2.1.2. Subunit II

Cytochrome c_{552}, which acts as the electron donor, interacts with the subunit II that forms a single TMH as well as being the only subunit with a polar (periplasmic) domain of β-barrels. The primary

[1] Unless otherwise stated, all numberings are based on the *T. thermophilus* sequences. Superscripts denote the species-specific numberings; P, *Paracoccus denitrificans*; R, *Rhodobacter sphaeroides*; B, bovine heart.

[2] The high-spin heme is mainly heme O and partly heme A.

site of electron entry into the whole complex is through the homodinuclear copper redox center, Cu_A. The first Cu atom (CU1) is coordinated by the Cys149, Cys153, His157 and Gln151 while the Cys149, Cys153, His114 and Met160 coordinate the second atom (CU2). Theoretical calculations postulated that the presence of two Cu atoms in cytochrome oxidases instead of one promotes the electron transfer from cytochrome *c* to the Cu_A due to a highly-delocalised electron hole and significantly-reduced electron transfer reorganization energy so as to ensure rapid electron transfer [39–43]. The bridging ligands between the Cu atoms are the two thiolate groups of the cysteines. In *P. denitrificans*, TrpII-121^P is the only entry point for electrons from the electron donor cytochrome *c* to redox center Cu_A and the interaction between the substrate and the binding site on the complex is electrostatic [44,45]; the Trp residue is most likely strictly-required due to steric reasons [46]. It has also been shown that two conserved arginine residues Arg481^R and Arg482^R play a key role in the intermolecular electron transfer in the *Rhodobacter sphaeroides* C*c*O [47]; substrate binding is also electrostatic [44]. Contrarily, the electron transfer is mediated by the Ala87 and Phe88 in *T. thermophilus* ba_3-oxidase [48], with a hydrophobic substrate–enzyme interaction [49]. This could be another adaptation by the thermophilic bacterium for growth as electrostatic interactions are weakened at elevated temperatures.

Pereira *et al.* noted that the subunit II of the HCO superfamily displays a family-specific loop of probable functional and, hence, evolutionary importance [18]. The loops of representative members are grouped into 'loop I' and 'loop II' types based on their actual position within the subunit II (Fig. 1). Found exclusively within the type A1 aa_3-oxidases and not in the other subtypes (viz. A1 caa_3- and quinol oxidases) are the loop II located between the two conserved aromatic residues (TrpII-121^P and 123^P) and the first Cu_A ligand HisII-181^P; neither of the *T. thermophilus* oxidases has such a loop, though they both have the conserved aromatic residues (PheII-88 and TyrII-90 in the ba_3-oxidase, and PheII-109 and TrpII-111 in the caa_3-oxidase). In the cyanobacterial A2 oxidases and type B oxidases, loop I is present directly between the transmembrane helix and the cupredoxin-like domain. The comparative structural positions of both loops are indicated in Fig. 1. However, the loop in the ba_3-oxidase is shorter than that in the cyanobacterial enzyme, perhaps as an adaptation to its elevated growth temperature. The loops connecting the subunit I TMH are also characteristically shortened. The absence of loop II in the other C*c*O and quinol oxidases has been argued to be a consequence of the lack of interaction between a soluble cytochrome *c* and the enzymes [18]. It may at least be due to the absence of electrostatic interactions (our interpretation). The authors' reasoning does not fully explain the absence of loop II in A2 aa_3 and B-type C*c*O, unless the loop I in these families is a structural and/or functional replacement of loop II. This might also be linked to the nature of substrate–enzyme interaction. The interaction between the *T. thermophilus* cytochrome c_{552} and ba_3-oxidase is not ionic strength-dependent but largely hydrophobic whereas the cyanobacterial cytochrome c_6-aa_3 oxidase interaction is greatly dependent on the ionic strength. However, there are conflicting reports on the actual effect of increased ionic strength in cyanobacterial substrate–enzyme interactions, and it has been suggested to be due to inherent differences between unicellular and filamentous species [50,51].

2.1.3. Subunit IIa

Prior to the determination of *T. thermophilus* ba_3-oxidase crystal structure, it was first described as a single-subunit enzyme [8] and

