Biochimica et Biophysica Acta 1817 (2012) 898–910

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

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

Review

Biogenesis of cbb_3-type cytochrome *c* oxidase in *Rhodobacter capsulatus* ☆

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ARTICLE INFO

Article history:
Received 24 September 2011
Accepted 31 October 2011
Available online 4 November 2011

Keywords:
cbb_3-type cytochrome *c* oxidase
Co- or post-translational cofactor insertion
Assembly of membrane proteins
Copper acquisition
Photosynthesis and respiration
Energy transduction

ABSTRACT

The cbb_3-type cytochrome *c* oxidases (cbb_3-Cox) constitute the second most abundant cytochrome *c* oxidase (Cox) group after the mitochondrial-like aa_3-type Cox. They are present in bacteria only, and are considered to represent a primordial innovation in the domain of Eubacteria due to their phylogenetic distribution and their similarity to nitric oxide (NO) reductases. They are crucial for the onset of many anaerobic biological processes, such as anoxygenic photosynthesis or nitrogen fixation. In addition, they are prevalent in many pathogenic bacteria, and important for colonizing low oxygen tissues. Studies related to cbb_3-Cox provide a fascinating paradigm for the biogenesis of sophisticated oligomeric membrane proteins. Complex subunit maturation and assembly machineries, producing the *c*-type cytochromes and the binuclear heme b_3-Cu_B center, have to be coordinated precisely both temporally and spatially to yield a functional cbb_3-Cox enzyme. In this review we summarize our current knowledge on the structure, regulation and assembly of cbb_3-Cox, and provide a highly tentative model for cbb_3-Cox assembly and formation of its heme b_3-Cu_B binuclear center. This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

1. Introduction

1.1. Generalities

The emerging ease of whole genome sequencing approaches has revealed the existence of multiple terminal oxidases in bacteria, which allow them to utilize efficiently varying oxygen (O_2) concentrations in their environments. Most of the terminal oxidases belong to the heme-Cu: O_2 reductase superfamily (heme-Cu: O_2 reductases); they reduce O_2 to H_2O and couple electron transfer with vectorial proton translocation across the membrane [1]. Members of this superfamily are present in eukarya, archaea and eubacteria. They are thought to be of monophyletic origin, possibly related to nitric oxide reductases (NOR) [2]. NOR catalyze reduction of NO to N_2O during denitrification. Although they do not reduce O_2 and do not generate a proton gradient [3], their structural organization is similar to heme-Cu: O_2 reductases.

The heme-Cu: O_2 reductases can be divided into different subgroups in respect to their electron donors: those that use a *c*-type cytochrome (*i. e.*, cytochrome *c* oxidase, Cox) and those that use a quinol (*i. e.*, quinol oxidase, Qox). Cox are universally conserved oligomeric membrane proteins that terminate the respiratory chains of aerobic and facultative aerobic organisms. The structure and function of Cox enzymes have been studied intensely because of their central role in energy metabolism. Given the complexity of these multi-subunit, multi-cofactor enzymes, their assembly also attracted much attention. Indeed, assembly defects in the human Cox are major causes of mitochondrial disorders, and are crucial for neurodegenerative diseases, cancer and aging [4–7]. Among the Cox enzymes, the aa_3-type Cox (aa_3-Cox) of mitochondria and many bacterial species are highly abundant and the best-studied group. However, the heme-Cu: O_2 reductases represent a diverse ensemble of enzymes with significantly different subunit compositions and cofactors [8]. In this review, we focus on the structure, subunit maturation and assembly of the cbb_3-type Cox (cbb_3-Cox) that represent the second most abundant group after the aa_3-Cox. Most of the studies on cbb_3-Cox biogenesis were performed with the facultative phototrophic α-proteobacterium *Rhodobacter capsulatus*. This species is used as a model organism for these studies as it contains only one heme-Cu: O_2 reductase, cbb_3-Cox. *R. capsulatus* is still capable of aerobic respiration in the absence of it because it also contains an unrelated terminal oxidase (*bd*-type quinol oxidase) (Fig. 1) [9,10].

1.2. Classification of heme-Cu: O_2 reductases

Members of heme-Cu: O_2 reductases are diverse in terms of their subunit composition, heme cofactor content, electron donor, and O_2 affinity (Table 1) [8,11,12]. All members have a conserved core subunit

Abbreviations: Cox, cytochrome *c* oxidase; Qox, quinol oxidase; Ccm, cytochrome *c* maturation; NOR, nitric oxide reductase; BN-PAGE, blue-native polyacrylamide gel electrophoresis; Cu, Copper

☆ This article is part of a Special Issue entitled: Biogenesis/Assembly of Respiratory Enzyme Complexes.

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doi:10.1016/j.bbabio.2011.10.011

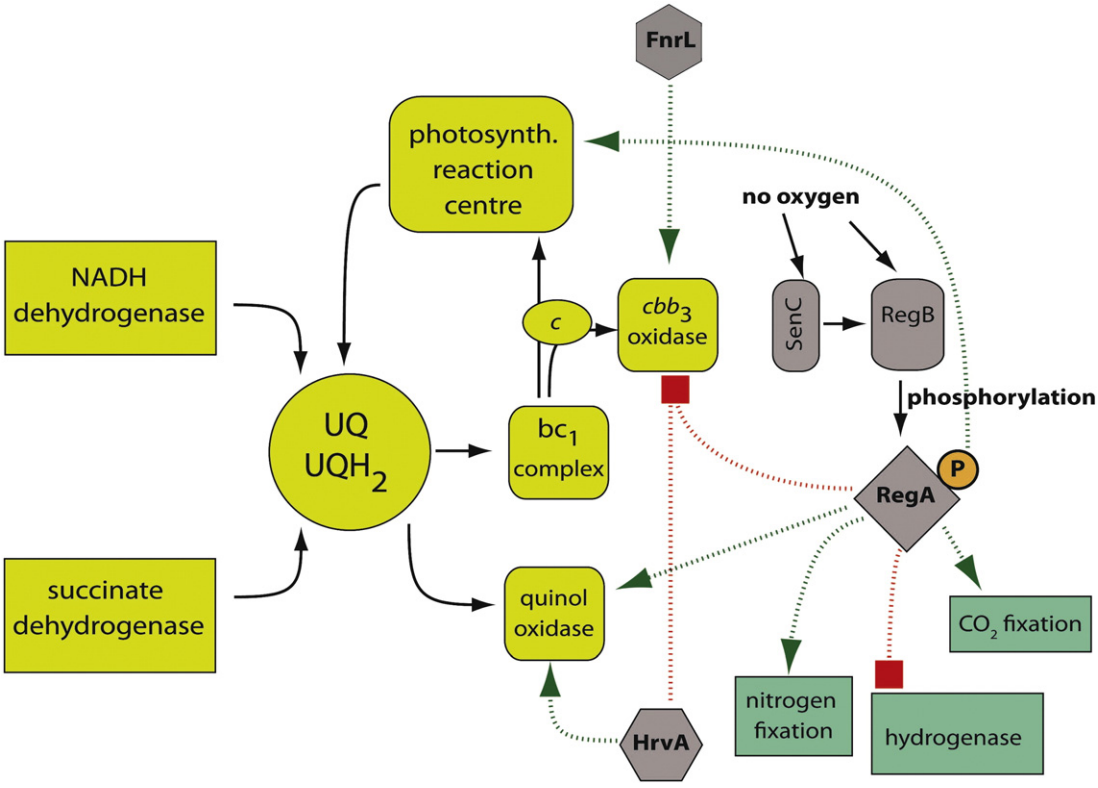

Fig. 1. The respiratory chain of *R. capsulatus* and its regulation in response to oxygen availability. The protein complexes and electron carrier of the respiratory chain are shown in yellow. UQ and UQH_2 correspond to the oxidized and reduced form of quinones. Soluble cytochrome c_2 and the membrane-bound cytochrome c_y (indicated as *c* in the figure) are electron donors for the photosynthetic reaction center and cbb_3-Cox. Metabolic processes which also consume redox equivalents are shown in blue. Regulatory proteins are shown in gray. RegB is a sensor kinase that phosphorylates the response regulator RegA under low oxygen concentrations. Phosphorylated RegA (RegA-P) inhibits (shown as the red square) expression of *ccoNOQP* (cbb_3-Cox) and *hupSLC* ([NiFe] hydrogenase), but stimulates the expression of the *bd*-type quinol oxidase and of genes required for photosynthesis. In addition, RegA-P stimulates (shown as an arrow) the expression of *nifHDK* (nitrogenase) and the cbb_1 and cbb_2 operons (CO_2 fixation) both directly and indirectly [154]. Under microaerobic and anaerobic conditions HvrA further represses *ccoNOQP* expression but stimulates *bd*-type quinol oxidase expression. Finally, FnrL probably activates *ccoNOQP* expression in the transition phase between aerobic and anaerobic conditions. *R. capsulatus* SenC and its *R. sphaeroides* homologue PrrC were suggested to be involved in oxygen-dependent expression of *ccoNOQP* and photosynthetic genes. FixLJ-K regulatory system discussed in the text is not shown in the figure.

