# Biogenesis of *cbb₃*-type cytochrome *c* oxidase in *Rhodobacter capsulatus*

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## Abstract

The *cbb₃*-type cytochrome *c* oxidases (*cbb₃*-Cox) constitute the second most abundant cytochrome *c* oxidase (Cox) group after the mitochondrial-like *aa₃*-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 *cbb₃*-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 *cbb₃*-Cox enzyme. In this review we summarize our current knowledge on the structure, regulation and assembly of *cbb₃*-Cox, and provide a highly tentative model for *cbb₃*-Cox assembly and formation of its heme $b_3$-$Cu_B$ binuclear center.

## Keywords

*cbb₃*-type cytochrome *c* oxidase; co- or post-translational cofactor insertion; assembly of membrane proteins; copper acquisition; photosynthesis and respiration; energy transduction

## 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

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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 ageing [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 (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 *Rhodobacter 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 *P. denitrificans*, *R. sphaeroides* and bovine heart mitochondria; $ba_3$-Cox from *T. thermophilus*, $bo_3$-Qox from *E. coli* and the $cbb_3$-Cox from *Pseudomonas stutzerii*) (Fig. 2A) [14–19] reveal a remarkably conserved Cu and heme cofactors

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 *B. 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 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 gonorrhoea* [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 ceacum being primarily colonized by obligate anaerobic bacteria, *C. jejuni* is likely to be exposed to anaerobic conditions transiently. 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 genes 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 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 *Pseudomonas stutzerii 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 the 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 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 CcoN α-helices. 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. stutzerii*, 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 *cbb₃*-Cox. It does not contain any cofactor, and its elimination does not completely abolish *cbb₃*-Cox activity. CcoQ is absent in *P. stutzerii* 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 cbb₃*-Cox is unknown, but it can be cross-linked to CcoP, suggesting that it is associated with this subunit [62].

## 3. Assembly of the *cbb₃*-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, *aa₃*-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 *aa₃*-Cox is possibly less complex, but still relies on specific components that mediate heme and Cu insertion into the subunits. Assembly of *cbb₃*-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 *cbb₃*-Cox containing bacteria [54, 66–68]. The roles of *ccoGHIS* products in the assembly of *cbb₃*-Cox are described below.

### 3.1 An assembly pathway for the subunits of *cbb₃*-Cox and role of CcoH

Assembly of *cbb₃*-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 *cbb₃*-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 cbb₃*-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 cbb₃*-Cox (Pawlik et al., unpublished results). Unlike the mitochondrial *aa₃*-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 *cbb₃*-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

#### 3.2.2 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 mutants 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 *B. 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. subtilus* the membrane protein YcnJ [85] were 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 (PIB 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 PIB is one of the largest and most widespread. PIB-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)- and the conserved histidine-proline (HP)-motifs [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 PIB-type ATPase from *Legionella pneumophila* revealed a putative docking platform for Cu chaperones [97]. The PIB-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 *Pseudomonas 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 *cbb₃*-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 *cbb₃*-Cox assembly, but a *Rubrivivax gelatinosus* mutant lacking the CcoI homologue CtpA seems to be defective in other Cu containing enzymes such as c*aa₃*-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 *cbb₃*-Cox subunits [66] (Pawlik et al., unpublished results). Cu supplementation does not suppress the *cbb₃*-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 *aa₃*-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 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 PCuAC, 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 stereo-specifically 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 in detail recently [103, 104]. Ccm-system I can be divided into three operation modules that are described briefly below.

**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].

**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 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]

**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 (Verissimo et al, unpublished results). 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 *cbb₃*-Cox assembly and electron transfer to *cbb₃*-Cox, respectively [126]. Whether this or other kinds of modification of *cbb₃*-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 *cbb₃*-Cox biogenesis

Several additional proteins of not yet fully defined roles, including SenC [127], PCuAC [128, 129], DsbA [130] and CcoA (Ekici et al., unpublished results) might be involved in *cbb₃*-Cox biogenesis, and described below.

**SenC (ScoI):** SenC is a membrane-anchored protein with a single transmembrane helix, and in its absence, *cbb₃*-Cox biogenesis in *R. capsulatus* [127] and in *P. aeroginosa* [131] is drastically decreased and *cbb₃*-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 *aa₃*-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 *aa₃*- but not *cbb₃*-Cox and showed reduced symbiotic $N_2$ fixation [134]. Similarly, *R. sphaeroides* mutants lacking PrrC (a SenC homologue) had no effect on *cbb₃*-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 *aa₃*-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 *cbb₃*-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 *cbb₃*-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 *cbb₃*-Cox assembly (Lohmeyer et al., unpublished results) (Fig. 3B).

