Building the CuA site of cytochrome c oxidase: a complicated, redox-dependent process driven by a surprisingly large complement of accessory proteins

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Running title: COX2 and CuA site maturation

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Abstract
Cytochrome c oxidase (COX) was initially purified more than 70 years ago. A tremendous amount of insight into its structure and function has since been gleaned from biochemical, biophysical, genetic and molecular studies. As a result, we now appreciate that COX relies on its redox-active metal centers (heme $a$ and $a_3$, CuA and CuB) to reduce oxygen and pump protons in a reaction essential for most eukaryotic life. Questions persist, however, about how individual structural subunits are assembled into a functional holoenzyme. Here, we focus on what is known and what remains to be learned about the accessory proteins that facilitate CuA site maturation.

COX is a multi-subunit enzyme of dual genetic origin
COX is a member of the A1 subgroup of a diverse superfamily of heme-copper oxidases. Embedded in the inner mitochondrial membrane, it is a multimeric protein complex comprised of structural subunits that are encoded by two distinct genomes. The three largest of these, COX1-3, are mitochondrially-encoded and form the catalytic core of the enzyme. COX1 contains the two heme ($a$, $a_3$) moieties and a mononuclear CuB center, all of which are buried within the lipid bilayer in the fully assembled holoenzyme. COX2 harbors a mixed valence, binuclear CuA site within a cupredoxin fold that is localized to the intermembrane space (IMS) and is solvent exposed. The CuA site accepts electrons from cytochrome c, and subsequent electron transfer steps to the heme $a$ and then the heme $a_3$-CuB metal centers of COX1 ultimately allow COX to convert molecular oxygen to water. Four protons are pumped across the membrane during each catalytic cycle, and contribute to the electrochemical gradient that is required for aerobic ATP production. The catalytic core is surrounded by a variable number of nuclear-encoded structural subunits (8 in yeast, 11 in humans), which function collectively to stabilize the holoenzyme, provide sites for the allosteric modulation of its catalytic activity, and facilitate its organization into higher order structures termed supercomplexes or respirasomes (reviewed in (1,2)). High resolution structures of mammalian COX (3) and of mammalian respirasomes that contain COX (8,(4) have been invaluable to advancing our understanding of how inter-subunit interactions impinge upon enzyme activity and dimerization, and the integration of COX into higher order structures.
COX is assembled in a modular fashion

COX assembly is a very complex process that requires the stoichiometric expression of its nuclear- and mitochondrially-encoded subunits and the ordered incorporation of its heme and copper prosthetic groups. The basic blueprint that defines the steps and mechanisms that facilitate COX assembly within the inner mitochondrial membrane has been derived almost exclusively from studies of mutant yeast strains and cell lines from patients who presented with an isolated COX deficiency. Elegant work done by Leo Nijtmans and colleagues (5) in the late 1990s established that the individual structural subunits of COX are matured and assembled in modules, with the formation of three assembly intermediates (S1-S3) preceding the biogenesis of the mature holoenzyme (S4). Subsequent studies identified the existence of unique maturation modules specific to COX1, COX2 and COX3 (6,7) and challenged the exact subunit composition of individual modules and the order in which they are added to the assembling holoenzyme (e.g. (8)). However, the original concept that COX is built using a modular blueprint remains intact.

Maturation of individual modules and progression through the various stages of holoenzyme assembly requires a surprisingly large number of accessory proteins, termed COX assembly factors. Pioneering studies of yeast nuclear petite (PET) mutant collections identified more than 30 different complementation groups that encode for gene products with unique functions in holoenzyme biogenesis (9,10), many of which are conserved in humans (11). At least six of these complementation groups encode for accessory proteins crucial to the maturation of COX2 and the matellation of its CuA site (Table 1). Additional, evolutionarily conserved COX assembly factors with poorly characterized functions like COA5 (12,13) and CMC2 (14,15) may also function in this pathway. Pathogenic mutations in COX2 (16-18), COX20 (19,20), COA6 (21-23), SCO1 (24-26) and SCO2 (27,28) all cause severe, early onset forms of fatal disease owing to an isolated COX deficiency (Table 1).

