Bacterial model systems for cytochrome c oxidase biogenesis

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Cytochrome c oxidase is the key player in cellular respiration, catalysing the reduction of molecular oxygen to water via its internal heme and copper redox centres. Biogenesis of the enzyme is a complex process involving up to 30 accessory proteins in higher eukaryotes. Factors directly involved in cofactor recruitment and insertion into the two core structural subunits I and II are also present in many bacteria and have been conserved during evolution. Herein we briefly review the chaperones required during early biogenesis steps, with special emphasis on the bacterial counterparts.

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Cytochrome c oxidase (COX) is the key enzyme in cellular respiration. As the terminal member of the electron transfer chain, it catalyses the reduction of dioxygen to water, transiently storing the free energy of this step in a transmembrane proton gradient to eventually power the uphill reaction of ATP formation by ATP synthase. Nature has evolved a large number of related oxidase frameworks, now summarised as the superfamily of heme-copper terminal oxidases (HCO), with their common denominator being a binuclear centre (BNC) of two redox-active metal ions deeply imbedded into the hydrophobic core of subunit I (su I) of this integral membrane protein complex. Figure 1 schematically depicts the arrangement of this active site, composed of a heme group, in many cases heme $a_3$, and a nearby copper ion termed Cu$_B$ that together allow O$_2$ binding and the concerted transfer of four electrons in a carefully controlled redox process. This BNC, positioned at approximately 1/3 in the depth of the membrane bilayer, most likely is also the site of coupling the redox reaction to the generation of an outward-directed proton gradient, a process still poorly understood in terms of its molecular mechanism and gating properties. A second heme in su I (heme $a$; Fig. 1) is the immediate donor of electrons to the BNC, making this a heme $aa_3$-type oxidase found in mitochondria and many bacteria. Several other bacterial variations in heme composition have also been described, such as $bb_3$, $bo_3$, or $ba_3$-type enzyme complexes. Similarly, the type of electron donor to the redox centres in su I may vary; the mitochondrial enzyme (again along with many bacterial enzymes; see Fig. 1) uses a dinuclear, mixed-valence copper centre termed Cu$_A$ which is

![Fig. 1—Schematic representation of the two core subunits found in $aa_3$-type oxidase complexes ($P$. denitrificans $aa_3$ oxidase, see ref. 70) and the position of their redox centres, the hemes $a$ and $a_3$ as well as their three copper ions, relative to the phospholipid bilayer. For further details, see text.]

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liganded by residues of su II; its large hydrophilic domain, tethered to the membrane by two transmembrane helices, is the major binding site for cytochrome c which delivers one electron at a time to CuA. Alternative electron entry sites and electron pathways into the redox centres of su I are found in other members of the HCO superfamily, such as in the quinol oxidases or the cbb3-type enzymes.

On studying structural, functional, or biogenesis (see below) aspects of HCO, a major complication is brought about by the fact that the traditionally addressed mitochondrial oxidase, isolated from mammalian sources, is composed of a large number of additional su which are encoded on two different genomes: su I-III genes are found on the mitochondrial genome, and up to further 10 su encoded on the nuclear genome make up the final oxidase complex, the latter being synthesised on cytoplasmic ribosomes and subsequently imported into the organelle and assembled into a functional enzyme. Both on a protein chemical level as well as for its genetic accessibility (such as in site-directed mutagenesis experiments), this complexity has seriously hampered progress in understanding this enzyme in the past.

Ever since interest arose in the bacterial oxidase counterparts some three decades ago, it soon became obvious that their extensive functional homology was paralleled by a much simpler subunit composition, making them attractive model systems to avoid some of the shortcomings of the far more complex mitochondrial enzyme(s). A typical bacterial oxidase complex is composed of 3 (+/-1) su, corresponding to the "mitochondrially coded" subunits of eukaryotic cells, once again highlighting the endosymbiotic relationship between bacteria and present-day mitochondria. Much of our present knowledge on oxidase structure and function has recently been gained by studying their bacterial versions, and they provide appealing model systems for biogenesis approaches as well.

Comparing the numerous well-documented metal cofactor chaperones as identified in yeast, to either of their human or bacterial counterparts in a pairwise fashion (Fig. 2, listing the top five ranks only), it is obvious, already from this introductory argument of sequence identity, that each bacterial version shares considerable similarity with their mitochondrial relatives. Both these models have provided specific information for the fact that yeast has long been recognized a genetically highly developed laboratory organism, while interest in the human counterparts arose from the fact that defects in individual biogenesis steps elicit severe, often lethal diseases in patients.

