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From respiratory supercomplexes to mega-complexes

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Abstract

Cellular energy is produced in mitochondria by respiratory enzymes in a process called oxidative phosphorylation. Respiratory enzymes are large proteins embedded in the inner mitochondrial membrane which can occur either as individual enzymes or organize into larger assemblies called super- and mega-complexes. Originally, supercomplex organization was not generally accepted, but nowadays, their existence is clear. However, the functional aspects are still in the dark. In this overview, we will briefly touch upon the history of supercomplex research, the variation of the supercomplex compositions and shed light on the challenges of studying their functional aspects.

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1. Introduction

Every cell in our bodies is a hub of activity fueled by cellular respiration. When your eyes are browsing through this short overview, your mitochondria are busy producing adenosine triphosphate (ATP) – the energy currency of the cell. In general, it is widely known that mitochondria are the powerhouses of the cells, but the details of the tiny molecular machines that carry out the processes are less known. These molecular machines, the respiratory enzymes, are a class of transmembrane proteins that participate in cellular respiration, more specifically the mitochondrial energy conversion.

Cell respiration is the series of chemical reactions in which nutrients, like glucose, are broken down to produce ATP. There are many metabolic pathways, both aerobic and anaerobic, all of which contain a series of chemical reactions but in this overview, we will focus on oxidative phosphorylation (OXPHOS), the culmination of cell respiration. OXPHOS comprises the electron transport chain (ETC) which generates proton electrochemical gradient across the inner mitochondrial membrane (alternatively bacterial membrane in prokaryotes lacking mitochondria) by oxidizing NADH obtained from Krebs cycle. The proton electrochemical gradient is required by F_1F_o-ATP synthase to produce ATP by phosphorylating ADP.

The ETC enzymes are called respiratory complexes I-IV; complex I (CI) or NADH:ubiquinone (UQ) oxidoreductase, complex II (CII) or succinate:UQ oxidoreductase, complex III (CIII) or cytochrome bc_1, and complex IV (CIV) or cytochrome *c* oxidase. Ubiquinone (UQ) and cytochrome *c* (cyt *c*) are mobile electron carriers crucial for OXPHOS located in the membrane and the external membrane surface, respectively. Complex I is the first and largest enzyme in ETC. It receives electrons from NADH and transfers them to UQ, while pumping protons across the inner mitochondrial membrane contributing to the proton gradient. Complex II does not directly contribute to the proton gradient, but it reduces UQ thereby having an indirect contribution. Complex III transfers electrons from UQ to cyt *c* while also pumping protons. Complex IV is the final step in the ETC, where electrons are transferred to oxygen, the final electron acceptor, producing water and pumping more protons to maintain the gradient. See Figure 1 for a schematic of the process.

The idea of a larger entities of respiratory complexes was proposed already decades ago (Chance et al., 1963; Keilin & Hartree, 1947). Roughly at the same time with these suggestions, individual functioning complexes were obtained (Hatefi et al., 1962). Therefore, the discussion of the organization of the ETC complexes in the membrane was initiated early on. On the absolute extreme ends of the scale, the two opposite theories for ETC complexes are the so called "fluid" and "solid" state models (Milenkovic et al., 2017). The "solid" state model considers the ETC complexes gathering into single units with coenzyme Q. On the contrary, the "fluid" model proposes that the ETC complexes act separately, with electron carriers like ubiquinone and cytochrome c shuttling electrons between them (Hackenbrock et al., 1986). In the "fluid" state model, the reaction catalysis is based on diffusion and random collisions of the enzymes and carriers in the membrane gave the name random collision model

(Hackenbrock et al., 1986). In fact, the random collision model became the accepted standard model for ETC until blue native polyacrylamide gel electrophoresis (BN-PAGE) analysis, which allowed researchers to isolate and visualize protein complexes in their native state, was developed. The analysis revealed the existence of large, stable assemblies of respiratory complexes in mammalian and yeast respiratory chains, later identified supercomplexes (SC) (Schagger & Pfeiffer, 2000). These findings challenged the traditional fluid model and suggested that the respiratory complexes might work together in a more organized manner than previously envisaged. It is noteworthy, that the existence of the larger entities does not remove the ability of the individual ETC complexes from catalyzing the reactions as well. While approximately 85-100 % of CI is estimated to be found in SC, the corresponding estimates are only 55-65 % for CIII and 15-25 % for CIV (Greggio et al., 2017; Schagger & Pfeiffer, 2001). The development of more and more advanced imaging techniques allowed the visualization of these SCs in unprecedented detail, up to the atomistic detail as of today.

