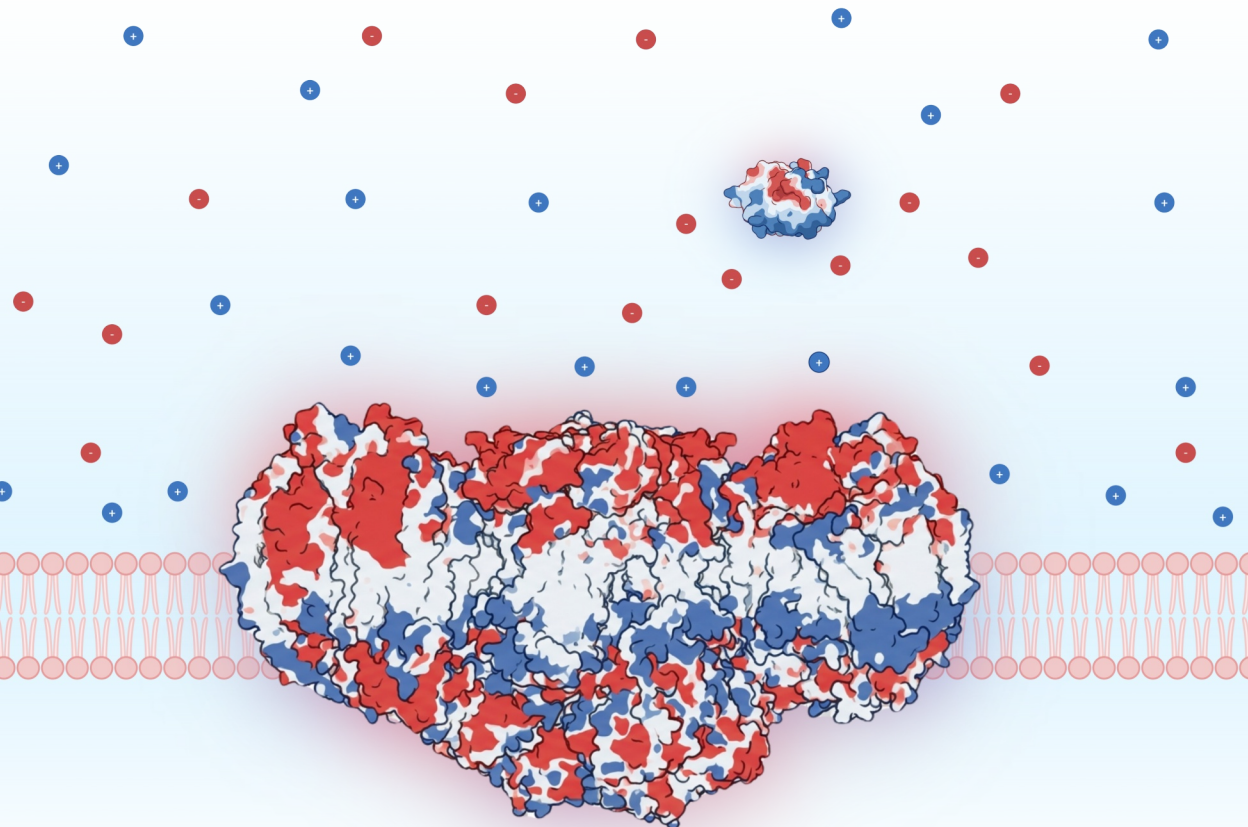


The intracellular electrostatic environment

Effect of ionic strength on protein-protein interactions: studies on respiratory supercomplexes

Ana Paula Lóbez Rodríguez



The intracellular electrostatic environment

Effect of ionic strength on protein-protein interactions: studies on respiratory supercomplexes

Ana Paula Lóbez Rodríguez

Academic dissertation for the Degree of Doctor of Philosophy in Biophysics at Stockholm University to be publicly defended on Friday 17 April 2026 at 09.00 in Magneli Hall, Arrhenius laboratory, Svante Arrhenius väg 16B.

Abstract

Protein-protein interactions are essential to maintain cellular function and organization and often occur by means of electrostatic interactions. These forces are determined by the electrostatic conditions within the cell, a dynamic and highly crowded environment containing ions and charged molecules.

In this thesis, the mitochondrial respiratory chain is employed as a model system to study how cellular electrostatics, specifically ionic strength, governs protein-protein interactions, with particular focus on interactions between cytochrome *c* and the *Saccharomyces cerevisiae* III₂-IV_{1/2} supercomplex. The electrostatic nature of this interaction provides an ideal framework for studying ionic strength-dependent effects.

Previous data showed that at the commonly assumed “physiological” ionic strength of 150 mM monovalent salt, electron transfer within the supercomplex is mediated by 2D diffusion of a single cytochrome *c* molecule between complexes III₂ and IV. However, recent findings indicate that a monovalent salt concentration of 20 mM more realistically mimics the intracellular conditions. Under these conditions, our data show that at least two cytochrome *c* molecules bind simultaneously to the supercomplex surface. Additionally, previously unresolved residues at the N and C termini of subunits Qcr6 and Qcr9, respectively, were observed.

The cytochrome *c*-supercomplex interactions were also studied at 20 and 150 mM monovalent salt in mitoplasts containing the supercomplex. Overall activity in mitoplasts was lower than in detergent-purified supercomplexes. The results show that supercomplex activity as a function of cytochrome *c* concentration was similar at 20 mM and 150 mM salt, contrasting the behavior observed in detergent-purified supercomplexes. This difference is explained by a shift in the rate-limiting step in membrane-bound supercomplexes.

The effect of ionic strength was further studied by measuring the supercomplex activity, both in solution and in mitoplasts, at increasing salt concentrations. Increasing ionic strength resulted in a monotonic decrease in cytochrome *c* affinity for the supercomplex, indicating a classical Debye-Hückel behavior that contrasts earlier studies with non-biological systems.

Finally, the structure of the *Mycobacterium smegmatis* supercomplex was resolved in native membranes, revealing a physical association with the enzyme malate:quinone oxidoreductase. Spectrophotometric analyses showed that malate:quinone oxidoreductase can transfer electrons from malate to the supercomplex, suggesting a connection between the Krebs cycle and aerobic respiration in mycobacteria.

Keywords: ionic strength, electrostatics, electron transfer, cytochrome *c* oxidase, respiratory supercomplex, bioenergetics.

Stockholm 2026

<http://urn.kb.se/resolve?urn=urn:nbn:se:su:diva-252914>

ISBN 978-91-8107-524-3
ISBN 978-91-8107-525-0



Stockholm
University

Department of Biochemistry and Biophysics

Stockholm University, 106 91 Stockholm

THE INTRACELLULAR ELECTROSTATIC ENVIRONMENT

Ana Paula Lóbez Rodríguez



The intracellular electrostatic environment

Effect of ionic strength on protein-protein interactions: studies on respiratory supercomplexes

Ana Paula Lóbez Rodríguez

©Ana Paula Lóbez Rodríguez, Stockholm University 2026

ISBN print 978-91-8107-524-3

ISBN PDF 978-91-8107-525-0

Printed in Sweden by Universitetservice US-AB, Stockholm 2026

A mis padres.

List of publications

- i. **Lobez AP***, Wu F*, Di Trani JM, et al. Electron transfer in the respiratory chain at low salinity. *Nat Commun.* 2024;15:8241.
- ii. **Lobez AP**, Oliveberg M, Brzezinski P. Electron transfer between complexes III and IV in native *S. cerevisiae* mitochondrial membranes. Submitted.
- iii. **Lobez AP**, Sato W, Oliveberg M, Brzezinski P. Effects of ionic strength on protein-protein interactions in the respiratory chain. Manuscript.
- iv. Di Trani JM, Yu J*, Courbon GM*, **Lobez AP***, Cheung CY* et al. Cryo-EM of native membranes reveals an intimate connection between the Krebs cycle and aerobic respiration in mycobacteria. *Proc Natl Acad Sci.* 2025;122:E2423761122

*These authors contributed equally

Author's contribution

- i. Purified supercomplex samples and performed activity assays. Participated in interpreting the activity data guided by A.M., P.B. and M.O. and prepared part of the figures.
- ii. Purified mitoplast samples and performed activity assays. Participated in writing the manuscript, analyzing and interpreting the activity data and prepared figures.
- iii. Purified mitoplast and supercomplex samples and performed activity assays. Participated in writing the manuscript, analyzing and interpreting the data and prepared figures.
- iv. Performed spectroscopy and stopped-flow spectroscopy measurements. Participated in analyzing and interpreting the spectroscopic the data.

Contents

Introduction.....	3
The cellular environment.....	5
Biomembranes.....	5
Membrane electrostatics.....	7
The intracellular environment.....	8
Intracellular electrostatics.....	8
Ion composition and electrostatic screening.....	11
Respiratory chain.....	12
Complexes III ₂ and IV: structure and electron transfer.....	14
Respiratory supercomplexes.....	16
III-IV supercomplexes.....	17
Electron transfer in the III ₂ -IV _{1/2} supercomplexes.....	21
Cytochrome <i>c</i> binding and diffusion.....	21
Experimental methods.....	24
Oxidoreductase activity measurements.....	24
Stopped-flow measurements.....	24
Summary of papers.....	26
Concluding remarks and future prospects.....	30
Popular science summary.....	31
Populärvetenskaplig sammanfattning.....	33
Resumen de divulgación científica.....	35
Acknowledgments.....	37
References.....	40

Abbreviations

ATP	adenosine triphosphate
cyt.	cytochrome
NADH	nicotinamide adenine dinucleotide
CI-V	complexes I-V
Q/QH ₂	quinone/quinol
FeS	iron-sulfur cluster
BN-PAGE	blue native polyacrylamide gel electrophoresis
n side/p side	negative/positive side of the membrane
Mqo	malate:quinone oxidoreductase
SOD	superoxide dismutase
3D/2D	three/two-dimensional

Introduction

The cellular interior is a crowded and dynamic environment, which contains a variety of proteins, nucleic acids and small molecules that interact with each other. Protein-protein interactions are essential to maintain cellular function and homeostasis, and commonly occur by means of long-range electrostatic interactions between charged surface groups¹. The surfaces of proteins contain acidic and basic residues that can be protonated or de-protonated, many of which have been found to be present at protein-protein interfaces², indicating that electrostatic interactions play a significant role in protein association and function.

Electrostatic forces are affected by the electrostatic conditions of the surrounding media. The intracellular environment is an electrolyte solution that contains mobile ions and other charged molecules. The ionic strength influences the way electrostatic interactions take place by affecting the range and magnitude of these forces³. As a consequence, the ionic strength modulates the long-range attraction and repulsion forces between proteins.

Another important factor that shapes cellular electrostatics is the presence of biological membranes. These carry a net negative charge on their surfaces, which yields a surface-localized electrostatic field that attracts positively charged ions and other molecules from the cytoplasm^{4,5}. This membrane surface charge has important implications for protein-protein interactions occurring near the membrane. Many peripheral cytosolic proteins contain charged residues on their surfaces and interact electrostatically with the integral proteins in the membrane to perform different cellular functions. The negative charge of the membrane affects this interactions, promoting the recruitment of positively charged proteins to the membrane and enhancing their interaction probabilities.

The effective membrane charge is also affected by the ionic strength of the intracellular environment since the ions in solution screen the membrane charges, changing the effective potential sensed by proteins. Therefore, protein-protein interactions in the membrane periphery are governed both by the membrane charge and the ionic strength of the intracellular environment, which highlights the importance of understanding these interactions within the context of cellular electrostatics.

One of the cellular machineries in which protein-protein interactions are important is the respiratory chain, a sequence of membrane-bound proteins that catalyse reactions leading to the production of adenosine triphosphate (ATP), the universal energy currency in living organisms. In these reactions electrons are transferred through a series of membrane-bound protein complexes, named complexes I-IV, and mobile electron carriers, to ultimately reach its final acceptor, molecular oxygen (O_2). The O_2 reduction is catalyzed by complex IV (CIV), a terminal oxidase that receives electrons from complex III₂ (CIII₂) via a mobile electron carrier, cytochrome (cyt.) *c*.