Apart from two enzymes acting in the last steps of heme a biosynthesis (Cox15 and Cox10; see Fig. 2), the remaining three chaperone proteins, CtaG/Cox11, Sco1/2, and Shy1/Surf1, are briefly reviewed in this article, mostly from a bacterial point of view.

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Fig. 2—The degree of sequence similarity for human and Paracoccus homologs of known COX assembly factors compared to the yeast sequences. The numbers given represent the E-values from local alignments (BLAST search) which are used as a measure for the degree of sequence similarity; the lower the E-value, the more significant the score. The E-value, or expectation value, is defined as the number of alignments with an equivalent or better score result that are expected to occur in a database search by chance. The graph shows that the bacterial model system is highly related to the human system and can be used to study COX biogenesis in terms of redox cofactor insertion.
Heme insertion into subunit I

Cytochrome c oxidase contains two heme a moieties as mentioned above, and starting from heme b, biosynthesis of the cofactor is achieved by two enzymatic steps. First, heme b is converted to heme o by the addition of a hydroxymethyl farnesyl side chain as catalysed by the enzyme heme o synthase (CtaB). In a second step, heme a synthase (CtaA) oxidises the C-8 methyl group of heme o to a formyl group present in heme a. CtaB and CtaA of Bacillus subtilis and Rhodobacter sphaeroides interact when coexpressed in E. coli and both enzymes are conserved from prokaryotes like Paracoccus to humans as exemplified in Fig. 2.

It is generally agreed that heme a is present within the membrane in a protein-bound form because it would be detrimental to the cell in its free form. The reason for this could be that heme a possesses a significantly higher redox potential compared to heme o, as shown by analysis of heme binding in designed heme protein maquettes. Studies on the regulation of the heme a biosynthetic pathway in yeast demonstrate strict regulation of Cox15 expression and activity. The same authors proposed that heme a liberation from Cox15 is likely to be controlled by COX su I or assembly intermediates because this would preclude uncontrolled release of potentially toxic heme a.

These results show that biosynthesis and incorporation of heme a into cytochrome c oxidase must be tightly controlled at any time to avoid unwanted redox chemistry by solvent-exposed heme. As discussed in the following sections, specific proteins apparently have been evolved to chaperone heme incorporation reactions into different heme a containing terminal oxidases.

Surf1 protein

The human Surf1 protein is located in the inner mitochondrial membrane. It has a molecular weight of ~30 kDa and contains two transmembrane helices that are connected by a large loop region facing the intermembrane space. Mutations in the surf1 gene leading to a functional loss of the protein cause Leigh syndrome which is a neurodegenerative disorder (first described in 1951), characterised by lesions in the central nervous system. In these cases, patients show 80-90 % loss of cytochrome c oxidase activity in all tissues, COX maturation and assembly is stalled at intermediate levels.

One of the best studied Surf1 proteins is the yeast homologue Shy1p (Surf1 homologue of yeast) which differs from other Surf1 proteins by an additional (approx. 65 amino acid long) part in the loop region facing the intermembrane space. Apart from its function in early COX assembly events, the yeast protein was also detected in high-molecular weight complexes that contain oxidase subunits, as well as in respiratory supercomplexes compromising oxidase and the cytochrome bc1 complex.

Sequence alignments demonstrate the presence of Surf homologs in eukaryotes and prokaryotes where the gene is often encoded within operons for terminal oxidase subunits. The Rhodobacter sphaeroides Surf1 protein was the first bacterial homolog to be studied in closer detail and upon deletion of the protein, oxidase complexes were obtained that were specifically diminished in their heme a3 content. These results were confirmed by studies in Paracoccus denitrificans. Here, two Surf1 genes were identified, one in an operon encoding subunits for the aa3-type cytochrome c oxidase (termed Surf1c), and, the other one in an operon for the ba3-type quinol oxidase subunits (termed Surf1q). Despite a 31 % amino acid sequence identity each Surf protein exclusively serves its cognate oxidase. This study was the first to describe a Surf protein (Surf1q) that functions in the biogenesis of an oxidase other than a cytochrome c oxidase. Results obtained from bacterial proteins are consistent with studies in yeast where Shy1p is also involved in chaperoning the formation of the heme a3 centre. Surprisingly, the Paracoccus homologs Surf1c and Surf1q were found to directly bind heme a both in vivo and in vitro, and isothermal titration calorimetry measurements revealed a 1:1 stoichiometry and binding affinities in the submicromolar range (Surf1c: $K_D = 303$ nM, Surf1q: $K_D = 650$ nM). Whether the eukaryotic Surf1 proteins are also able to bind heme a remains to be shown but an interaction with heme a synthase as observed for the Paracoccus proteins seems likely since Cox15 could be copurified with Shy1p.

