

Wiring Components of the Respiratory Chain

Modulation of the Respiratory Chain in Yeast and Bacteria

Jacob Schäfer

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Abstract

The enzyme complexes of the respiratory chain are organized in supramolecular assemblies, so-called respiratory supercomplexes. In the yeast *Saccharomyces cerevisiae*, these supercomplexes consist of two copies of complex III (bc_1 complex) and one or two copies of complex IV (cytochrome *c* oxidase, Cyt c O). Several factors, including lipids and small proteins, have been identified to facilitate or stabilize this organization.

Respiratory supercomplex factor (Rcf) 1 interacts with Cyt c O. In this work, we show that in the native *S. cerevisiae* mitochondrial membrane several forms of Cyt c O co-exist. Intact Cyt c O shows spectral and functional properties similar to those of Cyt c O from other organisms characterized earlier. A second population displayed a lower midpoint potential of heme a_3 as well as accelerated ligand binding, suggesting structural differences around the catalytic site. Severe structural changes of the catalytic site and the overall structure of the enzyme were found in a third population of Cyt c O. The fraction of the structurally altered Cyt c O increased upon removal of Rcf1. Here, a mechanism is proposed in which Rcf1 regulates function of the Cyt c O by altering the catalytic site so that electron transfer between heme *a* and heme a_3 is slowed, resulting in a more exergonic O₂-ligand binding. This scenario would in turn increase heat production on the expense of the proton electrochemical gradient.

Rcf1 was further shown to facilitate electron transfer from the bc_1 complex to Cyt c O in a supercomplex by interacting with the electron carrier cytochrome *c* (cyt. *c*).

In addition, we purified and structurally and functionally characterized the supercomplex of *Mycobacterium smegmatis*, which contains a membrane-anchored cyt. *c* as a subunit of the bcc_1 complex.

Keywords: *Cytochrome c oxidase, Electron transfer, Membrane protein, Ligand, Kinetics, Mechanism, Rcf1, Cytochrome c, Respiratory supercomplex, Cryo-electron microscopy.*

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The most exciting
phrase to hear in
science, the one that
heralds new discoveries,
is not "Eureka!" but
"That's funny..." - Isaac
Asimov

List of Publications

This thesis is based on the following publications, which will be referred to by their roman numerals:

- I. Schäfer, J., Dawitz, H., Ott, M., Ädelroth, P., Brzezinski, P. **Structural and functional heterogeneity of cytochrome *c* oxidase in *S. cerevisiae***, *Biochim. Biophys. Acta – Bioenergetics* **1859**, 699-704 (2018).
- II. Schäfer, J., Dawitz, H., Ott, M., Ädelroth, P., Brzezinski, P. **Regulation of cytochrome *c* oxidase activity by modulation of the catalytic site**, *Sci. Rep.* **8**, 11397 (2018).
- III. Sjöholm, J., Schäfer, J., Zhou, S., Rydström Lundin, C., Widengren, J., Ädelroth, P., Brzezinski, P. **A membrane bound anchor for cytochrome *c* in *S. cerevisiae*** (manuscript).
- IV. Nitharwal, R.G., Schäfer, J., Wiseman, B., Sjöstrand, D., Kuang, Q., Ädelroth, P., Brzezinski, P., Högbom, M. **Biochemical and structural characterization of a superoxide dismutase-containing respiratory supercomplex from *Mycobacterium smegmatis*** (submitted manuscript).

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Additional Publications:

Smirnova, I.A., Sjöstrand, D., Li, F., Björck, M., Schäfer, J., Östbye, H., Högbom, M., von Ballmoos, C., Lander, G.C., Ädelroth, P., Brzezinski, P. **Isolation of yeast complex IV in native lipid nanodiscs**, *Biochim. Biophys. Acta – Biomembranes* **1858**, 2987-2992 (2016).

Contents

| | |
|--|-----------|
| Introduction | 15 |
| The respiratory chain and ATP synthesis | 17 |
| Respiratory supercomplexes..... | 21 |
| Architecture of the respiratory chain..... | 21 |
| Supercomplex formation and factors | 23 |
| Structures of supercomplexes..... | 24 |
| Supercomplex function | 25 |
| The respiratory supercomplex factors..... | 29 |
| Cytochrome <i>c</i> oxidase..... | 33 |
| General features of Cyt _c O..... | 33 |
| Electron transfer | 35 |
| Proton Transfer..... | 37 |
| Catalytic cycle..... | 38 |
| Yeast Cyt _c O | 40 |
| Assembly of yeast Cyt _c O..... | 42 |
| Methods | 45 |
| Spectral features of Cyt _c O | 45 |
| Flash photolysis..... | 46 |
| Cyt _c O activity | 48 |
| Fluorescence correlation spectroscopy..... | 49 |
| Conclusion..... | 51 |
| Future work and speculation..... | 53 |

| | |
|---|-----------|
| Populärwissenschaftliche Zusammenfassung | 57 |
| Populärvetenskaplig sammanfattning | 59 |
| Acknowledgements | 61 |
| References | 63 |

Abbreviations

| | |
|-------------------|------------------------------|
| BNS | binuclear site |
| Cyt. <i>c</i> | cytochrome <i>c</i> |
| Cyt <i>c</i> O | cytochrome <i>c</i> oxidase |
| IMM | inner mitochondrial membrane |
| IMS | intermembrane space |
| PLS | proton-loading site |
| Q/QH ₂ | quinone/quinol |
| ROS | reactive oxygen species |
| SMP | submitochondrial particle |
| SOD | superoxide dismutase |
| TMH | transmembrane helix |

Introduction

Relax, take a deep breath and enjoy reading this thesis! Oxygen is filling your lungs, taken up by your blood and distributed to every cell of your body. In parallel, your last meal is metabolized and ultimately stored as chemical energy in small molecules. In the mitochondrion, the powerhouse of the cell, electrons pass along the respiratory chain, a series of enzymes (biological machines), while releasing chemical energy. In the majority of the machines of the respiratory chain, part of the energy is used to move protons, charged elementary particles, across biological membranes. This process can be compared to filling a water basin of a hydroelectric dam (see cover picture). At the final enzyme of the respiratory chain, the oxygen we breathe reacts with protons to form water. In this reaction, too, energy is released and used for the pumping of protons thereby generating a difference in concentration and charge between the two sides of the membrane. The ATP synthase, the “hydroelectric power plant” of the cell, uses the energy stored in this concentration and charge difference to create ATP, the universal energy carrier of the cell, very much like a turbine uses the energy of the height difference of the water, to convert the potential energy to mechanical energy.

This process is crucial for all respiring life and has been studied for decades. Molecular mechanisms of the machinery are now mostly understood. However, central aspects of the respiratory chain, for example its organization and interactions between the single enzymes, or the regulation of all these processes remain to be understood.

I hope that with my work described in this thesis I can add at least one puzzle piece to the big picture.

The respiratory chain and ATP synthesis

In the respiratory chain, chemical energy is transformed into potential energy¹ (Figure 1). Electron donors, such as NADH or succinate, deliver electrons to the first complexes of the respiratory chain. The electrons are then passed along the enzyme complexes of the respiratory chain to the final electron acceptor, molecular oxygen.