In the work presented in this thesis, the mitochondrial respiratory chain is used as a model system to study protein-protein interactions under different ionic conditions, with particular focus on supercomplexes, higher-order assemblies of respiratory complexes. Specifically, the interaction between the III-IV supercomplex and cyt. *c*, has been studied. Cyt. *c* carries a positively charged surface that interacts electrostatically with the negatively charged surface of the supercomplex. The electrostatic nature of this interaction offers an ideal tool for investigation of protein-protein interactions under a wide range of conditions.

To this end, the cyt. *c*-supercomplex interaction has been analysed under different ionic conditions in bulk solution, allowing direct assessment of ionic strength-dependent electrostatic effects. In addition, experiments have been conducted in the presence of the membrane in order to account for the effects of the membrane surface charge. Through this combined approach, this work aims to understand and elucidate how protein-protein interactions are governed and modulated by cellular electrostatics.

The cellular environment

The cell is a basic structural and functional unit of all living organisms. The cellular volume is delimited by the plasma membrane, which functions as a selective barrier that tightly regulates the movement of chemical substances in and out of the cell. Within this boundary lies the cytosol, a crowded and dynamic aqueous solution containing ions, metabolites and different macromolecules. This highly structured and densely packed environment is a determinant of cellular processes, many of which rely on protein-protein interactions.

Biomembranes

Biological membranes perform a wide range of functions that are essential for cellular life, including compartmentalization of the cellular interior, regulating the movement of substances inside and outside of the cell and mediating in cell-cell recognition and signaling, among others.

Based on their structural organization and degree of complexity, cells can be divided into two classes: prokaryotic and eukaryotic cells, both of which present a plasma membrane that delimits the intracellular environment. Prokaryotic cells differ from eukaryotic cells by the absence of a nuclear membrane enclosing the genetic material and by the lack of internal membrane-delimited compartments. In addition, many prokaryotes possess a cell wall, a semi-rigid outer layer that surrounds the plasma membrane and provides shape, support and protection to the cell. In eukaryotes, the presence of membranous compartments, called organelles, allows inside-cellular compartmentalization enabling biochemical processes to occur at the same time under specific and regulated conditions.

Regardless of the cell type or organelle, all cellular membranes share a conserved basic architecture consisting of a two-dimensional lipid bilayer (Fig. 1), into which proteins are embedded⁶. The bilayer is primarily composed of phospholipids, amphipathic molecules with a hydrophilic head group that contains a phosphate group attached to a glycerol backbone, and a hydrophobic tail composed of two fatty acids. The hydrophilic head groups are oriented toward the surrounding aqueous environment, whereas the hydrophobic tails

face inwards, forming the core of the bilayer. In addition to phospholipids, membranes incorporate other lipid species. Among these, sterols, such as cholesterol in mammals, are the most abundant in the plasma membrane, where they play a crucial role in modulating membrane fluidity and stability ⁷.

Cellular membranes also contain a large number of proteins, that play a significant role in molecular transport and signal transduction. These proteins can either span the lipid bilayer as transmembrane or integral proteins, or associate with the membrane surface as peripheral proteins. Both lipids and proteins can be glycosylated, meaning that a carbohydrate molecule is covalently attached to their extracellular side. Glycolipids and glycoproteins play a critical role in cell recognition, adhesion and signaling processes ⁸.

The protein and lipid content of cellular membranes differs substantially depending on membrane function and subcellular location. For instance, in the myelin sheath of neuronal axons, proteins constitute approximately 20% of the total membrane mass, whereas in the plasma membrane proteins account for approximately half of the total membrane mass ⁹. Furthermore, membrane lipids and proteins are not uniformly distributed, neither laterally within the plane of the membrane, nor between the two leaflets of the bilayer ¹⁰, a feature with important implications in membrane fluidity, permeability and electrostatics.

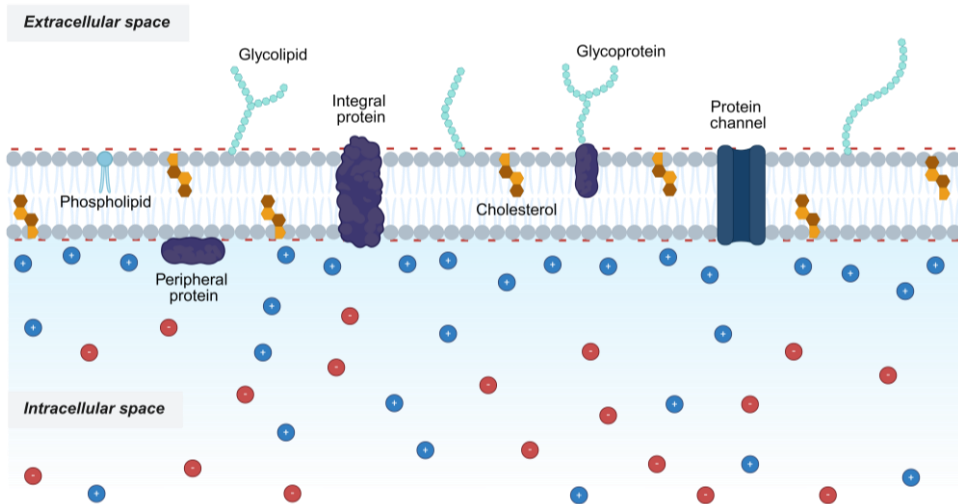


Figure 1: Schematic representation of a plasma membrane composed of a lipid bilayer in which different proteins are embedded.

Membrane electrostatics

Biological membranes are capable of sustaining a transmembrane potential difference due to their insulating and capacitive properties. The hydrophobic core of the lipid bilayer acts as a dielectric barrier with high resistance that restricts the flow of charges across the membrane. While small nonpolar molecules may diffuse freely across the bilayer, ions, charged and polar molecules require the presence of membrane proteins, i.e. ionic channels and transporters, to cross the membrane. The different active transporters establish and maintain ion concentration differences between the two sides of the membrane, creating different electrochemical environments on the inner and outer sides. For instance, in most cells, K^+ ions are more concentrated in the cytoplasm, whereas Na^+ , Ca^{2+} and Cl^- ions are found at higher concentrations in the extracellular space⁵. Furthermore, different ions exhibit different permeabilities, meaning that the membrane allows certain ions to cross at a higher rate than others.

By separating two conductive electrolyte solutions with a dielectric layer, membranes behave as capacitors, allowing charge accumulation on either side of the membrane¹⁰. Together, the selective permeability of the lipid bilayer, its high resistance and capacitive properties, enable the generation and maintenance of the transmembrane potential, which plays an essential role in a wide range of cellular processes, such as transport and bioenergetics.

In addition to the transmembrane potential, each membrane-solution interface has its own surface potential⁵. The hydrophilic heads of lipids typically carry negative charges and are oriented towards the aqueous surrounding solutions¹⁰. The accumulation of these negative charges at the membrane surfaces results in a local electrostatic potential that attracts positively charged ions from the cytoplasm^{5,4}. This leads to a non-uniform ion distribution, in which the concentration of cations is increased in the vicinity of the membrane.

The resulting charge distribution, known as diffuse double layer, together with the intrinsic surface charge of the membrane, modulates the strength and range of protein-protein electrostatic interactions occurring near the membrane. In fact, many peripheral cytosolic proteins possess charged residues that enable electrostatic interactions with integral membrane proteins. The net negative charge of the membrane modulates these interactions by promoting the recruitment and accumulation of positively charged proteins at the membrane

interface. Consequently, the local concentration of these cytosolic proteins is increased, which enhances the probability of productive protein-protein interactions.

Taken together, biological membranes constitute complex electrostatic structures in which the global transmembrane potential and the local surface potentials coexist. These electrostatic features shape the distribution of ions and charged molecules in the membrane vicinity, regulating fundamental cellular processes.

The intracellular environment

The cytosolic medium is composed of water, ions, small organic molecules and different macromolecules, that together comprises more than 50% of the cell's volume. Water diffuses over the plasma membrane and inside the cell, so the cytosolic compartment has a uniform osmotic pressure set by the extracellular solution and that is typically assumed to be equivalent to ~150 mM monovalent salt ¹¹.

Intracellular electrostatics

The intracellular environment is an electrolyte solution. Hence, each protein is surrounded by charged molecules and small ions. This electrostatic environment is defined by the ionic strength (I), that accounts for the molar concentration (c_i) and charge (z_i) of all ions in solution:

$$I = \frac{1}{2} \sum c_i z_i^2 \quad (1)$$

The behavior of electrolyte solutions is non-ideal. The electrostatic potential of the different ions in the solution is affected by the presence of other charged particles. Therefore, the ionic strength has an effect in the effective concentration of the different ionic species, known as activity (a_i). Due to the screening of other charged particles, a_i differs from the ions' actual molar concen-

trations. This difference can be measured using activity coefficients (γ), factors that account for the deviation of a mixture from its ideal behavior¹² and that are defined by the following equation:

$$\gamma_i = \frac{a_i}{c_i} \quad (2)$$

The relation between ionic strength and activity coefficients in dilute electrolyte solutions is modelled by the Debye-Hückel theory, which enables the calculation of activity coefficients for the different ionic species through the following equation:

$$\log \gamma_i = -Az_i^2 \sqrt{I} \quad (3)$$

where A is a constant that depends on the temperature and the dielectric properties of the solvent. As ionic strength increases, the activity coefficient of each ion decreases, meaning that their effective concentrations, i.e. activities, decrease too.

The effect of ionic strength on the electrostatic potential and, in turn, on ionic activity, affects the electrostatic interactions between the charges in solution. For two charged molecules, A and B, that interact with each other in an electrolyte solution, the ions in solution contribute to the screening of their potentials. As the ionic concentration, i.e. the ionic strength, increases, the screening of the potentials of A and B increases too. The Debye-Hückel theory predicts that electrostatic interactions in low density ionic solutions decay exponentially with distance¹³, meaning that in order for A and B to interact with each other, they should come in close-enough contact. The distance at which charged particles start to ‘feel’ and interact with one another is referred to as screening length and it is quantified by the Debye length (λ_D), defined as:

$$\lambda_D = \sqrt{\frac{\epsilon k_B T}{2e^2 N_A I}} \quad (4)$$

where ϵ is the permittivity of the solvent, k_B is the Boltzmann constant, T is the absolute temperature, e is the elemental charge and N_A is the Avogadro’s number^{32,4}.

According to this framework, at extremely high salt concentrations, e.g. 4M salt, the electrostatic screening is very strong, such that λ_D becomes smaller than an atom size, and the repulsive and attractive forces between charged molecules would effectively vanish (Fig.2). However, recent studies have found that repulsion forces are still measurable between similarly charged surfaces in ionic liquids and highly concentrated electrolytes^{3,14,15}. In these studies, the experimentally measured screening length, λ_s , recovers as the electrolyte concentration increases beyond 1.5 M monovalent salt, presumably due to ion-ion correlation (Fig.2). The recovery of λ_s at high ionic concentrations is modelled by the following equation¹⁶:

$$\lambda_s \sim \frac{2N_A e^2 a^3 I}{\epsilon k_B T} \quad (5)$$

where a is the ion diameter. Notably, the dependence of the screening length on the ionic strength is the opposite of that predicted by the classical Debye-Hückel theory. From this perspective, three different regimes can be identified. At concentrations below 0.5 M salt, the charge repulsion follows the conventional Debye decay (Eq.4); between 0.5 and 1.5 M salt, repulsion forces are severely mitigated; and above 1.5 M salt, these forces are restored and effective again (Eq.5). These different ranges have been called ‘Debye’, ‘eclipse’ and ‘ionic-liquid’ regimes¹⁷, as shown in figure 2.