2. Composition and architecture

The three main mammalian SC compositions in order of declining molecular mass are $CI_1CIII_2CIV_1$ (~1.7 MDa), CI_1CIII_2 (~1.5 MDa) and $CIII_2CIV_1$ (~0.7 MDa) (Letts & Sazanov, 2017) (See panels A-C in Figure 2). The $CI_1CIII_2CIV_1$ composition is special as it is the one most observed in BN-PAGE analyses and has been named the respirasome. The respirasome is considered a "base unit" of respiratory SCs and *in vitro*, it can carry out the entire NADH to O_2 oxidoreduction as presented linearly in Figure 1.

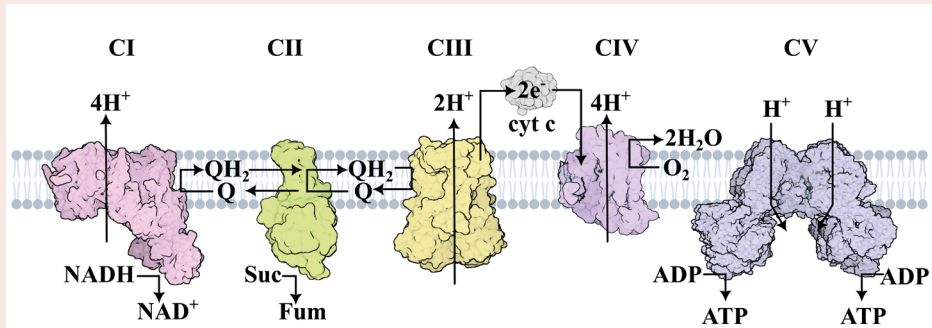

Figure 1. Linear view of the respiratory chain. H^+ represent proton and e- electron. The figure was created in BioRender.

While 2D projections and 3D maps of the SCs were published starting from mid 2000s (Althoff et al., 2011; Dudkina et al., 2005; Dudkina et al., 2011; Schafer et al., 2007; Schafer et al., 2006), it is noteworthy that the resolution was only around ~20 Å which means that atomistic details were not resolved. The first atomistic mammalian respirasome structure was published by Letts, Fiedorczuk and Sazanov from ovine mitochondria in 2016 (Letts et al., 2016). While there are reports of bacterial SCs, the research was heavily concentrated on mammalian respirasomes, and since high-resolution structures require a template structure, the lack of the atomistic mammalian CI structure until 2016 (Fiedorczuk et al., 2016; Zhu et al., 2016) was a bottleneck for resolving the respirasome structure as well. Therefore, the first respirasome structure was quickly followed by two others, a respirasome from porcine (Gu et al., 2016; Wu et al., 2016) and bovine (Sousa et al., 2016). In all the above mammalian structures, the overall architecture is similar with CI curved around the CIII dimer and CIV located at the antiporter end of the CI membrane arm. See Figure 2.

In addition to the respirasome, there are also larger respiratory assemblies. These assemblies are often referred to as megacomplexes. For example, the human mitochondrial megacomplex structure was published in 2017 in $CI_2CIII_2CIV_2$ composition (Guo et al., 2017). The architecture shares similarity with the respirasome with CI curving around CIII, but in the megacomplex two CI's surround the CIII from opposite sides almost like a ring around it. Recently, multiple megacomplex assemblies were observed in porcine mitochondria in situ (Zheng et al., 2024). These include $CI_1CIII_2CIV_1$, $CI_1CIII_2CIV_2$, $CI_2CIII_2CIV_2$ and $CI_2CIII_4CIV_2$ truly highlighting the variance of the mitochondrial super/megacomplexes.

Notably, CII is not a part of the SC compositions presented above. A recent study on ciliate protist *Tetrahymena thermophila* revealed a unique SC composition $CI_1CII_1CIII_1CIV_2$ where, for the first time, CII was observed as a part of a SC. Considering it has been suggested that CII also interacts with the proton pumping respiratory complexes, the result does not contradict earlier findings (Acin-Perez et al., 2008; Jiang et al., 2020; Lapuente-Brun et al., 2013; Schon & Dencher, 2009; Zhou et al., 2022). CII was also hypothesized as a part of the human megacomplex but not observed (Guo et al., 2017). While there are some similarities between the two assemblies, they are so different that direct comparison of the CII location is not straightforward.