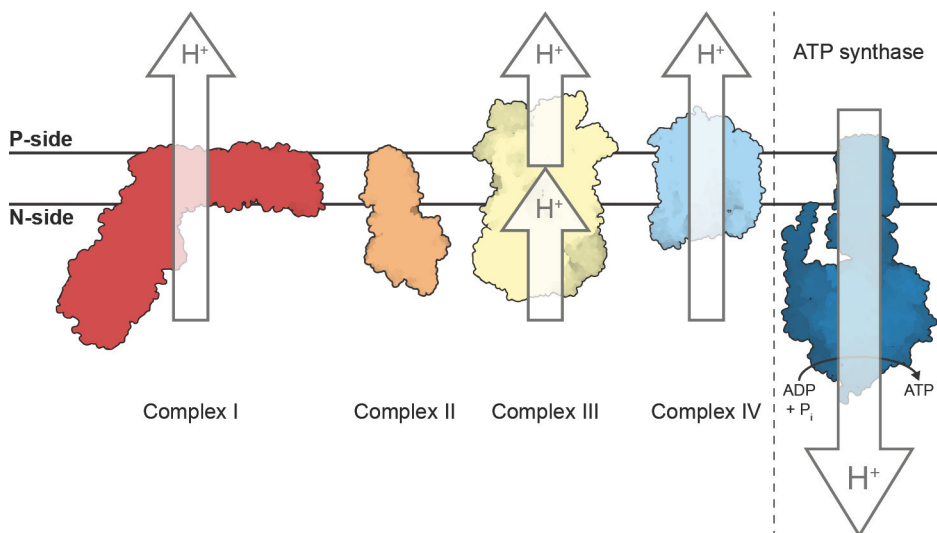


Figure 1: Schematic representation of the respiratory chain complexes and the ATP synthase. The respiratory complexes, with the exception of Complex II, are involved in the translocation of protons across the membrane thereby maintaining a proton electrochemical gradient, which is then utilized by the ATP synthase to create ATP².

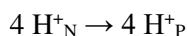
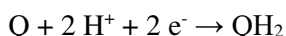
Using the energy released by this electron transport, the respiratory complexes translocate protons across the plasma membrane of bacteria or the inner mitochondrial membrane (IMM) of eukaryotes, respectively. These complexes maintain a proton electrochemical gradient to store energy, which is, for example,

used by the ATP synthase to provide the energy for creating ATP, the universal energy carrier of the cell², or for transmembrane transport³.

On the following pages, the components of the respiratory chain are introduced and discussed in more detail.

Complex I

Complex I, or NADH:ubiquinol oxidoreductase, is the first enzyme of the respiratory chain. It couples the oxidation of NADH to NAD⁺ and the reduction of quinone (Q) to quinol (QH₂). These redox reactions are linked to the translocation of four protons across the membrane⁴:



where P and N describe protons on the positive (inter membrane space (IMS)/extracellular) or negative (matrix/cytosol) side of the membrane, respectively.

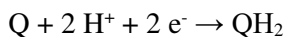
Complex I is the largest protein complex of the respiratory chain. It contains up to nine iron sulphur (Fe/S) clusters and one flavin mononucleotide (FMN)⁵. The FMN is located close to the NADH binding site and connects the NADH with the series of Fe/S clusters, which span the distance to the quinone-binding site. This binding site is located at the interface between the membrane and soluble domain of the complex⁶.

While the redox reactions catalyzed by complex I take place in the soluble domain of the enzyme, proton translocation occurs in the membrane domain.

In the yeast *Saccharomyces cerevisiae*, the respiratory chain does not contain a typical Complex I. Instead, alternative NADH dehydrogenases located on the P-side (external, Nde1 and Nde2) or N-side (internal, Ndi1) catalyze the electron transfer from NADH to quinone, but in these enzymes this reaction is not coupled to proton translocation⁷⁻⁹.

Complex II

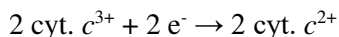
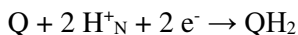
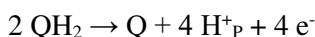
Respiratory Complex II, or succinate dehydrogenase (SDH), catalyzes the oxidation of succinate to fumarate, coupled with the reduction of Q to QH₂.



SDH is the only enzyme that is both part of the respiratory chain and the citric acid cycle. It is also the only enzyme of the respiratory chain that is not involved in the translocation of protons¹⁰.

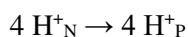
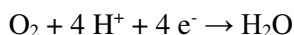
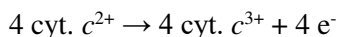
Complex III

Quinol, reduced by the respiratory complexes I and II, is re-oxidized by Complex III, also called co-enzyme Q:cytochrome *c* oxidoreductase or *bc*₁ complex. The *bc*₁ complex consists of four to eleven subunits in bacteria or mammalia, respectively¹¹. The three core subunits contain the cofactors heme *c*₁, two *b* hemes and a Fe/S-cluster. The Rieske iron sulfur protein subunit is flexible and shuttles electrons from the heme *c*₁-containing subunit cytochrome *c*₁ to the soluble electron carrier cytochrome *c* (cyt. *c*)¹¹. In the reaction catalyzed by the *bc*₁ complex, called the Q-cycle, two molecules of QH₂ are oxidized at the Q_o site of the protein, releasing four protons to the outside of the bacterial cell or the mitochondrial IMS. Two of the four electrons are transferred to two molecules of oxidized cyt. *c* via the Rieske iron sulfur cluster and cyt. *c*₁. The two other electrons are transferred to hemes *b*_L and *b*_H and finally to the Q_i site, where they reduce one molecule of Q. Two protons are taken up on the cytoplasmic side of bacteria or the mitochondrial matrix side. In summary, two protons are taken up on the N-side and four protons are released to the P-side^{12,13}. The overall reaction catalyzed by the *bc*₁ complex can be summarized as:



Complex IV

Complex IV, also called cytochrome *c* oxidase (Cyt*c*O), catalyzes the oxidation of cyt. *c* and the reduction of the final electron acceptor O₂ to water. In this reaction four protons (further referred to as substrate protons) are taken up from the bacterial cytoplasm or mitochondrial matrix. The reaction is coupled to the translocation of four (pumped) protons across the membrane.



The majority of Cyt*c*O_s consist of three core subunits and, depending on the organism, up to ten additional supernumerary subunits¹⁴⁻¹⁶. The core subunits harbor four metal cofactors involved in the electron transport from cyt. *c* to O₂. A binuclear copper site, Cu_A, is located close to the cyt. *c* binding site in subunit II. Buried within subunit I of the enzyme are a heme *a* and the active site, consisting of a heme *a*₃ and a mononuclear copper cofactor, Cu_B¹⁶. At least two proton pathways, so-called K- and D-pathway, named after key amino acids involved in proton translocation, connect the mitochondrial matrix with the active site of the enzyme¹⁷⁻²⁰. Cyt*c*O is described in more detail from page 33.

ATP Synthase

The proton electrochemical gradient maintained by the respiratory chain is utilized by the ATP synthase (sometimes called respiratory complex V) to form ATP from ADP and pyrophosphate (P_i)². The ATP produced in this reaction is the universal energy carrier of the cell and can be used to power a wide range of processes.

ATP synthase consists of two main domains. The motor domain, also called F_o domain, is located in the membrane. It is comprised of two parts, subunit *a* and a ring of *c*-subunits²¹. The catalytic domain, also called F₁ domain, contains three *α*- and three *β*-subunits as well as a *γ*-, *δ*- and *ε*-subunit that form the so-called central stalk. A peripheral stalk, containing two long, alpha-helical *b*-subunits, fixes the catalytic domain to the *a*-subunit²². Protons that move through the F_o domain downhill the proton gradient power a rotation of the *c*-ring, which is connected to the central stalk with its asymmetric *γ*-domain. Conformational changes induced by this rotation provide the free energy necessary for the catalysis of the reaction of ADP and P_i to ATP.