**PCuAC (DR1885):** The periplasmic CuA-chaperone (PCuAC) was first identified in *Deinococcus radiodurans* and its structural gene is located near ScoI [128]. In this species, PCuAC is thought to provide Cu to the $Cu_A$ center after ScoI reduces the appropriate disulfide bond at subunit II of *aa₃*-Cox. In the case of *T. thermophilus* PCuAC is involved in Cu transfer to $Cu_A$ of *ba₃*-Cox [129]. Homologues of PCuAC are also found in many species that lack *aa₃*-Cox such as *R. capsulatus*. Unlike *D. radiodurans*, in *R. capsulatus* the

structural gene of PCuAC is not located near that of SenC, but its product contains similar conserved metal binding motifs. In this species whether PCuAC affects $cbb_3$-Cox $Cu_B$ center assembly is unknown as no chromosomal PCuAC knockout mutant could be obtained so far (Fig. 3B).

**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.

**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) of 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 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 *Schizosaccahromyces 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 lipids environments were also shown to affect carbonmonoxide 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 PIB-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 PCuAC, 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

## 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 |

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**Highlights**

- Structure-function, regulation and biogenesis of $cbb_3$-type cytochrome $c$ oxidases are reviewed.

- An emerging multistep assembly pathway of the subunits of this membrane enzyme is discussed.

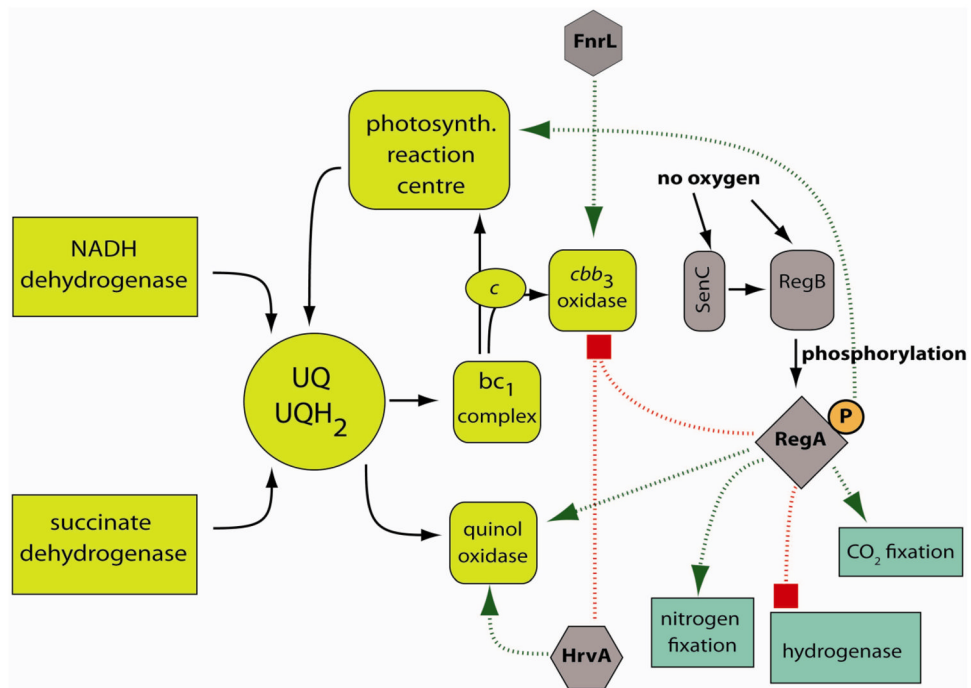- A tentative model for formation of the heme $b_3$-$Cu_B$ catalytic center of this enzyme is proposed.