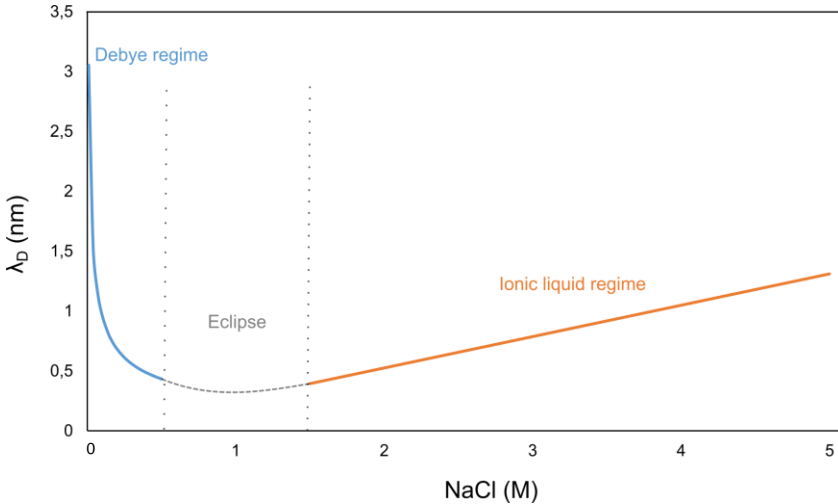


Figure 2: Electrolyte screening showing the three different regimes. Up to 0.5 M NaCl, the screening length follows the Debye decay (Eq. 4, blue), whereas for concentrations above 1.5 M, the screening length recovers (Eq. 5, orange). For NaCl in water, $a=0,3$ nm. Based on¹⁷.

Living organisms would presumably adapt more easily to the ‘Debye’ and ‘ionic-liquid’ regimes, as these regimes allow molecular and protein interactions. This idea would explain why some organisms, such as Halobacteria, live in extreme saline environments and have been able to adapt to sustain their essential cellular functions in cytosolic saturated-salt conditions, solutions that can be described as ionic liquids.

Ion composition and electrostatic screening

As discussed above, the intracellular ionic strength is typically assumed to be equivalent to ~150 mM monovalent salt. This salt concentration yields a Debye-screening length of $\lambda_D = 0.8$ nm. However, in addition to small ions, the cell contains a wide range of charged macromolecules. An analysis of the cell’s content has estimated that the concentration of small anions (Cl^- , HCO_3^- , H_2PO_4^-) is kept between 6 and 30 mM, while the concentration of small cations (Na^+ , K^+ , Mg^{2+}) is maintained between 80 and 150 mM¹⁸. The reason for this difference is that most negative charges are part of macromolecules in the form of large poly-anions.

As a consequence of this mismatch, the intracellular ionic strength is presumably lower than that assumed for a “physiological buffer” of 150 mM monovalent salt and it can be more realistically mimicked by a solution of 20 mM 1:1 electrolyte¹⁷. This ionic concentration yields a Debye screening length of $\lambda_D = 2.2$ nm, which is considerably longer than the $\lambda_D = 0.8$ nm at 150 mM. Consequently, inside the cell, charged proteins are able to “feel one another” over larger distances and these interactions are not properly reproduced by using a “physiological buffer” of 150 mM monovalent salt. From an experimental perspective, it is therefore essential to use ionic conditions that accurately reflect the screening length and electrostatic environment encountered by proteins *in vivo*.

Respiratory chain

Aerobic organisms obtain energy by linking the degradation of organic compounds to the production of ATP in the presence of oxygen. This process is called cellular respiration, which is key to maintaining the cell's functions and homeostasis.

In the last steps of respiration, electrons from NADH and succinate are transferred from low potential electron donors to high potential electron acceptors through a series of membrane-bound enzymes, that form the so called respiratory chain or electron transport chain. In this chain, free energy from electron transfer drives proton translocation across the membrane, maintaining an electrochemical gradient that is used for ATP synthesis.

The respiratory chain (Fig. 3) is formed by four protein complexes, named complexes I-IV. In eukaryotes, these proteins are located in the inner mitochondrial membrane, while in bacteria they are found in the cytoplasmic membrane. The components and fundamental mechanisms of respiratory chains are highly conserved throughout all domains of aerobic life, but there are still some differences between eukaryotic and prokaryotic organisms, as well as among prokaryotes. Bacteria often contain multiple membrane-bound dehydrogenases and oxidases that act as electron donors and acceptors, respectively, resulting in branched electron transport chains in which electrons enter and leave the system through multiple pathways, depending on the species and growth conditions^{19,20}.

In eukaryotes, electrons from NADH are transferred to NADH: ubiquinone oxidoreductase, also called complex I (CI), which catalyzes reduction of quinone (Q) to quinol (QH₂), a membrane bound electron carrier. CI couples electron transfer from NADH to Q, to proton pumping across the inner mitochondrial membrane. In many yeast species, e.g. in *Saccharomyces (S.) cerevisiae*, CI is not present and, instead, they possess alternative NADH dehydrogenases, which transfer electrons from NADH to quinone without proton pumping. The quinone pool is also reduced by the succinate ubiquinone oxidoreductase, also known as complex II (CII). This enzyme is part of the Krebs' cycle and couples succinate oxidation to fumarate with the reduction of Q to QH₂.

Reduced QH₂ diffuses within the membrane to the ubiquinol-cyt. *c* reductase, an obligate homodimer commonly referred to CIII₂ or cyt. *bc*₁ complex. Here, QH₂ undergoes oxidation and electrons are transferred to cyt. *c*, a water-soluble electron carrier located in the intermembrane space. CIII₂ releases protons to the periplasm upon QH₂ oxidation, contributing to charge separation across the membrane. Reduced cyt. *c* is oxidized by the cyt. *c* oxidase, i.e. CIV, which catalyzes reduction of O₂ to water, while pumping protons across the membrane. Reduced cyt. *c* is oxidized by the cyt. *c* oxidase, i.e. CIV, which catalyzes reduction of O₂ to water, while pumping protons across the membrane.

Proton translocation from the mitochondrial matrix (*n* side) to the intermembrane space (*p* side) by complexes I, III₂ and IV, results in a difference in voltage and proton concentration across the membrane which is known as electrochemical gradient or proton-motive force. The energy stored in this gradient is used by the ATP synthase, also called complex V (CV), for ATP synthesis.

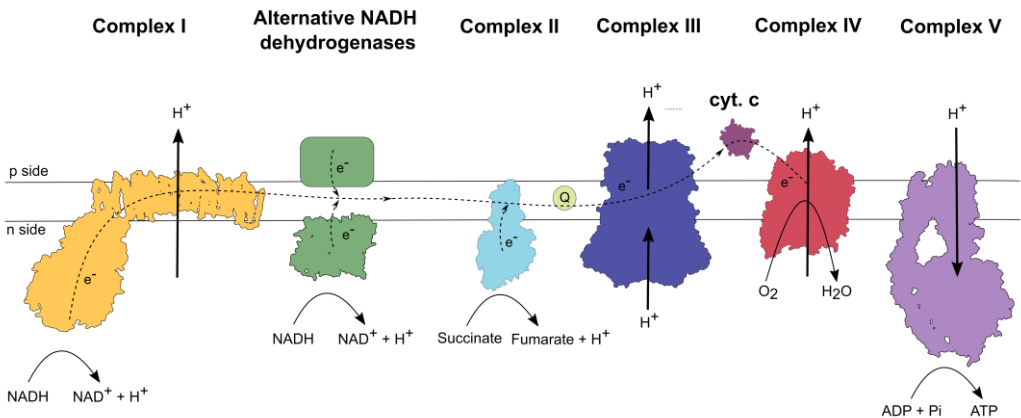


Figure 3: Schematic representation of the mitochondrial respiratory chain. Electron transfer is indicated by dashed lines and proton translocation is shown in black bold arrows. CI is not present in *S. cerevisiae*. Instead, alternative NADH dehydrogenases catalyze the NADH-oxidation:Q-reduction reaction.

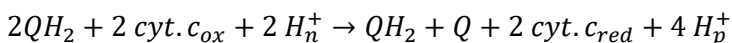
Complexes III₂ and IV: structure and electron transfer

As described above, in the last steps of cellular respiration in mitochondria, O₂ is reduced to H₂O by CIV, which receives electrons from CIII₂ via cyt. *c*.

Each CIII₂ monomer is composed of three main catalytic subunits that are highly conserved from bacteria to eukaryotes: cyt. *b*, cyt. *c*₁ and the Rieske iron-sulfur protein (FeS domain) (Fig. 4A). Cyt. *b* contains two *b* heme groups, the high-potential *b*_H and the lower-potential *b*_L, as well as two quinone-binding sites: Q_P, located near the *p* side of the membrane, and Q_N, near the *n* side. Cyt. *c*₁ harbours a *c*-type heme called heme *c*₁, and the Rieske protein contains the 2Fe-2S cluster^{21,22}.

In some bacteria, such as e.g. *Paracoccus denitrificans* or *Rhodobacter capsulatus*²¹, bc₁ complexes contain only the three main subunits, whereas additional subunits are found in mitochondrial CIII₂. For instance, in yeast *S. cerevisiae* this complex is composed of seven additional subunits, which are not directly involved in electron transfer but are believed to increase the stability of the complex and contribute to the complex assembly^{23,24}.

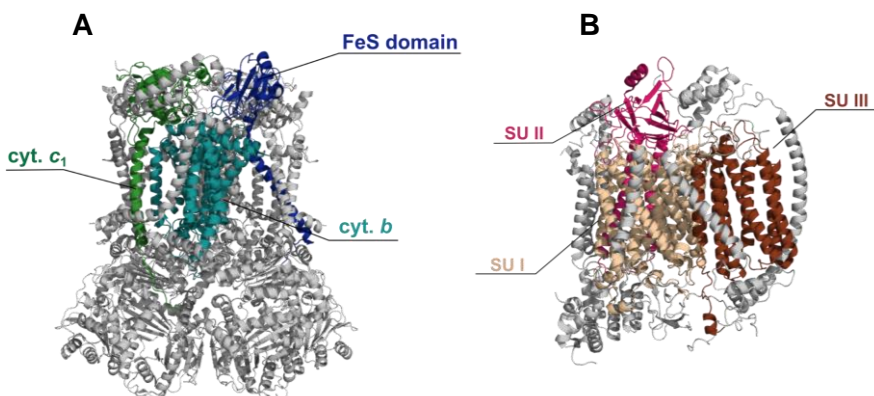
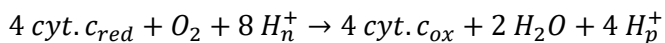
The mechanism by which CIII₂ links electron transfer to the release of protons to the *p* side is known as the Q cycle (Fig. 4C). QH₂ serves as an electron donor and binds at the Q_P site, where it is oxidized and two electrons are transferred through two different branches. The first electron is transferred along the so-called C branch via the FeS domain and heme *c*₁ to cyt. *c*. This electron transfer is coupled to the release of two protons to the *p* side of the membrane. The second electron is transferred along the B branch consecutively from heme *b*_L to heme *b*_H to reach a Q molecule in the Q_N site, which forms a semiquinone, SQ[•]. Another cycle of quinol oxidation in the Q_P site provides the second electron that reduces the semiquinone in the Q_N site to quinol, upon the uptake of two protons from the mitochondrial matrix (*n* side). The net product of the Q cycle is the release of four protons to the *p* side of the membrane, the uptake of two protons from the *n* side and the reduction of two cyt. *c* molecules in the intermembrane space²⁵. The overall reaction is thus:



In mitochondria, reduced *cyt. c* diffuses through the intermembrane space and delivers electrons to CIV, a heme-copper oxidase that catalyzes the final step of cell respiration. The catalytic core of CIV is formed by subunits I-III (Fig.4B), called Cox 1-3 in *S.cerevisiae*, and is conserved among mammals, yeast and many aerobic bacteria^{23,26,27}. Subunit I is homologous among members of the heme-copper oxidase family and contains three redox-active sites: heme *a*, heme *a*₃ and Cu_B. Heme *a*₃ and Cu_B form the active site where O₂ binds. Subunit II harbours the primary electron acceptor: the dinuclear copper centre Cu_A; while subunit III does not contain any redox-active cofactors.

In addition to the three core subunits, eukaryotic CIV contains other peripheral subunits. In *S. cerevisiae*, nine additional subunits have been identified and suggested to be involved in the regulation of electron transfer and proton pumping^{28,29}.