Characterization of human cell lines in which COX20 (29), COA6 (23), SCO1 (30) or SCO2 (31,32) function is impaired indicates stalling of COX assembly always occurs at the S2 stage, when fully matured COX2 is normally inserted into the assembling holoenzyme. Loss of function mutations in the remaining COX assembly factors with putative (OXAIL) or established (COX17, COX18) roles in COX2 maturation have yet to be described in COX-deficient patients (Table 1), despite candidate gene screening (e.g. (33,34)) and the advent of next generation sequencing technologies, suggesting that such variants may be embryonic lethal in humans. Consistent with this idea, Cox17 deletion in the mouse results in a severe COX deficiency at embryonic day 6.5 and lethality shortly thereafter (35). The clinically diverse forms of fatal disease caused by mutations that compromise the function of any member of the COX2 assembly module (Table 1) means that there is great interest from a biomedical perspective in defining the precise roles of the individual COX assembly factors, and identifying how they collaborate with one another at various stages as COX2 is matured. Such insight, once garnered, will be equally fascinating from a basic biological point of view, for it will clarify how each step in COX2 maturation ultimately leads to the metallation of its CuA site.

Co-translational insertion of apo-COX2 into the inner membrane is paired with
the translocation of its N-terminus into the IMS

COX2 is a polytopic protein with two transmembrane domains whose N- and C-termini protrude into the IMS. Because of its inherent hydrophobicity, COX2 insertion into the inner membrane is always coupled to mRNA translation. In yeast, a long 5' untranslated leader sequence is recognized by the translational activator Pet111, which recruits COX2 mRNA to specific ribosomes (Figure 1A). Mammalian COX2 mRNA is essentially devoid of a 5'-untranslated leader sequence, and searches for the ortholog of Pet111 based on amino acid identity have proved fruitless. However, it has long been postulated that mammalian mitochondrial mRNAs contain recognition motifs within their coding sequences that are similarly vital to their efficient translation, and this is now known to be true at least for COX1 (36), raising the intriguing possibility that a mammalian-specific COX2 translational activator remains to be discovered.

Co-translational insertion of yeast Cox2 into the membrane also requires Oxa1, a member of the conserved YidC/Alb3/Oxa1 family of insertases (37). The C-terminus of Oxa1 interacts with the large ribosomal subunit in close proximity to the polypeptide exit tunnel where it promotes protein insertion into the inner membrane (38,39). Oxa1 function in this regard is aided by Mba1, which acts to tether ribosomes to the inner membrane on the matrix side of the leaflet (40) (Figure 1A). The human homolog of Oxa1, OXA1L, also physically interacts with mitochondrial ribosomes via its C-terminal tail (41,42) and is able to restore COX assembly in an OXA1L null yeast strain, arguing that Oxa1-dependent insertion of the N-terminal transmembrane domain of Cox2 and the simultaneous translocation of its N-terminus into the IMS is an evolutionarily conserved function (43) (Figure 1B). However, OXA1L knockdown in HEK293 cells leads to a diminution in the abundance of Complexes I and V rather than COX (44). This has led to the suggestion that in mammals COX20 may be sufficient for insertion of the N-terminal transmembrane domain of COX2, given that its hydrophilic N-terminus is shorter than that of yeast Cox2 and does not require proteolytic processing upon membrane insertion (45). Further studies of OXA1L are required to discriminate between these distinct mechanistic possibilities, but are challenged by its broad function as an insertase of subunits of multiple complexes of oxidative phosphorylation.

COX20 stabilizes apo-COX2 in the inner membrane to protect it from degradation and promote the export of its C-terminus into the IMS by COX18

Although the mechanisms that facilitate export of the N-terminal tail of mammalian COX2 remain unclear, recent studies emphasize that COX20 fulfills an essential, evolutionarily conserved role during the early stages of COX2 maturation (Figures 1, 2). COX20 contains two transmembrane domains with N- and C-termini that also protrude into the IMS (29) and serves as a chaperone, stabilizing the newly synthesized polypeptide and protecting it from degradation (29,46). Consistent with this idea, deletion of COX20 in either yeast (46) or human (29) cells results in rapid COX2 turnover. In yeast, Cox20 interacts with Cox2 and Mba1 at the ribosome (47) (Figure 1A). Cox20 then presents the pre-protein to an inner membrane peptidase complex, which cleaves the N-terminal yeast leader sequence within the IMS to yield mature, apo-Cox2 (48) (Figure 1A).

