

Review Article

Assembly of mammalian oxidative phosphorylation complexes I–V and supercomplexes

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The assembly of the five oxidative phosphorylation system (OXPHOS) complexes in the inner mitochondrial membrane is an intricate process. The human enzymes comprise core proteins, performing the catalytic activities, and a large number of ‘supernumerary’ subunits that play essential roles in assembly, regulation and stability. The correct addition of prosthetic groups as well as chaperoning and incorporation of the structural components require a large number of factors, many of which have been found mutated in cases of mitochondrial disease. Nowadays, the mechanisms of assembly for each of the individual complexes are almost completely understood and the knowledge about the assembly factors involved is constantly increasing. On the other hand, it is now well established that complexes I, III and IV interact with each other, forming the so-called respiratory supercomplexes or ‘respirasomes’, although the pathways that lead to their formation are still not completely clear. This review is a summary of our current knowledge concerning the assembly of complexes I–V and of the supercomplexes.

Introduction

The oxidative phosphorylation system (OXPHOS) of the mitochondrial inner membrane is composed of five enzymes (complexes I–V; cI–V). In mammals, they are all multimeric and, except for cII, have subunits encoded both in the mitochondrial genome (mtDNA) and the nuclear genome (nDNA). The mtDNA-encoded subunits are hydrophobic and their translation happens close to the inner membrane to facilitate their translocation [1]. The nuclear-encoded structural subunits and many other factors necessary for the correct biogenesis of OXPHOS are expressed in the cytoplasm and imported inside the organelle [2].

Assembly of mitochondrial complexes II–V has been extensively studied in *Saccharomyces cerevisiae* [3–7], whereas research concerning cI has been carried out in *Yarrowia lipolytica* [8] and *Neurospora crassa* [9]. Many factors and mechanisms are conserved in mammals, and this has helped to identify genetic mutations associated with mitochondrial disease. However, it is now evident that there are specific factors in higher animals that are also involved in OXPHOS biogenesis and efforts are being made to understand their exact functions and implications in disease (see article by Ghezzi and Zeviani in this issue [201]). Moreover, studying assembly defects both in human cells and mouse disease models, has given highly valuable information about the assembly pathways and the proteins involved [10].

The OXPHOS complexes can interact with each other forming higher order structures, called supercomplexes or ‘respirasomes’ [11–13], whose functional role and assembly are still not completely understood [14–18].

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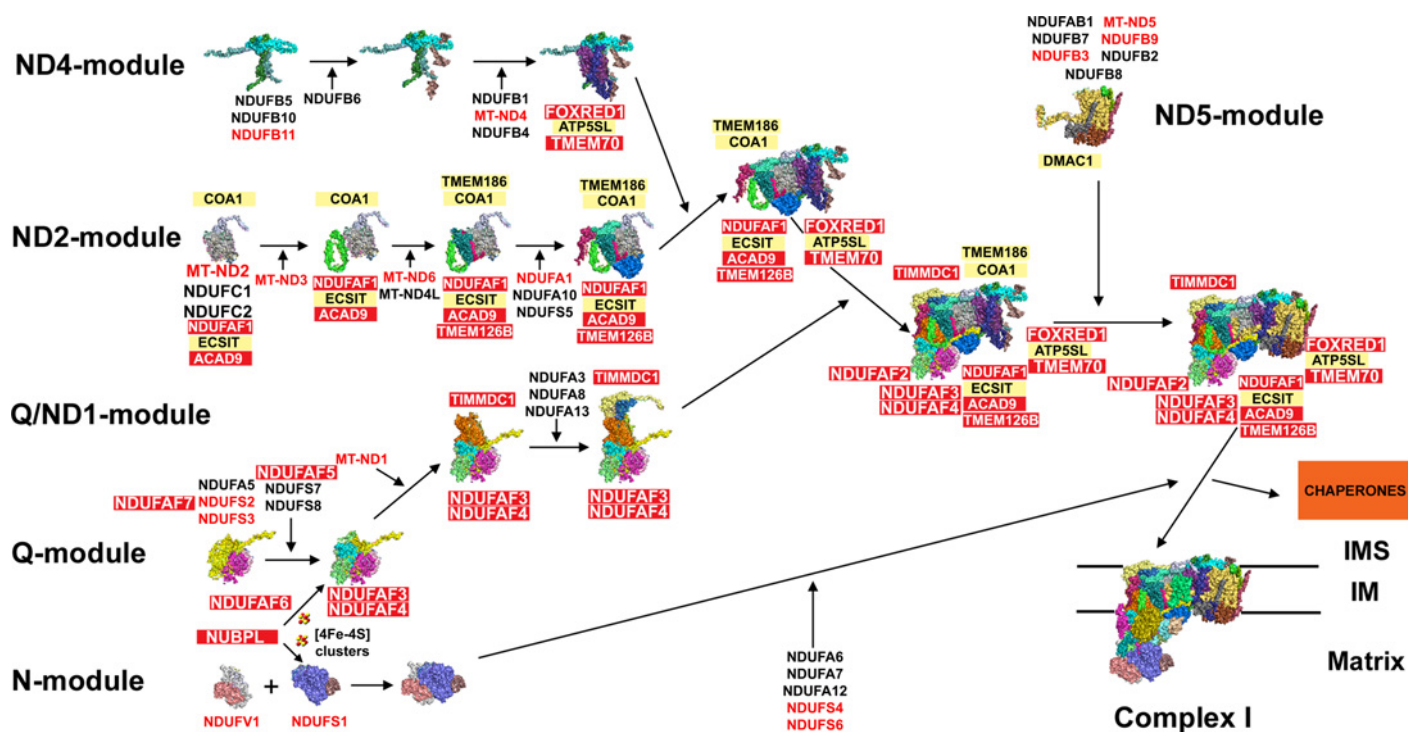