Respiratory supercomplexes

Architecture of the respiratory chain

The respiratory chain has been investigated for many decades. An early model of the organization of the respiratory chain was introduced by Chance and Williams in the 1950s. This so-called “solid-state model” (Figure 2A) proposed an assembly of all respiratory components into one complex (the so-called oxysome), carrying out all reactions in a sequence²³. The model was supported by the isolation and successive reconstitution of enzyme units containing complexes I and III or II and III²⁴ and collective isolation of these units²⁵.

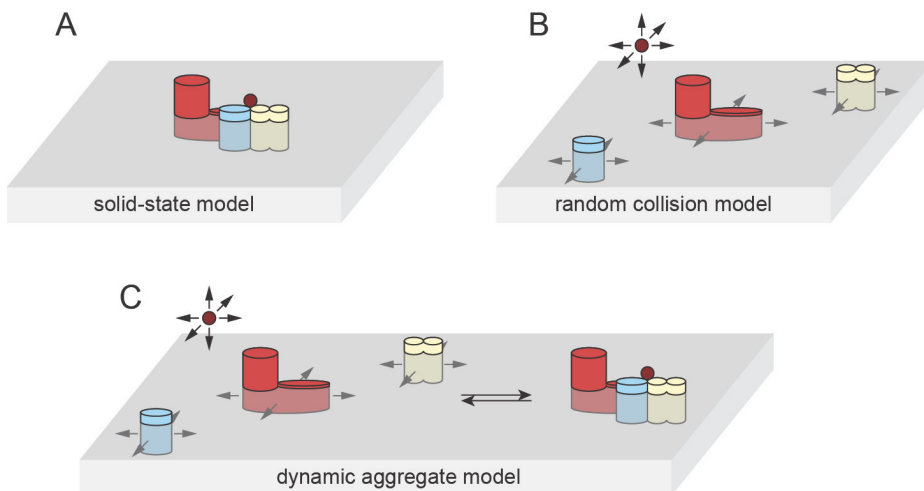
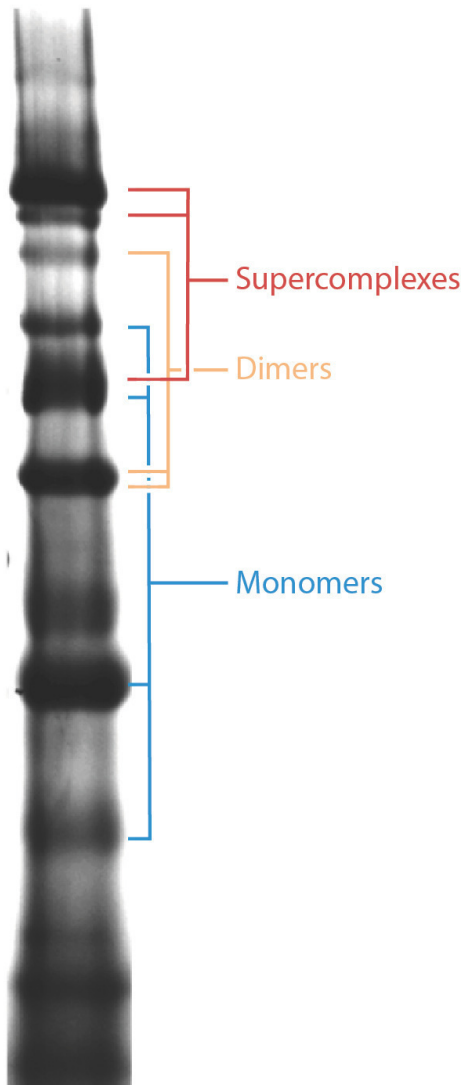


Figure 2: Models of the organization of the respiratory chain. (A) The “solid-state model” describes the respiratory complexes as a single structural and functional unit, the so-called oxysome, with the redox factors Q/QH₂ and cyt. *c* (dark red) bound to the complexes^{23–25}. (B) The “random collision model” describes the respiratory complexes as separate units that diffuse in two dimensions within the membrane. Redox factors like cyt. *c* can diffuse freely in the IMS^{26,27}. (C) In the dynamic aggregate model, the two states described in A and B exist in an equilibrium^{28–30}.

In this model, coenzyme Q is permanently bound to the respective components of the respiratory chain, preventing exchange of bound and free coenzyme Q^{31,32}.

This view was challenged in the 1970s by a model first proposed by Hackenbrock and colleagues (Figure 2B)^{26,27}. The so-called “random collision model”, proposed lateral diffusion of respiratory complexes within the inner mitochondrial membrane and redox components that diffuse randomly between the complexes.

BN PAGE



A few years later, a combination of these two models was presented as the “dynamic aggregate model”²⁸ (Figure 2C), but was first dismissed in favor of the “random collision model”. Nearly 20 years later, with the first purification of so-called respiratory supercomplexes, super-assemblies of the respiratory complexes with different compositions (Figure 3), this model was brought back to attention and re-introduced as the “plasticity model”^{29,30}.

Figure 3: Blue Native Gel showing the organization of respiratory complexes. BN PAGE analysis of Digitonin-solubilized mitochondria from ovine heart showing the coexistence of monomeric (blue) and dimeric (orange) forms of the respiratory complexes as well as the different supercomplexes (red) in one preparation (modified with permission from ³³).

Supercomplex formation and factors

With the introduction of Blue Native polyacrylamide gel electrophoresis (PAGE) it became possible to identify the supramolecular assemblies of the respiratory chain, termed respiratory supercomplexes³⁴. Initially observed in mitochondria from *S. cerevisiae* and bovine heart, supercomplexes were later found in many other organisms, including bacteria^{35–37}, and human tissues³⁸. Blue Native PAGE allows for the analysis of the distribution of free or associated respiratory complexes, showing that in yeast mitochondria, nearly all Cyt_cO is associated with the *bc*₁ complex³⁵. In bovine heart mitochondria, all complex I was shown to be associated with the *bc*₁ complex and Cyt_cO, but some Cyt_cO was found to be in a free form³⁴. The fraction of respiratory complexes assembled into supercomplexes varies between organisms, tissues and metabolic conditions^{35,39}.

The complexes of the respiratory chain are fully assembled before the supercomplexes are formed⁴⁰. Several factors are involved in the assembly and stabilization of respiratory supercomplexes. Cardiolipin, a lipid found in the inner mitochondrial membrane and the cytoplasmic membrane of bacteria, is associated with the *bc*₁ complex and Cyt_cO. Removal of cardiolipin results in a smaller fraction of supercomplexes in yeast mitochondria^{41,42}, indicating a stabilizing role of the lipid.

In addition to lipids, several proteins have been identified to be involved in the assembly and stabilization of respiratory supercomplexes. Among the first proteins assigned with this function in yeast were the respiratory supercomplex factors (Rcf) 1 and 2^{43–45} (see page 29). The alleged Cyt_cO subunit Cox26 is another protein affecting the organization of supercomplexes. Cox26 is a protein located in the IMM, which is associated with the respiratory supercomplex via Cyt_cO. Deletion of Cox26 in *S. cerevisiae* results in a decrease in the amount of supercomplexes and an increase in the amount of free Cyt_cO, as well as a small, but significant reduction in Cyt_cO activity⁴⁶.