(Subunit I), containing at least 12 transmembrane helices. This subunit contains a low spin heme (of *a*- or *b*-type), a binuclear metal center composed of a high spin heme (of *a*-, *o*-, or *b*-type heme, referred to as a_3, o_3 or b_3)-iron, and a Cu atom (Cu_B), as well as a tyrosine residue covalently linked to a histidine ligand of Cu_B [1,8]. Besides subunit I, which is the catalytic heart where O_2 is reduced to H_2O, heme-Cu: O_2 reductases have at least two other core subunits: Subunit II is the primary electron acceptor, and in some cases, it harbors extra cofactors like a binuclear Cu center (Cu_A), or is a *c*-type cytochrome. Subunit III in many cases does not contain any cofactor except in cbb_3-Cox where it is a diheme *c*-type cytochrome (Table 1) [1]. Most bacterial Cox are composed of these three core subunits, but mitochondrial Cox are more complex with 13 subunits of which only three (subunits I, II, and III) are encoded in the mitochondrial genome. An additional fourth subunit with a single transmembrane helix is also present in some bacterial Cox. However, this subunit is unlike any of the ten nuclear encoded mitochondrial subunits [13].

A classification, based on common features of the core subunits, and key residues in proton transfer pathways, defines three (A, B and C) types of heme-Cu: O_2 reductases (Table 1) (Fig. 2) [1]. Mitochondrial-aa_3-Cox (Type A), ba_3-Cox of *Thermus thermophilus* (Type B) and cbb_3-Cox of *R. capsulatus* (Type C) are the most representative members of these types.

Available three-dimensional (3D) structures of the heme-Cu: O_2 reductase family members (*i. e.*, the aa_3-Cox from *Paracoccus denitrificans, Rhodobacter sphaeroides* and bovine heart mitochondria; ba_3-Cox from *T. thermophilus*, bo_3-Qox from *Escherichia coli* and the cbb_3-Cox from *Pseudomonas stutzeri*) (Fig. 2A) [14–19] reveal a remarkably conserved Cu and heme cofactor arrangement in these enzymes. Subunit I of type A enzymes contain two proton-transfer pathways referred to as K- and D- channels (Table 1). The four electrons required for O_2 reduction at the binuclear heme-Cu_B center are conveyed sequentially via a non-covalently attached low spin heme, which itself receives electrons from the Cu_A center in subunit II [15,19]. Type B enzymes are present only in bacteria and archaea, but not in eukaryotes, and constitute the least abundant group [20]. The catalytic center of type B enzymes is similar to that of type A, except that they lack the D-channel for proton transfer [21]. The prototypes of type C are the cbb_3-Cox, which are present only in bacteria, and considered to represent the most distant members of heme-Cu: O_2 reductases. They also lack the D-channel for proton transfer [22–24], their subunits II and III are *c*-type cytochromes (Table 1), and they exhibit higher O_2 affinity as compared with other types of Cox enzymes.

2. The cbb_3-Cox

2.1. Distribution of cbb_3-Cox in bacteria and its role in pathogenesis

The cbb_3-Cox enzymes are common to proteobacteria and also found in the *cytophaga*, *flexibacter* and *bacteriodes* (CFB) group [12]. They were first described in the facultative symbiotic N_2-fixing *rhizobiacaea* [25,26]. In *Bradyrhizobium japonicum* expression of cbb_3-Cox is required for symbiotic N_2 fixation under very low O_2 conditions in soybean root nodules [26]. Due to their importance for symbiotic N_2 fixation, the four structural genes of cbb_3-Cox were initially termed *fixNOQP* [26]. Subsequent studies identified these genes and their

Table 1
Characteristic features of different Cox and NOR enzymes.

	cbb_3-Cox	NOR	ba_3-Cox	aa_3-Cox
Subunit composition	• CcoN[a] • Two *c*-type cytochromes: CcoP and CcoO • Two putative small subunits: CcoQ, CcoH	• NorB[a] • *c*-type cytochrome: NorC	• Subunit I[a] • Subunit II with or without Cu_A	• Subunit I[a] • Subunit II with Cu_A • Subunit III • Subunit IV • Other subunits
Catalytic binuclear center	• Heme b_3 • Heme *b* • Cu_B • Covalent His-Tyr • Ca^{2+} coordinating heme *b* and b_3 propionates	• Heme b_3 • Heme *b* • Non-heme iron Fe_B • No covalent His-Tyr • Ca^{2+} coordinating heme *b* and b_3 propionates	• Heme a_3 • Cu_B • Covalent His-Tyr	• Heme a_3 • Heme *a* • Cu_B • Covalent His-Tyr
Enzymatic properties	K_m ~ 7 nM (for O_2) K_m ~ 12 mM (for NO)		K_m ~ 0,1 μM	K_m 0.7–2 μM
Proton pathway	'K' pathway	Protons from periplasm	K pathway	K and D pathways

[a] Catalytic subunit of the enzyme

products also in non-symbiotic bacteria such as *R. capsulatus*, and referred to them as *ccoNOQP* [27–29]. A recent bioinformatics study, based on the occurrence of *ccoN* and *ccoO* genes, identified the *ccoNOQP* cluster in all bacterial species, except the *Thermotogales*, *Deinococcales* and *Firmicutes* [12]. So far, no cbb_3-Cox coding sequences were identified in archaea [30]. Interestingly, *ccoNO* genes were found recently in the last mitochondrial ancestor bacterium *Midichloria mitochondrii* [31].

The cbb_3-Cox is present in many microaerophilic pathogenic bacteria and implicated in host colonization (Table 2). In some species including *Campylobacter jejuni* [32], *Helicobacter pylori* [33,34], *Neisseria meningitidis* and *Neisseria gonorrhoeae* [35] cbb_3-Cox is the sole Cox, and it is suggested that its high O_2 affinity allows these pathogens to colonize low O_2 containing tissues [11]. Correlations between cbb_3-Cox and host colonization have so far been examined mainly in *C. jejuni*, which is a non-fermenting microaerophile that colonizes the small and large intestines of humans and animals [36], causing acute gastroenteritis in humans. *C. jejuni* has a branched aerobic electron transport chain, with a cbb_3-Cox of high O_2 affinity ($K_m = 40$ nM) and a quinol oxidase (CioAB) of low ($K_m = 800$ nM) O_2 affinity. Gene expression studies have shown that CioAB was mainly expressed at high O_2 conditions [37], while cbb_3-Cox was induced significantly in *C. jejuni* cells colonizing the chick caecum, which is a low O_2 environment [38]. *C. jejuni* mutants lacking cbb_3-Cox were unable to colonize the chick caecum, whereas mutants lacking CioAB were slightly impaired in colonization [38]. The caecum is primarily an anaerobic environment, hence *C. jejuni* is likely to be exposed transiently to anaerobic conditions. Probably, it tolerates anaerobiosis by using alternative electron acceptors like nitrate, nitrite, DMSO or TMAO [39], while its cbb_3-Cox would keep cellular O_2 concentrations low to protect its crucial O_2-labile enzymes and allow establishment of anaerobic respiration [40,41]. Indeed, *C. jejuni* mutants lacking nitrate or nitrite reductases are significantly attenuated for colonizing the chick caecum [39]. Thus, anaerobic respiration together with cbb_3-Cox seems to allow *C. jejuni* to adapt successfully to environmental changes at their ecological niches.

A cbb_3-Cox is also present in *Brucella suis*, which is an intracellular Gram-negative pathogen and the causative agent of brucellosis [42]. Under microaerobic conditions in liquid cultures, *B. suis* expresses mainly cbb_3-Cox. However inside macrophages, it relies almost exclusively on a *bd*-type quinol oxidase [42], which apparently has a higher O_2 affinity than cbb_3-Cox in this species. In *H. pylori* which is responsible for stomach ulcers, cbb_3-Cox is the sole respiratory terminal oxidase recognizable in its genome (Table 2). Transposon insertion mutants in *ccoN*, *ccoO* and *ccoP* genes of this species were obtained under microaerobiosis, indicating that cbb_3-Cox is not essential under these conditions [43]. Whether *H. pylori* needs cbb_3-Cox to colonize the intestine is unknown. The fact that *H. pylori* mutants that lack fumarate reductase fail to colonize the mouse stomach [44] indicates that anaerobic respiration is crucial for virulence of this and other microaerophilic pathogens.

Although not yet studied extensively, the role of cbb_3-Cox during pathogenesis might provide a potential antibacterial target for therapeutic interventions. Chemicals that interfere with cbb_3-Cox might act as powerful specific antibiotics with minimal side effects due to the absence of this type of Cox in mammals [45].