**Figure 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 grey. 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.
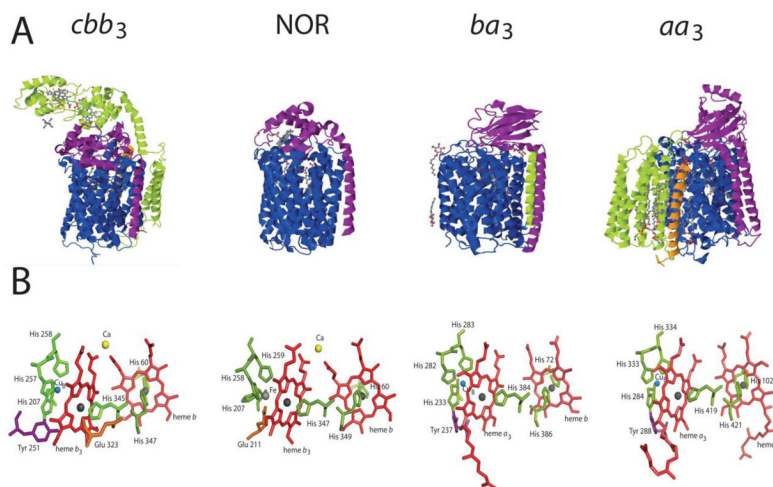
**Figure 2.**
**A.** 3D structures of different types of heme-Cu:$O_2$ reductases and NO reductase: Catalytic subunit I blue (*cbb₃*-Cox CcoN), subunit II (*cbb₃*-Cox CcoO) – magenta, subunit III (*cbb₃*-Cox CcoP) – green, additional subunit –orange. **B.** Architecture of the catalytic binuclear center 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 grey, non-heme iron – light grey, calcium – yellow. The structures depicted are taken from protein database (PDB) entries PDB ID: 3MK7 (*cbb₃*-Cox) [14], PDB ID: 3O0R (NOR ) [60], PDB ID: 1M56 (*aa₃*-Cox) [19], PDB ID: 1EHK (*ba₃*-Cox) [17] using Jmol software (http://www.jmol.org/).
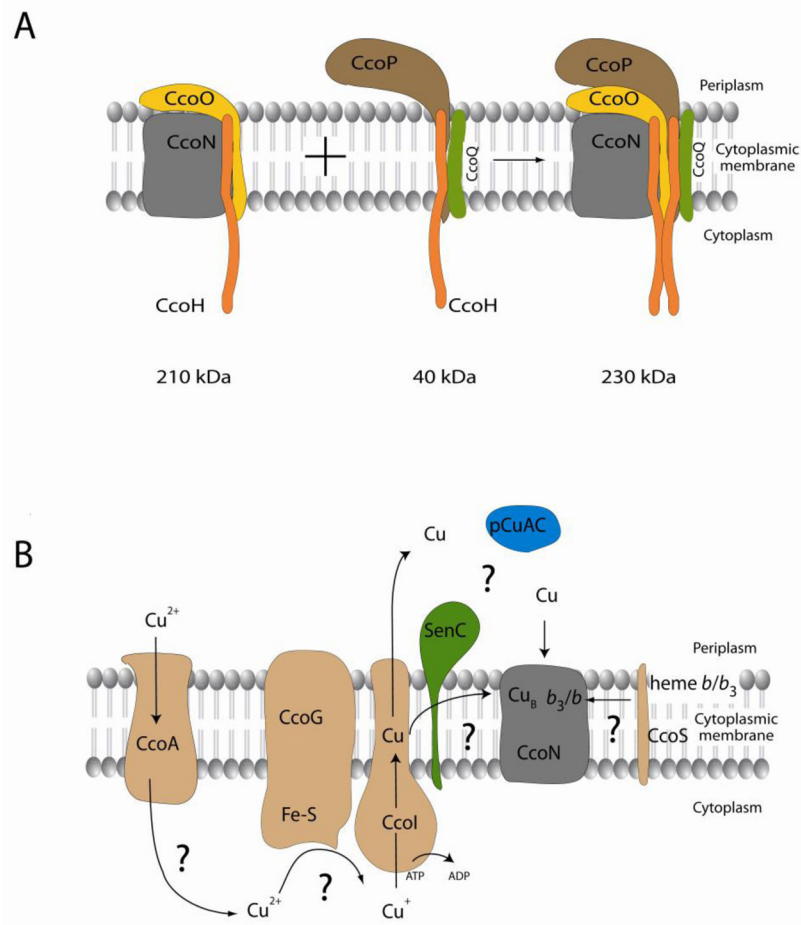
**Figure 3.**
**A.** An assembly scheme for *cbb₃*-Cox *in R. capsulatus*. Using BN-PAGE, active *cbb₃*-Cox is detectable in *R. capsulatus* membranes as a 230 kDa complex. Assembly of the 230 kDa complex proceeds via two inactive assembly intermediates: a 210 kDa subcomplex that contains the catalytic 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 *cbb₃*-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 *cbb₃*-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 PIB-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 PCuAC. The insertion of heme *b* and heme *b₃* into CcoN is probably mediated by the assembly factor CcoS.
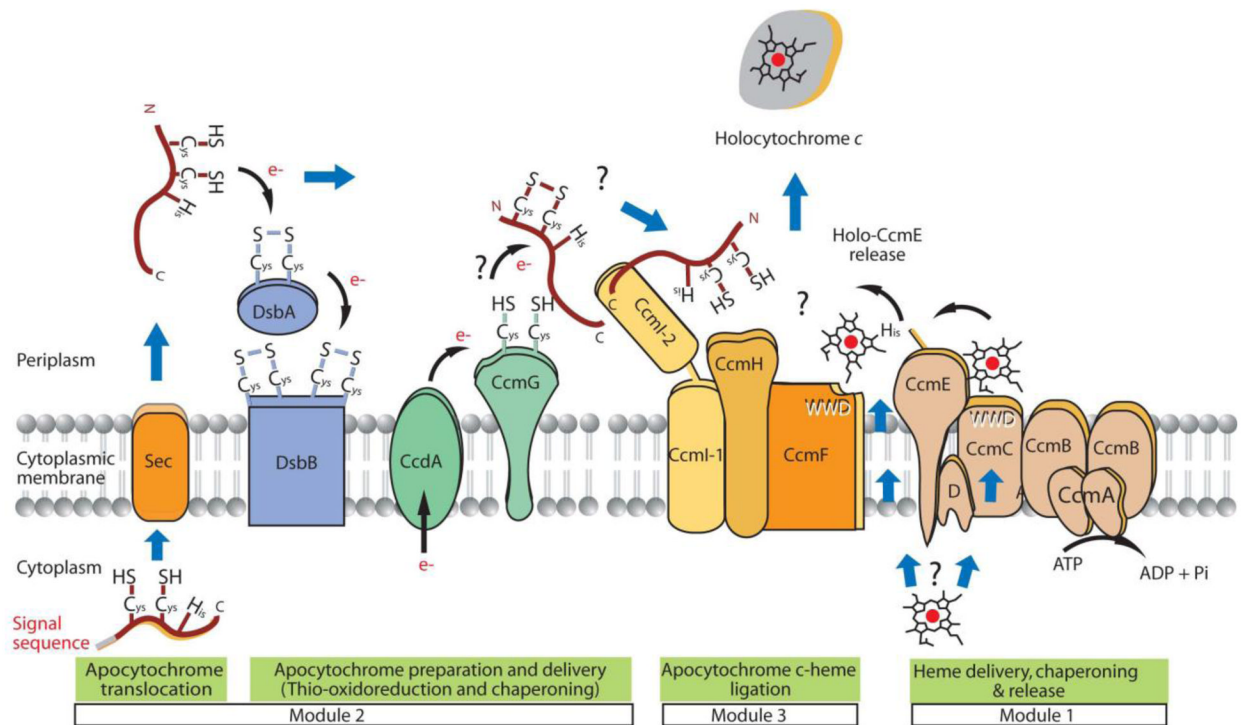
**Figure 4.**
Cytochrome *c* maturation (Ccm) system in *Rhodobacter capsulatus.* The cytochrome *c* maturation components can be divided into three modules. First module (right) is the transport of heme and its preparation for ligation to apocytochrome that involves CcmABCD proteins which forms a ABC-type 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])