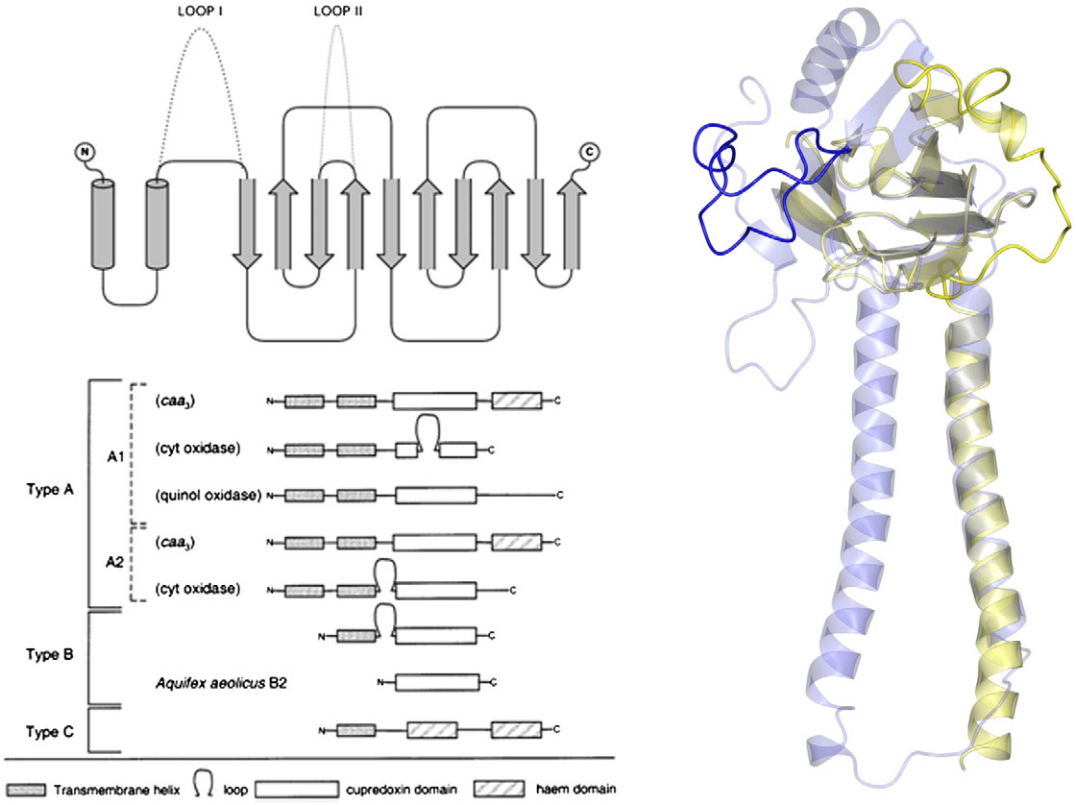

Fig. 1. Representation of loops within the subunit II of *Paracoccus denitrificans* aa_3- (PDB ID: 1AR1) and *Thermus thermophilus* ba_3-oxidases (PDB ID: 1EHK). Top left: The subunit consists of two helices and ten β-strands forming the cupredoxin fold, where the position of loops I and II is indicated. Redrawn from [18] using TopDraw [128] implemented within the CCP4 suite. Bottom: Presence of loops in the different oxidase families. The *T. thermophilus* caa_3- and ba_3-oxidases are of the A2 and B families, respectively, while the *Aquifex aeolicus* subunit II putatively consists of only the cupredoxin domain [129]. Adapted from [18]. Right: Superposition of subunit II of ba_3-oxidase (green) onto the aa_3-oxidase (blue). The loop positions are shown in the darker colors while the main chains are transparent. Figure created with CCP4mg [130].

subsequently shown to comprise two subunits [52]. The third subunit (IIa) was only identified later [9]. It is a short polypeptide of 34 residues and forms a TMH [53]. By searching for homologous sequences using Basic Local Alignment Search Tool (BLAST), the subunit IIa *per se* seems to exist only in *T. thermophilus*, *Thermus aquaticus* and *Meiothermus silvanus*, with all of them predicted to be with the same polarity across the membrane (Fig. 2); it is also 35% identical to the subunit IV of *Natronomonas pharaonis* ba_3-oxidase CcO [53].

Furthermore, the helical region of subunit IIa superimposes with the first TMH of subunit II of all the structurally-known CcO (bovine, *R. sphaeroides* and *P. denitrificans*) but with an opposite polarity (Fig. 3). The canonical number of TMH of the subunit II is two. Hence, it could be the 'missing' helix of *Thermus* subunit II and functionally/structurally complement the latter. Indeed, cloning experiments of the ba_3-oxidase in which only the subunits I and II were cloned and expressed did not provide overexpression. Only after the addition of subunit IIa to the overexpression vector could the recombinant ba_3-oxidase be obtained, signifying its subunit II-stabilizing function [28]. The peculiarities of ba_3-oxidase are not only limited to the hydrophobic interaction with its electron donor, the lower proton pumping efficiency and the presence of HEGG; there are also no other metal ions such as Mg^{2+} or Ca^{2+} in both the native [based on the inductively-coupled plasma-atomic emission spectroscopy (ICP-AES) and electron density map] [54] and recombinant ba_3-oxidase structures, in contrast to the bovine heart, *R. sphaeroides*, *P. denitrificans* CcO and *P. stutzeri* cbb_3-oxidase.

Soulimane *et al.* have previously questioned the role of a region within the gene encoding subunit IIa that lies between the promoter and the coding Met residue of the protein as this region also has a codon for a Met (Fig. 4) [53]. Given that the distance between the promoter and the Shine–Dalgarno (SD) sequence is ~50 nucleotides (the optimal distance being 5–20 nucleotides) and between SD and the initiation codon of the coding Met is ~7 nucleotides (optimal is 4–9 nucleotides), we now believe that the SD is within this region. An SD sequence between two initiation codons promotes the gene expression from the codon downstream the SD as long as it is the better initiation site. For this, the distance between the downstream codon and SD should be optimal and strong enough to bind to the ribosome, though an exact complementarity between the mRNA and the 16S rRNA is not a prerequisite for translation [55]. Moreover, the presence of SD itself has been shown not to be required for translation as not all gene sequences contain SD [56,57].

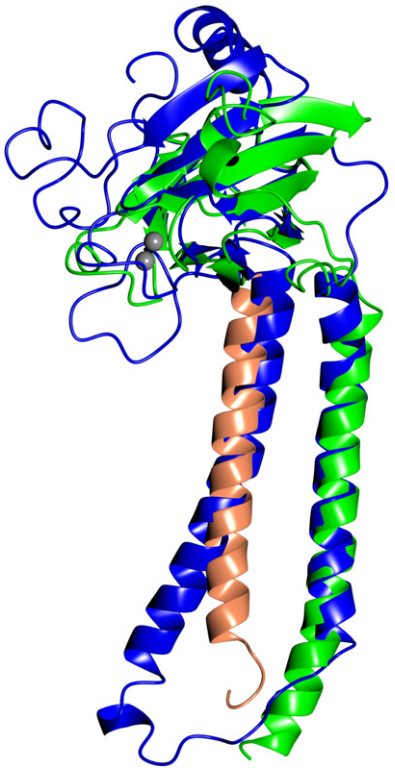

Fig. 3. Ribbon representations of *Thermus thermophilus* ba_3-oxidase subunits II (green) and IIa (coral) superposed with the *Paracoccus denitrificans* aa_3-oxidase subunit II (blue). The accession IDs are PDB ID: 1EHK and PDB ID: 1AR1, respectively. The *Thermus* subunit IIa structurally and functionally complements the single TMH of the subunit II. The two copper atoms in the homodinuclear Cu_A center are shown as gray spheres. Figure created with CCP4mg [130].