2.2. Regulation of cbb_3-Cox expression

In *R. capsulatus* and most other bacteria the steady-state concentration and activity of cbb_3-Cox is dependent on the environmental O_2 concentrations. This activity is high under microaerobic, low under fully aerobic, and even lower under fully anaerobic growth conditions [46]. Several components, including RegA (or PrrA), RegB (or PrrB), FnrL, HvrA, and FixLJ-K that respond to different O_2 and redox conditions, are thought to coordinately regulate this process (Fig. 1) [47]. RegA and RegB constitute a global two-component regulatory system that controls the expression of many genes involved in energy metabolism, including cbb_3-Cox, *bd*-quinol oxidase, and various genes related to photosynthesis, hydrogen utilization, nitrogen fixation and carbon assimilation [46,48–51]. RegA is a response regulator with a conserved helix-turn-helix DNA binding motif, whereas RegB is a sensor kinase proposed to contain a conserved region that monitors redox changes inside the cells. Under microaerobic and aerobic conditions the dephosphorylated form of RegA activates cbb_3-Cox expression. Conversely, under anaerobic conditions RegA is phosphorylated by the sensor kinase RegB, and phosphorylated RegA represses cbb_3-Cox and enhances photosynthesis genes expression [47]. In *R. sphaeroides*, it was proposed that cbb_3-Cox monitors electron flow through its CcoN subunit [52]. When this electron flow is high, it sends an inhibitory signal to PrrA and PrrB (homologues of RegA and RegB) to repress photosynthesis gene expression [52]. Thus, in this species abolishing cbb_3-Cox results in activation of photosynthetic genes under aerobic conditions [53].

Besides RegA and RegB, some other genes also regulate cbb_3-Cox expression. FnrL-like genes are found upstream of *ccoNOQP* in *R. capsulatus* [54], and upstream of both *ccoNOQP* and *ccoGHIS* (involved in the assembly of cbb_3-Cox, see below) in other organisms including *R. sphaeroides*, *P. denitrificans*, *B. japonicum*. Studies performed in *E. coli* established that FnrL controls switching from aerobic to anaerobic respiration [55]. Inactivation of FnrL in *R. capsulatus* decreases cbb_3-Cox activity by about 80% under anaerobic growth conditions, suggesting that it acts as an activator in the absence of O_2 [47]. This effect is counter balanced by the trans-acting regulatory protein HvrA, which functions as a repressor of *ccoNOQP* under microaerobic and anaerobic conditions (Fig. 1) [47]. Another major set of regulators of microaerophilic growth as well as photosynthesis is the FixLJ-K regulatory system. FixL is a histidine kinase that can bind O_2 via a heme *b* group, whereas FixJ is a transcription factor that undergoes phosphorylation by FixL under microaerophilic

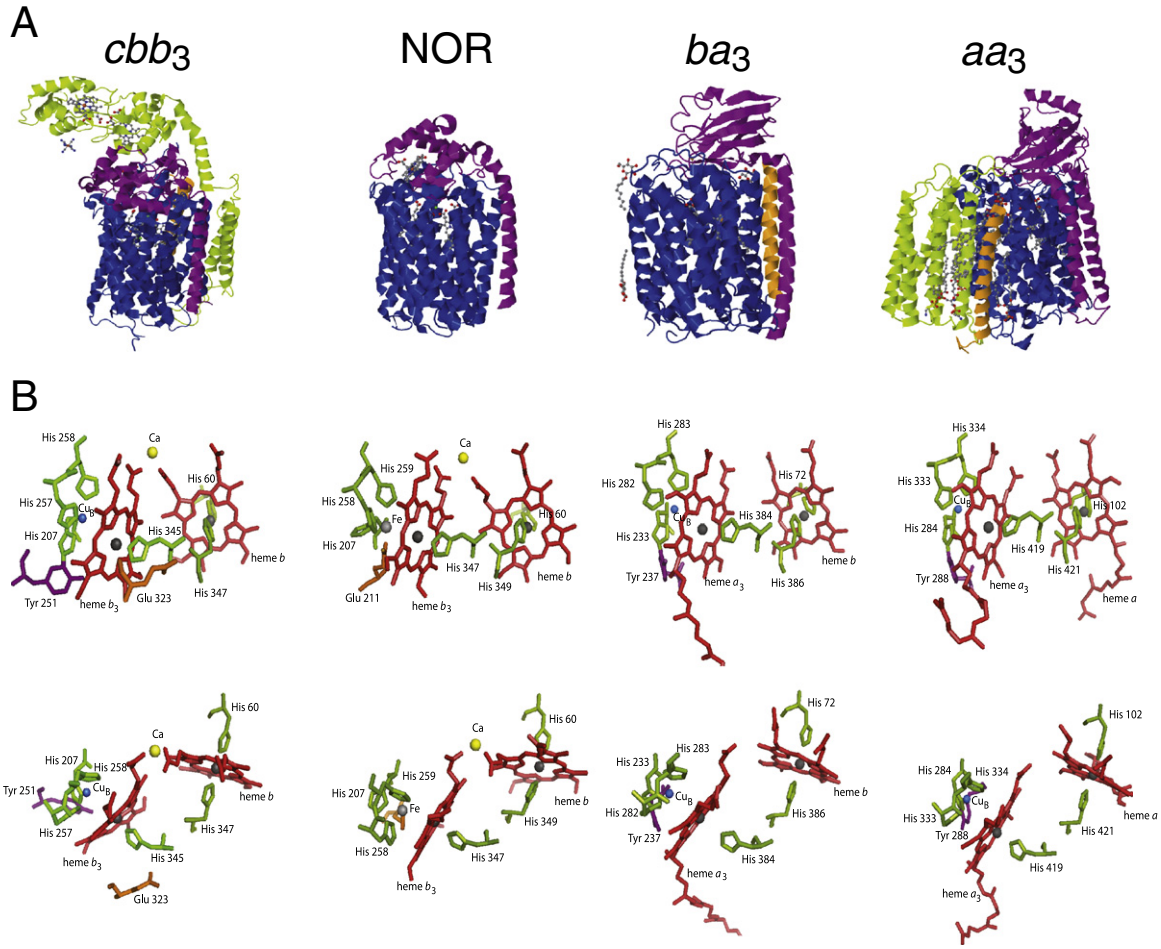

Fig. 2. A. 3D structures of different types of heme-Cu: O_2 reductases and NO reductase: Catalytic subunit I — blue (*cbb*$_3$-Cox CcoN), subunit II (*cbb*$_3$-Cox CcoO) — magenta, subunit III (*cbb*$_3$-Cox CcoP) — green, additional subunit — orange. B. Architecture of the catalytic binuclear center of the different types of heme-Cu:O_2 reductases and NO reductase. Heme — red, His — green, Tyr — violet, Glu — orange. Spheres: copper — blue, heme iron — dark gray, non-heme iron — light gray, calcium — yellow. The structures depicted are taken from protein database (PDB) entries PDB ID: 3MK7 (*cbb*$_3$-Cox) [14], PDB ID: 3O0R (NOR) [60], PDB ID: 1M56 (*aa*$_3$-Cox) [19], PDB ID: 1EHK (*ba*$_3$-Cox) [17] using Jmol software (http://www.jmol.org/).

conditions. Phosphorylated FixL then activates the expression of FixK, a downstream transcription regulator that binds specifically upstream of *ccoN* gene to activate its expression [56–58]. The FixLJ-K system regulating expression of *cbb*$_3$-Cox is also found in many other bacteria, including *B. japonicum*, *Caulobacter crescentus* and *Novosphingobium aromativorans* [59].

2.3. Subunit composition and structure of *cbb*$_3$-Cox

Usually, *cbb*$_3$-Cox enzymes are composed of CcoN (subunit I), CcoO (subunit II), CcoQ (subunit IV) and CcoP (subunit III) proteins (Table 1) [12]. Excitingly, the 3D structure of *P. stutzeri cbb*$_3$-Cox was solved recently at a resolution of 3.2 Å [14]. The structure revealed that the overall shape and size of the membrane-embedded part of *cbb*$_3$-Cox is similar to those of *R. sphaeroides aa*$_3$-Cox [19] and *T. thermophilus ba*$_3$-Cox, [17] whereas its periplasmic part, mainly formed by the heme containing domains of CcoO and CcoP, is more surface-exposed than in other Cox enzymes (Fig. 2A). Moreover, highlighting the close evolutionary relationship between the *cbb*$_3$-Cox and NOR enzymes, the 3D structure of CcoN was found very similar to that of *Pseudomonas aeruginosa* NOR [60], even though the amino acid sequence conservation between these two proteins is below 40%.

