Computational microscopy of the supramolecular organization of the respiratory chain complexes
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Introduction

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Mitochondria are essential intracellular structures found in most eukaryotic cells. These membrane-bound organelles synthesize most of the energy used by our cells to function. A complex mechanism involving a series of oxidoreduction reactions carried out by several enzymes embedded in the inner membrane of mitochondria is responsible for this synthesis, known as the respiratory chain. The whole mechanism has been shown to functionally depend on specific collective conformations of the proteins involved in the process, and on the composition of the surrounding lipid content.

This introductory chapter brushes through a description of the various molecular components, the different existing hypotheses and the current state of the knowledge available on the respiratory chain processes, and introduces the content of this thesis against that background.
“Conceptually simple, oxidative phosphorylation is mechanistically complex.”

Oxidative phosphorylation and the respiratory chain

Cells are manufacturing plants; as such, they require energy to function and produce.

The function of the cells is maintained through a high rate recycling of adenosine diphosphate (ADP) back into adenosine triphosphate (ATP). As an illustration, the sum of the energy required by all the cells of an average human body at rest is about 2000 kcal per day, which can be converted to about 80 kg of ATP per day. At any given time, however, the whole human body contains only about 250 g of ATP. In our (eukaryotic) case, this recycling takes place in the inner membrane of the mitochondria present in each cell of our organism. It is believed mitochondria got embedded in cells during an endosymbiotic event, in which the bacterial ancestor of mitochondria got internalized, lost part of its DNA making it incapable of independent living; in return, the host cell became dependent on the production of ATP provided by the mitochondria.

The ATP recycling process is known as oxidative phosphorylation (OxPhos), and is the result of electron transfers between various electron carriers and the concurrent movement of protons out and back into the mitochondria. During this process, ATP is formed from ADP. The dependency of our cells on the OxPhos system makes any mitochondrial disorder dramatic. More and more diseases are now related to mitochondria as our understanding of its biochemistry increases. Organs that are highly demanding in ATP (heart and nervous system for instance) are the most vulnerable to mutations in mitochondrial DNA and the resulting dysfunctions. The accumulation of mutations in mitochondrial DNA seems to directly contribute to the aging process, several types of cancer and several disorders such as myopathies for instance.

Amongst the proteins found in the mitochondrial membrane, three specific enzymes forming the respiratory chain are responsible for OxPhos. These three proteins, complex I (NADH:Q oxidoreductase or NADH dehydrogenase; shortened to CI in the rest of this thesis), complex III (QH₂:cytochrome c oxidoreductase or cytochrome bc₁; CIII) and complex IV (cytochrome c oxidase; CIV) act in unison to create the proton gradient required by the ATP synthase — often called the complex V of the respiratory chain — to convert ADP into ATP. Another complex (complex II, succinate dehydrogenase) is not directly involved in the building of the gradient and therefore not further described here.

The function of the respiratory chain complexes

The proton gradient used by the ATP synthase to construct ATP is the result of a series of electron transfers. Most of these transfers are carried out between specific redox centers included in, or diffusing between, the three respiratory chain protein complexes CI, CIII and CIV. Each of these proton-pumping enzymes and electron carriers has a specific role in the overall mechanism, which is detailed in the following subsections. The complete mechanism is summarized in Figure 1.1.

Complex I: CI oxidizes the reduced form of the nicotinamide adenine dinucleotide (NADH)
molecules present on the matrix side of the membrane. NADH is one of the products of glycolysis, fatty acid oxidation or citric acid cycle. Most of the protons released during the oxidation of NADH are directly pumped into the intermembrane space (cytoplasm), while some are used to reduce the cofactor ubiquinone (Q) into ubiquinol (QH$_2$). The ubiquinone reduction is possible due to the capture and transfer of the electrons released during the oxidation by various metallic centers (iron-sulfur clusters) located in the extramembranous section of CI. Once reduced into QH$_2$, the Q previously bound in a pocket of the protein complex is released in the membrane and diffuses to CIII, while oxidized NAD$^+$ will be reused in the above-mentioned biological cycles as precursor for NADH. The chemical reaction taking place in CI can thus be written:

$$\text{NADH} + \text{Q} + 5\text{H}^+ \rightarrow \text{NAD}^+ + \text{QH}_2 + 4\text{H}^+_{\text{cytoplasm}}$$