2.2. caa_3-type CcO

The four-subunit caa_3-type CcO is unusual in that the subunits are encoded as fused polypeptides — the subunit I with III (I/III) and the subunit II with the soluble electron donor, cytochrome c_{549} (II/c) [14,15] (Section 2.2.1). The genes are organized as part of a classical operon by the loci TTHA0311-0312 and TTC1671-1672 in HB8 and HB27, respectively (Table 1). Recently, an additional, third polypeptide (subunit IV) was found to co-purify and co-crystallize with caa_3 (T. Soulimane; unpublished results). As determined by SDS-PAGE, N-terminal sequencing and mass spectrometry, this 2-TMH, 7-kDa subunit is present in the purified caa_3-oxidase and single crystals grown in sitting drops; it shows a significant similarity exclusively to proteins encoded by members of the *Deinococcus–Thermus* phylum as discussed later (Section 2.2.2). Protein sequence analyses show that the subunits differ between the HB8 and HB27 strains; the Val175 of the subunit I/III in HB27 has been replaced with Ile (with respect to HB8) while the subunit II/c exhibit a six-residue difference (Table 1). To our best knowledge, no study has been performed to investigate the effects of these differences on the enzyme properties.

2.2.1. Subunits I/III and II/c

The subunit I/III contains the redox center heme *a* (low spin) and heme a_3 (high spin) and the Cu_B; O_2 reduction occurs at the a_3-Cu_B dinuclear center. The homodinuclear Cu center Cu_A is located within the subunit II/c, which also contains the covalently-linked heme *c* electron donor. No exact function has been assigned to the region encoding the subunit III. Topologically, there are 19 predicted TMH, of which 12 are based on the subunit I-like sequence [15]. The subunit II/c has two TMH and both the N- and C-termini are located on the periplasmic side, as expected [14]. As with the ba_3-oxidase, the *a*-type hemes of the caa_3-oxidase contain the HEGG side chain [33].

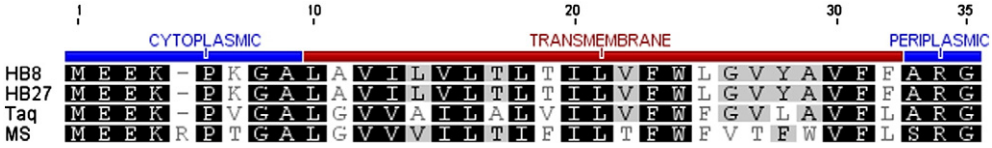

Fig. 2. Sequence alignment of *Thermus thermophilus* HB8 (HB8) ba_3-oxidase subunit IIa with other similar sequences in *T. thermophilus* HB27 (HB27), *T. aquaticus* (Taq) and *Meiothermus silvanus* (MS) using ClustalW. The topology of the subunit is indicated above the HB8 sequence based on the crystal structure of ba_3-oxidase. NCBI RefSeq ID: YP144399, YP004741, ZP03497340 and ZP04034717, respectively. Alignment created using ClustalX 2 [131] and modified with GeneDoc and GIMP.

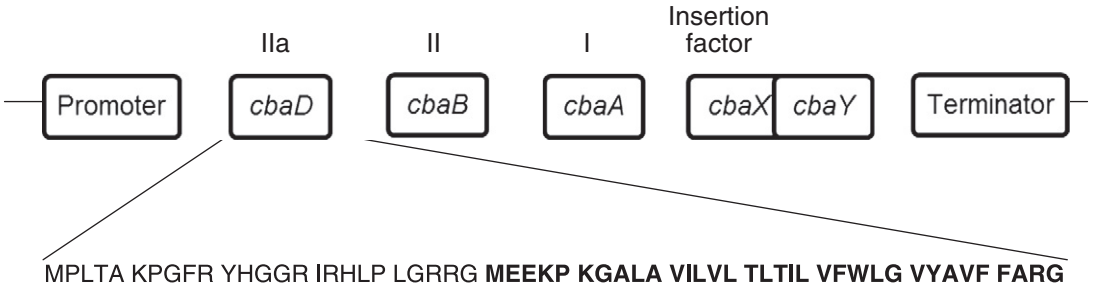

Fig. 4. Organization of the ba_3-oxidase operon. The sequence of *cbaD* is shown as enlarged with two possible Met residues, of which only the second one is translated into the subunit IIa (bold). The products of the other genes are indicated above the genes. The ORF of *cbaY* overlaps with *cbaX*. The figure is not drawn to scale with respect to the gene size and intergenic space.

The identity of the electron donor to the caa_3-oxidase has been a subject of controversy [58,59]. Recently, however, using a recombinant soluble cytochrome *c* domain of caa_3-oxidase, it has been determined that electrons can be received directly from the hydrophilic cytochrome *c* of *bc* (c_{bc}, or $c_{549/554}$, named after its spectroscopically-split α band). This is a largely non-ionic strength dependent process involving hydrophobic interactions [60], and is similar to the cytochrome c_{552}–ba_3–oxidase interaction (see Section 2.1). However, preliminary data from our laboratory shows the absence of an efficient direct interaction between the c_{bc} and the whole-complex caa_3-oxidase (T. Soulimane and A. Giuffrè, unpublished results); in addition, the caa_3-oxidase can also receive electrons from the cytochrome c_{552} [60]. While physiological studies are technically more challenging and sometimes unfeasible, it is doubtful that these *in vitro* studies reflect the actual electron transfer dynamics at low oxygen tension. For instance, fast and efficient electron transfer between the c_{bc} complex and caa_3-oxidase would exclude a necessity for the cytochrome c_{552} involvement. A complex interplay of rates, equilibrium constants, O_2 affinities, and protein expression dynamics exists that will then determine the proportion of electrons transferred directly to the caa_3-oxidase and through the cytochrome c_{552} to both the ba_3- and caa_3-oxidases.

In the A2-type *T. thermophilus* caa_3-oxidase, the Glu278^P is replaced by a highly-conserved Tyr-Ser pair, residues 248 and 249 (Fig. 5). This pair of residues probably act in a physicochemically similar way to the GluI-278^P, which is conserved in the A1-type. The thermohalophilic *Rhodothermus marinus* caa_3-oxidase belongs to the A2 group whereas the *Bacillus subtilis* caa_3-oxidase is of A1 type. As the YS motif is found in the evolutionarily deepest-branching organisms (based on 16S rRNA phylogeny) whereas the GluI-278^P-containing oxidases can only be found within purple bacteria, Gram-positive bacteria and mitochondria, the former oxidases may be ancestral to the latter [61].