Discussing the early oxidase assembly events, a threefold contribution of Surf1 is conceivable: Surf1 (i) modulates heme a synthase activity by abstracting the biosynthetic end product from the active site, (ii) avoids free heme a within the cell membrane and provides a readily available pool of the protein-bound cofactor, and, (iii) chaperones heme a to its target sites in terminal oxidases, especially to the high-spin site.
Several indications make us argue that heme incorporation into su I occurs co-translationally (Fig. 3). The 12-transmembrane helix bundle structure of su I is a rigid scaffold that may not allow incorporation of the bulky heme a moiety once it is folded, as much as fully assembled oxidase does not lose its heme groups even on prolonged chromatographic purification procedures. Having received the heme a cofactor from heme a synthase, Surf1 delivers it to the nascent su I polypeptide as schematically depicted in Fig. 3. After that it may have important additional functions in stabilising later oxidase assembly intermediates and even supercomplexes in eukaryotic systems.

Surf1 is not strictly essential for oxidase biogenesis since, after deletion, residual oxidase activity has been measured in all organisms studied so far. However, the sequence conservation from pro- to eukaryotes and the fact that loss of the protein causes Leigh syndrome in humans reveal its importance in oxidase biogenesis. In future, bacterial model systems may in particular help in elucidating individual heme incorporation events occurring in the early assembly events for terminal oxidases.

CbaX protein

Many aspects of heme incorporation into terminal oxidases are currently unknown, as outlined above. However, apart from the actual mechanism and the precise timing, it even seems that players involved in this process may vary.

One organism which differs in this respect is the thermophilic eubacterium, Thermus thermophilus. With the full genomic sequence at hand, only three proteins have been identified that are involved in oxidase metal centre biogenesis: the heme a biosynthetic enzymes prenyltransferase and heme a synthase, and, the copper chaperone Sco1, discussed in a later section. Most notably, while no Surf1 homolog has been found, a protein termed CbaX has recently been shown to be involved in the incorporation of heme a into the oxygen reducing site of the ba3 oxidase. This oxidase is a typical member of the heme-copper family of oxidases produced mainly under conditions of low oxygen tension. Heme b occupies the low-spin site, and heme a3 (with hydroxy-geranyl instead of the ubiquitous farnesyl sidechain) and Cu6 form the usual binuclear centre.

The operon encoding the ba3 oxidase does not only contain the three genes for its structural subunits but two additional downstream genes, the first of which, cbaX, was recently shown to be cotranscribed with the oxidase subunit genes. The pivotal role of CbaX for oxidase integrity was elucidated by creating a Thermus deletion strain.

Loss of functional CbaX causes a drastic decrease in activity to about 10% of the wt value for ba3 oxidase when isolated from this deletion strain. In line with this, the content of spectroscopically detectable heme a3, under native conditions, is lowered to about 10% while the amount of heme b occupying the low-spin site shows wild type level. Therefore, it is obvious that CbaX function exclusively addresses the a3 high-spin site, while assembly of the low-spin heme b site is unaffected. However, determining the heme bla content under denaturing conditions surprisingly resulted in a much higher heme a content while no difference was observed for heme b. This observation points to the specific role of CbaX in that it may position heme a for correct insertion into the binuclear site.

The functional assignment for CbaX was further corroborated by analysing the ba3 oxidase isolated from a complemented deletion strain: both activity and spectroscopic properties of this oxidase were fully restored. When, on the other hand, cbaX is placed under control of a strong promotor, a disturbance of the normal heme occupancy in the purified oxidase...
was observed. Heme \(a\) not only populates the high-spin site of the enzyme as anticipated, but about a third of heme \(b\) in the low-spin site was, in addition, replaced by heme \(a\) under these conditions (Fig. 4). We assume that heme \(a\) can not be correctly accommodated into the low-spin site, due to its bulky side chain, causing a concomitant drop in activity. It is therefore mandatory that the organism maintains, by so far unknown mechanisms, a controlled steady-state level of CbaX production to ensure complete heme \(a\) incorporation in the high-spin site, without compromising the occupancy of heme \(b\) in the low-spin site.