**Complex III:** CIII then oxidizes QH$_2$, releasing the protons into the inter-membrane space. It also captures the electrons through the iron centers of the internal hemes and transfers them to cytochrome c (Cyt$c$, a small heme-containing protein located in the intermembrane space of the mitochondria. Cyt$c_{\text{ox}}$ (carrying an extra electron) diffuses to CIV, while QH$_2$ oxidized into Q returns to CI for a new cycle. This ability of easily switching from one oxidoreduction state to the other is what gave the “ubiquitous” prefix to this compound. The chemical reaction involving CIII is as follows:

$$\text{QH}_2 + 2\text{Cyt}_{c_{\text{ox}}} + 2\text{H}^+_{\text{matrix}} \rightarrow Q + 2\text{Cyt}_{c_{\text{red}}} + 4\text{H}^+_{\text{cytoplasm}}$$

**Complex IV:** CIV is the last actor of the proton-pumping process. It uses the electrons received through Cyt$c_{\text{red}}$ together with protons from the proton pool available on the matrix side of the inner membrane, to reduce O$_2$ brought from the lungs by blood flow into water:

$$4\text{Cyt}_{c_{\text{red}}} + 8\text{H}^+ + \text{O}_2 \rightarrow 4\text{Cyt}_{c_{\text{ox}}} + 2\text{H}_2\text{O} + 4\text{H}^+_{\text{cytoplasm}}$$

With this last step the electrons initially freed are concealed and an effective proton gradient is built across the membrane. The membrane potential created by the difference of charge between the two sides of the membrane, as well as the local pH gra-
Figure 1.2 | Electron microscopy maps obtained for supercomplexes extracted from bovine heart [8], potato [3], and yeast [12] mitochondria (A), B) and C) respectively). In each case, atomistic structures of the complexes involved in the supercomplexes were fitted inside the densities: complex I (yellow or blue in A) and B) respectively), complex III (green, red or blue in A), B) and C) respectively) and complex IV (blue, yellow or pink). See the respective publications for more detailed information on the figures.

...gradient, compel the free protons to flow back into the matrix to equilibrate the charge; this flow is captured by the ATP synthase (which possesses proton channels within its transmembrane section) and drives the generation of ATP. Peter Mitchell was the first to propose this hypothesis in 1961 and was awarded the Nobel Prize in Chemistry in 1978 for his work.

Respiratory chain supercomplexes

In the previous section, we described that one cycle of the respiratory chain involves at least three protein complexes (CI, CIII and CIV) and two electron carriers (QH₂ and the Cytc_red). Some reactions even require more than one copy of these carriers to be completed. The build up of the proton gradient, and, by extension, the rate of ATP synthesis are thus limited by the encounter rate of these molecules and would depend critically on the large scale organization of the respiratory chain complexes. Up to date, the mechanism by which the respiratory chain complexes self-organize is highly debated.

The first mechanism, proposed by Hackenbrock et al. in 1986 [1], was based on a stochastic assembly of the enzymes. This model postulates that electron carriers — together with the complexes — are constantly and freely diffusing in the inner mitochondrial membrane, and the electron transfers are carried out according to the random contacts between the different actors of the respiratory chain. In the last decade, however, the existence of a higher-order organization of the respiratory chain has been demonstrated through various techniques, notably blue-native gel electrophoresis (BN-PAGE) and electron microscopy. The three protein complexes seem to assemble into larger structures named supercomplexes (together defining the respirasome) and have been observed in various organisms such as potato [2,3], plants [4-6], beef [7-10] and yeast — although in the last case CI is absent from the inner membrane of mitochondrial membrane and a slightly different organization of the supercomplexes has been reported [11,12]. Recently published electron density maps and resulting molecular models are reported in Figure 1.2. These observations lead to the emergence of a new model, describing the series of respiratory chain reactions within the frame of a static arrangement of complexes. The smaller molecular actors (QH₂/Q, and Cytc) could benefit from this optimized spatial ordering, being channeled by the close proximity of the enzymes, as proposed by Schägger and Pfeiffer when...
they showed the existence of such superstructures [13] (based on the ligand channeling model established by Kholodenko and Westerhoff [14]). As a direct consequence the whole process would have its production rate increased. Evidence exists for even higher levels of organization between these supercomplexes at specific conditions [3,15], hypothetically further increasing the efficiency of the respiratory chain.