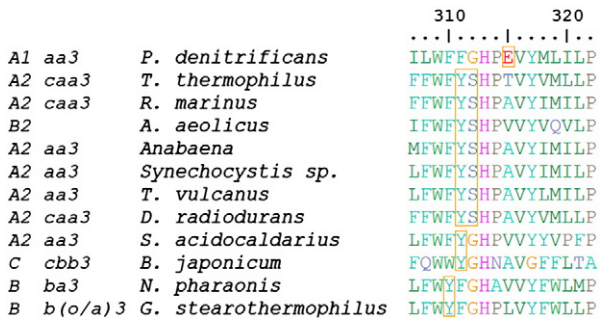

Fig. 5. Multiple sequence alignment of oxidases with the YS-motif and Y-only sequence. The *Paracoccus denitrificans* sequence shows the conservation of the Glu278^P involved in D-pathway whereas it is replaced by either Tyr and Ser or Tyr only in the other sequences. Accession numbers: *P. denitrificans* (UniProt ID: P08305), *Thermus thermophilus* (UniProt ID: P98005), *Rhodothermus marinus* (UniProt ID: CAC08532), *Aquifex aeolicus* (UniProt ID: O67935), *Anabaena* sp. (UniProt ID: CAB10935), *Synechocystis* sp. (UniProt ID: Q06473), *Thermosynechococcus vulcanus* (UniProt ID: P50676), *Deinococcus radiodurans* (UniProt ID: Q9RR77), *Sulfolobus acidocaldarius* (UniProt ID: P98004), *Bradyrhizobium japonicum* (UniProt ID: Q03073), *Natronomonas pharaonis* (UniProt ID: CAA71525), and *Geobacillus stearothermophilus* (UniProt ID: O82837). Alignment created using ClustalX 2 [131] and modified with GeneDoc.

2.2.2. Subunit IV

Evidently, the subunit IV of the aa_3- and caa_3-oxidases are not only located outside the main oxidase coding region but are also not required for their functionality [62,63]; for quinol oxidases, the subunit IV plays an indispensable role in the complex biosynthesis/activity [64–67]. The subunit IV of *P. denitrificans* (RefSeq ID: YP914242) is found on the chromosome 1 while the subunits I (RefSeq ID: YP915727), II (RefSeq ID: YP918081) and III (RefSeq ID: YP918077) are on the chromosomes 1, 2 and 2 respectively; these locations are in contrast to the results published ten years prior to the genome sequencing [68]. Similarly, in *R. sphaeroides*, the subunits I (RefSeq ID: YP351928), II (RefSeq ID: YP351875), III (RefSeq ID: YP351879) and IV (RefSeq ID: YP353819) are all on the chromosome 1 but not contiguously.[3]

Perhaps due to such a relaxed necessity, the subunit IV from the structurally-known oxidases does not display any sequence similarity to distantly-related organisms. For example, the *T. thermophilus* caa_3-oxidase subunit IV shows similarity only to sequences within the *Thermaceae* family; no sequence motifs have been identified (T. Soulimane, unpublished results). Since the subunit IV is not required for functional reasons, the question of why it is retained in diverse organisms arises; the *Deinococcus–Thermus* phylum is phylogenetically ancient whereas *Alphaproteobacteria*, of which *P. denitrificans* and *R. sphaeroides* are members, is as recent as to be the closest extant relative of mitochondria [69]. Interestingly, there are two TMH predicted in the *T. thermophilus* subunit IV while there is only one each in the bacterial oxidases; one of the helices could have been deleted over the course of the oxidase evolution. The presence of subunit IV instead of its complete loss could also be possibly related to the fact that their bovine counterpart has 13 subunits, of which 9 are supernumerary, and that these may be needed to reduce reactive oxygen species production and for expression regulation [70,71].

2.2.3. Heme *a* insertion factor

The uniqueness of the sequence similarities of the ba_3-oxidase subunit IIa and the caa_3-oxidase subunit IV (Sections 2.1.3 and 2.2.2) further extends to the heme *a* insertion factors for both oxidases. Sequence analyses of the genes within the putative caa_3-oxidase operon indicate the presence of fused polypeptides encoding the heme *a* insertion factor and the constituent subunits of the oxidase. The

[3] The *R. sphaeroides* structure was obtained from the strain JS100 but its genome sequence is not available, so the sequences from the type strain 2.4.1 are used here instead while Ref. [62] was based on strain 1222.

insertion of the two heme *a* moieties into the caa_3-oxidase apoprotein requires the action of an insertion factor, encoded by TTHA0310 (CtaA/B) in *T. thermophilus* HB8. The residues 12–294 of CtaA/B form the heme A synthase domain (CtaA) while the residues 340–598 form the heme O synthase (CtaB) domain. Heme A is synthesized through a three-step process; the *B. subtilis* CtaB is responsible for the farnesylation of heme B to heme O which is then acted upon by the monooxygenase/dehydrogenase CtaA in a two-step reaction [72,73].

A PSI-BLAST search for sequences homologous to the fused *T. thermophilus* CtaA/B limited to a length similar to that of the CtaA/B revealed the presence of such a sequence mostly only in the phylogenetically-ancient lineages of *Deinococcus–Thermus* and *Chloroflexi* (Table 2). Interestingly, *Bacteriovorax marinus* is a Gram-negative predatory bacterium that preys on other Gram-negative bacteria [74] and, consequently, the fused CtaA/B gene could have been horizontally transferred.

A similar situation exists for the subunit I/III, where the C*c*O subunit can only be found in the *Deinococcus–Thermus* and *Crenarchaeota* phyla (Table 3). Compared to the relatively more widespread presence of 'fused' CtaA/B, the 'fused' subunit I/III is only limited to the *Deinococcus–Thermus* phylum. Unexpectedly, the C*c*O of *Aeropyrum pernix* is of aa_3-type [75]. Possibly due to the architecture of the A1 and A2 caa_3-oxidases themselves, the subunits II and cytochrome *c* are encoded as fused polypeptides in a wide-range of organisms. It is found in both as phylogenetically-ancient lineages as the *Deinococcus–Thermus* and as phylogenetically-recent lineages as the *Proteobacteria*.

We, therefore, propose that at the beginning of aerobic respiration, the caa_3-oxidase subunits and the insertion factor were encoded as 'fused' polypeptides and were split more recently. The reason for the split is more likely to be due to an absence of any selection pressure to maintain them as fused polypeptides rather than because of evolutionary pressure to split them. Theoretically, a protein complex encoded by a single gene would be more efficient in terms of transcription, translation, complex assembly and function compared to two separately-encoded genes.