During CIV turnover, *cyt. c* transfers electrons via the Cu_A site and heme *a*, to the active heme *a*₃-Cu_B site, where O₂ is reduced to H₂O (Fig. 4C). A total of four electrons are transferred consecutively to reduce an O₂ molecule, with the uptake of four protons from the *n* side which results in a charge separation across the membrane. In addition, for each electron transferred to the catalytic site, one proton is pumped across the membrane from the *n* side to the *p* side, increasing the charge separation stoichiometry. Therefore, reduction of O₂ is linked to the uptake of eight protons from the *n* side, four of which are translocated to the periplasm. The overall reaction catalyzed by CIV is:



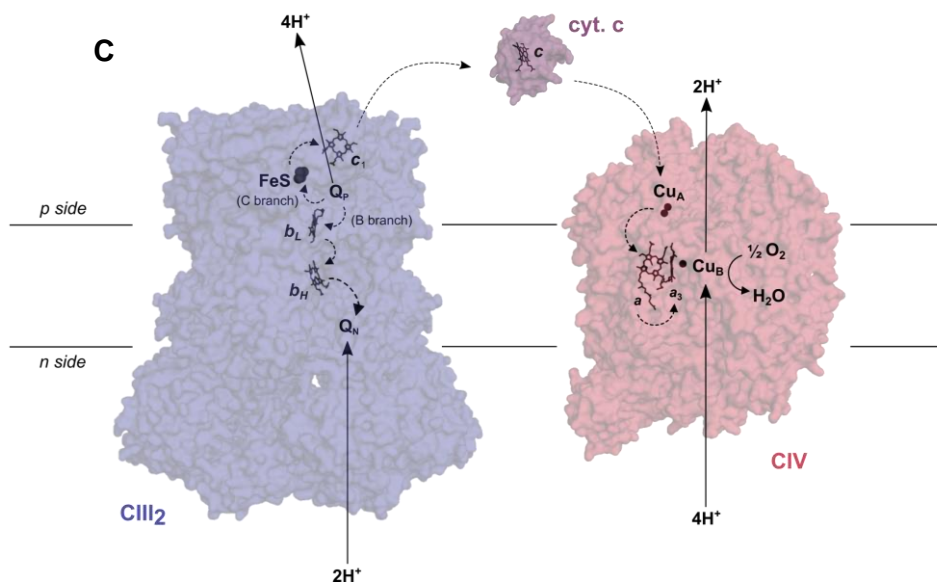


Figure 4: Structures from CIII₂ (A) and CIV (B) with their core subunits shown in color. (PDB:6HU9)²⁷. C Electron and proton transfer between one monomer of CIII₂ (blue) and CIV (red). Electron transfer is shown with dashed arrows and proton transfer with black arrows.

Respiratory supercomplexes

The structural organization of the respiratory chain is still a matter of debate and different models have been proposed over the years. From the solid-state model, in which respiratory enzymes were believed to form a single rigid unit, to the liquid-state model, which pictures these proteins diffusing freely in the membrane³⁰. The development of blue native polyacrylamide gel electrophoresis (BN-PAGE) made it possible to identify higher-order assemblies of the respiratory enzymes, called supercomplexes, in *S. cerevisiae* and bovine heart mitochondria³¹. These observations led to the plasticity model, which postulates a dynamic equilibrium between freely diffusing complexes and associated supercomplexes, enabling the respiratory chain to adapt to changes in the cellular environment and conditions.

Using more recently developed structural techniques, such as cryo electron tomography and microscopy, a large number of supercomplexes have been isolated and structurally characterized in different organisms, from bacteria to plants. The composition and abundance of these assemblies vary between organisms and tissues, depending on the metabolic and physiological conditions^{32,33}, as well as on the lipid content of the membrane^{34,35}. For example, *P. denitrificans* harbours a I-III₄-IV₄ supercomplex³⁶, while *Mycobacterium (M.) smegmatis*³⁷ contains an obligate III₂-IV₂ supercomplex. In plants, different assemblies have also been observed, like the I-III₂ supercomplex in *Arabidopsis thaliana*³⁸ or the III₂-IV supercomplex in *Vigna radiata*³⁹. The distribution of free complexes and supercomplexes in the membrane also differs between organisms. In the yeast *S. cerevisiae* almost all CIV is part of a III₂-IV_{1/2} supercomplex, but in mammals only around 20-30% of this CIV is in the form of supercomplexes³⁰.

Although the existence of supercomplexes is now widely accepted, their physiological function remains a topic for discussion. It has been observed that no clear structural changes arise from supercomplex formation, which suggests that the function of individual complexes remain unaltered upon the formation of these assemblies. Early studies proposed that supercomplex formation enables quinone channelling, maximizing the electron flux across the respiratory chain. However, this hypothesis was ruled out based on functional studies showing the lack of channels connecting the substrate binding sites in supercomplexes⁴⁰. Other roles that have been proposed include a lower rate of reactive oxygen species production^{41,42}, the prevention of protein aggregation in the membrane⁴³ or the stabilization of the individual complexes^{44,45}. None of these hypotheses is fully accepted and the functional advantage of supercomplex formation remains a topic of discussion and requires further research.

III-IV supercomplexes

As discussed previously, the composition of supercomplexes can vary between organisms and tissues. In many cases, CIII₂ interacts with one or two CIV monomers to form III₂-IV_{1/2} supercomplexes. These assemblies are found

in a wide range of organisms, including Gram negative⁴⁶ and Gram positive^{37,47} bacteria, as well as in yeast^{48,49}, plants³⁹ and mammals⁵⁰, although their arrangement is different between eukaryotes and bacteria.

Part of the work presented in this thesis focuses on the functional aspects of these supercomplexes, more specifically III-IV supercomplexes from *M. smegmatis* and *S. cerevisiae*.

The *Mycobacterium smegmatis* III₂-IV₂ supercomplex

M. smegmatis is a non-pathogenic Gram positive aerobic bacterium that belongs to the phylum Actinobacteria. It encodes many conserved mycobacterial gene orthologues, which makes it a useful model organism for studies of more pathogenic counterparts, such as e.g. *M. tuberculosis*.

As discussed above, the respiratory chains in bacteria in many cases differ from the canonical mitochondrial electron transport chain. For instance, Mycobacteria rely on menaquinone rather than ubiquinone and their lack of a mobile cyt. *c* requires of close contact between CIII₂ and CIV for electron transfer to take place. These organisms harbour an obligate supercomplex composed of a CIII₂ flanked by two monomers of CIV, one on each side^{37,51}. It has been shown that the equivalent of this supercomplex is essential for the growth of *M. tuberculosis* and its disruption caused impaired growth in *M. smegmatis*⁵².

Each CIII₂ monomer in the supercomplex is composed of the three core subunits, named QcrA, QcrB, and QcrC. The latter subunit harbours a membrane-bound diheme cyt. *cc* subunit that replaces both canonical cyt. *c*₁ and soluble cyt. *c*, and mediates electron transfer between CIII₂ and CIV. The *M. smegmatis* CIV is composed of only four subunits, called CtaC, CtaD, CtaE and CtaF (Fig. 5).

In addition to these subunits, the *M. smegmatis* supercomplex has a copper-containing superoxide dismutase (SodC) dimer as an integral part of the assembly^{37,51}. It is located in the extracellular part of the membrane, bound by its two N-terminal tails to the QcrA subunit of CIII₂. As other SodC enzymes, it catalyzes the dismutation of O₂⁻ into O₂ and H₂O₂, but its functional role

within the supercomplex is still unclear. The *M. smegmatis* SodC has a high sequence homology to that from *M. tuberculosis*, suggesting a similar organization and function in the pathogen.

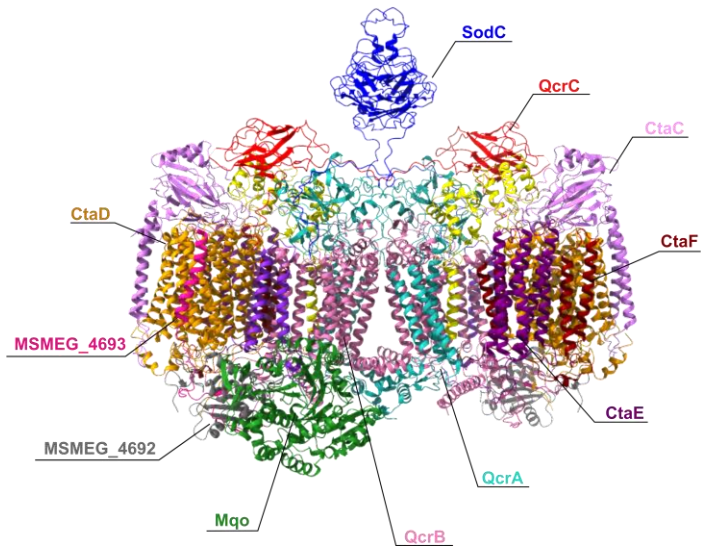


Figure 5: Structure of the *M. smegmatis* supercomplex (PDB: 9MD1)⁵⁴ with the different subunits colored, including the Mqo malate oxidase.

Additionally, the structural study presented in **paper IV**⁵³ of this thesis has revealed that in native membranes the enzyme malate:quinone oxidoreductase (Mqo) is also physically associated to the *M. smegmatis* supercomplex. This enzyme is bound between CIII₂ and CIV, making close contact with the MSMEG_4692 subunit, and part of it is also embedded in the lipid bilayer. Mqo is also present in *M. tuberculosis*, where its deletion makes the pathogen susceptible to oxidative and low pH stress⁵⁴. In *M. smegmatis* it is essential for growth on non-fermentable carbon sources⁵⁵. Mqo catalyzes the oxidation of malate to oxaloacetate, a critical step in the Krebs cycle. Associated with the *M. smegmatis* III₂-IV₂ supercomplex it is capable of transferring electrons from malate to the supercomplex⁵³, which would indicate a connection between the Krebs cycle and aerobic respiration in mycobacteria.

The *Saccharomyces cerevisiae* CIII₂-CIV_{1/2} supercomplex

The yeast *S. cerevisiae* is a unicellular eukaryote typically used as a model for studying the mitochondrial electron transport chain, since the respiratory enzymes of this organism are very similar to their mammalian counterparts^{56,57}. After the yeast *S. cerevisiae* respiratory supercomplex was identified by means of BN-PAGE³¹, many structures of this assembly have been reported. The yeast supercomplex is composed of CIII₂ flanked by either one or two monomers of CIV, the stoichiometry being dependant on the levels of expression of CIV⁵⁸.

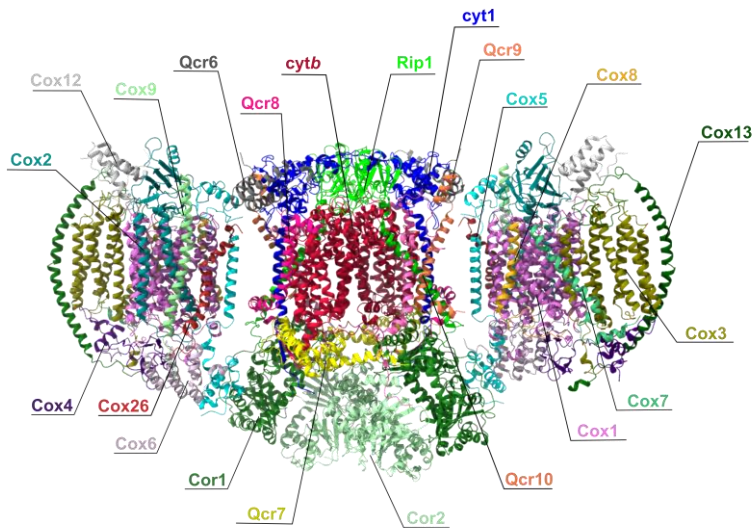


Figure 6: Structure of the *S. cerevisiae* supercomplex (PDB:6HU9)²⁷ with the different subunits colored.