Similar effects are observed upon removal of the protein Aim24, which is also located in the IMM, however not as a subunit of the Cyt_cO. Deletion of Aim24 results in severe growth defects on non-fermentable media in combination with a decrease in the amount of III₂IV₂ supercomplexes as well as a decrease in Cyt_cO activity⁴⁷.

In mammalia, an isoform of a Cyt_cO subunit, the protein COX7a2l, was proposed to play a major role in the assembly of respiratory supercomplexes. In the absence

of COX7a2l, Cyt_cO is not assembled into supercomplexes in certain mouse strains. It was therefore renamed to supercomplex assembly factor 1 (SCAF1)⁴⁸. The relevance of Cox7a2l was however questioned after a study found unaltered formation of supercomplexes in the absence of the protein⁴⁹. In a recent review, Letts and colleagues commented that the studies on respiratory supercomplexes were often carried out using different tissues and detergent concentrations, leading to contradictory results. They furthermore proposed the existence of at least two different assembly pathways of respiratory supercomplexes, either involving or lacking COX7a2l³³.

Structures of supercomplexes

To gain further understanding of the function of and interactions within respiratory supercomplexes, significant effort was put into the determination of molecular structures of the assemblies.

In recent years, advances in the field of single particle cryo-electron microscopy (cryo-EM) yielded more and more structural information. Whereas the first publications only showed the arrangement of the respective complexes in a supercomplex, recent studies revealed interactions between the complexes on the scale of amino acid residues.

Information about the general arrangement of mammalian respiratory supercomplexes was obtained using negative stain imaging, but the resolution was very low (36 Å)⁵⁰. A few years later, a 19 Å 3D map was published, allowing the fitting of previously determined crystal structures of the single respiratory complexes, and thus first proposing structural arrangements and predictions of the electron flow within respiratory supercomplexes from bovine heart⁵¹. Further studies yielded higher resolution maps, thus revealing information about subunits involved in the interactions and molecular mechanisms. Two major configurations of the mammalian respirasome (the supercomplex containing complex I, the *bc*₁ complex and Cyt_cO) were observed in two studies^{52,53}, differing in the orientation of the *bc*₁ complex dimer relative to complex I. The interactions in the two configurations, named “tight” and “loose”, are different. The functional relevance of such differences remains unclear, but it is an indication that the supramolecular assembly of the respiratory complexes is not necessarily well defined under all circumstances.

The structure of the III₂IV₂ supercomplex from *S. cerevisiae* has only been published at low resolution⁵⁴. In this arrangement, Cyt_cO subunit Cox5a can be identified at the interface between the complexes, which is further supported by cryo-EM experiments performed in our laboratory (Figure 4). Further progress towards an increase in resolution is required to obtain information about detailed interaction sites.

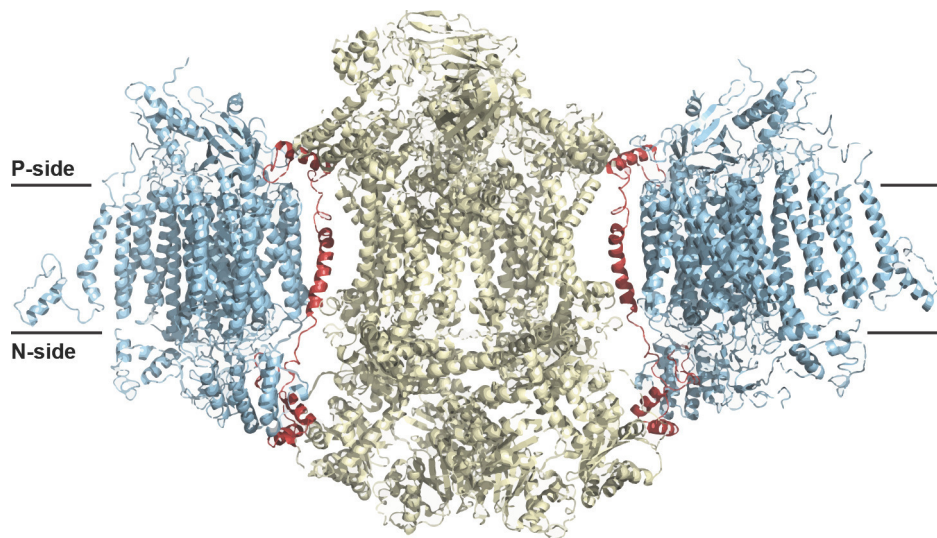


Figure 4: Arrangement of the *bc*₁ complex and Cyt_cO from *S. cerevisiae* in a III₂IV₂ supercomplex. A low-resolution electron density map (10.7 Å, unpublished work by Moe *et al.*, 2018) was fitted with the structural model of the *bc*₁ complex (light green, PMID: 1KYO) and a homology model of Cyt_cO (light blue, PMID: BBI46762-mmc3). Cyt_cO interacting subunit Cox5a is shown in red.

Supercomplex function

Although the existence of supercomplexes has been widely accepted, their function remains largely obscure.

In some organisms, the formation of supercomplexes is essential for respiration, because they do not express soluble, but only membrane bound cyt. *c*⁵⁵. The heme *c*-carrying protein can be incorporated as part of a subunit of the *bcc*₁ complex⁵⁶ or Cyt_cO^{57,58}, where cyt. *c* is fused into the sequence of the cyt. *c*₁ subunit of the *bc*₁ complex or subunit II of the *aa*₃ Cyt_cO. In these organisms,

close proximity of the respiratory chain complexes is required for electron transfer between the bc_1 complex and Cyt c O via the membrane-anchored cyt. c .

Several roles have been proposed for respiratory supercomplexes. One of these roles is the stabilization of the single respiratory enzymes. Mutations in the bc_1 complex have been shown to cause a decrease in abundance of complex I⁵⁹. Furthermore, knock-out of Cox10, a protein required for assembly of Cyt c O, leads to a decrease in the amount of complex I⁶⁰. It was concluded from these findings that the formation of supercomplexes is required for the assembly and stability of complex I. However, as pointed out by Milenkovic and colleagues⁶¹, this complex I deficiency could also be caused by indirect effects like an increased production of reactive oxygen species (ROS), which in turn affect assembly of complex I and formation of supercomplexes^{62,63}.

Another possible role of supercomplexes is to enhance respiration by substrate channeling. Several studies proposed the existence of multiple, sequestered substrate pools^{64,65}. However, no available structural information indicates the presence of substrate channels. Furthermore, several studies show the existence of a single quinone pool^{66,67}. Having the redox centers of the respiratory chain in close proximity increases the probability of a substrate reacting with the components of one respirasome, however, this is also simply given by the close proximity of the respiratory enzymes in the highly crowded mitochondrial inner membrane⁶¹.

It was furthermore proposed that the formation of respiratory supercomplexes serves to prevent unspecific binding interactions in the highly crowded protein environment of the IMM^{61,66}. A similar mechanism is used by proteins of the eye-lens of vertebrates, which form weak, reversible interactions to avoid irreversible nucleation^{68,69}.