Figure 1. Complex I assembly model (see main text for details) based on the bovine cI cryo-EM structure with Protein Data Bank (PDB) ID: 5LC5 [23] and the models proposed in references [33,34,199]

Red colour indicates proteins with described pathological mutations. Abbreviations: IM, inner membrane; IMS, intermembrane space.

Assembly of complex I

Complex I (EC 1.6.5.3) or NADH:ubiquinone reductase (H^+ translocating) with 45 subunits is the largest OXPHOS complex. It is an L-shaped enzyme with a hydrophilic arm protruding into the matrix, where electron transfer from NADH to coenzyme Q (CoQ) happens, and a proton translocating hydrophobic arm. The CoQ binding site is in the interface of both arms. Fourteen core subunits, conserved from bacteria to humans, perform the catalytic activities [19,20]. Seven core subunits in the hydrophilic arm contain the redox active centres: a non-covalently bound FMN and seven Fe–S clusters [21]. The other seven are all the cI subunits encoded in the mtDNA and are located in the hydrophobic arm, forming the proton channels [22]. The remaining 30 subunits are ‘supernumerary’ but important for assembly and stability [22–24].

Exhaustive research concerning human cI assembly has been carried out for 15 years [25–33]. However, several recent breakthroughs have granted a much deeper understanding about this process. Thus, we now know the complete mammalian cI structure [22,23] and how the subunits are organized in six modules (N, Q, ND1, ND2, ND4 and ND5) that, with the help of specific assembly factors, are brought together through five main subassemblies (Figure 1) [24,34].

The **N-module**, which is the tip of the hydrophilic arm and the last one to be incorporated [30,35], results from the assembly of NDUFV1, NDUFV2, NDUFV3 and NDUFV4 [34], to which NDUFV6, NDUFV7, NDUFV12, NDUFV4, NDUFV6 and NDUFV3 must be further associated with to complete the module [24].

The **Q-module** is built through the association of NDUFV5, NDUFV2 and NDUFV3 plus NDUFV7 and NDUFV8. The chaperones NDUFV3/C3ORF60 and NDUFV4/C6ORF66 [36,37] remain bound to this module until the final assembly steps [34]. NDUFV6/C8ORF38 [38] also seems to participate in the assembly of the Q-module [24,39]. NDUFV3, 4 and 6, are necessary to maintain normal MT-ND1 synthesis [40,41]. NDUFV5 adds a hydroxyl group to Arg⁷³ of NDUFV7 [42] and NDUFV7 dimethylates NDUFV2 in Arg⁸⁵ [43], an essential modification for cI assembly [44]. NUBPL/IND1 delivers [4Fe–4S] clusters specifically to the N- and Q-module subunits [45,46].

The **ND1-module** builds around the Q-module with the help of TIMMDC1/C3ORF1 [47,48], which remains bound to the Q/ND1 subassembly until the last maturation steps. MT-ND1 joins first and then NDUFA3, NDUFA8 and NDUFA13 are added [34].

The **ND2-module** is formed by an initial intermediate that contains MT-ND2, NDUFC1 and NDUFC2 bound to NDUFAF1/CIA30 [49,50], ECSIT [51] and ACAD9 [52,53]. Then, MT-ND3 is added together with TMEM126B [54], forming a larger intermediate to which subunits MT-ND6 and MT-ND4L bind. The latest assembly stages involve the incorporation of subunits NDUFA1, NDUFA10 and NDUF5 [24,34]. The stable association of the assembly factors NDUFAF1 + ECSIT + ACAD9 + TMEM126 was denominated Mitochondrial Complex I Assembly (MCIA) complex [48,54]. Two other chaperones were found interacting with this module: TMEM186 and COA1 [34], the latter being a well-known cIV assembly factor [55,56].

The main **ND4-module** intermediate binds NDUFB1, NDUFB4, NDUFB5, NDUFB6, NDUFB10, NDUFB11 and MT-ND4 together with FOXRED1 [46,57–59], ATP5SL [24,47] and also TMEM70, described as a cV assembly factor [34,60,61].