Furthermore, neither the structural subunits of the caa_3-oxidase nor the CtaA/B has significant sequence similarities to the ba_3-oxidase subunits and its heme *a* insertion factor CbaX [76], suggesting divergent evolutionary histories of the two oxidases. The CbaX, which only exists in the *Thermaceae* family, does not have any identifiable conserved domain (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) with no significant matches against *B. subtilis* in contrast to the CtaA/B. It is also considerably smaller than the CtaA/B (17 kDa vs. 66 kDa), raising questions on the differences between the synthesis and insertion of heme *a* moieties into the ba_3- and caa_3-oxidases, including the presence of two heme *a* moieties in the latter. The details of C*c*O biogenesis have been recently reviewed [77].

2.2.4. Loops

Transmembrane helices are connected to each other by loops. For thermophilic proteins, the loops tend to be shorter than their mesophilic counterparts (Section 2.1.1). However, the loop connecting the subunit I to the subunit III of the caa_3-oxidase is predicted to be of 72 residues and located on the cytoplasmic part (residues 483–555 as predicted by the TMHMM server http://www.cbs.dtu.dk/services/TMHMM-2.0/). This is longer than the average loop length of 20 residues predicted for the rest of the protein sequence and the structurally-determined length for the ba_3-oxidase subunits I and II; the loops predicted for the CtaA/B are also about 20 residues with the loop directly between CtaA and CtaB domains consisting of 37 residues. Moreover, this 72-residue loop also exists in the *A. pernix* C*c*O subunit I/III (Table 3) with a predicted 154 residues and in *Deinococcus radiodurans* with 75 residues.

Whether such a long loop is required to maintain a certain spatial distance between the subunits I and III or is involved in protein–protein interactions would require extensive mutagenesis including deletion of certain parts of the loop. It appears that this might be a feature common to all C*c*O where the subunits I/III are fused. That the *Thermus* loop is a thermophile feature can be excluded based on its presence in the closely-related *Deinococcus* species, though the thermophilic *A. pernix* (optimum growth temperature of 90–95 °C [78]) has a longer loop.

Table 2
Distribution of the fused CtaA/B. With the exception of *Proteobacteria*, all phyla are ancient. The hit of HB27 against the *T. thermophilus* HB8 is not included. Some of the protein sequences do not display complete conserved CtaA domain based on CD search but are instead truncated at the N-termini (a) or C-termini (b).

Phylum	Species name (RefSeq ID)	Identity (%)
Deinococcus–Thermus	*T. aquaticus* (ZP03496720)	86
	T. scotoductus (YP004201293)	88
Chloroflexi	*Roseiflexus castenholzii* (YP001434348)[a]	36
	Sphaerobacter thermophilus (YP003321390)[a]	36
	Thermomicrobium roseum (YP002523677)	37
Proteobacteria	*Bacteriovorax marinus* (CBW25150)	22
Euryarchaeota	*Halalkalicoccus jeotgali* (YP003737487)[b]	33
	Natrialba magadii (YP003480427)[b]	30

Table 3
Distribution of the fused subunit I/III. The subunit is only found fused in the *Deinococcus–Thermus* phylum, and the *Aeropyrum pernix* of which the C*c*O is aa_3-type.

Phylum	Species name (RefSeq ID)	Identity (%)
Deinococcus–Thermus	*Deinococcus deserti* (YP002786574)	58
	Deinococcus geothermalis (YP603878)	58
	Deinococcus radiodurans (NP296339)	58
	Meiothermus ruber (YP003506497)	56
	Meiothermus silvanus (YP003684057)	66
	T. aquaticus (ZP03496722)	95
	T. scotoductus (ADW20746)	93
	Oceanithermus profundus (YP004057123)	73
Crenarchaeota	*Aeropyrum pernix* (NP147500)	34

3. Mechanistic analysis (electron transfer and proton pumping)

3.1. Overview of ba_3-oxidase functional mechanism

Three processes central to the ba_3-oxidase catalysis are the electron transfer from the *bc* complex *via* the soluble cytochrome c_{552}, proton pumping across the membrane to generate the gradient required for ATP generation and the reduction of molecular oxygen to water.

3.2. Intermolecular electron transfer (from substrate to enzyme complex)

Residues important for C*c*O function, such as the heme and Cu ligands as well as those involved in the electron transfer described above, are highly conserved among all the structurally-determined C*c*O sequences. The electron transfer pathway between the cytochrome c_{552} and the Cu_A has been elucidated by nuclear magnetic resonance (NMR), where it is proposed that the electron from the porphyrin ring in the cytochrome c_{552} is sequentially transferred *via* the residues Ala87, Phe88 and His114 before reaching the CU2 atom of Cu_A [48]. The edge-to-edge distance between the heme and the imizadole of His114 is 10.9 Å. An analysis of structures in PDB shows that the majority of edge-to-edge distances between redox cofactors are less than 14 Å, thereby minimizing mutational or thermal fluctuation effects [79]. Alternatively, longer electron jumps through space could occur directly to Phe88, His114 and the Cu atoms; it is also possible to bypass the His residue. With a total distance of 13.5 Å, the rate would be very low.

3.3. Intramolecular electron transfer (from Cu_A to Cu_B)

The electrons from Cu_A are transferred to Cu_B, probably *via* heme *b* and possibly *via* heme a_{s3}. His114, Arg449 and Arg450 together with the propionates of heme *b* provide a pathway for electron transfer between Cu_A and heme *b*; either Phe385 or His384 and His386 (heme a_3 and *b* ligands, respectively) can further transfer the electrons to the high-spin heme. These residues are all conserved in the *Thermus* ba_3-oxidase compared to the other C*c*O. Alternatively, electron tunneling may allow for the transfer from Cu_A through Gln151 to Tyr136, Trp229 (a through-space jump using hydrogen bond) and His283 (Cu_B ligand) before reaching the Cu_B itself. With the exception of cbb_3-oxidase that has no Cu_A center, the distance between Cu_A and Cu_B is ~22 Å in all the structurally-determined C*c*O, even greater than the Cu_A to $Fe_{a/b}$ distance of 19 Å. Direct electron transfer from the two Cu centers will be very slow compared to the indirect pathways.