Although the recent structural evidences are in favor of this static model, making it largely accepted nowadays, several hypotheses concerning its stability and its functionality need yet to be demonstrated. The increased efficiency brought about by such supramolecular assemblies has not been clearly quantified and is still an assumption of the model. The proximity of the electron carrier reactive sites in the existing supercomplex structures seems to point towards a channeling function. But a close look at the conformations reveals the existence of a gap separating the complexes, presumably filled with immobilized lipids in real membranes, and preventing fast diffusion rates. Furthermore, the stoichiometry of the complexes involved in supercomplexes is not matching their relative respective concentrations in the membrane, indicating a pool of freely diffusing complexes is still present. This last observation suggests a potential equilibrium between free complexes and associated supercomplexes, which is not taken into account by the static model. The presence of CIVs not associated in supercomplexes (large majority) but still participating in the respiratory chain mechanism, and the known fact they can receive electrons from other sources than these three complexes, are additional lines of evidence that the static model is too simplistic. Recent studies showed that the membrane potential could be governing the association and dissociation of supercomplexes: at low potential (small difference in proton concentration on each side of the membrane) the complexes might be associated, while at high potential (large concentration difference resulting from proton pumping) they might dissociate to avoid physiological problems [16-18].

Together, these observations led to a recently proposed mechanism, mixing the features of both the dynamic and the static hypotheses: the plasticity model, first suggested by Acín-Pérez et al. in 2008 [19]. This model postulates that electron transfer can occur in both supercomplexes and by random collisions of the mobile independent components. One could imagine that the balance between static and dynamic (re)arrangements of the supercomplexes is condition dependent, similar to the switching behavior observed for the photosynthetic complexes in the thylakoid membrane [20].

**Cardiolipin, signature lipid of the mitochondrial membrane**

The respiratory chain complexes are localized in the mitochondrial inner membrane, which separates two compartments necessary to build the electrochemical gradient. This membrane embeds a high number of proteins, having only about 50% of its mass as lipids. The lipid environment includes a specific variety of head groups, with three major components being phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipins (CL). The latter is very abundant (~20% of the lipidic phosphate moiety in bovine heart mitochondria for instance [21]) and found almost exclusively in mitochondrial membranes; it is often mentioned as the signature lipid of mitochondria and was used in the past to locate mitochondria in cells. CL was first isolated from cells extracted from bovine heart (explaining the name “cardiolipin”) and characterized in the early 1940s by Mary Pangborn [22]. Its structure includes two phosphatidyl groups linked by a glycerol moiety; each phosphatidyl supports the regular aliphatic lipidic tail bound by two glycerol linkers (Fig. 1.3).
is asymmetric; in mitochondria extracted from bovine heart, for instance, about 40% of the total PC and PE lipids, and at least ~75% of CL, is found on the matrix side of the inner membrane [21]. The lower concentration of CL on the intermembrane space leaflet is compensated by a higher number of PC and PE lipids. The specific lipid composition of the different membranes is crucial for the metabolic processes occurring in their vicinity and the inner membrane of mitochondria is not an exception; its composition, as well as the asymmetric lipid distribution, have been shown to play significant roles in different processes. The importance of this asymmetry can be illustrated by the role of CL in triggering cell apoptosis for instance. Under specific biological circumstances, a major flip-flop of the CLs from the matrix leaflet to the intermembrane space leaflet of the inner membrane is triggered. The higher CL concentration is recognized by a specific enzyme (from the oxygenase family), which transfers oxygen from the O2 present in the intermembrane space to CL, in the OxPhos process, most notably CL, which has been shown to have an effect on both stability and functionality of the whole respiratory chain. Its absence from the membrane, or a drastically modified structure (shorter aliphatic tails for instance, or peroxides formed during apoptosis), systematically results in a dysfunctional respiratory chain [25,26]. In Barth syndrome (mutation leading to cardiomyopathy, skeletal myopathy, growth retardation), the mitochondrial inner membrane shows a lower content of CL and/or a spread in the composition of their alkyl tails [27]. BN-PAGE analyses performed on such cells report a decrease in supercomplex formation and an increase in monomeric free complexes. Thus, CL seems to be involved at every level of the respiratory chain process: maintaining the functionality of independent complexes, allowing the formation of supercomplexes and stabilizing them, and preserving the functionality of these assemblies [10,28-32].