The CbaX protein consists of 156 amino acids and the prediction of three transmembrane helices strongly suggests that it is membrane-bound, which may also reflect its function in assisting or promoting the incorporation of heme \(a\) into the high-spin site. The amino acid composition disfavours CbaX being a carrier/storage protein for heme \(a\) itself, since no histidine residues are present in the protein as typical ligands for heme moieties. One of several possible speculations is that CbaX maintains su I of oxidase in a state competent for heme \(a\) insertion. At the moment no further information about the specific role of CbaX can be given as no sequence identities to known biogenesis factors are evident. It is tempting to speculate that CbaX has separately evolved, since it lacks sequence similarity with Surf1, to cope with the specific requirements of heme \(a\) incorporation under thermophilic conditions.

**Copper insertion into subunit I**

**Cox11/CtaG**

The copper chaperone Cox11 was first described for yeast mitochondria to contribute to COX assembly. Initially a coordinative function in the assembly of the structural subunits was proposed\(^{39}\), whereas later studies even suggested Cox11 participating in heme \(a\) biosynthesis\(^{40}\). In contrast to other known assembly factors like Surf1 or Sco1/2, no disease relation has been described for Cox11 so far. The protein seems to be essential for organisms relying on aerobic respiration making it an optimal candidate for studies in bacterial systems that can also maintain anaerobic growth or rely on alternative oxidases.

Studies in *P. denitrificans* showed a decreased iron and copper content upon deletion of the Cox11 homolog CtaG\(^{41}\). At the same time the role of Cox11 was investigated in *R. sphaeroides* where a specific loss of the copper ion in the Cu\(_B\) centre of oxidase was described, indicating that Cox11 might be a copper chaperone for COX su I\(^{42}\). Indeed, the mitochondrial protein was shown to bind Cu(I), and two cysteine residues within a highly conserved CFCF motif were associated with its function\(^{43}\). This is in good agreement with studies on the *Paracoccus* homolog where reconstitution of the apo-protein can only be achieved using Cu(I) ions\(^{44}\). Cox11 has a single transmembrane helix, and the C-terminal hydrophilic domain faces the intermembrane space of mitochondria\(^{45}\), namely, the periplasm in bacteria. The hydrophilic domain houses the above mentioned CFCF motif that is essential for function of the protein. The N-termini of different Cox11 homologs vary greatly in length. In *Schizosaccharomyces pombe*, for example, this domain shows homology to the mitoribosomal protein Rsm22\(^{45}\) and there is also certain evidence for the association of Cox11 with the mitochondrial translation machinery\(^{46}\). Therefore, a co-translational insertion of the copper ion into su I seems most likely\(^{11}\).

The solution structure of the hydrophilic domain of *Sinorhizobium melliloti* Cox11 was solved in 2004 in its copper-free form, revealing a \(\beta\)-immunoglobulin-like fold\(^{47}\). Interestingly the structure shows high flexibility in the region of the CFCF motif. EXAFS studies (extended X-ray absorption fine structure) of
Cox11 in yeast as well as in S. melliloti show that a dimer of Cox11 binds two Cu(I) ions each liganded by three sulphur atoms. Dimerisation upon copper binding is also observed for the Paracoccus protein. Structures of a dimer are currently limited to models based on computer protein docking methods, where it is only possible to successfully model a dimer if the monomers face each other in an anti-parallel way. In this situation the CFCF copper binding motif must point towards the surface of the membrane (Fig. 5). The structural orientation together with the proposal of a co-translational insertion mechanism for the CuB ion into su I makes it very difficult to study the actual transfer events.

The coordination state of the copper ion and the number of cysteine residues involved is still under debate, and varying results have been obtained for Cox11 proteins isolated from different organisms. In R. sphaeroides, 1.5 copper ions per monomer have been measured whereas for P. denitrificans, the Cu:protein ratio is 1 (ref. 50). In order to investigate functionally important residues, site-directed mutagenesis studies have been carried out in yeast, Rhodobacter, and Paracoccus, all of them showing the importance of the cysteine residues in the CFCF motif. Mutations of the phenylalanines within this motif only slightly decrease the copper content and most likely only have a function in fine-tuning the metal binding. A mutation of a further conserved cysteine residue at the periplasmic end of the transmembrane helix has different effects in Rhodobacter and Paracoccus: in the latter it contributes to copper binding, while in the former it does not but is essential for generating an active oxidase. This finding led to the proposal that this residue is involved in the copper transfer from the CFCF copper binding motif to the CuB centre in su I. In our current model this residues contributes to copper binding (Fig. 5), which does not exclude an additional function in copper transfer.