CcoN forms a "clamshell"-like structure, with its amino (N-) and carboxyl (C-) termini located close to each other on the cytoplasmic side. CcoN also has two β strands that form a hairpin loop between the helices V and VI. Two calcium atoms with a proposed stabilizing role are found in the structure (Fig. 2B). One of these atoms interacts directly with the heme b_3-Cu_B catalytic center, while the second one is located between the loops IV and V at the edge of the structure. The clamshell structure surrounds the catalytic center located near the outer membrane surface and consists of a low spin heme *b*, a high spin heme b_3 and a Cu (Cu_B) atom. The open edge of the clamshell is close to heme *b*, whereas heme b_3 and Cu_B are at its distal end. Like in NOR, the hemes *b* and b_3 are linked together by a calcium atom, which is coordinated to the carboxyl groups of pyrrole D rings of both hemes. Like other Cox enzymes, three histidine residues coordinate the Cu_B atom, and one of them is covalently ligated to a tyrosine residue on helix VII (Fig. 2B). This linkage is absent in NOR, and in *aa*$_3$-Cox the ligating tyrosine residue is located on helix VI (Table 1) [19]. Like the type B enzymes, *cbb*$_3$-Cox contains only one proton pathway that is positioned similar to the K-channel in the type A enzymes. In *cbb*$_3$-Cox, hydrophobic residues block the equivalent of the D-channel, and moreover, none

Table 2
Various terminal oxidases and NOR in some pathogenic bacteria.

	cbb$_3$-Cox	NOR	*aa*$_3$-Cox	*bd*-type Qox
Campylobacter jejuni	✓	✓		✓
Helicobacter pylori J99	✓			
Neisseria gonorrhoeae	✓	✓		
Neisseria meningitidis	✓	✓		
Pseudomonas aeruginosa PAO1	✓	✓	✓	✓
Vibrio cholera	✓			✓

of the residues of the proposed proton pathway of cbb_3-Cox (Ser240, Tyr223, His243, Tyr317, Thr215, Tyr251 of CcoN and Glu49 of CcoP) is conserved in type A heme-Cu: O_2 reductases. However, Tyr223 and Tyr317 can be found in the predicted proton channel of type B enzyme ba_3-Cox of *T. thermophilus* [17]. Similar proton pathways have not been identified in NOR which does not pump protons, but it is noteworthy that some cbb_3-Cox also exhibit low NOR activity (Table 1) [61].

CcoO is a mono-heme *c*-type cytochrome, and has an N-terminally located transmembrane helix as an anchor (Table 1). It is thought to convey electrons to heme *b* of CcoN, and makes strong contacts with the α-helices of CcoN. In particular, CcoO contacts the calcium atom that coordinates heme *b* and heme b_3 at the catalytic center via its Ser102 residue. Of the two cavities that are visible on the 3D structure of cbb_3-Cox, one is close to the periplasmic face of the membrane, between CcoO and CcoN. This cavity is located at a position equivalent to the end of the D-channel of type A enzymes, and is proposed to provide an exit path for protons and water molecules from the catalytic site to the periplasm. The second cavity is membrane-embedded and connected by narrow and hydrophobic channels to the catalytic site, possibly providing an O_2 access pathway. An equivalent hydrophobic channel is also present in the NOR structure, and is suggested to allow access of NO to the catalytic site of this enzyme [60].

CcoP is a di-heme *c*-type cytochrome with both of its heme-groups solvent exposed. In the case of *P. stutzeri*, CcoP has two transmembrane helices connected via a long linker that makes multiple contacts to the cytoplasmic part of CcoN (Table 1). In some other organisms, including *R. capsulatus*, CcoP probably contains only one transmembrane helix as its anchor. The distal heme group of CcoP is thought to accept electrons from a soluble or membrane bound electron donor (*e. g.*, cytochrome c_2 or cytochrome c_y in *R. capsulatus*) and transfers them to the proximal heme of this subunit. This latter heme then conveys electrons to the heme group of CcoO, which transfers them to the catalytic center in CcoN.

CcoQ is a small subunit formed by a single transmembrane helix, and is not present in all cbb_3-Cox. It does not contain any cofactor, and its elimination does not completely abolish cbb_3-Cox activity. CcoQ is absent in *P. stutzeri* 3D structure, which instead contains an unassigned α-helix, located close to helices IX and XI of CcoN. The location of CcoQ in *R. capsulatus* cbb_3-Cox is unknown, but it can be cross-linked to CcoP, suggesting that it is associated with this subunit [62].

3. Assembly of the cbb_3-Cox

In general, Cox assembly is an intrinsically complex process because this oligomeric enzyme is membrane embedded and contains multiple cofactors [63]. Membrane insertion and maturation of individual subunits, insertion of cofactors, and assembly of cognate partners have to be coordinated to produce an active enzyme [63,64]. In eukaryotes, aa_3-Cox assembly involves more than 30 factors, which provide timely availability of mitochondrially-encoded subunits, or which associate transiently with various assembly intermediates [13,65]. Assembly of bacterial aa_3-Cox is possibly less complex, but still relies on specific components that mediate heme and Cu insertion into the subunits. Assembly of cbb_3-Cox is particularly challenging because the maturation of CcoO and CcoP require the *c*-type cytochrome maturation (Ccm) machinery, whereas CcoN relies on heme *b* and Cu atom insertion processes. A set of genes, *ccoGHIS* are located immediately downstream of *ccoNOQP* operon in most cbb_3-Cox containing bacteria [54,66–68]. The roles of *ccoGHIS* products in the assembly of cbb_3-Cox are described below.

3.1. An assembly pathway for the subunits of cbb_3-Cox and role of CcoH

Assembly of cbb_3-Cox has been studied in *R. capsulatus* membranes using blue-native polyacrylamide gel electrophoresis (BN-PAGE) analyses (Fig. 3A) [62,67,69]. This technique allows identification of membrane protein complexes in their native state, and is particularly suitable for examining abundant and stable respiratory chain complexes [70]. In *R. capsulatus* membranes, BN-PAGE, activity staining and immunodetection analyses showed that cbb_3-Cox forms an active complex of 230 kDa, which contains all four (CcoNOQP) structural subunits [67]. However, BN-PAGE does not necessarily reflect the correct molecular mass of a protein complex, as binding of lipids, detergent and Coomassie blue can interfere with the migration on BN-PAGE [71]. Indeed *R. capsulatus* cbb_3-Cox purified after detergent-solubilization migrates as an active 160 kDa complex on BN-PAGE (Pawlik et al., unpublished data).

Later on, it was also found that the putative assembly factor CcoH is a part of the 230 kDa complex (Fig. 3A) [69]. CcoH encodes a single-spanning membrane protein with an extended periplasmic domain that is suggested to serve as a dimerization domain [69]. CcoH was also found in the purified *R. capsulatus* cbb_3-Cox (Pawlik et al., unpublished results). Unlike the mitochondrial aa_3-Cox, which forms together with other respiratory complexes a network of supercomplexes [70], no similar large macromolecular assemblies were so far observed in the case of cbb_3-Cox in *R. capsulatus* membranes [67]. In addition to the active 230 kDa complex, using BN-PAGE one large (210 kDa) and one small (~40 kDa) inactive assembly intermediates were also detected in *R. capsulatus* membranes (Fig. 3A). The 210 kDa complex contained CcoN, CcoO and CcoH, but lacked CcoP [67,69], and the ~40 kDa complex had CcoP [67], CcoQ and CcoH [62,69]. The CcoO subunit in the 210 kDa, and the CcoP subunit in the ~40 kDa complexes contained their covalently attached heme groups as indicated by their peroxidase activity [67]. Thus, maturation of the *c*-type cytochrome subunits occurred prior to their assembly into cbb_3-Cox. Genetic data supported these findings, as mutants expressing truncated CcoP derivatives contained only the 210 kDa, and not the 230 kDa complex [67], and mutations abolishing the *c*-type cytochrome maturation process also prevented cbb_3-Cox assembly [72]. Although not essential, CcoQ seemed to improve cbb_3-Cox assembly as in its absence active enzymes were produced at reduced amounts. More recent chemical cross-linking data suggest that interactions between CcoQ and CcoP favor assembly of the ~40 kDa complex with the 210 kDa complex [62]. The surprising finding that CcoH is associated with the assembly intermediates of cbb_3-Cox [69] suggested that this protein behaved more like a *bona fide* subunit of *R. capsulatus* enzyme rather than an assembly factor. This is further supported by the observation that the steady-state stability of CcoH is strictly dependent on the presence of CcoNOQP [69]. Available data suggest that cbb_3-Cox assembly might occur via a fusion between the CcoNOH and CcoQPH subcomplexes mediated by CcoH (Fig. 3A) [67,69]. The 3D structure of cbb_3-Cox indicates that CcoO is sandwiched between CcoN and CcoP [14]. A similar assembly pathway has also been proposed for *B. japonicum* cbb_3-Cox, although CcoH was not included in this model [73].

3.2. Maturation of cbb_3-Cox subunits

3.2.1. CcoN: Roles of CcoS, CcoI and CcoG in hemes b and Cu incorporation

CcoN is an integral membrane protein and is most likely co-translationally inserted into *R. capsulatus* cytoplasmic membrane [74,75]. The two heme *b* and the Cu cofactor are deeply buried within CcoN, and their incorporation into this subunit might be critical for proper folding and stabilization of cbb_3-Cox. However, whether these cofactors are inserted co-translationally into CcoN, and if so, in which order, is unknown. A co-translational insertion process for hemes *a* and Cu cofactors for aa_3-Cox has been proposed [76], but not yet established.