Cleavage of the N-terminal leader sequence results in Mba1 dissociation from the Cox20-Cox2 complex (47) and the subsequent recruitment of Cox18, which
promotes the insertion of the C-terminal transmembrane domain and concomitant export of the long soluble, C-terminal tail of Cox2 (49) (Figure 2A). In yeast, deletion of COX18 stalls Cox2 maturation and leads to the accumulation of a species in which the N-terminal transmembrane domain is inserted into the membrane and the leader sequence has been cleaved (50). Deletion of COX18 in human cells similarly leads to the accumulation of a stalled COX2-COX20 complex (45). Although COX18 belongs to the OXA1 protein family (50), COX2 is its only known substrate in both yeast and mammals (45). Yeast experiments in which Oxa1 was overexpressed in a COX18 null strain failed to restore growth on a non-fermentable carbon source even though some export of the C-terminal tail of Cox2 was observed, emphasizing that Cox18 translocates the tail across the membrane and delivers it in a state competent for metallation of its CuA site (51). In yeast, Cox18 depends on a physical association with two additional factors, Mss2 and Pnt1 (50,52), for its function, while it is thought that COX18 acts alone in mammals to promote insertion of the C-terminal transmembrane domain of COX2 and the export of its tail into the IMS (45) (Figure 2B).

**COA6, SCO1 and SCO2 form a metallochaperone module that interacts with the COX20-COX2 complex to metallate the CuA site**

Following the export of its C-terminal tail into the IMS, COX2 remains bound to COX20 and is competent for CuA site metallation (29,45). Physical interaction studies indicate that dissociation of COX18 from the COX20-COX2 complex coincides with the recruitment of a metallochaperone module composed of SCO1, SCO2 and COA6 (45). This trio of proteins then functions as a collective to deliver and insert copper into the CuA site (Figure 3), the ligands for which are two conserved cysteines, two histidines, a glutamate and a methionine contained within a cupredoxin fold (53).

Metallation of the CuA site of COX2 by its metallochaperone module requires a dedicated upstream copper donor within the IMS, and COX17 fulfills this role in both yeast and humans (Figure 3). COX17 was the first member to be identified from a surprisingly large family of soluble proteins with twin Cx9C motifs localized to the IMS (54). The cysteinyl sulfurs of these twin Cx9C motifs are recognized by the MIA40-ERV1 import pathway within the IMS, which oxidizes them to form a pair of disulfide bonds that convert an intrinsically disordered protein into one with a helical hairpin structure (55-58). Mutagenesis studies in yeast revealed that two additional, conserved cysteines at the N-terminus of Cox17 are responsible for the coordination of Cu(I) ions (59,60). Results from a comprehensive suite of functional genetic experiments argue that COX17 receives its copper from an evolutionarily conserved, labile Cu(I) pool that is housed in the matrix (61). Although Cobine and co-workers have provided some important insight into how Cu(I) is trafficked to and within mitochondria (62,63), the mechanisms that regulate IMS copper translocation and transfer to COX17 have yet to be identified. It is clear, however, that both yeast Cox17 and human COX17 transfer copper to both SCO1 and SCO2 (Figure 3). Elegant work from the Winge group used *in vitro* and *in vivo* copper transfer studies to demonstrate that Cox17 requires a cysteine within one of its twin Cx9C motifs, Cys57, to be competent to transfer copper to Sco1 (64). Equally elegant work by the Bertini group used NMR to directly demonstrate that COX17 transfers Cu(I) to both SCO1 and SCO2, and that this copper transfer step to SCO1 may...
be coupled to electron transfer, which serves to reduce the copper-binding site and promote ligand exchange (65).

SCO1 and SCO2 are paralogs that must be able to bind copper to promote CuA site maturation (30,31,66). Both proteins were originally identified in yeast as high copy suppressors of a COX17 point mutant strain (54). Subsequent studies, however, found that only deletion of SCO1 resulted in an inability to grow on a non-fermentable carbon source (67,68) and, until recently, the function of yeast Sco2 remained unknown (see below, (69)). Both SCO proteins bind Cu(I) via a conserved CxxxC motif and a conserved histidine (70,71), and rely on an additional, conserved aspartate residue to coordinate Cu(II) (71). One copper atom is bound per monomer, and mutations that impair Cu(I) or Cu(II) binding result in a non-functional SCO protein, arguing that this property is crucial to their roles in COX assembly (71). The importance of copper binding to SCO1 function is emphasized by two parallel studies that both found the pathogenic P174L substitution in SCO1 severely compromises COX17-dependent copper transfer (72,73). Whether mutations in SCO2 also impair copper transfer from COX17 remains unclear. However, the most common pathogenic variant of SCO2, SCO2 E140K, rescues the COX deficiency in SCO2 patients when overexpressed, arguing that it is competent for metallation (31).