A fourth proposed role for the formation of respiratory supercomplexes is the decrease of production of or limitation of damage by ROS. The term ROS is used for several different oxygen radicals. In the respiratory chain, mostly superoxide ($O_2^{\cdot-}$) is created by incomplete reduction of molecular oxygen. Complex I^{62,70} and the bc_1 complex^{63,71} are the major source of superoxide in the mitochondrion. Release of complex I from bovine heart supercomplexes results in a significant increase in superoxide production by complex I⁷². Furthermore it has been proposed that superoxide production in supercomplexes is decreased by restriction of the movement of the Rieske Fe/S subunit of the bc_1 complex, preventing electron transfer from one quinol binding site to the Fe/S center and thus minimizing electron leakage³³.

The topic is highly controversial and arguments for⁷³ and against⁶⁶ the kinetic relevance of respiratory supercomplexes have been brought forward.

A possible reason for the seemingly opposing results might be the use of different strains of organisms and tissue, different growth conditions as well as different detergent concentrations. It seems that small variations in the conditions can have major impact on the organization of the respiratory chain, illustrating its high degree of adaptation to metabolic changes.

The respiratory supercomplex factors

Among the first proteins reported to be involved in the assembly and stabilization of respiratory supercomplexes in *S. cerevisiae* are Rcf1 and Rcf2, formerly Aim31 and Aim38, respectively.

The two proteins are members of the hypoxia-induced gene 1 (Hig1) protein family. Rcf2 is specific to yeast, while Rcf1 is conserved among a wide range of species, from alphaproteobacteria to humans⁴³. The Hig1 homology domain of Rcf1 is located in the N-terminal region, while the C-terminus is unique to fungi.

Rcf1 and Rcf2 are integral membrane proteins, located in the IMM. A structural prediction of Rcf1 indicated the presence of two transmembrane helices (TMHs) in the Hig1 domain of the protein⁴³. In the predicted structure of Rcf1, both C- and N-termini are located in the IMS. A more recent determination of the structure of Rcf1 using solution NMR shows the protein as a homodimer⁷⁴ (for comparison of the structures see Figure 5). In this study, Rcf1 was expressed in *Escherichia coli*, purified from inclusion bodies and re-folded into DPC micelles. Each monomer consists of five TMHs. Two of the α -helices are consistent with the predicted transmembrane helical structures. The other three helices contain many charged residues and form the dimer interface. The charged regions have been proposed to form the interaction site with Cyt_cO in the monomeric form of Rcf1. The loop between TMHs 4 and 5 contains several charged residues that show interactions with cyt. *c* (Figure 5)⁷⁴. In the solution NMR structure of Rcf1, the C- and N-termini are located on opposite sides of the membrane. Although the orientation of the protein in native membranes was not determined in this form, the most probable orientation can be suggested by the location of the loop interacting with cyt. *c*, which has to be located in the IMS. Since this loop is on the same side as the N-terminus and opposite of the C-terminus, a C_N-N_P orientation is most probable for the dimer.

The two proteins Rcf1 and Rcf2 independently interact with the *bc*₁ complex and Cyt_cO in yeast. Rcf1 co-purifies with Cyt_cO subunit Cox3 independently of the

assembly of the subunit into Cyt c O⁴³. Genetic removal of Rcf1 results in a lack of assembly into Cyt c O of the supernumerary subunit Cox13, a membrane spanning subunit on the opposite side of the bc_1 complex-Cyt c O interface. Loss of Cox13 has similar effects on oxygen consumption of mitochondria as loss of Rcf1⁴⁴. Deletion of both Rcf1 and Cox13, however, results in a more severe growth phenotype^{43,45} and an almost complete loss of III₂IV₂ supercomplexes⁴³.

Rcf1 is not a stoichiometric subunit of Cyt c O. Based on the co-purification of Rcf1 with Cyt c O or with Cyt c O assembly intermediates it was proposed that Rcf1 is a subunit of Cyt c O. However, Garlich and colleagues showed, using quantitative mass spectrometry of different Cyt c O subpopulations, that only a fraction of Cyt c O is bound to Rcf1⁷⁵.

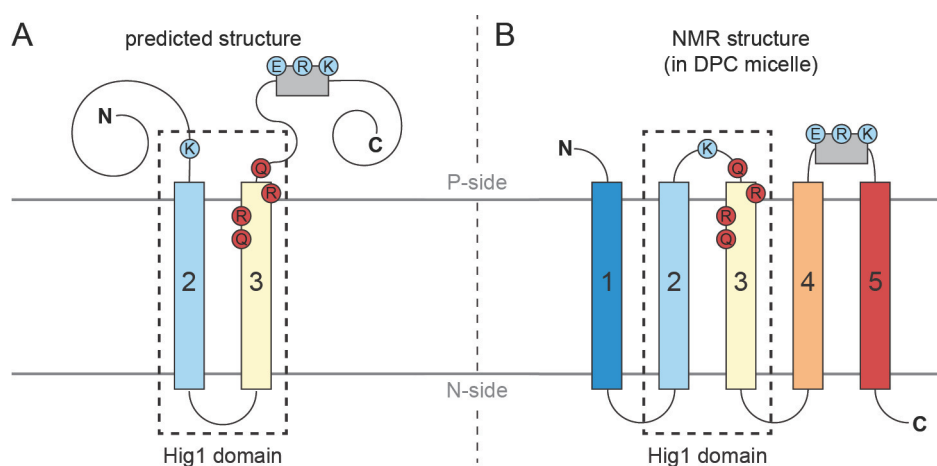


Figure 5: Solution NMR and predicted structure of Rcf1. (A) In the predicted structure of Rcf1⁴³, both C- and N-termini are located on the P-side of the membrane. (B) The structure of Rcf1 in a DPC micelle, determined by solution NMR⁷⁴, consists of five TMHs, with C- and N-termini at opposite sides of the membrane. The QRRQ motif (see page 31) and interacting residues with cyt. c (see page 29) are indicated in red and light blue, respectively.

Several roles have been proposed for Rcf1. Loss of the protein results in a shift in the equilibrium of the different supercomplex forms towards the smaller III₂IV₁. Furthermore, Rcf1 is involved in the assembly of Cyt c O subunit Cox13 and the supercomplex factor Rcf2 into the Cyt c O complex. Cox13 is not assembled into supercomplexes in the absence of Rcf1, while assembly of Rcf2 is blocked when either Cox13 or Rcf1 are absent⁴⁴.

In addition to the proposed role in the assembly and stabilization of respiratory supercomplexes, Rcf1 also plays a role in regulation of Cyt_cO activity⁷⁵⁻⁷⁷. Upon removal of Rcf1, the activity of Cyt_cO in mitochondria decreased to 30-60% of that measured with the wild-type Cyt_cO^{43-45,76}.

Hig1 type 2 proteins, including Rcf1, show a conserved motif, (Q/I)X₃(R/H)XRX₃Q, termed QRRQ (Figure 5). Mutation of the second arginine to an alanine in Rcf1 (R67A) leads to a tighter association of Rcf1 and Cyt_cO, possibly via Cox3. In contrast, mutations of both glutamates to alanines (Q61A, Q71A) weaken this interaction. The assembly of supercomplexes was not affected in these variants⁷⁵. It should be noted that this study was conducted with mitochondria lacking Rcf2, the role of which is still not understood.

A regulatory role has also been shown for the mammalian homolog of Rcf1, Higd1a. Addition of recombinant Higd1a to purified bovine Cyt_cO resulted in an increase in Cyt_cO activity, proposed to be linked to structural changes around heme *a*. Furthermore, knock-down of Higd1a in mouse cardiomyocytes resulted in a significant decrease in Cyt_cO activity, which could be rescued by overexpression of Higd1a⁷⁸.