The **ND5-module** corresponds to the distal part of the membrane arm and it is composed of MT-ND5, NDUFB2, NDUFB3, NDUFB7, NDUFB8, NDUFB9 and NDUFAB1 [24,34]. DMAC1/TMEM261 is implicated in its stabilization and/or assembly [24].

The ND2- and the ND4-modules get together first, with still all the chaperones bound to them. Later on, the Q/ND1 and the ND5-modules join the nascent complex. This intermediate only lacking the N-module is stabilized by NDUFAF2/NDUFA12L/B17.2L [24,35,62]. In the last step, the pre-assembled N-module becomes attached and the chaperones released [34].

Assembly of complex II

Complex II (EC 1.3.5.1) or succinate dehydrogenase (quinone) is shared between the TCA cycle and the ETC and has no proton pumping activity. It is composed of four nDNA-encoded subunits. The two hydrophilic catalytic subunits are SDHA/SDH1 and SDHB/SDH2. Hydrophobic subunits SDHC/SDH3 and SDHD/SDH4 constitute the cII membrane anchor, containing a haem *b* group and two CoQ binding sites [63–65]. The two electrons from succinate oxidation are transferred to a FAD covalently bound to SDHA, then to the three different Fe–S clusters in SDHB and finally to CoQ [65,66].

Complex II assembly (Figure 2) happens through the independent maturation of SDHA, SDHB and SDHC + SDHD mediated by subunit-specific chaperones [7].

SDHA is flavinylation before assembly into cII, and SDHAF2/Sdh5 mediates this step [67,68]. Following FAD incorporation, SDHA binds to SDHAF4/Sdh8, which keeps the subunit stable and competent for assembly with SDHB, while protecting it from auto-oxidation [69].

SDHB also incorporates its Fe–S clusters before joining the rest of the subunits. Fe–S clusters are synthesized in the mitochondrial matrix [70,71] and then transferred to the apoprotein. This step is mediated by SDHAF1, necessary also for SDHB stability [72–74]. SDHAF3/ACN9/LYRM10 is another protein involved in SDHB stability and oxidative damage protection after insertion of the Fe–S clusters [7,75,76].

When both SDHA and SDHB acquire their respective prosthetic groups they join together, liberating SDHAF4 but keeping the binding with SDHAF1 and SDHAF3 [7,75].

SDHC and **SDHD** are assembled together in the inner membrane by a yet unknown mechanism. The haem *b* group, co-ordinated in the interface of both subunits, does not play any catalytic role but is required for their stability [77,78]. Another factor that influences the dimerization of SDHC and SDHD, as well as their stability, is the presence of both hydrophilic subunits [68,75].

Assembly of complex III

Complex III (EC 1.10.2.2) or quinol-cytochrome *c* reductase performs electron transfer coupled to proton pumping using the ‘Q-cycle’ mechanism [79,80]. Structurally, it is a tightly bound symmetrical dimer (cIII₂), being each ‘monomer’ composed of three catalytic core (MT-CYB, CYC1 and UQCRFS1) and seven supernumerary subunits [81,82]. The 78-amino acid mitochondrial targeting sequence (MTS) cleaved off from UQCRFS1 was considered an extra subunit [81,83], but it needs to be cleared out to maintain cIII₂ structural and functional fitness [84,85]. MT-CYB contains two *b*-type haems with different redox potential as well as two CoQ binding sites. There is one [2Fe–2S] cluster inserted in the C-terminal end of UQCRFS1, and CYC1 binds a haem *c*1 group that transfers the electrons to the mobile electron carrier cytochrome *c*. The supernumerary subunits are not involved in the catalysis, but are important for correct assembly and/or stability of the enzyme [86,87].