In *R. capsulatus*, CcoS is a small membrane protein of 56 amino acids with no putative heme or Cu binding motifs, which is highly tolerant towards mutations of its conserved residues (Pawlik et al., unpublished results). A mutant lacking CcoS assembles an inactive cbb_3-Cox variant of 230 kDa that lacks both of the heme *b* cofactors

of CcoN, but contains properly matured CcoO and CcoP subunits [66]. This finding indicates that *c*-type cytochrome maturation is not affected, and further supports that the CcoN and *c*-type cytochrome maturation processes occur independently of each other [66]. Whether the inactive cbb_3-Cox variant present in a *R. capsulatus ccoS* knock out mutant contains Cu_B is unknown. Clearly, CcoS is required for proper maturation of CcoN, but defining its precise role in cbb_3-Cox assembly deserves further studies.

In both mitochondria and bacteria, heme insertion into aa_3-Cox is mediated by Surf1 (called Shy1 in yeast), which accepts heme *a* directly from heme *a* synthase (CtaA) and is thought to transfer it to subunit I [77]. In the absence of Surf1, aa_3-Cox assembly is significantly reduced, but not completely abolished [78–80] implying that Surf1 is important, but not essential for heme *a* insertion into the subunit I. Moreover, Surf1 homologues are not present in all species that contain heme *a* containing Cox (*e. g.* they are missing in *Bacillus subtilis* and *T. thermophilus*). In the case of *T. thermophilus*, a protein called CbaX, which has no homology to Surf1, was implicated in heme *a* insertion into ba_3-Cox [81]. A Surf1 homologue is absent in *R. capsulatus* genome, and a *R. sphaeroides* mutant that lacks SurfI is impaired only in the assembly of aa_3- but not cbb_3-Cox [78]. Available data indicate that Surf1 is dedicated to heme *a*, and not involved in heme *b* insertion. In fact, how heme *b* is inserted into any membrane protein is still enigmatic.

Copper is an essential cofactor of many enzymes such as heme-Cu: O_2 reductases, Cu-Zn superoxide dismutase, multicopper oxidase (or laccase) and tyrosinase. Like many metals, free Cu is basically undetectable in cells, minimizing the risk of reactive oxygen species production. Hence, Cu acquisition, trafficking, storage, and delivery to the target sites are strictly controlled processes [82]. Cu can be found in two redox states: the reduced Cu^+ is considered to be the transported form while the oxidized Cu^{+2} is probably the catalytically active form. In eukaryotes, the Cu transporter (Ctr) proteins represent the main Cu importers, and they have been studied intensely [83,84]. How Cu is imported into the bacterial cytoplasm is not clear. In the Gram-positive *Enterococcus hirae* a universally conserved P-type ATPase [84], and in *B. subtilis* the membrane protein YcnJ [85] was suggested to import Cu. In addition, two P-type ATPases are required to import Cu into cyanobacteria (*i. e.,* CtaA and PacS) [86,87] and into the thylakoids of plant chloroplasts (*i. e.,* PAA1/HMA6 and PAA2/HMA8) [86,87]. Very recently, CcoA, a novel member of the major facilitator superfamily (MFS) of membrane proteins, was implicated in Cu import in *R. capsulatus* (Ekici et al., unpublished results, see below) and *Schizosaccharomyces pombe* [88]. The MFS-type transporters are structurally and functionally distinct from the Ctr-type Cu transporters, and might illustrate a novel Cu uptake system.

Different to Cu import pathways, bacterial Cu efflux pathways are intensely studied, mainly motivated by the toxicity of Cu for all organisms. A major Cu efflux mechanism is provided by P-type ATPases (P1B subgroup). P-type ATPases are integral membrane pumps that hydrolyse ATP for maintaining ion homeostasis, electrochemical gradients and lipid asymmetry [89]. The P-type ATPase superfamily is composed of 11 distinct subgroups of which the P1B is one of the largest and most widespread. P1B-type ATPases use ATP hydrolysis for Cu extrusion across the cytoplasmic membrane [90–93], and contain the characteristic motifs of P-type ATPases, including the ATP binding domain, the phosphorylation domain, and the phosphatase domain [94]. They also contain an N-terminal heavy metal binding domain (HMBD) with the conserved Cu-binding motif (MXCXXC), the membrane embedded ion translocation (CPX)-motif and the conserved histidine-proline (HP)-motif [94]. The mechanism of loading cytosolic Cu to Cu-ATPases is not well understood, but HMBD domains are thought to interact with specific metallochaperones to deliver Cu atoms to these transporters [95,96]. The recent resolution of the first 3D structure of a P1B-type ATPase from *Legionella pneumophila* revealed a putative docking platform for Cu chaperones [97]. The P1B-type ATPases pump Cu^+ out of the bacterial cytoplasm, but their specific roles seem to be determined by their rate of Cu efflux [98]. For example two of them, CopA1 and CopA2 of *P. aeruginosa*, are highly homologous to each other (35% identity and 50% similarity), yet their functions differ. CopA1 has a faster Cu efflux rate and is required for Cu detoxification as its absence renders cells highly sensitive to Cu. CopA2 has a much slower Cu efflux rate and its absence does not affect cellular Cu sensitivity, but leads to the loss of cbb_3-Cox activity [99].

CcoI is a homologue of CopA2, and is the product of *ccoI* gene located in the *ccoGHIS* cluster in many species [54,68]. In *B. japonicum* and *R. capsulatus*, CcoI appears to be specifically required for cbb_3-Cox assembly, but a *Rubrivivax gelatinosus* mutant lacking the CcoI homologue CtpA seems to be defective in other Cu containing enzymes such as caa_3-Cox and N_2O reductase as well [100]. In *R. capsulatus*, absence of CcoI [66] or mutations in its N-terminal HMBD domain or CPC motifs, drastically reduce the steady-state amounts of cbb_3-Cox subunits [66] (Pawlik et al., unpublished results). Cu supplementation does not suppress the cbb_3-Cox defect of a mutant lacking CcoI, which is also not sensitive to Cu [66]. Whether Cu_B is inserted into CcoN before or after heme *b* is not known. In case of aa_3-Cox, the availability of enzyme variants containing heme *a*, but lacking Cu_B suggests that heme *a* is likely to be inserted before Cu_B [78].

The essential role of CcoI for cbb_3-Cox assembly suggests a model that involves transport of Cu^+ from the cytoplasm to the periplasm, then its concomitant or subsequent insertion into CcoN (Fig. 3B). However it is intriguing that a mutant lacking CcoI is not rescued by exogenous Cu addition, because Cu is thought to be diffused freely across the outer membrane into the periplasm. A possibility is that either Cu^+ transported by CcoI is inserted into CcoN without its full transport across membrane, or that it is delivered upon transport to specific periplasmic Cu chaperones (perhaps SenC or PCu_AC, see below), which then supplies it to CcoN (Fig. 3B). This model would rationalize the cytoplasmic origin of Cu, and suggests that in the absence of CcoI, its loading onto the specific chaperone might be inefficient.

Among the *R. capsulatus* mutants lacking any one of the *ccoGHIS* products, those devoid of CcoG exhibited the mildest effect on cbb_3-Cox assembly. In the absence of CcoG, all subunits were present at quasi wild type levels, and the enzyme activity was not significantly reduced [66]. CcoG is an integral membrane protein, which has five predicted transmembrane helices and two putative [4Fe-4S] cluster-binding motifs, but whether it contains an iron-sulfur cluster has not been shown experimentally. The exact function of CcoG remains unknown. It has been proposed that it might be involved in intracellular oxidation of Cu^+ to Cu^{2+} [68]. Another possibility is that CcoG might reduce Cu^{2+} to Cu^+ to provide the substrate for CcoI. If so, then CcoG might work together with the putative MFS-type Cu importer CcoA, provided that CcoA imports Cu^{2+} into the cytoplasm (as suggested by the insensitivity of *ccoA* mutant to silver which mimics Cu^+) (Ekici et al., unpublished results) (Fig. 3B). Clearly, both the structure and function of CcoG are poorly defined and warrant further studies.