In the fungal ageing model *Podospora anserine*, deletion of Rcf1 severely decreased the life span and growth rate of the organism and increased oxidative protein damage⁷⁹.

Less is known about Rcf2. As Rcf1, also Rcf2 is located in the IMM. Assembly of Rcf2 into the Cyt_cO complex is blocked in the absence of Cox13 or Rcf1⁴⁴. Unlike Rcf1, removal of Rcf2 has no negative effects on supercomplex formation or Cyt_cO activity. Instead, upon removal of Rcf2, Cyt_cO activity in mitochondria is slightly increased⁸⁰. However, removal of Rcf2 resulted in a significant increase in ROS production⁴⁴. Rcf2 interacts with Cyt_cO through its C-terminal region⁸⁰. This C-terminus is homologous to Rcf1, containing the conserved Hig1 domain. The N-terminus is homologous to a recently characterized third respiratory supercomplex factor, Rcf3, whose function overlaps with that of Rcf2⁸⁰. As Rcf1, Rcf2 forms a complex in DPC micelles, with each monomer consisting of five TMHs⁸¹.

Cytochrome *c* oxidase

General features of Cyt_cO

Cytochrome *c* oxidase is the final enzyme of the respiratory chain. Electrons are transferred from reduced cyt. *c* to the final electron acceptor, molecular oxygen, which is reduced to water. This electron-transfer process is coupled to translocation of four protons per four electrons across the membrane.

More than 90% of oxygen consumed by living organisms on earth is processed by heme copper oxidases⁸². This class of enzymes is divided into three subclasses⁸³. Here, we focus on the A-type oxidases, which include Cyt_cO_s from mitochondria, *Paracoccus denitrificans*, *Rhodobacter sphaeroides* and *E. coli* (for a structural comparison of bacterial and mitochondrial Cyt_cO_s see Figure 6).

Cyt_cO contains three core subunits, which in most eukaryotes are encoded by mitochondrial DNA. A notable exception are some species of leguminous plants, in which subunit II is nuclear encoded⁸⁴. Subunits I and II harbor the redox-active metal cofactors (Figure 7). Subunit III, which is exclusively found in A-type oxidases⁸⁵, does not take part in the catalytic reaction. A number of accessory subunits, ranging from one in bacteria like *R. sphaeroides* to ten in mammalian Cyt_cO, are part of the structure. Some of these accessory subunits have regulatory roles^{86,87}. The accessory subunits of eukaryotic Cyt_cO are encoded by nuclear DNA.

The structure of subunit I of Cyt_cO is highly conserved. It consists of twelve TMHs, which are slightly tilted with respect to the membrane plane. A separation in three clusters of four TMHs yields a quasi-3-fold symmetry¹⁶, forming three hydrophilic pores filled with polar amino acids and water molecules. Two of these clusters harbor the well-characterized K- and D-proton transfer pathways, the third harbors the proposed H proton transfer pathway (see page 37). The iron atoms of both hemes as well as Cu_B are coordinated by a total of six histidine residues in subunit I (Figure 7C,E)⁸³. The outer TMHs interact with TMHs of

other subunits and the lipids of the membrane. These interactions are mostly non-polar.

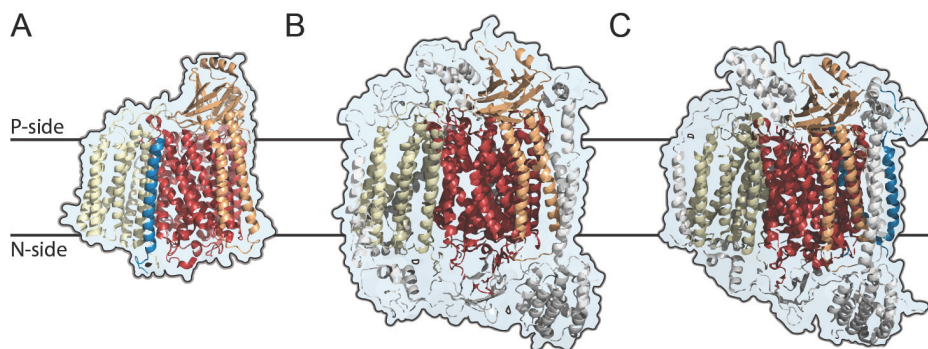


Figure 6: Subunit composition of Cyt c O. The core subunits I-III of Cyt c O are shown in red, light orange and light green, respectively. (A) Cyt c O from *R. sphaeroides* (PMID: 1M56)¹⁴. The only accessory subunit, subunit IV, is shown in blue. (B) Cyt c O from *S. cerevisiae* (PMID: BBI46762-mmc3)¹⁵, with its eight accessory subunits shown in grey. (C) Cyt c O from *Bos Taurus* (PMID: 1V54)⁸⁸. The eight subunits homologous to the yeast subunits are shown in grey. Two additional subunits, VIIb and VIII, only present in mammalian Cyt c O are shown in blue.

Subunit I contains the catalytic site of the enzyme, also called the binuclear site (BNS), formed by two magnetically coupled metal cofactors: a high-spin heme, heme a_3 , and a single copper ion, Cu_B (Figure 7). The iron atom of heme a_3 is coordinated by a highly conserved histidine, H376 (bovine Cyt c O numbering is used in this chapter unless specified otherwise) in helix X of subunit I. The propionates of the heme interact electrostatically with residues (R438 and R439) of TMHs in subunit I. Mutations of these residues alter rates of electron transfer and proton pumping in Cyt c O^{17,18,89–91}. The copper ion is located approximately 5 Å from the iron of heme a_3 (Figure 7A), leaving space for small diatomic ligands like O_2 , CO or CN^- to bind to the heme. Cu_B is ligated by three histidines, one of which (His240) is covalently linked to a tyrosine (Y244) (Figure 7C,E). This tyrosine plays an important role in the catalytic cycle of Cyt c O (see page 38).

Subunit II consists of two TMHs and a cluster of 10 β -sheets. The β -sheets hold the binuclear Cu_A , which is coordinated by two histidines, two cysteines, a glutamate and a methionine (Figure 7C,D). Electrons are transferred from bound cyt. c to Cu_A , which is the primary electron acceptor in Cyt c O (Figure 7B). The TMHs of subunit II are in close contact with TMHs of subunit I. The residue E62

of subunit II serves as an entry point for protons to the K-pathway (see page 37)^{19,20,92}.

As for subunit I, the structure of subunit III is highly conserved. However, this subunit is exclusive to A-type oxidases. It consists of seven TMHs, forming two clusters of two and five TMHs, respectively. These two clusters typically form a V-shaped structure, which acts as a channel for oxygen leading from the protein-membrane interface to the catalytic site in subunit I^{93,94}. Several lipid molecules have been found bound to subunit III. The head groups of these lipids influence the rate of proton transport in subunit I by decreasing the pK_a of D91 and perturbing the water structure in the D-pathway (see page 37)⁸⁵. Subunit III does not contain any redox cofactors, but removal of this subunit results in suicide inactivation of the enzyme during turnover⁹⁵⁻⁹⁷.

Electron transfer

To enable electron transfer in proteins at biochemically relevant rates, redox-cofactors must be no more than 25 Å from each other (although there are exceptions to that rule)⁹⁸, which is fulfilled in the electron-transfer reactions of Cyt c O (Figure 7A).