3. Functional aspects

Since SCs have been observed in many compositions and architectures, what are their functional implications? Thorough lists and analyses can be found for example in reviews by Letts & Sazanov and Milenkovic et al. (Letts & Sazanov, 2017; Milenkovic et al., 2017) but in this small overview, we will only briefly cover a few of the different aspects and more detailed consideration is left to the reader.

The most logical explanation for the existence of SCs would be substrate channeling since the structures bring the enzymes close together with possibility to even have separate substrate pools from

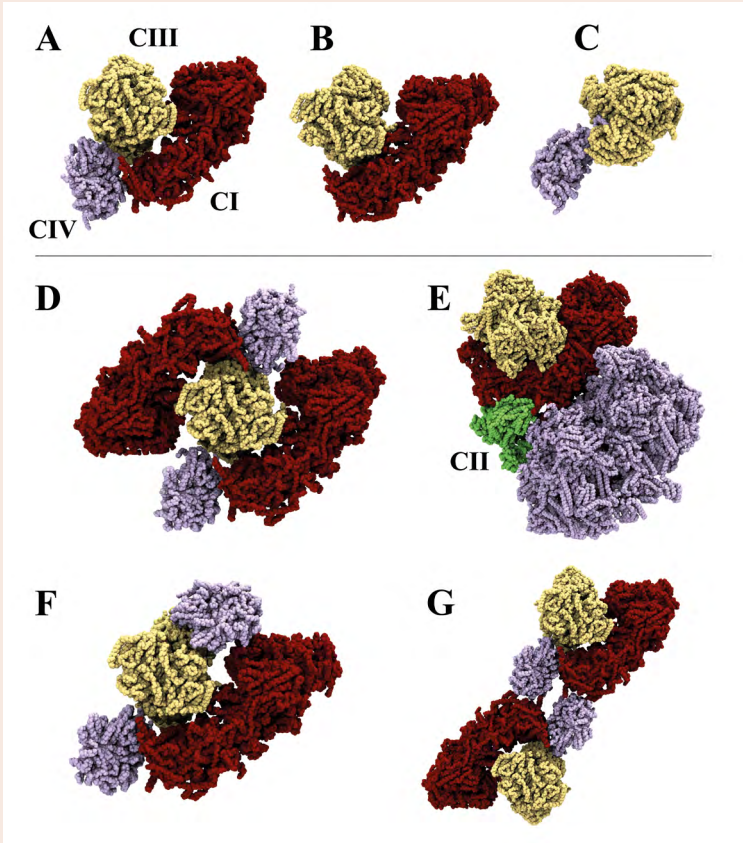

Figure 2. Examples of supercomplex (A-C) and megacomplex (D-G) architectures. In all panels, CI is shown in maroon, CII in lime green, CIII in pale yellow and CIV in lavender. The PDBids of the structures used are: (A) 5j4z (Letts et al., 2016) (B) 6qbx (Letts et al., 2019) (C) 7o3c (Vercellino & Sazanov, 2021) (D) 5xti (Guo et al., 2017)(E) 8b6f, 8b6g, 8b6h, 8b6j (Muhleip et al., 2023) (F)8ugj (G) 8ugr (Zheng et al., 2024).

the bulk pool in the membrane. This theory has evidence both for and against it (Althoff et al., 2011; Dudkina et al., 2011; Sousa et al., 2016). Eventually it has been rendered as rather unlikely (Letts & Sazanov, 2017; Milenkovic et al., 2017). For example, the substrate channeling has been proposed based on flux-control analysis (Bianchi et al., 2004) but there are issues with reproducibility of the results and the results are highly dependent on the choice of detergent (Blaza et al., 2014). Additional evidence against separate substrate pools come from diffusion experiments of the substrates (Gupte et al., 1984; Trouillard et al., 2011). Additionally, restricting the movement of the substrates would require protein structures that could "trap" substrates, but these have not been observed.

Reduction of reactive oxygen species (ROS) production has been suggested as one possible function or benefit of SC formation. ROS are unfortunate side products in OXPHOS – small molecules that contain oxygen and are highly reactive in cells potentially in a destructive manner. They are primarily produced by CI and CIII (Murphy, 2009). However, the experimental setups to measure ROS production are challenging in terms of designing a setup that is not dependent on factors such as other substrate or enzyme concentrations or the choice detergents since multiple ROS production sites are present in both CI and CIII (Maranzana et al., 2013; Sarewicz & Osyczka, 2015).