3.4. Coupling of electron transfer to proton pumping

For more than a billion years, since oxygen appeared in the atmosphere, respiratory complexes have evolved to sustain energetically-efficient mechanisms. High-energy intermediates in these complexes can be avoided through the proton-coupled electron transfer (PCET) and pathways in which electron transfer is followed by proton transfer [80]. The proton gradient required for the ATP generation by the ATP synthase complex is generated by the pumping of protons across the membrane into the periplasmic/intermembrane space by a series of oxidoreductases. The leakage of protons back into the cytoplasm/mitochondrial matrix is thermodynamically favored. Molecular dynamics simulation (MDS) has identified the putative valve-like residue GluI-242^B (GluI-278^P) as being responsible to prevent this intraprotein back leakage [81].

In the A-type (mitochondrial-like) C*c*O, two proton pathways, the D- and K-pathways have been identified. The D-pathway leads from the AspI-124^P to the GluI-278^P through which six or seven protons are transferred per catalytic cycle (four of which are pumped across the membrane). The K-pathway leads from a LysI-354^P to the cross-linked HisI-276^P/TyrI-280^P in the active site and transfers one or two protons to Cu_B center for O_2 reduction (reviewed in [21]). Both pathways are functional prerequisites for full enzyme function. In addition, a possible H-pathway, composed of a network of hydrogen bonds and waters, was identified by an examination of the crystal structure of bovine heart C*c*O [82]. Its existence in *P. denitrificans* [83,84] and *R. sphaeroides* [85] is controversial, but it is argued by Shimokata *et al.* [86] that the presently available mutagenesis studies do not disprove the existence of such H-pathways.

Soulimane *et al.* have previously identified three potential proton pumping pathways based on the ba_3-oxidase crystal structure [9]. Two are spatially analogous to the D- and K-pathways and the third (the Q-pathway) leads from a Gln254 to the heme a_{s3} axial His ligands [9]. Of all the B-type C*c*O sequences analyzed, only the residues forming the K-pathway analogue [22] are conserved. This contrasts with the high conservation of similar K-pathway residues within the A-type and implies that only a K-analogue is functional in the B family. A series of mutagenesis studies has identified the residues important for catalysis and electron transfer/proton pumping coupling. These have been mapped onto the ba_3-oxidase structure to identify those with the potential to form a K-analogue pathway. It leads from the GluII-15 to the subunit I residues Thr315, Tyr248, Thr312, Ser309, Tyr244 and Tyr237. When mutated to non-polar, non-H-bonding residues, the capability to reduce O_2 is abolished. Similar mutations on the possible D- or Q-pathway residues in the ba_3-oxidase generally affect neither catalysis nor proton pumping. The presence of an unknown proton pathway can be excluded as mutants with a blocked K-analogue pathway show retarded reduction and oxidation rates for the hemes *b* and a_{s3}. The *Thermus* ba_3-oxidase has a reduced proton-pumping efficiency, as only 0.4–0.5 protons are pumped per electron transferred compared to the standard 1 H^+/e^- in classical heme-copper oxidases. A single H^+/e^- is necessarily used for water formation [87].

3.5. Water pool in ba_3-oxidase

Water molecules accumulate above the heme propionates in the oxidase structure (PDB ID: 1EHK) to create a water 'pool' as in the C*c*O from *P. denitrificans*, *R. sphaeroides* and bovine heart mitochondria; the surrounding residues are also conserved [82,88,89]. Unfortunately, the low reported resolutions of the cbb_3-oxidase (PDB ID: 3MK7) [38] and bo_3 quinol oxidase (PDB ID: 1FFT) [67] at 3.2 and 3.5 Å, respectively, do not allow water pool comparisons with the other oxidases. By being connected to the bulk solvent in the periplasmic space, a fast equilibrium between the water pool and the bulk solvent could occur, thereby facilitating proton exit and rapid acceptance of both pumped protons and water molecules formed at the active site [90]. In the *E. coli* bo_3 quinol oxidase, the stabilization of the Δ-propionate of high-spin heme o_3 is a consequence of the electrostatic interactions by (i) the conserved Arg481$^{E.\ coli}$ and Arg482$^{E.\ coli}$ (equivalent to Arg438^B and Arg439^B), (ii) H bonding of Arg481$^{E.\ coli}$ (Arg438^B) and Trp126^B, and (iii) a water molecule residing between the propionate and His291^B that coordinates as axial Cu_B ligand [90]. Proton pumping capability is removed by mutating both Arg residues, implicating the Δ-propionate in proton pumping. The three proposed pathways for proton acceptance by the Δ-propionate are (i) directly from Glu242^B [91], (ii) from the Glu242^B *via* the bound water molecules [92] and (iii) from the Glu242^B *via* the water molecules and the Cu_B ligand His291^B [93].

Similarly in the *P. denitrificans* aa_3-type C*c*O, water-filled cavities are present in both the D- and K-pathways. The non-conserved (compared to the D-pathway in bovine C*c*O [82]), nonpolar Ala and Gly residues line the D-pathway in *P. denitrificans*, and, as such, water molecules might behave as polar 'side chains' to facilitate the proton translocation [88]. In contrast, MDS results imply that the idea of water-assisted proton transfer might be misguided; it seems energetically expensive to use water molecules to transfer protons between acidic residues [94]. In our opinion, proteins, especially those as large as respiratory complexes, could have regions of energetically-unfavorable interactions but still maintain net favorable interactions when considered wholly.