How can CL be so important? The existing molecular models available for these protein complexes feature co-crystallized CLs. Since successful crystallization requires most of the time lipids to be washed away with surfactants, these CLs can only be preserved if their binding to the protein is strong. Without any exceptions, these co-crystallized lipids are found embedded within the protein, in close proximity of electron carrier binding sites and proton pumping pathways (see Fig. 3.1 and Fig. 4.8 of this thesis). These observations led to a hypothetical role for CL: electron transfers being coupled with proton movements. As CL can easily trap and release protons [33], it can thus provide a pool of available protons in direct vicinity of these reaction centers [34], and act as transfer antenna for proton translocation (see Discussion section of reference [35] or Chapter III of this thesis). This hypothesis could explain the mandatory presence of CL associated with the function of independent complexes.

Cardiolipin and the respiratory chain

The composition of the inner mitochondrial membrane plays a critical role converting them into peroxides and inducing a change in CL conformation. This modification results in a migration of CL to the outer membrane where it associates with cytochrome c to form pores [23]. The release of cytochrome c in the cell cytoplasm activates receptors stimulating the release of calcium ions, which once above a critical concentration trigger cell death [24].
Another important role for CL is to stabilize supercomplexes [28]. From the existing models of supercomplexes available in the literature, the 3D electron density maps obtained by tomography show a clear gap between complexes associated in supercomplexes; this gap is undoubtedly filled by lipids in real membranes, indicating protein/lipid interactions are partly responsible of the association of these proteins into supercomplexes. The observation that, again, the presence of CL is absolutely mandatory to reconstitute supercomplexes [10,28-32] suggests a major role for CL in the assembly. Zhang et al. labeled CL as the “glue holding supercomplexes together” [28]. This comment can be pushed even further: amongst the major lipids present in the inner membrane of mitochondria, only CL is ionic, the PC and PE lipids being zwitterionic. Recent studies have shown that lowering the pH (resulting in the complete protonation of the acidic groups of the lipid moiety) causes supercomplex dissociation in plant mitochondria [36]. We can then safely infer that electrostatic interactions are a major driving force for supercomplex association, and are being mediated by CL in the membranous sections of the supercomplexes. However, the bulky tails of CL are also important, since other charged lipids — such as phosphatidylglycerol (PG), present in lesser proportions than CL in the inner membrane of mitochondria — do not support supercomplex association [32].

Aim and contents of this thesis

The work described in this thesis uses a computational approach to test some of the hypotheses discussed in the preceding sections. In particular, the role of CL in the respiratory chain is being investigated and two main hypotheses are tested here:

Experimental studies suggest a direct involvement of CL in the proton transfer process. The locations of the reaction centers and proton pathways on each complex are known; CL should then have specific binding sites in close vicinity of those. This hypothesis was tested through molecular dynamics (MD) simulations of independent CIII and CIV embedded in a mitochondrial model membrane; the results are presented and discussed in Chapter III and Chapter IV.

Common conformation of supercomplexes have been obtained and published by several research groups for various organisms. Because of its demonstrated involvement in supercomplex formation and stability we can anticipate the presence or absence of CL to affect the interfaces formed between complexes. This hypothesis was tested by large-scale MD simulations, and is presented in the Chapter V of this thesis.

The size of protein complexes and the timescale of the mechanisms studied in this thesis are also challenges for the modeling techniques I used to perform this work. These techniques, together with the inherent limitations caused by these challenges, are presented in the next chapter (Chapter II). As an attempt to address this problem, a modification of the current technique is proposed in Chapter VI, theoretically allowing and facilitating the study of even larger systems in future computational studies. In the concluding Chapter VII, I provide a perspective on the thesis as a whole.