In most Cyt c O_s, weak electrostatic interactions in the formation of a cyt. c – Cyt c O complex result in rapid association and dissociation of cyt. c , enabling electron transfer to Cu_A⁹⁹. In some organisms, cyt. c is covalently bound to either Cyt c O^{57,58} or the bc_1 complex^{55,56}.

In the reaction of Cyt c O, electrons are transferred transiently from bound cyt. c , first to Cu_A, to heme a and finally to the BNS (Figure 7B). The rates of electron transfer from cyt. c to Cu_A and further to heme a could be determined using external electron sources, where release of electrons could be triggered by short light flashes¹⁰⁰⁻¹⁰³. Electron transfer from heme a to the BNS, however, occurs over a much faster time scale. To measure this rate, flash photolysis experiments (see page 46) were conducted starting with the so-called mixed valence Cyt c O, in which heme a_3 and Cu_B are reduced and CO is bound to heme a_3 . Upon laser flash-induced dissociation of CO, the midpoint potential of heme a_3 is lowered, inducing electron back-flow to heme a and Cu_A and thus allowing determining the rates of these events^{104,105}.

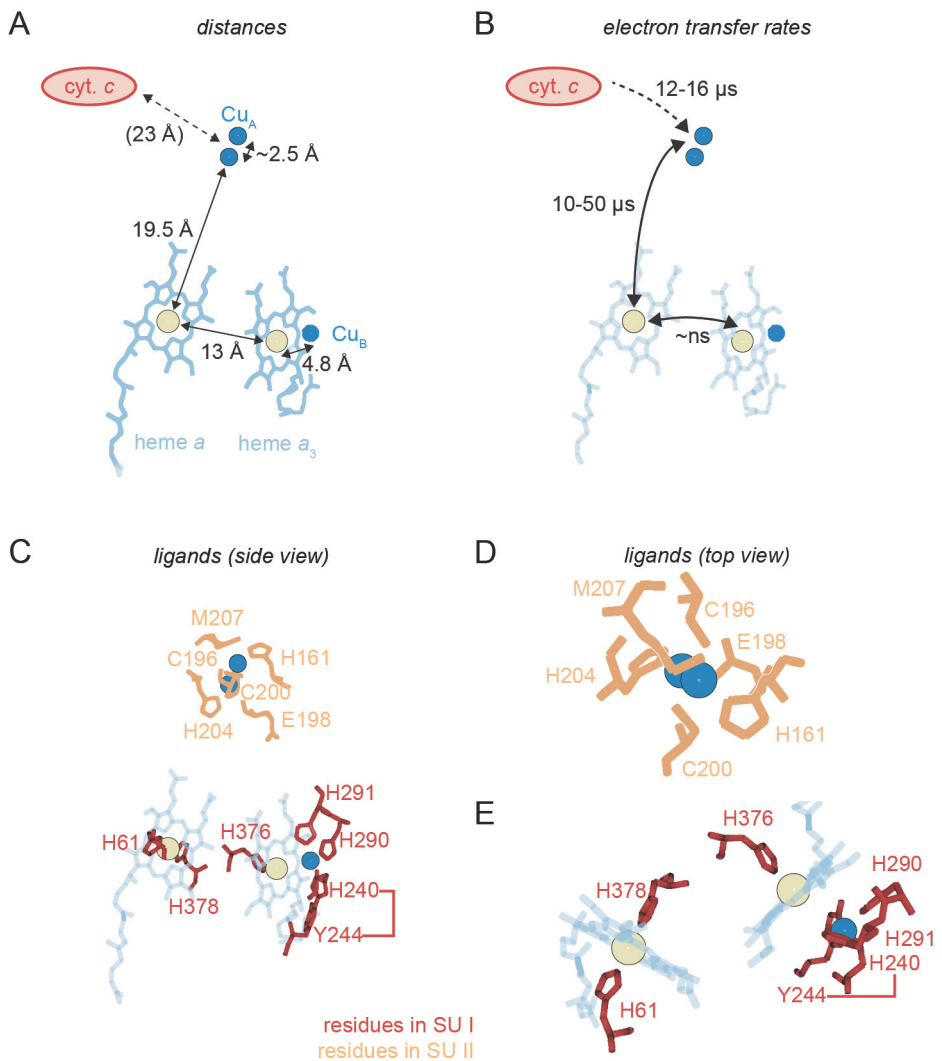


Figure 7: Redox cofactors of bovine CytcO. Cu_A , the electron acceptor from *cyt. c* (distance not drawn to scale), is located in subunit II. The remaining cofactors, heme *a*, heme *a*₃ and Cu_B , are located in subunit I. **(A)** Metal-to-metal distances between redox centers^{16,99,105}. **(B)** Electron transfer rates between the redox centers^{101,104,105}. **(C-E)** Residues ligating metal centers with indicated position in subunit I (red) or II (light orange)⁸⁸. **(C)** View from the membrane plane. **(D)** Ligands of Cu_A from the IMS. **(E)** Ligands of heme *a* and *a*₃ and Cu_B from the IMS.

Proton transfer

Protons are often transferred utilizing the so-called “Grotthus mechanism”. This process describes the diffusion of a proton through a hydrogen-bonded network in which covalent bonds are broken and formed¹⁰⁶.

In Cyt c O the pumping of protons requires at least two components of a proton pathway: one part leads from the N-side of the membrane to a proton-loading site (PLS) buried in the enzyme, from which the proton is released to the P-side of the membrane. The other part leads from the PLS to the P-side of the membrane. While the former component is well characterized for most Cyt c O_s, the location of the latter is still unclear. Two main pathways have been identified to translocate protons from the N-side to the proposed region containing the PLS (Figure 8).

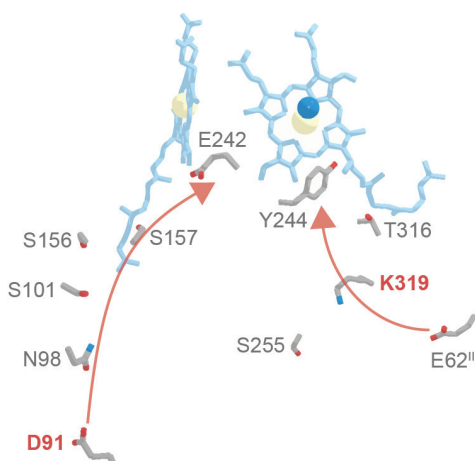


Figure 8: Proton pathways in Cyt c O. The key residues of the two main proton pathways in Cyt c O, the D- and K-pathway are shown. All residues except E62^{II} are located in subunit I.

Protons enter the so-called **D-pathway** (named after D91) on the N-side of subunit I. The pathway itself consists of a series of polar residues and bound water molecules, ending at a glutamate (E242), approximately halfway into the membrane. It is yet unclear how protons are translocated from the glutamate to the BNS or to the PLS and further. The D-pathway is used to translocate at least two substrate protons to the active site, as well as all four pumped protons^{15,107}.

In bovine Cyt c O, another pathway, called the H-pathway, was proposed to be involved in the translocation of pumped protons⁸⁸.

The second well-characterized proton pathway in Cyt c O is the **K-pathway**, named after a highly conserved lysine (K319). The proton uptake site for this pathway is a glutamate, E62^{II}, located on the N-side of subunit II^{19,108}. The pathway ends on the highly conserved tyrosine (Y244) close to the catalytic site, which is linked to the hydroxyethyl farnesyl of heme a_3 . The K-pathway transfers one or two substrate protons during the reductive phase of the catalytic cycle (Figure 9). It is not yet understood why A-type oxidases contain two proton pathways, while the B- and C-type oxidases only have one K-pathway analogue, responsible for the translocation of both pumped and substrate protons^{109,110}.