**Table 1**

Characteristic features of different Cox and NOR enzymes

| | *cbb₃*-Cox | NOR | *ba₃*-Cox | *aa₃*-Cox |
|---|---|---|---|---|
| Subunit composition | • [a] CcoN<br>• Two *c*-type cytochromes: CcoP and CcoO<br>• Two putative small subunits: CcoQ, CcoH | • [a] NorB<br>• *c*-type cytochrome: NorC | • [a] Subunit I<br>• Subunit II with or without $Cu_A$ | • [a] Subunit I<br>• Subunit II with $Cu_A$<br>• Subunit III<br>• Subunit IV<br>• Other subunits |
| Catalytic binuclear centre | • Heme $b_3$<br>• Heme *b*<br>• $Cu_B$<br>• Covalent His-Tyr<br>• $Ca^{2+}$ coordinating heme *b* and $b_3$ propionates | • Heme $b_3$<br>• Heme *b*<br>• Non-heme iron $Fe_B$<br>• No covalent His-Tyr<br>• $Ca^{2+}$ coordinating heme *b* and $b_3$ propionates | • Heme $a_3$<br>• $Cu_B$<br>• Covalent His-Tyr | • Heme $a_3$<br>• Heme *a*<br>• $Cu_B$<br>• Covalent His- Tyr |
| Enzymatic Properties | $K_m \sim$ 7 nM (for $O_2$) $K_m \sim$ 12 mM (for $K_m$ NO) | | $K_m \sim$ 0,1μM | $K_m$ 0.7 - 2 μM |
| Proton pathway | 'K' pathway | Protons from periplasm | K pathway | K and D pathways |

[a] catalytic subunit of the enzyme

**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 gonorrhoea* | ✓ | ✓ |  |  |
| *Neisseria meningitidis* | ✓ | ✓ |  |  |
| *Pseudomonas aeruginosa PAO1* | ✓ | ✓ | ✓ | ✓ |
| *Vibrio cholera* | ✓ |  |  | ✓ |