In addition to the three main catalytic subunits, each CIII₂ monomer is composed of seven supplementary subunits, named Qcr6-10 and Cor1-2; while CIV contains nine additional subunits, referred to as Cox4-9, Cox12-13 and Cox26 (Fig.6). The surface of interaction between the complexes is small and it is mainly maintained by hydrophobic protein-protein interactions in the matrix side between the N-terminal domains of CIII₂ Cor1 subunit and CIV Cox5 subunit^{59,60}. The C-terminal domains of these subunits also interact on the periplasmic side of the membrane. Subunit Cox5 has two isoforms, Cox5A and Cox5B, but the residues that are involved in the interactions with Cor1 are conserved in the two isoforms and, therefore, the change of isoform does not

affect the supercomplex structure or formation⁵⁸. Interestingly, *S. cerevisiae* has two cyt. *c* isoforms too, namely iso-1 and iso-2, which are encoded by two different genes, *cyc1* and *cyc7*, respectively, and that share approximately 84% sequence identity⁶¹. It has been shown that the isoform gene pairs *cyc1/Cox5a* and *cyc7/Cox5b* are co-expressed depending on the oxygen concentration, the former being predominant under aerobic conditions and the latest under hypoxic conditions^{62,63}.

Electron transfer in the III₂-IV_{1/2} supercomplexes

Cytochrome *c* binding and diffusion

In mitochondria, electron transfer between CIII₂ and CIV requires a water-soluble cyt. *c*, a small ~12 kDa globular protein (Fig. 7) with a dipole moment and a net positive charge^{64,65}. The *c* heme group of cyt. *c* is located in a hydrophobic pocket, surrounded by a number of conserved lysine residues⁶⁶, and its edge is positioned towards the positively charged surface of cyt. *c*, that docks with the negatively charged surfaces of CIII₂ or CIV, respectively. The orientation of cyt. *c* is the same when binding either of the complexes⁶⁷. Early data suggested the presence of a single cyt. *c* binding site in CIII₂, but recently a secondary low-affinity binding site has been reported in the cyt. *c*₁ subunit in plants⁶⁸. Two binding sites in CIV have also been suggested based on different activity and kinetic studies in mammals, plants and yeast^{66,69,70,71}.

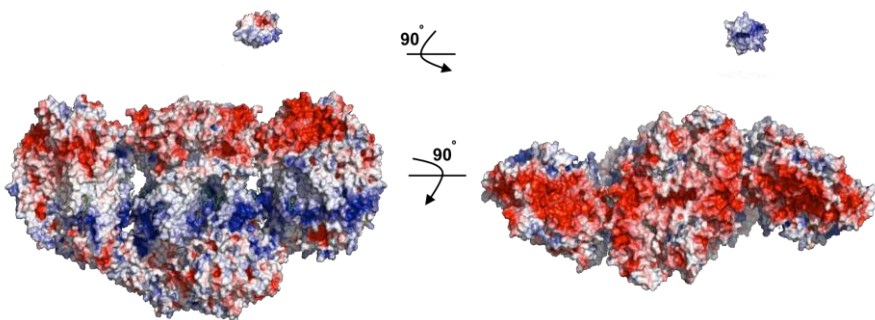


Figure 7: Surface representation of the electrostatic potential in the *S. cerevisiae* supercomplex (PDB:6HU9)²⁷ and cyt. *c* (PDB: 1YCC)⁶⁶.

As discussed above, cyt. *c* receives electrons from CIII₂ cyt. *c*₁ subunit and transfers them to Cu_A in CIV. When CIII₂ and CIV diffuse independently in the membrane, this reaction takes place via cyt. *c* 3D diffusion within the intermembrane space. Within the III-IV supercomplexes, the cyt. *c* binding sites in CIII₂ and CIV are 60-100 Å apart^{60,72,73}, which is too far to allow direct electron transfer by one bound cyt. *c* molecule. Different scenarios (Fig.8) were proposed in which electron transfer within the supercomplex would occur either via 3D diffusion of cyt. *c* through the bulk solvent, lateral 2D diffusion along the supercomplex surface or via the binding of several cyt. *c* molecules simultaneously, referred to as bridging.

Under the assumption that electrons are transferred within the supercomplex via 3D diffusion of cyt. *c*, the formation of this assembly would have a kinetic advantage by decreasing the cyt. *c* diffusion distance between the binding sites⁷³. It was seen that electron transfer is not dependent on the cyt. *c* diffusion time itself, but rather on its equilibration time with the cyt. *c* pool⁷³. This time increases with decreasing cyt. *c* concentration. However, in the mitochondrial intermembrane space there is a fixed concentration of cyt. *c*, e.g. in *S. cerevisiae* it is equivalent to 2-3 cyt. *c* molecules per III-IV pair⁷³. Hence, the equilibration time can be decreased by decreasing the distance between the complexes. This is accomplished through formation of supercomplexes, which implies a kinetic advantage when considering cyt. *c* 3D diffusion.

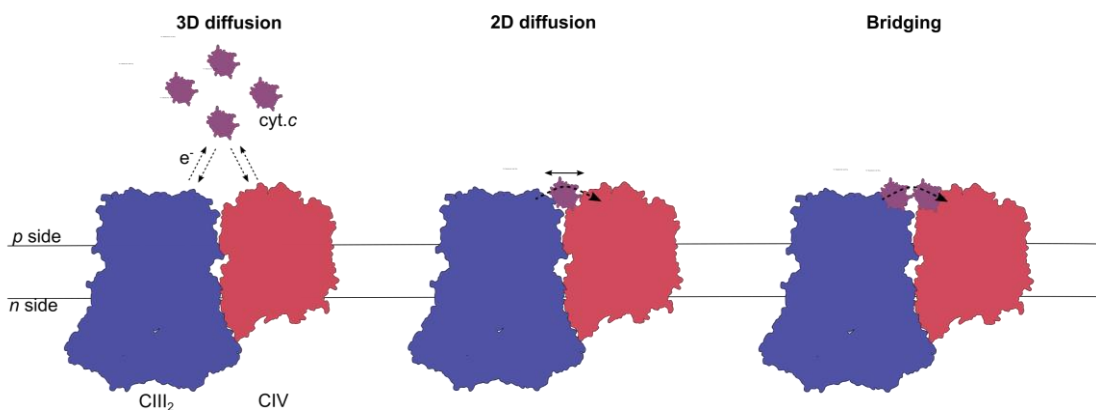


Figure 8: Possible scenarios of electron transfer within the III₂-IV supercomplex, either via 3D diffusion, 2D diffusion of a single cyt. *c* or via a bridge formed by two or more bound cyt. *c*.

Another possible scenario is for electron transfer to be mediated via 2D diffusion of cyt. *c* along the supercomplex surface^{69,74}. The formation of III-IV supercomplexes results in the formation of an overall negatively charged path that spans between the cyt. *c* binding sites of complexes III₂ and IV. According to computational studies^{75,75,76}, this path could facilitate the lateral diffusion of cyt. *c* across the supercomplex surface. This mechanism was studied experimentally in *S. cerevisiae*⁴⁸, in which the obtained supercomplex structure revealed that cyt. *c* was bound to either CIII₂, CIV or to an intermediate position, each with nearly equal occupancy. In addition, kinetic measurements of the supercomplex QH₂:O₂ oxidoreductase activity as a function of cyt. *c* concentration showed an increase in the turnover rate up to a cyt.*c*:supercomplex ratio of 1. The rate was almost unaltered at ratios from 1 to 10, but it increased again at high excess of cyt. *c*, i.e., cyt. *c*: supercomplex ratios >10. This results show that cyt. *c* diffusion is the rate limiting step of electron transfer within the supercomplex.

The same behaviour was observed for the *S. pombe*⁴⁹ III-IV supercomplex, where CIV is rotated 45° in relation to that of the *S. cereveisiae* supercomplex, resulting in a longer distance between the cyt. *c* binding sites. The *S. pombe* supercomplex structure showed cyt. *c* bound at four different positions along one of two symmetrical branches, indicating the possibility of 2D diffusion in this organisms too.

Experimental methods

The work presented in this thesis has involved the use of standard biochemical methods such as, for example, the growth of yeast, protein purification via gravity flow column and size exclusion chromatography and conventional protein characterization techniques such as spectrophotometric and activity measurements. Additionally, different specialized techniques for the study of respiratory enzymes and protein interactions have been used and are described below.

Oxidoreductase activity measurements

Oxidative phosphorylation is based on series of reactions that couple electron transfer to the generation of a transmembrane proton motive force. In aerobic organisms, the ultimate electron acceptor is molecular oxygen, which is reduced to water by CIV. The oxygen reduction rate can be measured by using a Clark-type oxygen electrode system, that enables accurate respirometry measurements in biological samples.

The instrument consists of a platinum electrode combined with a silver electrode set in an epoxy resin disc in contact with a saturated KCl solution diluted to 50%. When a small voltage is applied across the electrodes, the platinum electrode becomes negatively charged in relation to the silver electrode. Once there is enough overpotential to overcome kinetic limitations, i.e. the voltage reaches around 700 mV, oxygen in the sample is reduced to hydrogen peroxide (H_2O_2) at the platinum surface. The current of electrons that flow from the anode (silver electrode) to the cathode (platinum electrode) is directly proportional to the oxygen concentration⁷⁷.

Stopped-flow measurements

Many biochemical reactions take place over time ranges of milliseconds, too short time scales to allow analysis using standard laboratory equipment. Therefore, rapid measurement techniques are needed such as stopped-flow instrumentation. This device enables the study of solution-based kinetics in the

milliseconds to seconds timescale by rapid mixing of the reactants. The reaction is followed in time by measuring changes in the optical properties of the reactants, either absorbance or fluorescence.

Small volumes of solutions, contained in separate syringes, are rapidly driven into a mixing chamber. The resultant mixed solution flows into the cuvette, replacing its content and pushing it into a stop syringe, which is used to limit the volume of solution used in each experiment and to trigger data collection. The fresh reactants in the observation cell are irradiated with monochromatic light at a specific wavelength. As the reaction proceeds, the change in absorbance is recorded as a function of time^{78,79}.

In the present work, this technique was used to determine the electron transfer kinetics between the malate oxidase and the *M. smegmatis* supercomplex by following absorbance changes resulting from the reduction of the heme groups present in the supercomplex at the corresponding wavelengths.

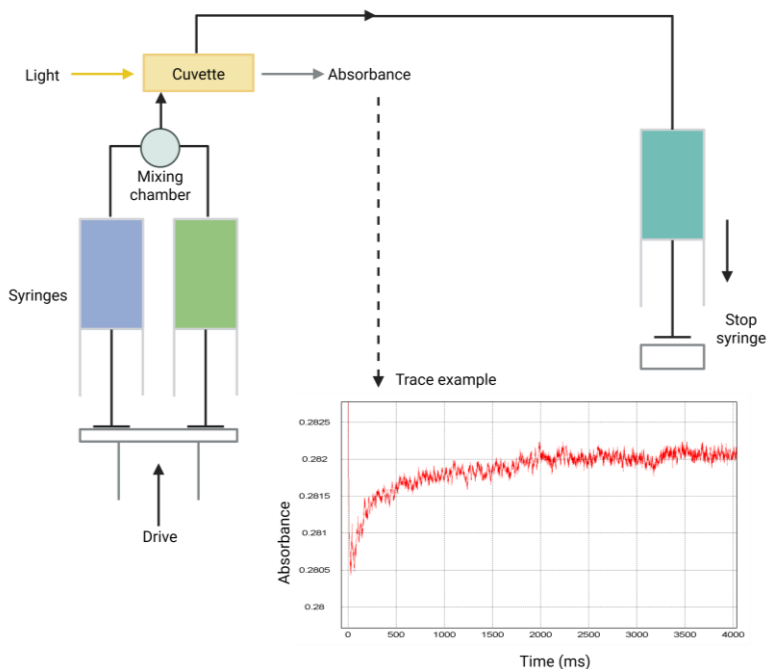


Figure 9: Schematic representation of stopped-flow experimental setup.

Summary of papers

The intracellular ionic strength is typically assumed to be equivalent to ~150 mM monovalent salt. However, as mentioned above, recent studies have shown that a more realistic mimic of the intracellular environment is ~20 mM monovalent salt solution. The interaction between cyt. *c* and the supercomplex is of electrostatic nature and is, therefore, determined by the electrostatic conditions of the intracellular environment. Consequently, a change in ionic strength is likely to affect this interaction, which, at 20 mM monovalent salt, is likely to be stronger than previously assumed.