Although SCO1 and SCO2 are part of the same metallochaperone module, each protein physically interacts with COA6 (23,69,74) to fulfill a distinct function during the metallation of the CuA site of COX2 (30,31,66) (Figure 3). Yeast studies were the first to show that Sco1 physically interacts with Cox2 (75), and mapped the functional interaction between the two proteins to a conserved sequence motif within loop 8 of Sco1 (76), which is solvent exposed and undergoes structural rearrangement when Sco1 transitions from an apo- to a metallated conformer (77). Mutations in residues within this loop 8 motif do not affect physical interactions with Cox17 or copper-binding, arguing that Sco1 uses distinct interfaces to interact with Cox17 and Cox2 (76). Pulse-chase labeling of mitochondrial translation products in human cells established that impaired SCO1 function did not affect COX2 synthesis but resulted in accelerated turnover of the newly synthesized protein (73). In contrast, COX2 synthesis is greatly reduced in SCO2 patient cells, yet the residual protein that is made is much more stable than in control cells (31). These results led Shoubridge and co-workers to propose that SCO2 acts upstream of SCO1 to stabilize newly synthesized COX2, and that the subsequent maturation of COX2 is contingent upon the formation of a ternary complex containing both SCO proteins and COX2 (31). However, it is now clear that another small, soluble twin Cx9C motif-containing protein, COA6, is also an essential part of the metallochaperone module that associates with the COX20-COX2 complex to catalyze copper insertion into the CuA site of COX2 (23,45,69,74,78) (Figure 3).

The fine mechanistic details of CuA site metallation in higher eukaryotes remain to be collected, but recent years have yielded a wealth of new information that brings much sharper focus to how the metallochaperone module functions as a unit. Elegant work from the Vila group has established that SCO1 and SCO2 are sufficient for CuA site maturation in vitro, with SCO2 functioning as a thiol-disulfide oxidoreductase to reduce the cysteinyl sulfurs of COX2 and SCO1 acting as the copper donor (66). However, in this system SCO2 performs its redox function in a copper-bound state rather than through a simple disulfide exchange reaction (66), and questions remain with respect to the fate of
its Cu(II) ion after SCO2 acts as an electron donor (Figure 3). The cysteines of the CxxxC motif of SCO1 also exist as a mixed population comprised of both oxidized disulfides and reduced thiols in vivo (26,31), and how the cysteinyl sulfurs of SCO1 are oxidized to drive copper insertion into the CuA site in the IMS is also unclear. Excitingly, the findings from a flurry of recent studies strongly suggest that COA6 might in fact bridge these distinct aspects of SCO1 and SCO2 function to directly support CuA site maturation. COA6 is reported to be a copper-binding protein (23,74), raising the intriguing possibility that it might transition from an apo- to a copper-loaded conformer upon SCO2-dependent reduction of the cysteinyl sulfurs of the CuA site. Consistent with this idea, human COA6 and SCO2 form a complex that is essential for COX2 maturation and COX assembly (74) and a yeast COA6/SCO2 double mutant is less fit than the single mutants when grown on a non-fermentable carbon source, arguing that SCO2 and COA6 fulfill overlapping functions in CuA site metallation (69). Yeast Coa6 and human COA6 also physically interact with both Cox2/COX2 and Sco1/SCO1, and these interactions are crucial for the stability of newly synthesized polypeptide (23,69).