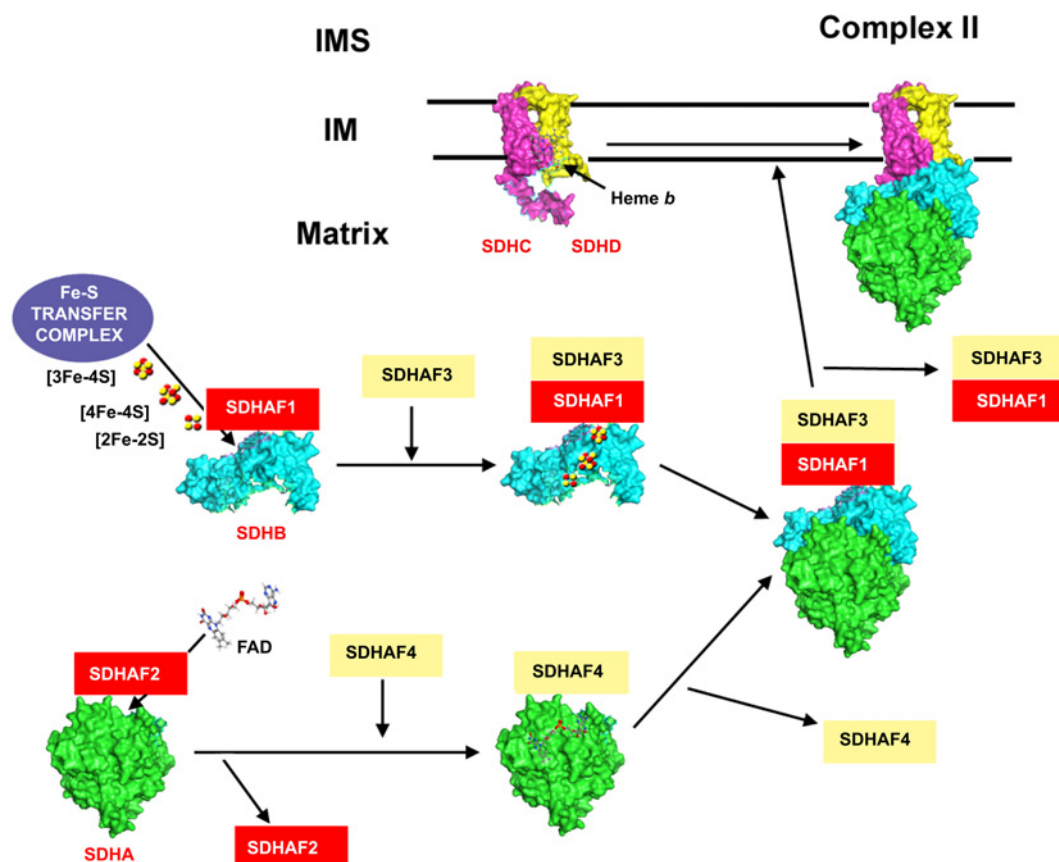


Figure 2. Complex II assembly model (see main text for details) based on the porcine cII crystal structure with PDB ID: 1ZOY [65] and the model proposed in reference [7]

Red colour indicates proteins with described pathological mutations. Abbreviations: IM, inner membrane; IMS, intermembrane space.

Yeast cIII assembly starts with the synthesis of **cytochrome b** (MT-CYB in human nomenclature) by mitochondrial ribosomes and its insertion into the inner membrane, mediated by Cbp3/UQCC1 and Cbp6/UQCC2 that remain bound to MT-CYB once it is completely synthesized. Cbp4/UQCC3 joins after the first haem-*b* (*b_L*) but before the second one (*b_H*) is incorporated [88–90]. Once the first structural subunits (UQCRB and UQCRQ) are incorporated, UQCC1–UQCC2 detach and go back to act as translational activators [88,89]. These first steps in cIII assembly (Figure 3) are supposedly conserved, because the three factors are present in humans and mutations in *UQCC2* produce deficient MT-CYB synthesis [91,92].

Maturation of cIII occurs, both in yeast and humans, with the addition of the **Rieske Fe–S protein** (Rip1/UQCRFS1) and of the smallest subunit (Qcr10/UQCR11) to an already dimeric pre-complex III (pre-cIII₂) [93–95]. After import into mitochondria, UQCRFS1 is bound and stabilized in the matrix by MZM1L/LYRM7 [96–98] that also mediates binding to the Fe–S cluster transfer complex [99]. Incorporation of UQCRFS1 to pre-cIII₂ is mediated by Bcs1/BCS1L [93,94,100,101]. In human and mouse mitochondria, TTC19 [102] binds fully assembled cIII₂ and favours the elimination of UQCRFS1 N-terminal fragments to maintain normal activity levels [84].

The intermediate steps of cIII₂ assembly are not known in humans. However, being that the initial and the final stages are the same and the assembly factors involved are orthologous proteins, it is assumed that they will share very many similarities [103]. The order of incorporation in *S. cerevisiae* was determined by creating yeast strains deleting one structural subunit at a time and studying the stability of the remaining cIII components [104–107]. Up to now, there are no described assembly factors involved in the incorporation or stabilization of cIII₂ intermediate subunits and transitional subcomplexes.