The impact of this change in ionic strength on cyt. *c*-supercomplex interactions was studied in **paper I**, in which kinetic and structural studies were done at 20 mM salt concentration with the *S. cerevisiae* III₂-IV_{1/2} supercomplex. The supercomplex oxidoreductase activity was measured as a function of cyt. *c* concentration and the results were compared to those obtained at 150 mM salt⁴⁸. At cyt. *c*: supercomplex ratios of 1-10, the activity at 20 mM salt was lower than that observed at 150 mM salt, but at ratios above 10, the activity at low salt increased sharply. The data at 20 mM salt in the range 0.03-1 μM cyt. *c* was fitted with a Hill equation, which yielded a Hill coefficient of $n = 5 \pm 3$, suggesting that at least two cyt. *c* molecules bind to the supercomplex surface to facilitate electron transfer between each monomer of CIII₂ and CIV. These results are supported by structural data, which show that at low salinity and a cyt. *c*: supercomplex ratio of 12, a subpopulation of the particles harbor two cyt. *c* molecules bound simultaneously at different positions along the negatively charged surface of the supercomplex. The binding of cyt. *c* at two positions for each complex at low ionic strength was previously observed with the III-IV mammalian⁶⁹ and plant⁶⁸ supercomplexes.

Furthermore, upon lowering the ionic strength we observed additional residues at the N and C termini of subunits Qcr6 and Qcr9, respectively, which had not been resolved previously. The additional residues at the N-terminus of Qcr6 are in close contact with cyt. *c* when it is near CIII₂, while those of the C-terminus of subunit Qcr9 interact with cyt. *c* bound at CIV. These data, together with a re-analysis of the cryo-EM data at 150 mM monovalent salt (42), showing cyt. *c* interacting with both Qcr6 and Qcr9 at an intermediate position, suggest that Qcr6 and Qcr9 could facilitate rotation of cyt. *c* while moving along the negatively charged surface of the supercomplex.

The study in paper I was carried out with detergent-purified supercomplexes, but as mentioned above, *in vivo* respiratory supercomplexes are located in the inner mitochondrial membrane. The impact of the inner mitochondrial membrane on the cyt. *c*-supercomplex electrostatic interaction was studied in papers II and III.

In **paper II**, mitoplasts containing the *S. cerevisiae* supercomplex were used and the kinetic analysis at 20 and 150 mM KCl was repeated. The catalytic activities of CIII₂ and CIV in mitoplasts were also measured separately at 50 μM cyt. *c*. Data were compared to those obtained with detergent-purified supercomplexes. In general, the activities of the detergent-purified supercomplexes and individual complexes are higher than those measured in mitoplasts, which is explained by the detergent-induced release of a steroid ligand from a binding site near the K proton pathway of CIV. When bound, this ligand would slow the rate-limiting proton uptake through this pathway, resulting in a lower activity compared to that with the empty binding site⁸⁰.

In addition, the data in paper II show that, in mitoplasts, the supercomplex activities as a function of cyt. *c* concentration were very similar at 20 mM and 150 mM salt. This observation contrasts the data with detergent-purified supercomplexes, where the supercomplex activity is limited by the binding and diffusion of cyt. *c*. The different behavior observed with mitoplasts and detergent-purified supercomplexes is explained by the shift in the rate-limiting step outlined above. Between 20 nM and 30 nM cyt. *c*, i.e. approximately a cyt. *c*: supercomplex ratio of unity, the supercomplex activity increases steeply, indicating that electron transfer is limited by cyt. *c* binding to the supercomplex surface. In this cyt. *c* concentration range, each CIII₂ monomer-CIV pair recruits one cyt. *c* at its cyt. *c* binding site. Up to a cyt. *c*: supercomplex ratio of 50, the supercomplex activity remains almost constant, which indicates that at these cyt. *c* concentrations, the rate is limited by the maximum catalytic activities of the individual complexes. At higher cyt. *c* concentrations, above 1 μM cyt. *c*, the activity increases again, which is explained by 3D diffusion of cyt. *c* molecules to CIV from the fraction of CIII₂ that is not part of the supercomplex.

The effects of ionic strength and membrane potential on electron transfer within the yeast supercomplex was further investigated in **paper III**. The activity of detergent-purified and membrane bound supercomplexes, as well as

that of the individual complexes, was measured as a function of salt concentration. As in paper II, activities in detergent-purified conditions were higher than those in mitoplasts. For both conditions, the supercomplex activity increased with increasing salt concentration, up to 200 mM monovalent salt. Above this concentration range that the activity decreased, reaching, at 2M NaCl, a nearly zero activity for supercomplexes in solution and around 50% of their maximum activity in mitoplasts. The catalytic activities of the individual complexes were also measured as a function of ionic strength and the same behavior as for the supercomplex activity was observed.

These data indicate that increasing the ionic strength results in the decrease of the affinity between cyt. *c* and the supercomplex due to the screening of charges at the interaction surface. However, the maximum activity is not observed at very low ion concentrations, which is explained by the formation of a stable complex between cyt. *c* and the supercomplex, leading to a restricted access of reduced cyt. *c* to its binding sites.

Overall, the data presented in papers I-III show that the cyt. *c*: supercomplex electrostatic interactions are highly responsive to the electrostatic environment and that electron transfer within the supercomplex is critically dependent on the screening length.

In **paper IV**, the structure of the *M. smegmatis* supercomplex was obtained in native membranes, revealing a physical association with the enzyme Mqo, which was not seen in detergent-purified supercomplexes. This enzyme catalyzes the oxidation of malate to oxaloacetate, a critical step in the Krebs cycle. It is bound between CIII₂ and CIV, making close contact with the MSMEG_4692 subunit, and part of the Mqo is also embedded in the lipid bilayer. To study whether Mqo is capable of transferring electrons directly to the supercomplex, spectrophotometric studies were done by adding malate or NADH to reduce the sample. A conventional spectrophotometer was used to perform long time scale measurements, in which a reduced minus oxidized difference spectrum of the sample was obtained. These experiments showed that, over ~30 min, malate was capable of reducing ~75% of the *c* hemes (ΔA_{554}) and ~67% of the *b* hemes (ΔA_{562}) in the sample, while addition of NADH led to the reduction of ~69% of the *c* hemes and ~55% of the *b* hemes. Absorbance changes attributed to *d* and *a* hemes were negligible. In addition, stopped-flow spectrophotometric measurements were done to study heme reduction over short time scales. Results showed that ~30% of the *c* hemes were

reduced within ~500 msec on addition of malate, while addition of NADH did not reduce the *c* hemes of the sample on this timescale. Altogether, this data show that, on its association with the *M. smegmatis* III₂-IV₂ supercomplex, Mqo is capable of transferring electrons from malate to the supercomplex, which would indicate a connection between the Krebs cycle and aerobic respiration in mycobacteria.

Concluding remarks and future prospects

The cellular function relies on the interaction of proteins with their physiological partners. An example is the mitochondrial respiratory chain, a set of enzyme complexes that interact with different electron carriers to ultimately synthesize ATP that is required for the cell's survival. These proteins coexist in the crowded, dynamic and concentrated environment that is the cell interior, surrounded by different charged molecules and small ions.

These conditions influence the way protein-protein interactions take place and, therefore, mimicking them is key for depicting the functional and structural aspects of respiratory complexes and cellular respiration. The work presented in this thesis reveals how electrostatic conditions affect the interaction and behaviour of respiratory supercomplexes, highlighting the importance of using an appropriate ionic strength to resemble the screening length inside the cell.

Nevertheless, the conditions used throughout the experiments still differ from the actual intracellular environment and further research is needed to fully understand how protein-protein interactions take place *in vivo* and, specifically, how these affect the function and structure of the respiratory chain. This thesis focuses on ionic strength but, as mentioned above, the cell interior is a highly-crowded environment in which many molecules coexists and interact with each other. Therefore, increasing the molecular crowding conditions, reconstituting the complete respiratory chain and performing experiments in whole living cells, would better mimic the true conditions inside the cell.

Popular science summary

The cell is a basic structural and functional unit of all living organisms, within which different biomolecules, such as proteins, nucleic acids, carbohydrates and lipids, coexist. Proteins play a central role in cellular function and organization, interacting with each other and with other macromolecules to carry out a wide range of functions. Protein-protein interactions are commonly of electrostatic origin and are therefore determined by the electrostatic conditions within the cell. This environment is dynamic and highly crowded, containing many different ions and charged molecules. Determining the effect of these charges on protein-protein interactions and replicating the intracellular conditions are essential for depicting the functional and structural aspects of the cell.

One of the cellular machineries in which protein-protein interactions play a crucial role is the respiratory chain, a series of membrane proteins that catalyze chemical reactions which result in the formation of ATP, a molecule that stores and supplies energy to sustain all basic cellular functions. In mitochondria, these reactions take place in the inner mitochondrial membrane, where electrons derived from different sources are transferred through a series of protein complexes and mobile carriers to a final electron acceptor, oxygen. This electron transfer is coupled to the transfer of positively charged protons across the membrane, leading to the formation of an electrochemical gradient, which provides energy for ATP synthesis.

In the work presented in this thesis, the respiratory chain is used as a model system to study protein-protein interactions under different ionic conditions, with a special focus on supercomplexes, higher-order assemblies of respiratory complexes. Specifically, the interaction between the supercomplex and the mobile electron carrier, cytochrome *c*, has been studied. Cytochrome *c* carries a positively charged surface that interacts with the negatively charged surface of the supercomplex. The electrostatic nature of this interaction offers an ideal tool for investigation of protein-protein interactions under a wide range of physiological conditions.

The results have revealed that changing the ion concentration of the medium has a clear effect on the supercomplex-cytochrome *c* interaction, thereby modifying the mechanism of electron transfer within the supercomplex. Overall,

the data show that the composition of the solution has a strong impact on protein behavior and highlights the importance of understanding and replication the cellular interior to obtain results that reflect the true functioning of the cell.

Populärvetenskaplig sammanfattning

Cellen utgör den grundläggande strukturella och funktionella enheten i alla levande organismer. Inuti cellen samexisterar olika biomolekyler, såsom proteiner, nukleinsyror, kolhydrater och lipider, vilka tillsammans möjliggör livets kemiska processer. I dessa processer spelar proteiner en central roll genom att interagera med varandra samt med andra makromolekyler för att utföra en mängd olika funktioner. Dessa protein–protein-interaktioner är ofta av elektrostatiske natur och påverkas därför av den komplexa intracellulära miljön, som innehåller en mängd joner och laddade makromolekyler. För att förstå cellens funktionella egenskaper är det därför avgörande att experimentellt kunna fastställa hur dessa laddningar påverkar interaktionerna och att efterlikna de naturliga förhållandena i cellen.

Ett exempel på reaktioner där protein–protein-interaktioner spelar en viktig roll är cellandningen, där cellen producerar ATP – en molekyl som driver alla energikrävande processer. Andningskedjan består av en serie membranproteiner i det inre mitokondriemembranet vilka katalyserar en sekvens av kemiska reaktioner. Elektroner transporteras stegvis genom flera proteinkomplex via mobila elektronbärare till den slutliga elektronacceptorn, syre. Denna elektrontransport är kopplad till överföring av positivt laddade protoner över membranet, vilket upprätthåller en elektrokemisk gradient över membranet. Den energi som är lagrad i denna gradient utnyttjas för att syntetisera ATP.

I denna avhandling används andningskedjan som ett modellsystem för att studera protein–protein-interaktioner under varierande jonstyrka, med särskilt fokus på så kallade superkomplex vilka består av två eller flera respiratoriska proteinkomplex. Specifikt har interaktionen mellan ett sådant superkomplex och dess mobila elektronbärare, cytokrom *c*, undersökts. Cytokrom *c* har en positivt laddad yta som interagerar elektrostatiske med den negativa ytan hos superkomplexet. Därför är detta system särskilt väl lämpat för analys av hur elektrostatiske krafter påverkar proteininteraktioner under fysiologiskt relevanta betingelser.

Resultaten visar att variationer i jonstyrkan i omgivningen påverkar interaktionen mellan superkomplexet och cytokrom *c*. Jonkoncentrationen påverkar bindning av cytokrom *c* vilket i sin tur påverkar mekanismen för elektronöverföring inom superkomplexet. Resultaten visar på hur den

omgivande miljön påverkar komplexa kemiska processer i cellen och understryker vikten av att noggrant efterlikna cellens naturliga förhållanden för att kunna dra tillförlitliga slutsatser rörande dess funktion.