**Future directions**

Our current understanding of mammalian COX2 maturation and CuA site metallation involves at least four distinct stages (Figures 1-3). First, the N-terminal tail of COX2 is exported into the IMS as its N-terminal transmembrane domain is inserted into the inner membrane, in a translocation reaction that may involve OXA1L and/or COX20 but that has yet to be fully defined (1,45). Second, COX20 associates with COX2 as it is co-translationally inserted into the membrane to stabilize the polypeptide and prevent its degradation. COX18 then promotes the simultaneous insertion of the C-terminal transmembrane domain of COX2 and translocation of its C-terminal tail into the IMS. Third, COX18 release from the COX20-COX2 complex coincides with the recruitment of the metallochaperone module composed of SCO1, SCO2 and COA6. Finally, the metallochaperone module functions collectively to reduce the cysteinyl sulfurs of COX2, with a SCO1 homodimer (30) inserting two copper ions into COX2 that it originally received from COX17 to form the CuA site. Whether COA6 oxidizes the CxxxC motif of SCO1 to promote insertion of copper into the CuA site, thereby priming its cysteinyl sulfurs to accept copper from SCO2, and whether this copper is in turn transferred to apo-SCO1 to prime SCO1 for a subsequent round of metallation are important, open and exciting questions.

Despite tremendous, recent progress, many other questions remain with respect to the maturation of COX2 and the biogenesis of its CuA site. The first, and most obvious, question is whether the full complement of proteins that participates in this process has been identified. The twin Cx9C-motif containing family of proteins contains a surprisingly large number of soluble factors that are essential for COX assembly in yeast (14), and several of these including Cmc1 (79), Cox19 (80) and Cox23 (81) are conserved in humans and have been shown to play a role in mitochondrial copper metabolism. However, recent studies indicate that all three of these factors fulfill important functions in delivering copper to COX1 rather than COX2 (82-84). Other family members with poorly understood but evolutionarily conserved roles in COX assembly, such as CMC2 and COA5, nonetheless remain and may facilitate CuA site maturation directly or indirectly by regulating IMS copper trafficking (Figure 3). It is equally plausible that unrelated or
novel, mammalian-specific factors relevant to this process have yet to be identified.

A second, critical question is whether the relative timing and composition of protein constituents that make up the various subassembly modules critical to COX2 maturation and Cu$_A$ site formation is constant. For example, it remains to be seen whether the metallochaperone module is homogeneous in its composition or whether there is dynamic exchange of proteins in and out of this module as COX2 is matured. This is an important consideration because there is some discord in the literature with respect to the COX assembly factors that have been identified within this module using affinity purification methods (e.g. (26) versus (78)). However, it is important to note that some of these discrepancies may be explained by inter-lab variation in methodology, which may favour the enrichment and therefore capture of one complex over another (e.g. $^{35}$S-pulse chase labeling protocols, (45)). The mitochondrial proteome also differs across tissues (85,86), and it is therefore worth considering the underexplored possibility that the constituents of the COX2 assembly module may in fact be specific to different cell types and/or species (11).

Other, more nuanced but equally important mechanistic questions also persist, independent of the complement of proteins that catalyzes Cu$_A$ site maturation and its relative conservation across cell types. Why, for example, do both SCO proteins need to be able to coordinate Cu(I) and Cu(II), and how does this relate to the biogenesis of the mixed valence, binuclear Cu$_A$ site? Redox functions have also been proposed for both human SCO1 (87) and SCO2 (31) based on in vivo and structural data, yet teasing apart the functional significance of their redox and copper-binding properties to Cu$_A$ site maturation has yet to be realized. In fact, our general knowledge of redox homeostasis in the IMS and its impact on the redox state of cysteiny1 sulfurs critical to protein function remains limited (92,93). Beautiful work from the Riemer group has established that the IMS is not an oxidizing environment as was once thought, and that its glutathione pool in fact exhibits the same redox potential as that of the cytosol (92). However, they have since demonstrated that thiol oxidation occurs within the reducing environment of the IMS because glutaredoxin levels are rate-limiting (93). The potential interplay between glutaredoxin and the metallochaperone module has obvious, but as yet poorly understood, consequences for maturation of the Cu$_A$ site. More specifically, it remains unclear whether glutaredoxin abundance may be altered within the IMS and, if it can be, how this may impinge upon the redox state of cysteines within the CxxxC motif of SCO proteins and those within the twin Cx6C motifs of COA6 to modulate the function of the metallochaperone module. We impatiently await the mechanistic insight that will be afforded by future studies that tackle these big and small questions.
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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