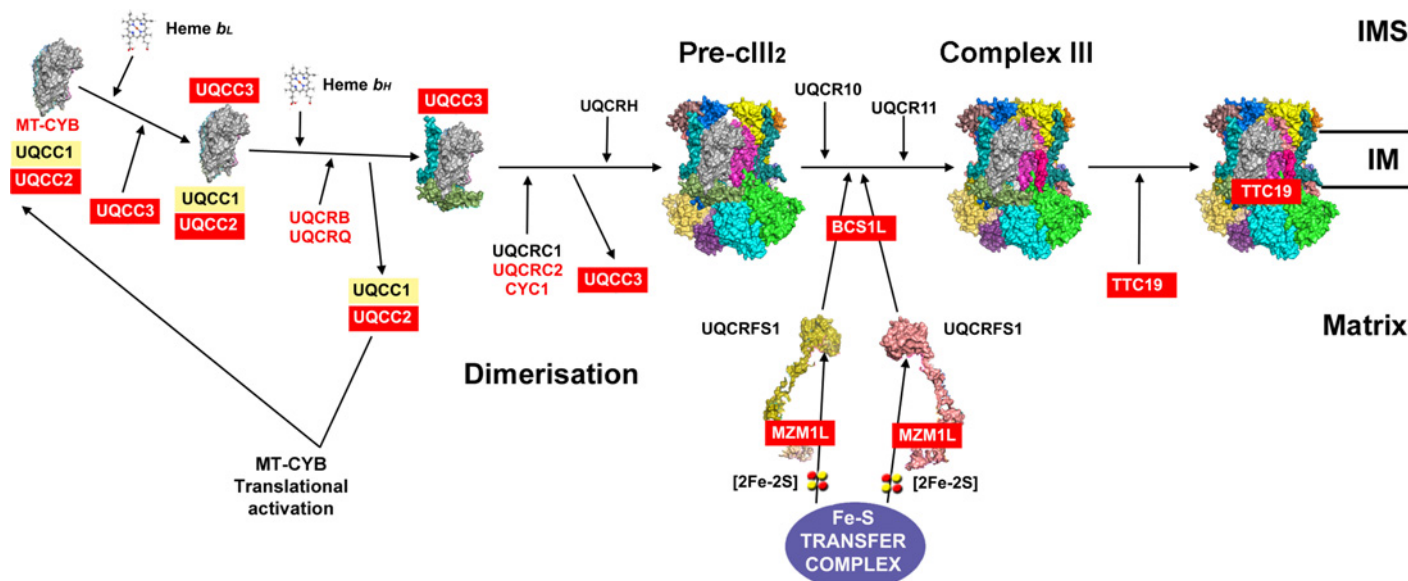


Figure 3. Complex III assembly model (see main text for details) based on the bovine cIII₂ crystal structure with PDB ID: 1BGY [81] and the models proposed in references [85,103]

Red colour indicates proteins with described pathological mutations. Abbreviations: IM, inner membrane; IMS, intermembrane space.

Assembly of complex IV

Complex IV (EC 1.9.3.1) or cytochrome *c* oxidase (COX) catalyses the oxidation of cytochrome *c* and the reduction of oxygen to water, coupled to proton translocation [108]. Mammalian cIV contains 13 or 14 subunits [109–111]. MT-CO1 is the largest catalytic subunit containing a haem *a* group and a binuclear haem *a*₃-Cu_B centre. MT-CO2 is the second core subunit and holds the Cu_A centre. MT-CO3, the third core subunit, plays no direct catalytic role [108]. The rest of subunits (supernumerary) are thought to be important for the stabilization of the catalytic core and regulation of its activity [112–117]. Complex IV is the only OXPHOS complex containing tissue-specific and developmentally regulated isoforms [118,119], reflecting the importance of an exquisite regulation of COX activity.

The first model of subunit incorporation for human COX [120], basically still stands with minor modifications [115,121–124]. According to this model, **MT-CO1** is the ‘seed’ around which the rest of the subunits build up, starting with COX4I1 and COX5A. The stable subassemblies created during this process were named S1–S4, S4 being the fully assembled holoenzyme [10,120]. Proteomics analyses of a MT-CO3-deficient cell line, with a very prominent subcomplex accumulation, completed the view about subunit incorporation (Figure 4), which happens in groups or ‘modules’, defined by each one of the core subunits [123], as it does in yeast [125].

The **initial COX subassembly** appears to be the association of COX4I1 + COX5A [123]. This early subcomplex also contains HIGD1A [123], one of the human homologues of yeast Rcf1 [126–129].

The **MT-CO1 module** contains the many chaperones and assembly factors involved in its maturation and stabilization, and it is also known as ‘MITRAC’ for mitochondrial translation regulation assembly intermediate of cytochrome *c* oxidase [56,130]. COX14/C12ORF62 [55,131] and COA3/CCDC56/MITRAC12 [56,132] bind nascent MT-CO1 and are implicated in assembly regulation either by translational [133] or post-translational mechanisms [134]. In human mitochondria, MT-CO1 expression is especially sensitive to defects in the mitochondrial RNA-binding protein LRPPRC [135–137] and requires the specific translational activator TACO1 [138,139]. Later on, CMC1 binds MT-CO1 + COA3 + COX14 before or during addition of the prosthetic groups [134]. Haem A biosynthesis is carried out by COX10 [140,141] and COX15 [142]. The exact molecular function of SURF1 [143,144] remains unclear, but its involvement in haem A delivery has been proposed [124]. A role for PET117 in this same process has been suggested due to its interaction with COX15 [145]. Cu_B assembly requires the metallochaperone COX11 [146,147], with COX17 donating the coppers [148,149], and COX19 maintaining COX11 in the right redox state [150]. CMC1 is released prior to the addition of COA1/C7ORF44/MITRAC15 [55,56,151] and SURF1. MITRAC7/SMIM20 is another factor described to stabilize MT-CO1 in early assembly stages [130].