Since there are different SC compositions, perhaps their formation and disassembly are per-requirement processes. In fact, it has been suggested that exercise increases the amount of SCs in muscle tissue (Greggio et al., 2017). As per requirements of the tissue change in terms of metabolism, forming or breaking down SCs could be a regulatory mechanism for respiration. However, "correlation does not equate to causation" (Milenkovic et al., 2017). Cell respiration is a complicated process related to other cell functions making it difficult to draw reliable conclusions of a single phenomenon alone.

The respiratory chain is embedded in a phospholipid membrane raising the question about the effect of SCs on the membrane. Specifically for the mammalian systems, the inner mitochondrial membrane has protrusions called cristae thereby having highly curved regions. The traditional view has been that the ATP synthase dimers (CV) form rows along the curved edges and the ETC enzymes reside in the flatter membrane regions (Blum et al., 2019; Davies et al., 2012; Davies et al., 2011; Muhleip et al., 2017). However, this view was recently challenged by Mühleip et al. (Muhleip et al., 2023). Ciliate supercomplex $CI_1CII_1CIII_1CIV_2$ was observed in highly curved architecture shaping tubular cristae of ~40 nm diameter. Notably, the cristae shape is dependent on the organism and ciliates specifically harbor the tubular ones. Nevertheless, this was the first observation of the SC formation shaping the bioenergetic membrane. Observations of mammalian SCs do indicate membrane curving, but the scales are totally different to the bending observed in ciliate SC (Zheng et al., 2024).

There is clear structural evidence that SCs exist, but their functional implications are not yet resolved. Experimental setups on respiratory enzymes are complicated, and as can be seen in the examples above, creating a setup to answer a specific research question in an isolated manner is not simple.

4. Computational research of supercomplexes

In addition to experimental research, there is some available computational research on the SCs as well. As the experimental setups suffer from complexity, the computational ones suffer from size.

One of the most popular computational methods in protein research is molecular dynamics (MD) simulations which are also widely used for studying the respiratory enzymes. However, the ability

to study the atomistic details of SCs was not properly available until the first atomistic structures of the respirasome in 2016, because protein structures are essential in constructing simulation systems. In addition to the lack of structures, computational studies of large systems, as the SCs encompass multiple proteins with hundreds of subunits, suffer from the computational cost of the calculations. To lower the cost, different levels of coarse-graining may be used depending on the research question. For example, the ciliate supercomplex $CI_1CII_1CIII_1CIV_2$ embedded in a membrane was recently studied using coarse-grained MD simulations with Martini3 forcefield (Muhleip et al., 2023). Similarly, respirasome from *Euglena gracilis* was computationally studied using the same forcefield (He et al., 2024). Although in both cases coarse-graining was used to limit the computational load, the simulation systems still comprised millions of beads.

Utilizing computational methods to complement in the experimental research will positively impact the bioenergetic field in terms of shedding light on the interactions between the individual enzymes and their substrates and the environment they are in. Both method types have their pros and cons but together they form a powerful toolset.

5. Final remarks

Respiratory supercomplexes are a fascinating extension of the studies on individual respiratory complexes. Their significance in biology, medicine and physiology is clear but there are still many mysteries and questions about them. Leaps in experimental, structural and computational methods have already been a significant impact on the research as we saw with BN-PAGE analyses and the atomistic structures. Who knows if the next big breakthrough in methodology will finally reveal the secrets of supercomplexes?

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Outi Haapanen is a Postdoctoral researcher at the University of Helsinki. Her work focuses on studying mitochondrial proteins, particularly respiratory enzymes, using computational methods.

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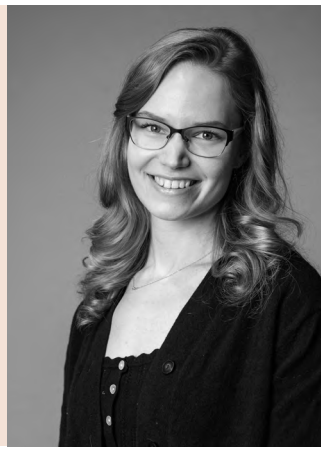

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