3.6. O_2 reduction through O_2 input pathway

Hydrophobic cavities within protein structures can be identified using Xe and Kr, and have been employed for more than forty years [95,96]. As O_2 is more soluble in organic solvents [96], it enters and diffuses within the lipid bilayer [97,98]. The input pathway of O_2 into the dinuclear center is the Xe1 site. Although CO is transferred to the high-spin heme from the Cu_B [96], and by extension the physico-chemically-similar O_2, [99], two residues (Trp229 and His283) block direct access from the Xe1 site to the Cu_B [30]. Mostly hydrophobic residues line the O_2 access pathway that is favorable to O_2 but not to the produced water molecules. The authors suggested that water molecules are repelled by these residues and attracted by the hydrophilic 'vent' leading to the water pool [30]. In addition, the other structurally-determined C*c*O display features obstructing O_2 access — there is a constriction point in *R. sphaeroides* (pathway diameter is reduced to ~1.7 Å), with conservation of the same residues in *P. denitrificans* and bovine heart mitochondria (Fig. 6); it is very likely that they require structural rearrangements for O_2 access. As mentioned previously, the ba_3-oxidase is a high-affinity C*c*O and the lack of such constricting features along with a large cavity volume (390 Å^3, compared to 140, 409 and 87 Å^3 in the *P. denitrificans*, *R. sphaeroides* and bovine heart enzymes) and a

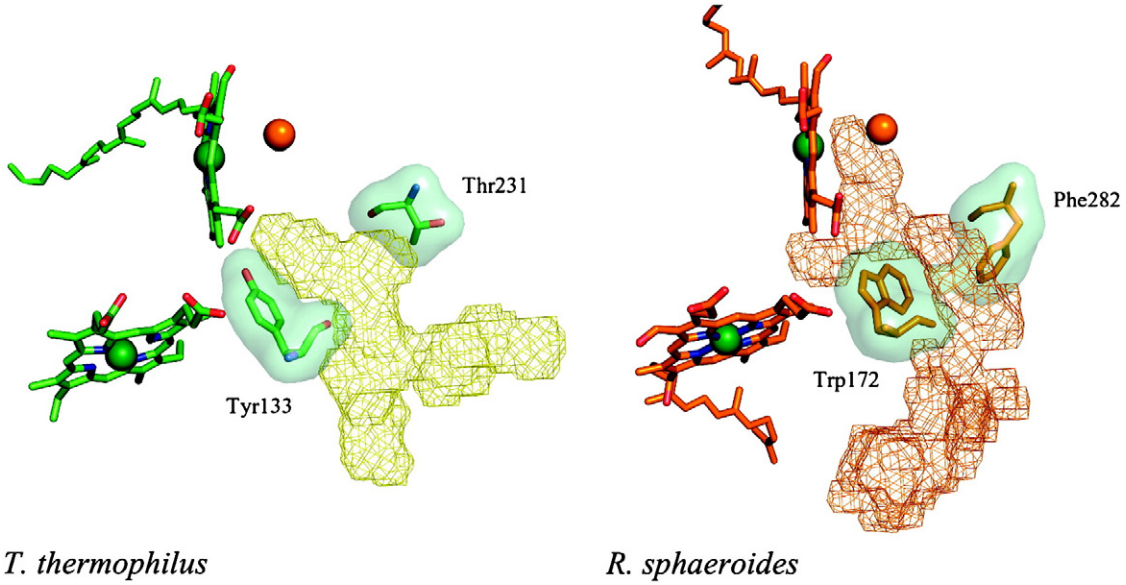

Fig. 6. Spatial accessibility of molecular oxygen to the active site of CcO of *Thermus thermophilus* and *Rhodobacter sphaeroides*. In the latter, a constriction point prevents direct access to the active site without structural rearrangement, a feature not present in the former. Reprinted with permission from [30]. Copyright 2008 American Chemical Society.

bifurcated oxygen entrance pathway [30] may partly explain its properties. Furthermore, the hydrophilic Glu278^P in *P. denitrificans* is substituted by the hydrophobic Ile235 in *T. thermophilus*; the residue replacement is also similar where the hydrophobic Val is present in the thermoacidophilic archaeon *Sulfolobus acidocaldarius* caa_3-quinol oxidase and the moderately-thermophilic haloalkaliphilic archaeon *N. pharaonis* ba_3-cytochrome oxidase [9,100].

3.7. Overall O_2 chemistry and reaction mechanism of ba_3-oxidase

Density functional theory (DFT) is a computational method that allows the modeling of the electronic structures, energetics and properties of atoms and molecules in a complex system. Initially used in solid state physics, it is now used in computational chemistry, and can be applied to biological systems (see Refs. [101–107] for the theory of DFT and its applications in biological contexts). Although experimental evidence for the exact ba_3-oxidase reaction mechanism is still scarce, a 14-step catalytic mechanism has been proposed using DFT in combination with experimental data [108]. It should be noted that the spectral properties of heme moieties do not allow for the investigation of Cu_B states. The ba_3-oxidase is known to pump H^+ with an experimental stoichiometry of only 0.5 H^+ per electron transferred, and the proposed mechanism does not conform to this stoichiometry. This inconsistency could be attributed to the enzyme preparations themselves that undergo a harsh purification process as well as the non-optimal experimental temperatures used. It is well-known that many thermophilic proteins display temperature-dependent activity.

3.8. Mechanistic analysis of caa_3-oxidase

Without the availability of its 3D structure, detailed mechanistic analysis of caa_3-oxidase is impossible. *In surfo* and *in meso* crystals have been generated [109]; the diffraction quality and radiation sensitivity problems were solved and the structure is forthcoming (J. A. Lyons, D. Aragão, T. Soulimane, M. Caffrey, PDB ID:2YEV). However, a few biophysical methods have been used to probe its active site and electrochemical properties. Resonance Raman spectroscopy indicates that this enzyme is fundamentally similar to the aa_3-oxidases of bovine heart and *P. denitrificans* [110]. As such, their mechanisms would also very likely be similar. Interestingly, the side chain of heme *a* moieties in the bacterial *R. marinus* is HEGG and, therefore, of A_s type [111]. The investigation into its proton pumping activity has shown that the *Thermus* caa_3-oxidase has a stoichiometry of 1 H^+/e^- [112,113].

Homology modeling of *R. marinus* caa_3-oxidase suggests that the hydroxyl of the highly-conserved Tyr (YS motif) spatially replaces the carboxyl of Glu278^P residue and that the Ser could play a role in the proton transfer [114]; the Glu278^P-like residue is also missing in *T. thermophilus* caa_3-oxidase. In an elegant experiment, when the GluI-278^P in *P. denitrificans* was mutated to Ala, proton pumping and activity were both abolished as was the G275S mutation; none of the mutations resulted in a wild type-like activity although proton pumping was restored as long as the mutation in GluI-278^P was complemented by F274Y with or without G275S [115]. This shows that while the Glu can be replaced by a Tyr, the Ser is not required. The pK_a of Tyr side chain is ~10.5; at physiological pH (~7.2), the side chain would be mostly protonated and unable to further transfer protons. There are two possible explanations regarding the high pK_a of Tyr in solution and its protonation — (i) since pK_a values of residues are dependent on their exact environment, the Tyr pK_a within the protein could well differ from the in-solution value [116], or (ii) the Tyr is not functionally replacing Glu278 but might orient chains of water molecules [114]. Notwithstanding these observations regarding the *R. marinus* caa_3-oxidase, Fourier-transform infrared (FTIR) technique revealed the protonation of a Tyr upon reduction of the *Thermus* enzyme [117].