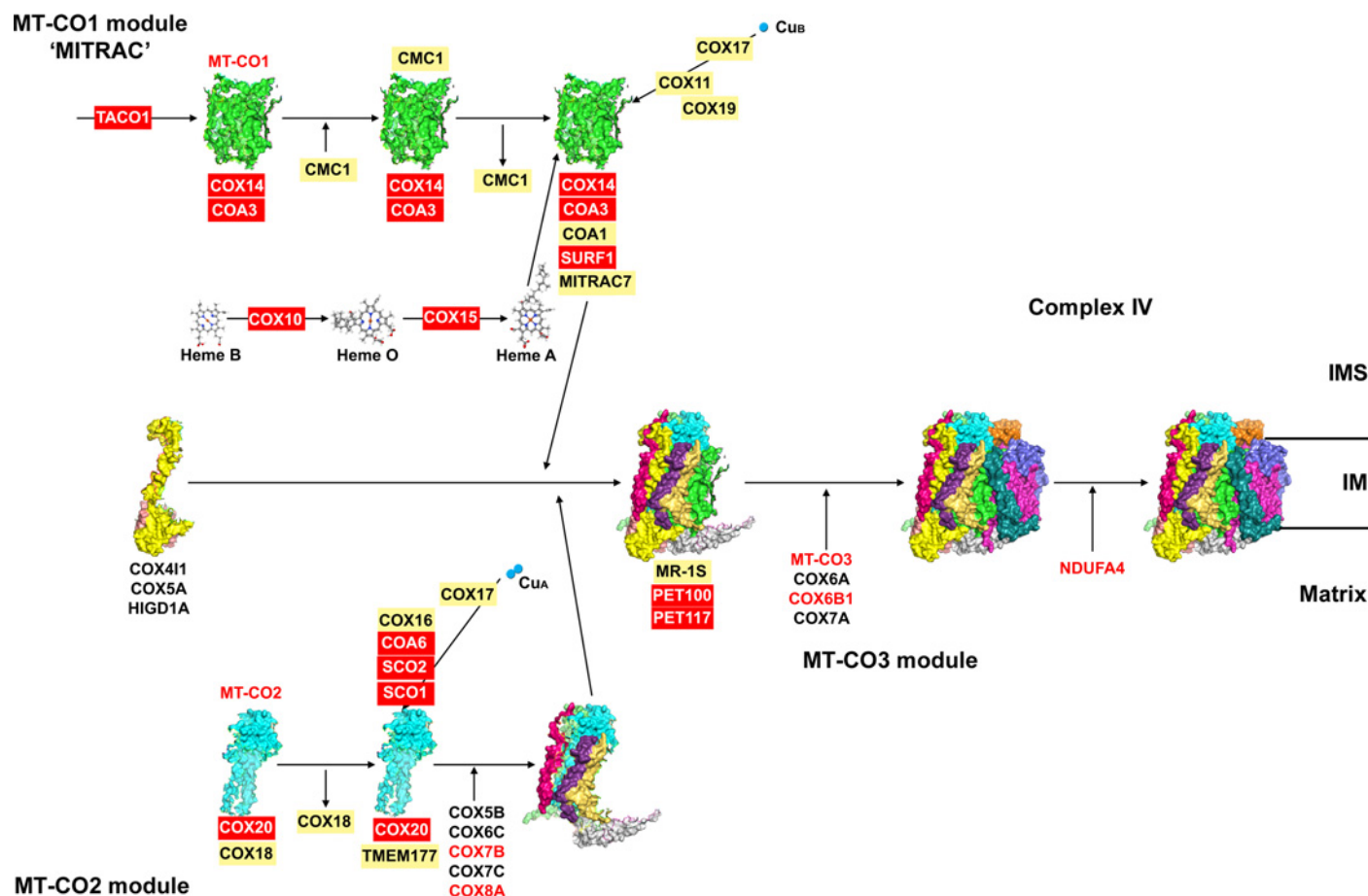


Figure 4. Complex IV assembly model (see main text for details) based on the bovine cIV crystal structure with PDB ID: 2OCC [109] and the model proposed in reference [123]

Red colour indicates proteins with described pathological mutations. Abbreviations: IM, inner membrane; IMS, intermembrane space.

The intermediate step in COX assembly is the joining of COX4I1 + COX5A, MT-CO1 and the **MT-CO2 module** (MT-CO2 + COX5B + COX6C + COX7C + COX8A and, most probably COX7B), equalling the 'S3' intermediary [120] minus MT-CO3 [123]. MT-CO2 requires COX18 for membrane translocation [152] and COX20/FAM36A and TMEM177 for stabilization [153–155]. Copper-binding proteins COX17, SCO1 and SCO2 [156–158] together with COA6 [159–161] and COX16 [162–164], are involved in the assembly of the Cu_A centre. MR-1S is a vertebrate-specific COX chaperone that interacts with the highly conserved factors PET100 [165–167] and PET117 [168,169] during assembly of the MT-CO2 module [123].

The incorporation of the **MT-CO3 module** (MT-CO3 + COX6A1 + COX6B1 + COX7A2) completes the assembly of the 13 canonical COX subunits [109,123]. No specific assembly factors for this module are currently known.