3.2.2. CcoO and CcoP: c-type cytochrome maturation (Ccm) process

The CcoO and CcoP subunits of cbb_3-Cox are matured by a *c*-type cytochrome maturation (Ccm) process, which operates independently from CcoN maturation. Ccm is a post-translation and post-translocation process that occurs on the periplasmic face of the cytoplasmic membrane (Fig. 4). During this process, heme *b* groups are covalently and stereospecifically attached by thioether bonds formed between their vinyl groups and the cysteine thiols of conserved CXXCH motifs of apocytochromes [101,102]. Several Ccm systems are encountered in nature, and in α- and γ-proteobacteria and *Deinococcus* species maturation of the CcoO and CcoP subunits relies on Ccm-system I, which was reviewed

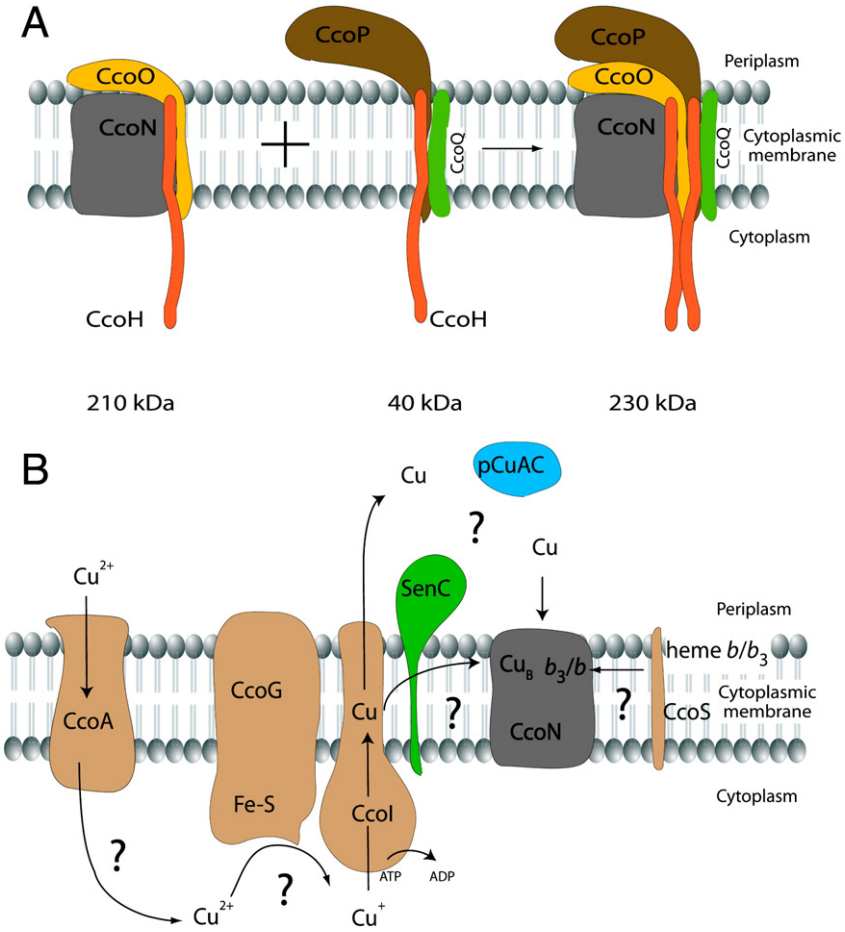

Fig. 3. A. An assembly scheme for cbb_3-Cox *in R. capsulatus*. Using BN-PAGE, active cbb_3-Cox is detectable in *R. capsulatus* membranes as a 230 kDa complex. Assembly of the 230 kDa complex proceeds via two inactive assembly intermediates: a 210 kDa subcomplex that contains the catalytic subunit CcoN, the mono-heme cytochrome *c* subunit CcoO and the single-spanning membrane protein CcoH, and a 40 kDa subcomplex composed of the di-heme cytochrome *c* subunit CcoP, the small subunit CcoQ and a second copy of CcoH. Full assembly of cbb_3-Cox is probably achieved by the dimerization of the cytoplasmic domains of the two CcoH. CcoH was originally considered to function as an assembly factor, but recent data demonstrate that it is a stable component of the fully assembled cbb_3-Cox. The *c*-type cytochromes in both assembly subcomplexes have their heme groups attached indicating that *c*-type cytochrome maturation preceded the formation of the assembly intermediates. B. A tentative model for the maturation of CcoN subunit. The maturation of CcoN requires Cu and heme *b* insertions. Cu is probably imported into the cytoplasm of *R. capsulatus* by the major facilitator superfamily (MFS) protein CcoA as oxidized Cu^{2+}. On the cytoplasmic face of the membrane Cu^{2+} is reduced to Cu^{+} by the ferredoxin-like protein CcoG and transported back to the periplasmic face of the membrane by the P1B-type ATPase CcoI. CcoI then supplies Cu^{+} to CcoN either directly within the membrane or from the periplasm, or via some putative periplasmic Cu binding proteins such as SenC and PCu_AC. The insertion of heme *b* and heme b_3 into CcoN is probably mediated by the assembly factor CcoS.

in detail recently [103,104]. Ccm-system I can be divided into three operation modules that are described briefly below.

3.2.2.1. Module 1: Transport and relay of heme. The function of this module is to translocate cytoplasmically synthesized heme *b* to the periplasm, and to prepare it for ligation to apo-cytochromes (Fig. 4, right). This module consists of five proteins, named CcmABCDE. CcmABCD is suggested to form an ATP binding cassette (ABC)-type transporter involved in loading heme *b* to CcmE. CcmE has a single membrane-anchoring helix and a conserved histidine residue that covalently binds heme, which then acts as a heme donor to apocytochromes. CcmA has an ATP binding domain and Walker A and B motifs needed for ATPase activity. CcmB is an integral membrane protein with six transmembrane helices, required for membrane localization of CcmA. CcmC contains a conserved a tryptophan-rich WWD motif and is involved in loading heme to CcmE, and apparently CcmD enhances this process. A recent bioinformatics study grouped CcmC and its homologues as the "heme handling proteins" (HHP) [105]. To what extent CcmAB and CcmCD function together or separately for loading heme onto CcmE and delivering heme-loaded CcmE to the heme ligation complex is unclear. A possibility is that CcmC and CcmD are necessary for attachment of heme to CcmE [106–108], and that CcmAB is required for ATP dependent release of heme-loaded CcmE from CcmCD [109]. On the other hand, whether CcmAB has another, yet to be defined, role in this process is unknown [110,111].

3.2.2.2. Module 2: Apocytochrome thio-oxidoreduction and chaperoning. Like most *c*-type apocytochromes, CcoO and CcoP are thought to be secreted via the SecYEG secretory pathway (Fig. 4, left). Their signal-anchor sequences are not processed, and serve as their N-terminal membrane-anchors. Upon translocation across the cytoplasmic membrane, the DsbA-DsbB dependent oxidative protein-folding pathway [112,113] is thought to rapidly oxidize the thiol groups of apocytochromes to possibly avoid their proteolytic degradation. The disulfide bonds formed between the cysteines at the conserved heme binding (CXXCH) motifs need to be reduced prior to heme *b* attachment. Thus, a Ccm-specific thioreduction pathway involving CcdA, CcmG and CcmH proteins has been proposed [114,115]. CcdA is an integral membrane protein with six transmembrane helices and is responsible for conveying from the cytoplasm to the periplasm reducing equivalents required for this process. CcmG and CcmH are membrane-anchored thioredoxin-like proteins that contain a single CXXC domain facing the periplasm [115], and thought to reduce the