Resumen de divulgación científica

La célula es la unidad estructural y funcional básica de todo ser vivo, en cuyo interior coexisten diferentes biomoléculas como proteínas, ácidos nucleicos, carbohidratos y lípidos. Las proteínas desempeñan un papel central en el funcionamiento y organización celular, interactuando entre sí y con otras macromoléculas para llevar a cabo una amplia variedad de funciones. Las interacciones proteína-proteína son comúnmente de origen electrostático y, por lo tanto, están determinadas por las condiciones electrostáticas dentro de la célula. Este es un entorno dinámico y concentrado que contiene una gran cantidad de iones y moléculas con carga. Determinar el efecto de estas cargas sobre las interacciones entre proteínas y replicar las condiciones intracelulares son elementos esenciales para comprender los aspectos funcionales y estructurales de la célula.

Una de las maquinarias celulares en la que las interacciones proteína-proteína desempeñan un papel crucial es la cadena respiratoria, un conjunto de proteínas de membrana que catalizan reacciones químicas a través de las cuales se forma ATP, una molécula que almacena y suministra energía para sostener las funciones celulares básicas. En las mitocondrias, estas reacciones tienen lugar en la membrana mitocondrial interna, donde electrones procedentes de diferentes fuentes son transferidos a través de una serie de complejos proteicos y transportadores móviles hasta un aceptor final, el oxígeno. Esta transferencia de electrones causa el movimiento de protones, cargados positivamente, a través de la membrana, generando así un gradiente electroquímico cuya energía es utilizada para la síntesis de ATP.

En el trabajo presentado en esta tesis se hace uso de la cadena respiratoria como modelo de estudio de las interacciones entre proteínas bajo diferentes condiciones iónicas, con especial foco en los supercomplejos, agrupaciones estructurales de los complejos respiratorios. Concretamente, se ha estudiado la interacción entre el supercomplejo y el citocromo *c*, un transportador móvil de electrones. El citocromo *c* presenta una superficie con carga positiva que interactúa con la superficie del supercomplejo cargada negativamente. La naturaleza electrostática de esta interacción ofrece una herramienta ideal para el estudio de las interacciones proteína-proteína bajo una gran cantidad de condiciones fisiológicas.

Los resultados obtenidos muestran que los cambios en la concentración iónica del medio tienen un claro efecto sobre la interacción entre el supercomplejo y el citocromo *c*, modificando el mecanismo de transferencia de electrones a través del supercomplejo. En conjunto, los datos muestran que la composición del medio tiene un fuerte impacto en el comportamiento proteico y destacan la importancia de comprender y reproducir el entorno celular para obtener resultados que reflejen el funcionamiento real de la célula.

Acknowledgments

Finally, I would like to thank everyone who has helped and supported me throughout these years and without whom this work would not have been possible.

First and foremost, **Peter**. Thank you for your guidance, encouragement and generosity, for your enthusiasm, and for always having your door open. You are the best supervisor I could have ever had, and I am deeply grateful to you for supporting and listening to me when I needed it most.

Pia, thank you for all your input, interesting discussions and for making me feel welcome from the very beginning.

To current and former members of the PBPÄ group. **Agnes**, thank you for introducing me to the lab and everything you taught me. Definitely, this work wouldn't have been possible without you, your work and advice. **Markus**, thank you for all your help during the first years, for always answering my questions and for recommending me what has become my favourite restaurant, Ai ramen. **Irina**, thank you for being a great office colleague, for caring about me and for the great scientific (and non-scientific) discussions. Thanks to **Mateusz**, **Sofia**, **Johanna** and **Sylwia** for being so generous and supportive, and for always making the lab a fun and safe space. **Finja**, it has been great to share this last year with you, thank you for your honesty, commitment and trust. Thanks to all other PBPÄ group members that I had the chance to work with: **Olga**, **Fei** and **Parul**. It was really nice meeting you all.

I would also like to express my gratitude to: **Mikael**, for guiding me during my TA sessions, for your collaboration and help with all the papers, and above all, for your kindness and generosity; **Ville**, for your help during the different evaluation points and for all the interesting and challenging questions; and our collaborators in **John Rubinstein's group**.

A special thanks to **Bea**. Conocerme me ha hecho sentir más cerca de casa. Gracias por acordarte siempre de mí, por tu amabilidad y cariño, y por hacerme recordar lo bonita que es nuestra tierra y su gente.

Finally, I would like to thank my friends and family.

Cholitas, **Kiwis**, gracias por hacer que todo siga igual a pesar del tiempo y la distancia. Me siento muy afortunada de haber crecido a vuestro lado y poder

llamaros familia. Gracias especialmente a **Irene**, por escucharme y aconsejarme, por haber estado y estar siempre, por ser mi lugar seguro.

María, qué suerte la mía de coincidir contigo en esta aventura. Gracias por haber sido un apoyo incondicional, por tu sensibilidad y comprensión, por siempre tener una sonrisa para mí. Llevaré a nuestra familia gatuna siempre conmigo.

Gracias a mis cuñadas, **Raquel** y **Marian**, por ayudarme cuando lo he necesitado y, en especial a mis hermanos, **José Javier** y **Jesús**, por demostrarme que, a pesar de la distancia, siempre están y estarán presentes y dispuestos a ayudarme. A mis sobrinos, **Ariadna**, **Pilar**, **Daniel** y **Hugo**, por alegrarse cada vez que volvía a casa. Lo más difícil de estar lejos ha sido no poder veros crecer durante estos años, pero espero que aun así hayáis sentido lo mucho que os quiero y sepáis que tenéis a vuestra tía para todo lo que necesitéis.

Un agradecimiento muy especial a mi **tío José** y mi **tía Ana**. Sois como unos padres para mí y no hay palabras suficientes que puedan expresar lo agradecida que estoy por teneros en mi vida y lo mucho que os quiero. Gracias por cuidarme, enseñarme y quererme, por hacerme feliz y por ayudarme a convertirme en la persona que soy hoy.

Por supuesto, tengo que agradecer a los mejores compañeros de vida que podría tener. **Blue**, eres un gato y esto no lo puedes leer, pero te escribo igualmente porque llegaste en el momento en el que más lo necesitaba, haciéndome jugar cuando quería llorar y salir a la calle cuando solo quería encerrarme. Eres y siempre serás mi familia y mi hogar. **Jorge**, eres mi persona favorita del mundo. Gracias por hacerme reír como nadie, por animarme a no tirar la toalla y por decidir acompañarme durante estos cuatro años. Soy consciente de los mil viajes que te has hecho para estar aquí conmigo y no sabes lo agradecida y afortunada que me siento. Gracias por tu confianza, por abrirme tu corazón y, por encima de todo, gracias por construir esta pequeña gran familia junto a mí. Sabes de sobra que sin vosotros dos esta tesis no estaría terminada. Te quiero con toda mi alma.

Obviamente no me puedo olvidar de las dos personas a las que les debo todo y más, que son mi luz cada día, y sin cuyo amor, apoyo y esfuerzo yo no sería quién soy ni estaría donde estoy. **Papá**, gracias por ser ejemplo y enseñarme que mi salud y mi bienestar siempre tienen que ser lo primero, por animarme a luchar por lo que quiero y por siempre tener las palabras justas y necesarias para cada momento. **Mamá**, gracias por enseñarme a no rendirme nunca, por nuestras conversaciones infinitas que me han salvado durante estos años, por esta relación madre-hija tan especial que tenemos y por esa confianza y cone-

xión tan increíble que solo tú y yo entendemos. Papá, mamá, gracias por hacerme tan feliz, y por dejarme ser, crecer y aprender de forma libre. Os admiro y os quiero con locura.

Finalmente, quiero terminar esta tesis agradeciendo a la personita que está escribiendo esto. Llegar hasta aquí no ha sido nada fácil pues los últimos diez años han estado llenos de mucho esfuerzo y una gran lucha personal. Ahora empiezo una nueva etapa desde un lugar más sano, más fuerte y mucho más feliz, con ganas de vivir y lista para afrontar todo lo que venga y más.

References

1. Ngounou, A. G. *et al.* Protein – protein interactions : switch from classical methods to proteomics and bioinformatics-based approaches Electron transfer dissociation. *Cell. Mol. Life Sci.* **71**, 205–228 (2014).
2. Sheinerman, F. B., Norel, R. & Honig, B. Electrostatic aspects of protein – protein interactions. *Curr. Opin. Struct. Biol.* **10**, 153–159 (2000).
3. Smith, A. M., Lee, A. A. & Perkin, S. The electrostatic screening length in concentrated electrolytes increases with concentration. *J. Phys. Chem. Lett.* **7**, 2157–2163 (2016).
4. Cevc, G. Membrane electrostatics. *BBA* **3**, 311–382 (1990).
5. Ma, Y., Poole, K., Goyette, J. & Gaus, K. Introducing membrane charge and membrane potential to T cell signaling. *Front. cell Dev. Biol.* **8**, 1–11 (2017).
6. Nicolson, G. L. & Mattos, G. F. De. A brief introduction to some aspects of the fluid – mosaic model of cell membrane structure and its importance in membrane lipid replacement. *Membranes (Basel)*. **11**, (2021).
7. Bernardino, J., Serna, D., Schütz, G. J. & Eggeling, C. There is no simple model of the plasma membrane organization. *Front. cell Dev. Biol.* **4**, 1–17 (2016).
8. Cheng, X. & Smith, J. C. Biological membrane organization and cellular signaling. *Chem. Rev.* **119**, 5849–5880 (2019).
9. Dupuy, A. D. & Engelman, D. M. Protein area occupancy at the center of the red blood cell membrane. *PNAS* **2007**, 0–4 (2008).
10. White, S. H., von Heijne, G. & Engelman, D. M. Lipid bilayers in *Cell Boundaries. How membranes and their proteins work*, 51–95 (2022).
11. Record, T. M., Courtenay, E. S., Cayley, S. & Guttman, H. J. Biophysical compensation mechanisms buffering E. coli protein-nucleic acid interactions against changing environments. *Trends Biochem. Sci.* **23**, 190–194 (1998).
12. Stokes, R. H. Thermodynamics of solutions. in *Activity coefficients in electrolyte solutions*, 1–29 (1991).
13. Zarubin, D. P., Pavlov, A. N., Zarubin, D. P. & Pavlov, A. N. A closer look at the Debye-Hückel theory and its modification in the SiS model of electrolyte solutions electrolyte solutions. *Mol. Phys.* **118**, 13

- (2020).
14. Gaddam, P. & Ducker, W. Electrostatic screening length in concentrated salt solutions. *Langmuir* **35**, 5719–5727 (2019).
 15. Gebbie, M. A., Dobbs, H. A., Valtiner, M. & Israelachvili, J. N. Long-range electrostatic screening in ionic liquids. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 7432–7437 (2015).
 16. Paulson, J. A. *et al.* Scaling analysis of the screening length in concentrated electrolytes. *Phys. Rev. Lett.* **026002**, 1–5 (2017).
 17. Wennerström, H., Vallina, E., Danielsson, J. & Oliveberg, M. Colloidal stability of the living cell. *PNAS* **117**, 10113–10121 (2020).
 18. Schultz, S. G. & Wilson, N. L. Cation transport in *Escherichia coli* II. Intracellular chloride concentration. *J. Gen. Physiol.* **46**, 159–166 (1962).
 19. Kaila, V. R. I. & Wikström, M. Architecture of bacterial respiratory chains. *Nat. Rev. Microbiol.* **19**, 319–330 (2021).
 20. White, D. C. & Sinclair, P. R. Branched electron transport systems in bacteria. *Adv. Microb. Physiol.* **5**, 173–211 (1971).
 21. Sarewicz, M. *et al.* Catalytic reactions and energy conservation in the cytochrome bc 1 and b 6 f complexes of energy-transducing membranes. *Chem. Rev.* **121** (4), 2020–2108 (2021)
 22. Vercellino, I. & Sazanov, L. A. The assembly, regulation and function of the mitochondrial respiratory chain. *Nat. Rev Mol Cell Biol.* **23**, 141–161.(2022).
 23. Brzezinski, P., Moe, A. & Adelroth, P. Structure and mechanism of respiratory iii-iv supercomplexes in bioenergetic membranes. *Chem. Rev.* **121**, 9644–9673 (2021).
 24. Kim, H. J., Khalimonchek, O., Pamela, S. M. & Winge, D. R. Structure, function, and assembly of heme centers in mitochondrial respiratory complexes. *Biochim. Biophys. Acta - Bioenerg.* **1823**, 1604–1616 (2012).
 25. Hunte, C., Palsdottir, H. & Trumpower, B. L. Protonmotive pathways and mechanisms in the cytochrome bc 1 complex. *FEBS Lett.* **545**, 39–46 (2003).
 26. Brzezinski, P. & Gennis, R. B. Cytochrome c oxidase: exciting progress and remaining mysteries. *J Bioenerg Biomembr.* **40**, 521–531 (2008)
 27. Hartley, A. M. *et al.* Structure of yeast cytochrome c oxidase in a supercomplex with cytochrome bc1. *Nat. Struct. Mol. Biol.* **26**, (2019).