The **last subunit** to be incorporated is NDUFA4, initially thought to be part of complex I [170], but later assigned to complex IV [110,117].

More proteins than those described here are required for cIV assembly [124] but their exact molecular role is still not understood.

Assembly of complex V

Complex V (EC 3.6.14), H⁺-transporting two-sector ATPase or F₀F₁-ATPase, is the enzyme that synthesizes ATP using the proton motive force generated by cI, III and IV. It is composed of two topological and functional distinct domains: membrane-extrinsic and matrix-facing F₁ plus membrane-intrinsic F₀, with a central axis and a peripheral stalk connecting them [171]. Subunits a (MT-ATP6) and A6L (MT-ATP8) of the F₀ domain are encoded in the

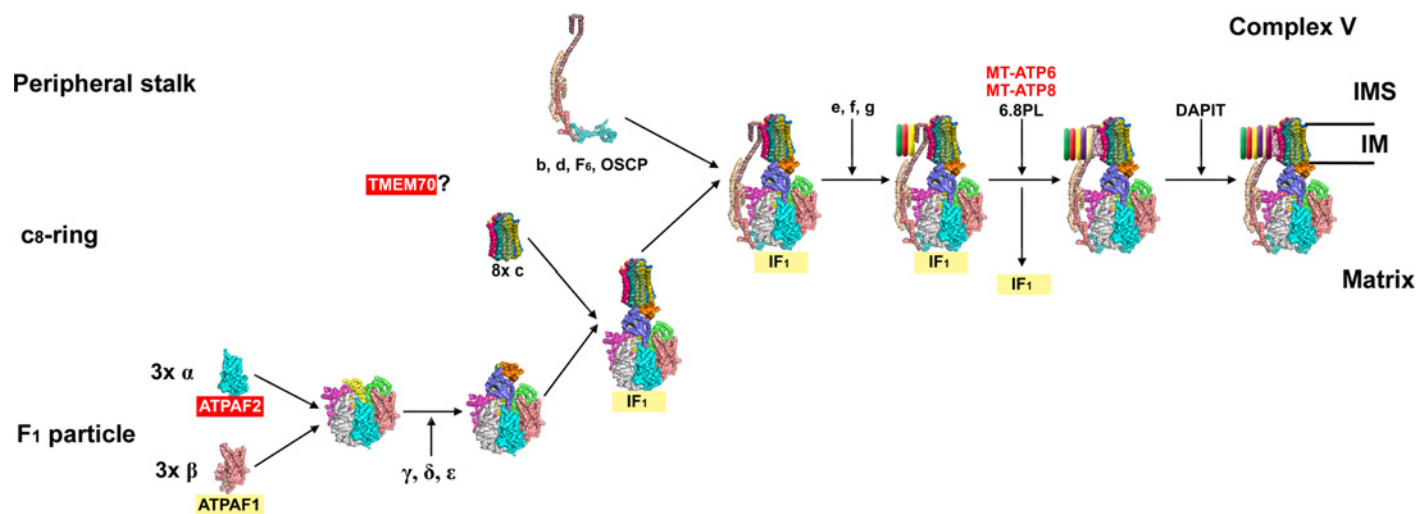


Figure 5. Complex V assembly model (see main text for details) based on the bovine cV cryo-EM structure with PDB ID: 5ARA [200] and the model proposed in references [172,180]

Red colour indicates proteins with described pathological mutations. Abbreviations: IM, inner membrane; IMS, intermembrane space.

mtDNA, whereas all the rest of cV components are nDNA encoded [172]. Protons coming back to the matrix through F_o produce a rotational movement providing the energy for ADP+Pi condensation in the F_1 domain [171,173].

Assembly of cV has been studied using subunit incorporation dynamics [174], analysis of mtDNA-deficient cell lines [175,176] and more recently by creating knockout cell lines for specific cV subunits [177–180]. As depicted in Figure 5, this complex is also put together by assembling three pre-formed modules corresponding to: F_1 particle, c_8 -ring (a ring composed by eight copies of the c-subunit) and peripheral stalk [172].

The **F_1 subcomplex**, composed of three copies of the α subunit/ATP5A1, three β subunits/ATP5B together with the central stalk subunits γ /ATP5C1, δ /ATP5D and ϵ /ATP5E, is assembled with the assistance of chaperones ATPAF1/ATP11 and ATPAF2/ATP12, which bind ATP5B and ATP5A1, respectively [181–185]. The **c_8 -ring**, encoded by *ATPG1*, *ATPG2* and *ATPG3*, is assembled in the membrane by still unknown mechanisms [172]. A subcomplex containing subunits of the **peripheral stalk** is also pre-formed [177,180].