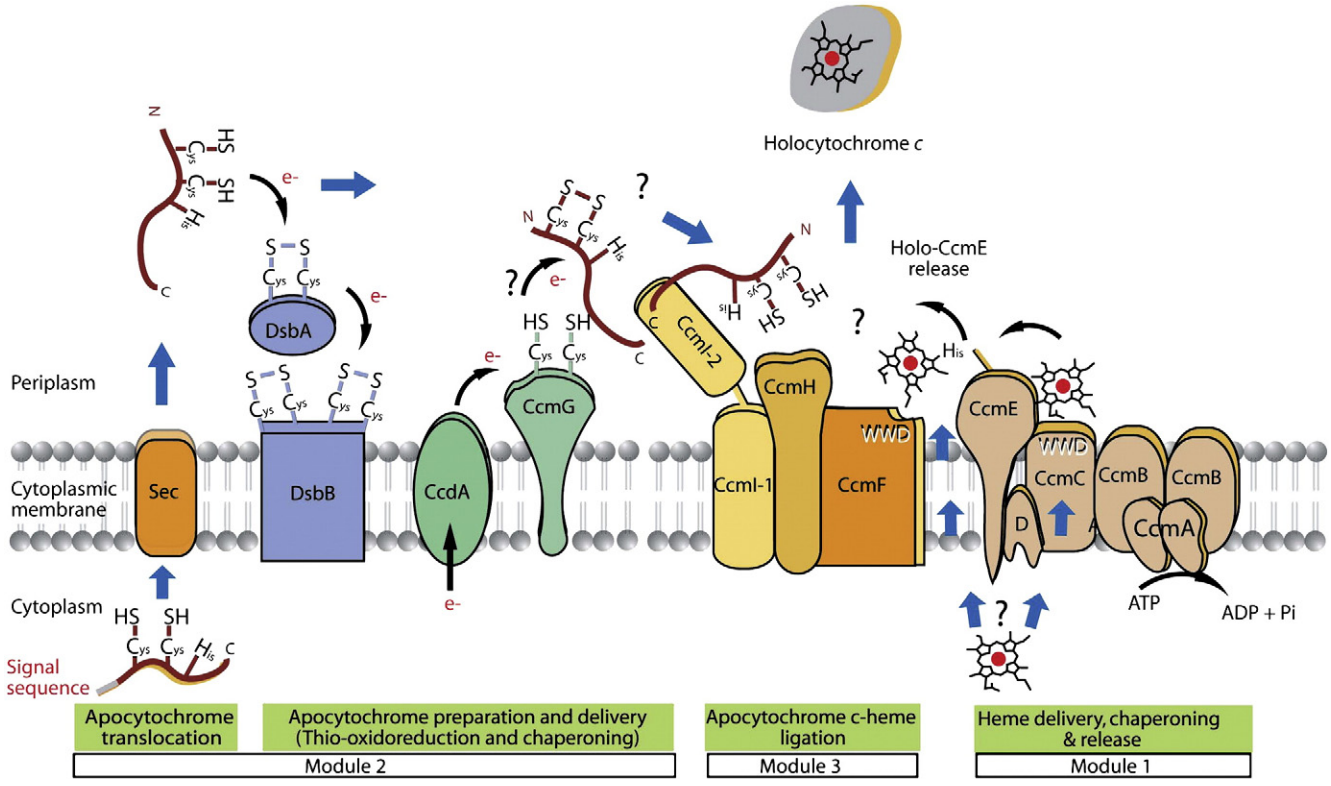

Fig. 4. Cytochrome *c* maturation (Ccm) system in *R. capsulatus*. The cytochrome *c* maturation components can be divided into three modules. First module (right) is the transport of heme and its preparation for ligation to apocytochrome that involves CcmABCD proteins which forms a ABC-type transporter that has a role in the delivery of heme to the heme chaperone CcmE before the ligation. It is unknown whether heme is transported via CcmABCD complex or by another unknown protein. CcmA and CcmB are required for the release of heme-bound CcmE from CcmC and CcmD. CcmC and CcmD are involved in the heme attachment to CcmE. Second module (left) involves the apocytochrome thioredox and chaperoning processes. After the translocation of apocytochrome into the periplasm, the cysteine thiols are first oxidized by DsbA-DsbB, then reduced by CcdA, CcmG and/or CcmH. Third module (middle) consists of CcmHIF which is the heme ligation core complex. CcmI binds to C-terminal part of apocytochrome to deliver it to the core complex for the catalysis of the thioether bond formation between reduced apocytochrome and heme vinyl groups forming the mature cytochrome *c* (adapted from [155]).

disulfide bonds at the heme binding sites. Of the three components, CcdA and CcmG are not required in the absence of DsbA-DsbB dependent thio-oxidation pathway, but CcmH is still required for *c*-type cytochrome production, suggesting that it has an additional role. Moreover, the 3D structure of CcmG showed that, in addition to its canonical thioredoxin fold, it has a cavity that might bind apocytochromes which is in accordance with a putative holdase role of CcmG as shown in *R. capsulatus* [116]

3.2.2.3. Module 3: Apocytochrome and heme b ligation. In *R. capsulatus,* CcmF, CcmH and CcmI proteins have been proposed to form a heme ligation core complex (Fig. 4, middle), bases on reciprocal co-purification experiments documenting protein–protein interactions between these components [117]. Of these proteins, CcmF is a large integral membrane protein with 11 transmembrane helices, with a trytophan-rich (WWD) signature motif and four conserved histidine residues facing the periplasm proposed to bind heme *b* before its ligation to apocytochromes [106]. It interacts with heme-loaded CcmE [118], is a heme handling protein (HHP) like CcmC [106]. CcmI is a bipartite protein that contains a membrane embedded N-terminal region with two transmembrane helices and a cytoplasmic leucine-zipper-like motif containing loop (CcmI-1 domain) and a large periplasmic C-terminal extension with tetratricopeptide repeat (TPR)-like motifs (CcmI-2 domain) [119–121]. Unlike most other bacteria, in *E. coli* the homologue of CcmI-2 domain is fused to the C-terminal end of CcmH, leaving only CcmF and a modified "CcmH" as components of heme ligation core complex [122]. *R. capsulatus*, mutants lacking CcmI can be suppressed by overproduction of CcmF and CcmH or CcmG. Complementation studies with two distinct domains of CcmI indicated that CcmF and CcmH overproduction relates to the functional role of CcmI-1 domain [123], whereas CcmG overproduction to that of CcmI-2 [120]. These findings suggested that CcmI might be a junction point between the CcdA-CcmG dependent thio-reduction and CcmF-CcmH dependent heme ligation processes. Indeed, CcmI was initially proposed to chaperone apocytochromes to the heme ligation complex [121], and very recently *in vitro* studies provided strong biochemical evidence to support this proposal [156]. Protein–protein interaction studies conducted using purified CcmI and purified apocytochrome c_2 indicated that the C-terminal CcmI-2 domain of CcmI recognizes and binds tightly the most C-terminal helix of apocytochrome c_2 in the absence of heme *b*. The folding process of cytochrome *c* (and cytochrome c_2) [124] indicates that their most C-terminal helix interacts with their most N-terminal heme binding helix to form a stable folding intermediate that can trap heme *b* non-covalently [125]. Altogether, these observations led to the proposal that at least some *c*-type apocytochromes are first recognized via their C-terminal helices by the periplasmic CcmI-2 domain of the CcmFHI core complex (Fig. 4, middle), and then released from this domain upon transfer of heme *b* from CcmF to apocytochrome, and subsequently, the thioether bonds are formed. Once the thioether bonds are formed, cytochrome *c* folds into its final structure.

Recently, another type of post-translational modification was seen in CcoP of pathogenic *Neisseria* species [126]. The CcoP subunit of *N. gonorrhoeae* contains a O-linked glycosylation in its periplasmic moiety [126]. The role of this glycosylation, perhaps important for pathogenesis, remains to be determined. It is noteworthy that O-linked glycosylation was also observed for ScoI (see below SenC) and cytochrome CycB, which are involved in cbb_3-Cox assembly and electron transfer to cbb_3-Cox, respectively [126]. Whether this or other kinds of modification of cbb_3-Cox subunits also occurs in other species,

and whether this affects biogenesis of this enzyme deserves further studies.

3.2.3. Additional proteins involved in cbb_3-Cox biogenesis

Several additional proteins of not yet fully defined roles, including SenC [127], PCu_AC [128,129], DsbA [130] and CcoA (Ekici et al., unpublished results) might be involved in cbb_3-Cox biogenesis, and described below.

3.2.3.1. SenC (ScoI). SenC is a membrane-anchored protein with a single transmembrane helix, and in its absence, cbb_3-Cox biogenesis in *R. capsulatus* [127] and in *P. aeruginosa* [131] is drastically decreased and cbb_3-Cox activity is regained upon addition of exogenous Cu. Its periplasmic domain contains a thioredoxin fold and a conserved Cu binding motif comprising CxxxC and a His ligand [127]. SenC is a close homologue of the universally conserved ScoI protein, which has been implicated mainly into the assembly of the Cu_A center of subunit II of aa_3-Cox. Whether ScoI acts as a direct donor of Cu to subunit II [132], or is a thiol:disulfide oxidoreductase reducing appropriate cysteines of subunit II for subsequent Cu delivery by another Cu chaperone [129] is not yet clear [133]. *B. japonicum* mutants lacking SenC were impaired in the assembly of aa_3- but not cbb_3-Cox and showed reduced symbiotic N_2 fixation [134]. Similarly, *R. sphaeroides* mutants lacking PrrC (a SenC homologue) had no effect on cbb_3-Cox assembly although they were defective in photosynthetic growth [52]. Like *R. capsulatus senC, R. sphaeroides prrC* is located next to *prrAB* (homologues of *R. capsulatus regAB*) genes that encode a two component regulatory system controlling energy processes, including photosynthesis [46,135]. Whether *prrC* mutations have any polar effect on downstream *prrB* gene, indirectly interfering with cellular amounts of PrrB, is not known (Fig. 1).

Several studies have shown that the role of prokaryotic ScoI homologues is not restricted to their involvement in aa_3-Cox assembly. A role of these proteins in oxidative stress response [136] or photosynthetic gene regulation [52] has been proposed. Bioinformatics based genome surveys indicated that ScoI homologues are also present in many bacterial species, like *R. capsulatus*, which do not have a Cu_A containing Cox [133]. *R. capsulatus* mutants lacking SenC produce very low amounts of cbb_3-type Cox [127,137], a phenotype which was rescued by addition of exogenous Cu [127]. *In vitro* experiments showed that in a mutant lacking SenC cbb_3-Cox assembly proceeded at a significantly reduced level (Lohmeyer et al., unpublished results). Very recent chemical cross-linking studies indicated that SenC interacts with CcoP and CcoH *in vivo*, suggesting that it is involved directly in cbb_3-Cox assembly (Lohmeyer et al., unpublished results) (Fig. 3B).