It should also be noted that though the bound cytochrome *c* is recognized as a substrate for the *Rhodothermus* enzyme, it displays a ten-fold higher activity with a *h*igh-*p*otential *i*ron-sulfur *p*rotein (HiPIP) as the electron donor [118]. As an implication, drawing any similarity of electron transfer mechanism between the two enzymes should be treated cautiously.

4. Overall functional and evolutionary significance

4.1. Possible additional functions

While the existence of multiple aerobic respiratory enzymes has been known for some time [119], there is no conclusive evidence to explain such redundancy; there is even no study suggesting an explanation for *T. thermophilus*. This is a critical issue since thermophiles

have comparatively much smaller genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). In the mesophilic *E. coli* (genome size of non-pathogenic strains of ~4.6 Mbp) and *Pseudomonas putida* (~6 Mbp), three (cytochrome *bd*-I, *bd*-II and *bo*; see [120] and references therein) and five terminal oxidases [121] have been identified, respectively. Hence, we speculate that the existence of two terminal oxidases in this extreme thermophile extends beyond their affinities toward oxygen. It is very likely that the O_2 affinity anticorrelates to the proton pumping stoichiometry for structural and/or functional reasons. As noted previously [21], a comparison of the exact physiological stoichiometries is hampered by the large deviance of the experimental procedures from the physiological conditions, such as the different lipidic environments, pH values and the ionic strengths. In addition, the additional functions of the terminal oxidases, including the scavenging of reactive oxygen species similar to *E. coli* [122], should be investigated.

4.2. Functional relationship with other CcO

For both the ba_3- and caa_3-oxidases, a significant divergence of their actual mechanisms is not expected since the heterodinuclear redox centers are Fe_{a3}-Cu_B, and are likely to be similar to what is already known. In fact, substitution of the high-spin heme at the heterodinuclear center generally inactivates the enzyme [36] but replacing the low-spin heme has no functional effect [123]; an exception does exist [124]. In contrast, the cbb_3-type oxidase has a heme b_3 as the heme at the O_2-reducing center; it is highly divergent from the other two types of CcO [18,125]. Of the four predicted cbb_3-oxidase subunits, only three could be purified as the canonical 'cbb_3-oxidase' complex [125]. Nevertheless, only some of the residues that act as K-analogue pathway in the ba_3-oxidase are conserved in the cbb_3-oxidase [18], while the crystal structure shows a blocked D-pathway [38]. Other features indicative of its different evolutionary lineage from the other types of CcO include the absence of Cu_A (which is characteristic of quinol oxidases) and the presence of multiple heme moieties functioning as redox centers. Among the striking structural elements of the cbb_3-oxidase is a novel periplasmic cavity, very likely acting as a water exit pathway, and a membrane cavity connecting O_2 entry to the high-spin heme b_3. These, and the unique conformation of redox-linked moieties, exemplify nature's engineering to create a high O_2-affinity enzyme. The 'best' model organism to study all the three types in a thermophilic physiology would be *R. marinus* as it possesses the A2- [126], B- [127] and C-type enzymes [111], and is genetically tractable. As stated above, the substrate–enzyme (electron donor-CcO) interactions are hydrophobic at higher growth temperatures.

5. Conclusion

An important point that must be recognized is that the determination of a crystal structure of any protein is merely a starting point to rationalize pre-determination experimental data. While having a structure could answer many questions, it could give rise to equally more questions. It has been about ten years since the publication of the ba_3-oxidase structure by Soulimane *et al.* [9], yet our understanding of the enzyme is still incomplete. Reasonable progress in this area is illustrated by the wealth of available data, both experimental and computational. We now know that the ba_3-oxidase interacts hydrophobically with the cytochrome c_{552} and the identities of the residues involved in the electron transfer from the substrate to the dinuclear center. Further adding to its 'aberrant' nature is the use of a single proton pathway for catalysis. For the caa_3-oxidase, very limited data are available. Under low oxygen tension, it is coexpressed with the ba_3-oxidase, therefore making available multiple pathways for electron transfer between the *bc* complex and the CcO.

Among the other questions are (i) whether the true ba_3-oxidase proton pumping efficiency reaches 1 H^+/e^- with assays performed at the *T. thermophilus* optimal growth temperature of 70 °C instead of at 'mesophilic' temperatures and the delipidation following the extensive purification, (ii) possible dynamic regulation of both CcO expressions as an adaptation by the bacterium under differential O_2 tension and (iii) the structure–function relationship of the caa_3-oxidase.

The sequence and structure analyses of the ba_3- and caa_3-oxidases indicate various interesting aspects from the protein thermostability and thermophilic bioenergetics perspectives. It would be extremely difficult to define the precise borders between these two adaptations. Nonetheless, a summary is provided here based on the comparison with their mesophilic counterparts; (i) the *Thermus* ba_3-oxidase has a higher O_2 affinity provided by the larger O_2 cavity as an adaptation to the reduced O_2 solubility at high temperatures, (ii) the ba_3- and caa_3-oxidases have shortened loops as a thermostability adaptation although there is an extended fusion linker connecting the caa_3-oxidase subunits I/III, (iii) the substrate–enzyme interaction between the cytochrome c_{552} and ba_3-oxidase is of hydrophobic nature. Beyond the investigation into the *T. thermophilus* oxidases, more detailed characterization studies involving other thermophilic oxidases should be undertaken to increase our understanding of thermophile-specific features and the actual diversity of this important family of enzymes. Obviously, these will eventually be directly linked to their physiology.

Note added in the proof

Recently, Han *et al.* (doi:10.1073/pnas.1018958108) demonstrated that the caa_3- and ba_3- oxidases have proton pumping stoichiometries of 1 and 0.5 H^+/e^- at room temperature. Therefore, the stoichiometry is not temperature-dependent and the latter does indeed pump protons with a lower efficiency.

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