28. Hartley, A. M. *et al.* Structure of yeast cytochrome c oxidase in a supercomplex with cytochrome bc 1. *Nat Struct Mol Biol.* **26**, 78–83 (2019).
29. Kadenbach, B. & Hüttemann, M. The subunit composition and function of mammalian cytochrome c oxidase. *Mitochondrion* **24**, 64–76 (2015).
30. Lobo-jarne, T. & Ugalde, C. Respiratory chain supercomplexes: Structures, Function and Biogenesis. *Semin Cell Dev B* **76**, 179–190 (2019).
31. Schägger, Hermann and Pfeiffer, K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J.* **19**, 1777–1783 (2000).
32. Greggio, C. *et al.* Enhanced respiratory chain supercomplex formation in response to exercise in human clinical and translational report enhanced respiratory chain supercomplex formation in response to exercise in human skeletal muscle. *Cell Metab.* **25**, 301–311 (2017)
33. Ramírez-aguilar, S. J. *et al.* The composition of plant mitochondrial supercomplexes changes with oxygen availability. *J. Biol. Chem.* **286**, 43045–43053 (2011).
34. Zhang, M., Mileykovskaya, E. & Dowhan, W. Gluing the respiratory chain. Formation in the inner mitochondrial. *J. Biol. Chem.* **277**, 43553–43556 (2002).
35. Böttinger, L. *et al.* Phosphatidylethanolamine and cardiolipin differentially affect the stability of mitochondrial respiratory chain supercomplexes. *J. Mol. Biol.* **423**, 677–686 (2012).
36. Stroh, A. *et al.* Assembly of respiratory complexes I , III , and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*. *J. Biol. Chem.* **279**, 5000–5007 (2004).
37. Wiseman, B. *et al.* Structure of a functional obligate complex III₂IV₂ respiratory supercomplex from *Mycobacterium smegmatis*. *Nat. Struct. Mol. Biol.* **25**, (2018).
38. Klusch, N. *et al.* Cryo-EM structure of the respiratory I + III ₂ supercomplex from *Arabidopsis thaliana* at 2 Å resolution. *Nat. Plants.* **9**, (2023).
39. Maldonado, M., Guo, F. & Letts, J. A. Atomic structures of respiratory complex III ₂ , complex IV , and supercomplex III ₂ -IV from vascular plants. *Elife.* **10**:e62047, 1–34 (2021).
40. Milenkovic D. *et al.* The enigma of the respiratory chain supercomplex. *Cell Metab.*, **25**, 765–776 (2017)

41. Winge, D. R. Sealing the mitochondrial respirasome. *MCB* **32**, 2647–2652 (2012).
42. Maranzana, E., Barbero, G., Falasca, A. I., Lenaz, G. & Genova, M. L. Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I. *Discoveries* **19**, (2013).
43. Blaza, J. N., Serreli, R., Jones, A. J. Y., Mohammed, K. & Hirst, J. Kinetic evidence against partitioning of the ubiquinone pool and the catalytic relevance of respiratory-chain supercomplexes. *PNAS*, **111**, 15735-15740 (2014)
44. Acín-pérez, R. *et al.* Respiratory complex III is required to maintain complex I in mammalian mitochondria. *Mol. Cell* **13**, 805–815 (2004).
45. Arenas, J., Barrientos, A. & Ugalde, C. Mitochondrial complex I plays an essential role in human respirasome assembly. *Cell Metab.* **15**, 324–335 (2012).
46. Sun, C. & et al. Structure of the alternative complex III in a supercomplex with cytochrome oxidase. *Nature* **557**, 123–126 (2018).
47. Moe, A. *et al.* The respiratory supercomplex from *C. glutamicum*. *Structure* **30**, 338–349 (2022).
48. Moe, A., Trani, J. Di, Rubinstein, J. L. & Brzezinski, P. Cryo-EM structure and kinetics reveal electron transfer by 2D diffusion of cytochrome c in the yeast III-IV respiratory supercomplex. *Proc. Natl. Acad. Sci. U. S. A.* **118**, 1–9 (2021).
49. Moe, A., Dimogkioka, A.-R., Rapaport, D., Näsвик, L. & Brzezinski, P. Structure and function of the *S.pombe* III-IV supercomplex. *PNAS* **120**, 1–9 (2023).
50. Vercellino, I. & Sazanov, L. A. Structure and assembly of the mammalian mitochondrial supercomplex CIII2CIV. *Nature* **598**, 364–367 (2021).
51. Yang, X. *et al.* An electron transfer path connects subunits of a mycobacterial respiratory supercomplex. *Science*, **362** (6418), (2018).
52. Matsoso, L. G. *et al.* Function of the Cytochrome bc₁ - aa₃ Branch of the respiratory network in mycobacteria and network adaptation occurring in response to its disruption. *J. Bacteriol.* **187**, 6300–6308 (2005).
53. Di, J. M., Yu, J., Courbon, G. M., Lobež, A. P. & Brzezinski, P. Cryo-EM of native membranes reveals an intimate connection between the Krebs cycle and aerobic respiration in mycobacteria. *PNAS* **122**, 1–10 (2025).

54. Kumar, R. *et al.* Malate : quinone oxidoreductase knockout makes Mycobacterium tuberculosis susceptible to stress and affects its in vivo survival. *Microbes Infect.* **26**, 105215 (2024).
55. Harold, L. K. *et al.* Deciphering functional redundancy and energetics of malate oxidation in mycobacteria. *J. Biol. Chem.* **298**, 101859 (2022).
56. Karathia, H., Vilapinyo, E., Sorribas, A. & Alves, R. *Saccharomyces cerevisiae* as a model organism : A Comparative Study. *PLoS One* **6**, 1–10 (2011).
57. Vanderwaeren, L., Dok, R., Voordeckers, K., Nuyts, S. & Verstrepen, K. J. *Saccharomyces cerevisiae* as a model system for eukaryotic cell biology , from cell cycle control to DNA damage response. *Int. J. Mol. Sci.* **23**, 2–26 (2022).
58. Hartley, A. M., Meunier, B., Pinotsis, N. & Maréchal, A. Rcf2 revealed in cryo-EM structures of hypoxic isoforms of mature mitochondrial. *PNAS*, **117**, 9329–9337 (2020).
59. Sousa, J. S. & Vonck, J. Respiratory supercomplexes III 2 IV 2 come into focus. *Nat. Struct. Mol. Biol.* **26**, 87–89 (2019).
60. Rathore, S. *et al.* Cryo-EM structure of the yeast respiratory supercomplex. *Nat. Struct. Mol. Biol.* **26**, 50–57 (2019).
61. Guerra-castellano, A. *et al.* The two yeast cytochrome c isoforms differentially regulate supercomplex assembly and mitochondrial electron flow. *International J. Biol. Macromol.* **313**, 1–14 (2025).
62. Oyton, R. O. O. P. Oxygen sensing in yeast : Evidence for the involvement of the respiratory chain in regulating the transcription of a subset of hypoxic genes. *PNAS* **96**, 5446–5451 (1999).
63. Burke, P. V, Raitt, D. C., Allen, L. A., Kellogg, E. A. & Poyton, R. O. Effects of oxygen concentration on the expression of cytochrome c and cytochrome c oxidase genes in yeast. *J. Biol. Chem.* **272**, 14705–14712 (1997).
64. Koppenol, W. H., Rush, J. D., Mills, J. D. & Margoliash, E. The dipole moment of cytochrome c. *Mol. Biol. Evol.* **8**, 545–558 (1991).
65. Louie, G. V & Brayer, G. D. High-resolution refinement of yeast iso-1-cytochrome c and comparisons with other eukaryotic cytochromes c. *J. Mol. Biol.* **527**, 527–555 (1990).
66. Capaldi, R. A., Darley-Usmar, V., Fuller, S. & Millett, F. Structural and functional features of the interaction of cytochrome c with complex III and cytochrome c oxidase. *FEBS Lett.* **138**, 1–7 (1982).
67. Speck, S. H., Ferguson-millert, S., Osheroff, N. & Margoliash, E.

- Definition of cytochrome c binding domains by chemical modification : Kinetics of reaction with beef mitochondrial reductase and functional organization of the respiratory chain. *PNAS* **76**, 155–159 (1979).
68. Moreno-beltrán, B. *et al.* Cytochrome c 1 exhibits two binding sites for cytochrome c in plants. *BBA - Bioenerg.* **1837**, 1717–1729 (2014).
 69. Moreno-beltrán, B. *et al.* Respiratory complexes III and IV can each bind two molecules of cytochrome c at low ionic strength. *FEBS Lett.* **589**, 476–483 (2015).
 70. Ferguson Miller, S., Brautigan, D. L. & Margoliash, E. Correlation of the kinetics of electron transfer activity of various eukaryotic cytochromes c with binding to mitochondrial cytochrome c oxidase. *J. Biol. Chem.* **251**, 1104–1115 (1976).
 71. Kang, C. H. & Margoliash, E. Steady state kinetics and binding of eukaryotic cytochromes c with yeast cytochrome c peroxidase. *J. Biol. Chem.* **252**, 919–926 (1977).
 72. Mileykovskaya, E. *et al.* Arrangement of the respiratory chain complexes in *Saccharomyces cerevisiae* supercomplex III₂IV₂ revealed by single particle cryo-electron microscopy. *J. Biol. Chem.* **287**, 23095–23103 (2012).
 73. Stuchebrukhov, A., Schäfer, J., Berg, J. & Brzezinski, P. Kinetic advantage of forming respiratory supercomplexes. *BBA - Bioenerg.* **1861**, 148193 (2020).
 74. Pérez-mejías, G., Guerra-castellano, A., Díaz-quintana, A., Rosa, M. A. De & Díaz-moreno, I. Cytochrome c: surfing off of the mitochondrial membrane on the tops of complexes III and IV. *Comput. Struct. Biotechnol. J.* **17**, 654–660 (2019).
 75. March, M. De & Geremia, S. A general exit strategy of monoheme cytochromes c and c₂ in electron transfer complexes? *Int. Union Biochem. Mol. Biol.* **67**, 694–700 (2015).
 76. Spaar, A., Flo, D. & Helms, V. Association of Cytochrome c with membrane-bound cytochrome c oxidase proceeds parallel to the membrane rather than in bulk solution. *Biophys. J.* **96**, 1721–1732 (2009).
 77. Kral, A., Aplin, F. & Maier, H. Advanced concepts physical chemistry: electrodes and electrolytes in *Prostheses for the brain* (2021).
 78. Corporation, K. Chemical Reaction Kinetics: The methods. <https://kintekcorp.com/methods>.
 79. Gore, M. G. & Bottomley, S. P. Stopped-flow fluorescence

spectroscopy in *Spectrophotometry and spectrofluorimetry: A practical approach* (ed. Gore, M. G.) 241–264 (Oxford University Press, 2000).

80. Smirnova, I., Wu, F. & Brzezinski, P. Stimulation of cytochrome c oxidase activity by detergents. *BBA - Bioenerg.* **1866**, 149509 (2025).