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Table 1. COX2 and COX assembly factors required for polypeptide maturation and Cu$_A$ site formation.

| Gene   | Function            | Clinical Course(s) of Disease                                      |
|--------|---------------------|-------------------------------------------------------------------|
| COX2   | Catalytic core      | Myopathy (16), Encephalopathy (17), Multisystem disorder (18)    |
| OXA1L  | N-terminal insertase? | N/A (33)                                                           |
| COX18  | C-terminal insertase | N/A (34)                                                           |
| COX20  | Chaperone           | Dystonia and ataxia (19)                                           |
| COX17  | IMS Cu trafficking  | N/A (35)                                                           |
| COA6   | Cu$_A$ site maturation | Hypertrophic cardiomyopathy (21,22)                             |
| SCO1   | Cu$_A$ site maturation | Hypertrophic cardiomyopathy and encephalopathy (24, Hepatopathy (25), Encephalopathy (26) |
| SCO2   | Cu$_A$ site maturation | Hypertrophic cardiomyopathy and encephalopathy (27,28)             |

N/A: Not Applicable, IMS: intermembrane space
Figure 1: Co-translational insertion of COX2 and export of its N-terminus. A, In yeast Pet111 binds to Cox2 mRNA to stimulate its translation. Translation is coupled with polypeptide insertion into the inner mitochondrial membrane (IMM), in a process that is facilitated by both Oxa1 and Mba1. The C-terminus of Oxa1 interacts with the large ribosomal subunit, and its interaction with Mba1 positions it near the exit tunnel where Oxa1 promotes Cox2 insertion and export of its N-terminus into the intermembrane space (IMS). Cox20 interacts with Mba1 and Cox2 to stabilize and prevent degradation of the pre-protein. The N-terminus of the pre-protein is then proteolytically processed to yield mature Cox2 by a peptidase complex composed of Imp1, Imp2, and Som1. B, In mammals, the hydrophilic N-terminus of COX2 is much shorter than that of yeast Cox2, and does not require proteolytic processing upon membrane insertion. Although OXA1L physically interacts with mitochondrial ribosomes and functions as an insertase, questions remain about whether it promotes co-translational insertion of COX2 into the IMM or whether this function is fulfilled by COX20. The outer mitochondrial membrane is represented by the dashed line in both panels.
Figure 2: Insertion and export of the C-terminus of COX2. 

A, In yeast insertion and export of the C-terminal transmembrane domain of Cox2 requires recruitment of Cox18 to the Cox2-Cox20 complex. The insertase function of Cox18 then depends on its physical association with Mss2 and Pnt1. 

B, In mammals, COX18 is similarly recruited to the COX2-COX20 complex where it is thought to function alone to promote insertion of the C-terminal transmembrane domain of COX2 and export of its C-terminus into the intermembrane space (IMS). The outer mitochondrial membrane is represented by the dashed line in both panels. IMM, inner mitochondrial membrane.
Figure 3: Cu_A site maturation of COX2. In yeast (A) and mammals (B) Cox17 and COX17, respectively, transfer Cu(I) ions to Sco1/SCO1 and Sco2/SCO2 to load the metallochaperone module. Because SCO1 and SCO2 are homodimers (30), this requires two successive rounds of COX17-dependent Cu(I) delivery to fully metallate each protein. In vitro studies support a model whereby copper-loaded SCO2 functions as a thiol-disulfide oxidoreductase to reduce the cysteinyl sulfurs of COX2 (66), in a reaction that converts Cu(I) (pink circle) to Cu(II) (purple circle) and requires COA6 function in vivo. In yeast, reduction of the cysteinyl sulfurs of Cox2 also requires Coa6 and Sco2, as well as Cox12 (69). Whether the human homolog of Cox12, COX6B, is similarly important for Cu_A site maturation is unclear. Upon priming of the cysteinyl sulfurs of Cox2/COX2, the Cu_A site is metallated by Sco1/SCO1. Whether other, evolutionarily conserved COX assembly factors with poorly defined roles in COX assembly also participate in Cu_A site maturation remains unknown. The outer mitochondrial membrane is represented by the dashed line in both panels. IMM, inner mitochondrial membrane.
Building the Cu\textsubscript{A} site of cytochrome c oxidase: a complicated, redox-dependent process driven by a surprisingly large complement of accessory proteins

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