After the c_8 -ring and the F_1 subcomplex come together, the peripheral stalk is incorporated in two steps: first subunits b/ATP5F1, d/ATPH, F_6 /ATP5J and OSCP/ATP5O and then e/ATP5I, g/ATP5L and f/ATP5J [172,180]. The peripheral stalk can also join the F_1 subcomplex in absence of the c_8 -ring [179,180]. During these initial steps, the inhibitor protein IF1 is bound to the intermediates, being liberated with the insertion of the two mtDNA-encoded subunits [178–180]. In the cases in which a/MT-ATP6 and A6L/MT-ATP8 are missing, the previous assembly intermediate is readily accumulated [174,176,179]. The interaction of the last subunits is stabilized by 6.8L/MLQ/C14ORF2 and the peripheral subunit DAPIT/USMG5 is incorporated to finish cV assembly [180].

One of the few proteins known to be involved in cV biogenesis is TMEM70 and although its exact function is still not known, mutations in the gene encoding this factor have recurrently been associated with ATP synthase deficiency [60,186].

Assembly of respiratory supercomplexes

The OXPHOS complexes interact with each other forming higher order structures, which have been called supercomplexes. Complexes IV and V can form dimers and oligomers [11,187,188]. In addition, defined associations of complexes I, III and IV are reproducibly found when mitochondrial membrane extracts are solubilized with digitonin and separated through Blue Native Gel Electrophoresis [11,12]. Thus, according to their molecular size and subunit composition, the main supercomplexes have been assigned the following stoichiometries: III_2IV_1 , I_1III_2 , $I_1III_2IV_1$, and $I_2III_2IV_{1-2}$. Supercomplex $I_1III_2IV_1$ is the ‘respirasome’ and supercomplex $I_2III_2IV_2$ has been named as ‘respiratory megacomplex’ [189]. High-resolution Cryo-EM structures of the respirasome of several mammalian species,

including human, have been recently resolved [189–193]. The association of the individual complexes into these structurally defined supercomplexes is now very well established but their specific functional role still needs to be clarified [14–18].

Two alternative views exist to explain respirasome assembly. The first possibility is that the individual complexes are completely assembled before they join together in the supercomplexes [12,34]. This mode of action would permit the dynamic association-dissociation of the complexes to adapt to varying energy demands, if the role of the supercomplexes were to increase the efficiency of electron transfer, as proposed by the ‘plasticity model’ [12,194]. However, there are also evidences pointing to the co-assembly of subunits from the different complexes before completion of the single enzymes. Accordingly, maturation of cI would not happen unless cIII₂ and cIV are bound to a ‘pre-cI’ scaffold [195]. Also, incomplete complexes have been found assembled together in cultured cells and tissues from patients carrying mutations in different structural subunits and assembly factors implicated in the last steps of cI and cIII assembly [10]. The fact that COA1, a well-characterized cIV chaperone, is bound to cI assembly intermediates [34] could also reflect co-assembly of at least cI and cIV, although the authors of this report did not provide evidence as to whether MT-CO1 is also bound to the same subcomplexes.

COX7A2L/COX7R/SCAFI is an orthologue of the cIV structural subunit COX7A that was first described as a supercomplex assembly factor because of being necessary for the incorporation of cIV into supercomplex structures [194]. However, more recent evidences have demonstrated a role for this protein for the formation of III₂IV₁ but not for the incorporation of cIV into the respirasomes [188,196,197]. The dynamic interchange between the three types of COX7A proteins, COX7A2L (SCAFI), COX7A1 (muscle-type structural subunit) and COX7A2 (liver-type structural subunit) could potentially determine whether cIV stays as a monomer, oligomerizes or forms the III₂IV₁ supercomplex, as well as the mode of binding to cI [13,198].

Final remarks

Assembly of the OXPHOS system is an intricate process that we still do not completely understand, despite the great efforts of many research teams and the spectacular advances described here. It is important to continue studying the processes governing the assembly of each of the complexes and of the supercomplexes, as well as the exact molecular role of the proteins involved in its basic assembly and fine regulation. This will help us understand the mechanisms regulating this central part of metabolism in health and disease. For a detailed explanation of the pathologies associated with mutations in the described assembly factors, see the accompanying article in this issue: ‘Human diseases associated with defects in assembly of OXPHOS complexes’ Ghezzi and Zeviani [201].

Summary

- Assembly of the OXPHOS complexes requires a significant amount of ancillary proteins.
- Many assembly factors are conserved from yeast to humans, but some are specific for higher animals.
- Complex I is the largest OXPHOS enzyme and its assembly occurs through modules, each of which requires specific assembly factors.
- Despite being the smallest OXPHOS component, complex II assembly is assisted by, at least, four different chaperones.
- Up to now, only the first and last steps of complex III assembly are well understood.
- Complex IV assembly is highly regulated, with more than 30 known assembly factors, involved mainly in the maturation of the catalytic core.
- The order of incorporation of the 17 subunits of complex V is well known, but only 3 assembly factors have been identified so far.
- The OXPHOS complexes interact with each other in the supercomplexes or ‘respirasomes’, although the way they assemble together is still not known.

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Competing interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

CoQ, coenzyme Q; COX, cytochrome c oxidase; MITRAC, mitochondrial translation regulation assembly intermediate of cytochrome c oxidase; MTS, mitochondrial targeting sequence; OXPHOS, oxidative phosphorylation system.

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