3.2.3.2. PCu_AC (DR1885). The periplasmic Cu_A-chaperone (PCu_AC) was first identified in *Deinococcus radiodurans* and its structural gene is located near ScoI [128]. In this species, PCu_AC is thought to provide Cu to the Cu_A center after ScoI reduces the appropriate disulfide bond at subunit II of aa_3-Cox. In the case of *T. thermophilus* PCu_AC is involved in Cu transfer to Cu_A of ba_3-Cox [129]. Homologues of PCu_AC are also found in many species that lack aa_3-Cox such as *R. capsulatus*. Unlike *D. radiodurans*, in *R. capsulatus* the structural gene of PCu_AC is not located near that of SenC, but its product contains similar conserved metal binding motifs. In this species whether PCu_AC affects cbb_3-Cox Cu_B center assembly is unknown as no chromosomal PCu_AC knockout mutant could be obtained so far (Fig. 3B).

3.2.3.3. DsbA. *R. capsulatus* mutants lacking the periplasmic thiol:disulfide oxidoreductase DsbA, primarily involved in oxidative protein folding pathway [112], overproduce the periplasmic protease DegP [138]. They have pleiotropic phenotypes, including temperature sensitivity for growth (35 °C), osmosensitivity, filamentation and decreased respiratory capabilities. Even at permissive growth temperature (25 ° C), they exhibit reduced cbb_3-Cox activity. Supplementation of the growth medium with redox active chemicals like cysteine/cystine or Cu^{2+}, restores both temperature sensitive growth and cbb_3-Cox defects of mutants lacking DsbA. Remarkably, DsbA knock out mutants revert frequently to regain their growth ability at 35 °C without any need for redox active supplements, and concomitantly, they restore their cbb_3-Cox production. These revertants acquire mutations in *degP* that decrease drastically the protease activity of DegP [138]. These findings point to important links between the formation of disulfide bond, degradation of misfolded periplasmic proteins, and production of active cbb_3-Cox. In the absence of DsbA, whether exogenously supplied Cu^{2+} acts exclusively as a source of oxidant, or also eases acquisition of Cu as a missing cofactor for cbb_3-Cox production, or both is unknown. Future studies might clarify these emerging links between major cellular processes and cbb_3-Cox biogenesis.

3.2.3.4. CcoA. Very recent studies of *R. capsulatus* mutants that exhibit "Cu supplement dependent cbb_3-Cox production" phenotype yielded a novel component, CcoA involved in cbb_3-Cox biogenesis (Ekici et al., unpublished results). These mutants lacked cbb_3-Cox activity on Cu-depleted media, but regained it upon exogenous Cu^{2+} supplementation. Molecular genetic studies established that these mutants were defective in an open reading frame (ORF) of previously unknown function, now named *ccoA* due to its role in cbb_3-Cox biogenesis. CcoA is an integral membrane protein formed by two subdomains of six transmembrane helices each, which are separated by a large cytoplasmic loop. Its sequence is highly homologous to the Major Facilitator Superfamily (MFS) type transporters [139]. It contains the AVYGRR and ARFGRE between its second-third and eight-nine transmembrane helices, respectively. These motifs are highly similar to DRXGRR motifs that characterize members of the MFS family. In addition, unlike many other MFS members, CcoA contains a putative metal (Cu) binding (MX_xM) motif as found in Ctr type Cu importers [140], suggesting a role in metal transport. In *R. capsulatus* mutants lacking CcoA, transcription and translation initiation of *ccoNOQP* occur, but the steady-state amounts of cbb_3-Cox subunits are quasi-absent (Ekici et al., unpublished results). Thus, absence of CcoA perturbs a co- or post-translational step(s) of cbb_3-Cox biogenesis. Remarkably, supplementation with Cu^{2+}, but not with other metals like Mn^{2+}, Zn^{2+} or Fe^{3+}, palliates the cbb_3-Cox production defect. CcoA knock out mutants are not sensitive to Cu^{2+}, but their cellular Cu content is lower than in normal cells, and they revert very frequently to restore their cbb_3-Cox activity. These by-pass suppressors restore normal cellular Cu content and production of cbb_3-Cox. Surprisingly, they also become hypersensitive to Cu^{2+} but not to other metals, including Ag^{+}. These findings suggest that the MFS-type transporter CcoA may be involved in Cu acquisition by a hitherto unknown pathway, which seems needed for normal biogenesis of cbb_3-Cox. Very recently, the Mfc1 protein of *Schizosaccharomyces pombe*, which is also a MFS-type transporter with a MX_xM motif and homologous to CcoA, was shown to import Cu across the plasma membrane [88]. In *S. pombe*, the Ctr-type transporters are thought to mediate high affinity Cu uptake, and Mfc1 might provide an additional low affinity Cu import route. Clearly, future studies of this novel family of MFS-type putative Cu importers might provide invaluable information about bacterial Cu acquisition pathways and their link to cbb_3-Cox biogenesis.

3.2.4. Role of membrane lipids for cbb_3-Cox biogenesis

Membrane lipids, especially those that act as "lipochaperones", are important determinants for membrane protein structure and activity [141]. These lipids bind to specific locations of proteins in stochiometric amounts, and influence their folding, stability, steady-state amounts and activity [142]. Phospholipids were shown to co-crystallize with heme-Cu: O_2 reductases from many different species [143–145], and

the stability and organization of respiratory chain super complexes were significantly influenced by some phospholipids like cardiolipin [146,147]. A specific role of lipids on biogenesis and catalytic activity of cbb_3-Cox is also likely, as changes in membrane lipid composition seem to affect the amounts of cbb_3-Cox found in *R. capsulatus* membranes [148]. Moreover, different lipid environments were also shown to affect carbon monoxide binding to CcoP [149]. *R. capsulatus* mutants that were defective in ornithine lipid (OL) biosynthesis lacked cbb_3-Cox [148]. Studies of these mutants defined two genes, *olsA* and *olsB* coding for N-acyltransferase and O-acyltransferase, respectively, which are both required for OL biosynthesis [148,150]. OL is a non-phosphorus membrane lipid that usually accounts only for a small fraction of the total lipids found in bacteria. In some species, OL are synthesized under phosphate limiting conditions, and are used as replacement for phosphate containing lipids [151–153]. In *R. capsulatus* OL biosynthesis is not regulated by phosphate availability, but absence of OL affects the steady-amounts of a group of membrane proteins, including most of the *c*-type cytochromes. Mutants unable to synthesize OL have very low amounts of cbb_3-Cox and the electron carrier cytochromes c_2 and c_y. They contain a small amount of cytochrome c_1, and consequently, very low ubiquinol: cytochrome c_2 oxidoreductase (cytochrome bc_1) activity. Interestingly, these defects are both temperature- and growth medium-dependent. Especially on enriched media at regular growth temperature (35 °C), mutants lacking OL are photosynthesis-incompetent and have no cbb_3-Cox. Pulse-chase studies conducted using cytochrome c_y indicated that absence of OL decreased drastically its cellular amount [148], suggesting that in the absence of OL, a group of proteins including cbb_3-Cox, may be more prone to misfolding and enhanced degradation.

4. Summary and future prospects

In recent years, enormous amount of progress has been accomplished in studies aimed at understanding the biogenesis of cbb_3-Cox. This second most common class of heme-Cu: O_2 reductases is becoming attractive for future studies due to the availability of an excellent model system like *R. capsulatus* providing molecular genetic analyses, a 3D structure at high resolution allowing refined structural and functional inquiries, and exquisite biochemical approaches developed and practiced in various laboratories. Salient recent accomplishments on this topic include an emerging subunit assembly process highlighting the indispensible role of CcoH as a "subcomplex assembler", the better defined role of CcoI subgroup among the P1B-type ATPases as a Cu exporter to cbb_3-Cox, and possibly to a few other Cu containing enzymes. Furthermore, the discovery of a periplasmic Cu chaperone PCu_AC, the emerging interactions between the ScoI homologue SenC/PrrC and cbb_3-Cox subunits and its assembly factors, as well as the novel MFS-type putative Cu importer CcoA, whose Cu acquisition functions correlate with the production of cbb_3-Cox, provide exciting novel insight into the complexity of binuclear center assembly. An attempt was made to organize all known components into a highly speculative working scheme that outlines an emerging path of Cu delivery to the heme-Cu_B center of cbb_3-Cox (Fig. 3B). Indeed, much work is needed to probe experimentally the validity of the various facets of this working model under construction. Currently, we are reaching a stage where the crucial players required for cbb_3-Cox assembly are becoming identified, and ongoing molecular genetic and biochemical studies are now initiating research aimed to untangle the sophisticated and unique biogenesis process of cbb_3-Cox.

Acknowledgments

This work was supported by grants DOE 91ER20052 and NIH GM38239 to F.D., and by grants from the German Science Foundation (DFG-GRK1478) and from the German-French PhD college (DFH/UFA) on "Membranes and Membrane Proteins" to H.G.K.

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