A current problem in protein characterisations of Cox11 is the oligomerisation state of the protein. To date monomer-dimer scenarios have been discussed as mentioned above, but in gel filtration experiments with all Cox11 variants studied, even higher oligomerisation states have been observed, and at least for Rhodobacter shown to bind different amounts of copper. It is unknown whether oligomers beyond dimers play a physiological role. It may well be a methodical artefact when working with only the hydrophilic C-terminal fragment. So far nobody has investigated the full length protein with its transmembrane helical part with respect to its copper binding stoichiometry, oligomerisation state, or structure.

Although some indirect evidence has been provided for a co-translational insertion of copper into su I as discussed above, a physical interaction between the copper chaperone and its proposed target remains to be shown. The Paracoccus chaperone may be suitable for that because it shows a copper-dependent spectroscopic signal at 385 nm (which is not described for other Cox11 proteins) that can be used to follow the copper transfer.

Copper insertion into subunit II Sco proteins

Initially studied in S. cerevisiae, two related proteins have been implicated in copper delivery to oxidase su II to form the CuA centre, the paralogs Sco1 and Sco2. Both proteins carry an N-terminal transmembrane helix that anchors them to the mitochondrial inner membrane, with a large hydrophilic domain facing the intermembrane space, and a direct interaction of yeast Sco1 with su II of oxidase was demonstrated. In a functional assay, expression of soluble fragments of Sco1 could not complement a sco1 deletion, nor could extraneously
added copper ions rescue the deletion phenotype\(^5\), pointing at the crucial role of the yeast Sco1 as a copper transfer protein (Fig. 6). Receiving a Cu(I) ion from the mitochondrial chaperone Cox17, the metal in Sco is coordinated by the motif CxxxCP along with a conserved histidine\(^5\). This latter ligand seems critical for redox property modulation of Sco proteins studied from a number of different sources, and probably accounts for their unexpected Cu(II) affinity in some cases\(^57-59\).

Related Sco proteins have been identified in a large number of organisms from all kingdoms\(^6\), with varying gene copy numbers and genetic context. Furthermore, their appearance is not strictly correlated with the presence of a Cu\(_A\) centre-containing terminal oxidase in any particular organism. From early sequence motif predictions\(^6\) and several later 3-D structure determinations of soluble Sco fragments\(^6\)\(^2\)\(^-\)\(^6\)\(^5\), it turned out that Sco proteins possess a thioredoxin fold, pointing at other or additional catalytic functions next to target protein metallation. Unlike in yeast, where deletion of Sco2 has no immediate deleterious effect on oxidase phenotype (see ref. 66 for a recent review), individual inactivations in each sco gene in human cells proved detrimental to oxidase function. A model has been put forward in which the human Sco2 supports oxidase su II synthesis\(^66,67\), but at the same time works as a thiol disulfide reductase acting on Sco1; further suggestions even assign a more general role for human Sco proteins in cellular copper homeostasis\(^68\).

An even more pronounced situation is encountered in T. thermophilus, where the single Sco protein present in this thermophilic bacterium seems to have lost its metallation properties altogether, and appears functionally restricted to maintaining the two Cu\(_A\)-liganding cysteine side chain sulphurs of su II in a reduced state, ready for copper insertion\(^69\). For this system, the PCu\(_A\)C protein was suggested as the immediate copper donor chaperone (see also Fig. 6).

Analysing the P. denitrificans genome, two paralogs are found, with their products presently termed ScoA and ScoB, with 216 and 210 amino acids, respectively. Both genes were deleted separately, and subsequently complemented. From these initial experiments, inactivation of scoA seems to have only a moderate effect on COX activity (65 % residual activity as measured in intact membranes), while no COX phenotypic alteration is detected in the ΔscoB strain. The double deletion, however, shows a more distinct loss of COX activity (down to 32 %), pointing at an overlapping, possibly synergistic function of the two chaperones, not restricted to copper delivery alone. Both proteins are expressed as tagged versions in E. coli, and the purified proteins are shown, both by UV-vis and by inductively-coupled plasma emission spectroscopy, to bind copper to about 60 % on a molar basis. The observation that even in the sco double deletion strain active oxidase is synthesised, leaves enough room for speculations that the two copper ions for Cu\(_A\) site metallation may be delivered by additional chaperone components (see Fig. 6).

Conclusions

Defects in COX biogenesis often lead to severe respiratory deficiencies and are the cause of numerous human diseases. Over the last few decades yeast has proven to be an important model system for the identification of a plethora of factors that are involved in COX biogenesis. Chaperone proteins that act directly in the recruitment and insertion of the heme and copper redox centres of COX are also found in bacteria and show a distinct sequence similarity to higher eukaryotes. Studying such chaperones in less complex bacterial model systems will focus on those fundamental steps of COX biogenesis common to all organisms.
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