Catalytic cycle

Intermediates of the catalytic cycle of Cyt c O have been observed using different techniques^{100–105,111}. The reaction of Cyt c O proceeds via different intermediates, depending on the initial reduction state of the enzyme. In many experiments, the reaction is initiated using the fully reduced Cyt c O. During turnover in the native system, the full reduction of all redox cofactors is probably never achieved. The native mechanism can be mimicked by reducing only heme a_3 and Cu $_B$ (“mixed-valence” state)¹¹². An overview of the intermediates and their transitions is shown in Figure 9.

The oxidative phase of the catalytic cycle involves the binding of oxygen and the successive oxidation of the catalytic site. In the reduced state (state **R**), the iron ion of heme a_3 is in a five-coordinated ferrous state (Fe a_3^{2+}) and Cu $_B$ is in a cuprous state (Cu $_B^+$). The heme iron is thus able to bind small ligands such as oxygen or carbon monoxide.

In the presence of oxygen, state **A** (named after compound A, initially proposed by Chance *et al.*¹¹²) is formed^{113,114}. The binding of oxygen to heme a_3 is only possible in the presence of reduced Cu $_B$, possibly by removal of a blocking ligand of Cu $_B$ upon reduction¹¹⁵, enabling oxygen to bind “end-on” to the heme¹¹⁶. The affinity of oxygen to isolated heme a_3 is very low, which is incompatible with respiration at the low oxygen concentrations at which the respiratory chain operates¹¹⁷. Tight binding of oxygen to heme a_3 in Cyt c O is achieved by kinetic trapping of the oxygen by an electron transferred from heme a . This mechanism yields a high apparent K_M ^{118,119}.

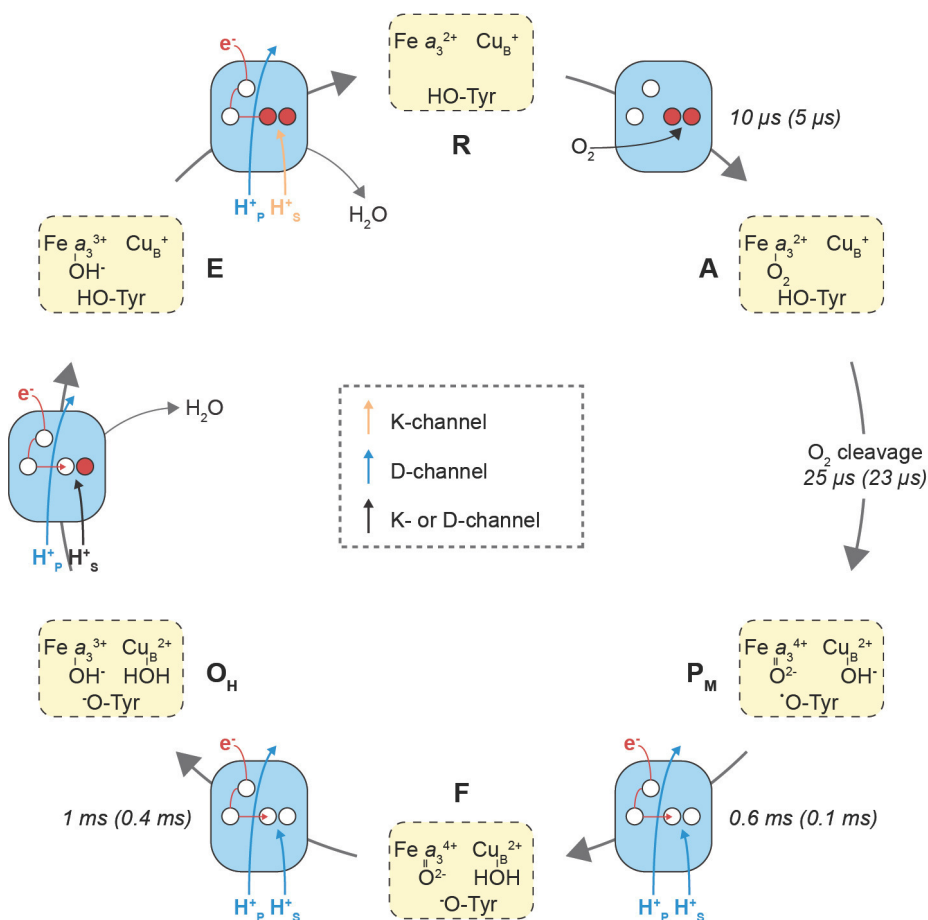


Figure 9: Catalytic cycle of CytcO. The reaction of CytcO with oxygen proceeds in several steps. Electrons are transferred to the catalytic site, and protons are transferred to the catalytic site as well as pumped across the membrane. Protons are transferred via the D-pathway (blue) or K-pathway (orange). Reduction of the cofactors is indicated by red colour of the respective cofactor. The rates of the reactions are indicated for bovine and yeast (in parentheses) CytcO.

In the next step, state **P** (for historical reasons named peroxy), is formed. The intermediate formed in this step depends on the initial reduction level of the enzyme^{116,120}. In case of the “mixed valence” state, the more native form, the state **P_M** is formed. In the formation of this state, the electrons required to split the O-O bond are transferred from heme a₃, Cu_B and the covalently bound tyrosine (Y244), which forms a neutral radical. In a fully reduced enzyme, state **P_R** is formed, with oxygen receiving electrons from heme a instead of the tyrosine¹²¹.

A proton is provided by the tyrosine residue, resulting in the formation of a hydroxyl ligand on Cu_B.

Upon protonation of state P, state **F** (named after the ferryl state of the heme *a*₃ iron) is formed. In addition to the proton provided to form the water ligand of Cu_B, a proton is pumped across the membrane during the P-to-F transition¹²².

State **O** (for oxidized) is the state in which Cyt_cO is usually isolated. The high-spin heme *a*₃ is in a ferric state (Fe *a*₃³⁺) and Cu_B is in a cupric state (Cu_B²⁺)^{113,114}. In the absence of electron donors, e.g. after isolation, this state exists in a “relaxed form”. The catalytically active form of the state is called **O_H**, formed after reduction and consecutive re-oxidation of the enzyme^{123,124}. In the F-to-O transition, one proton is provided for the reaction, while another proton is pumped across the membrane¹²⁵.

In the reductive phase of the catalytic cycle, first the one-electron intermediate **E_H** is formed, and then state R, with ferrous Fe *a*₃ and cuprous Cu_B. Each of these transitions is accompanied by pumping of one proton in each reaction^{123,126}.

Yeast Cyt_cO

The baker’s yeast *S. cerevisiae* is an excellent model organism for the study of eukaryotic systems. Yeast does not rely only on respiration, but can also grow using fermentation, enabling the construction of strains containing otherwise fatal variants of respiratory chain complexes. A high sequence similarity to bovine Cyt_cO allows to draw conclusions about the mammalian Cyt_cO from studies on the yeast system^{127–129}. In *S. cerevisiae*, as in mammalian Cyt_cO, the core subunits are encoded by the mitochondrial DNA, while the supernumerary subunits are encoded by the nuclear DNA. While manipulation of the mitochondrial genome is a difficult challenge in mammalian cell lines¹³⁰, introducing mutations into the mitochondrial genome of *S. cerevisiae* is possible and commonly done^{131,132}.

The core subunits of *S. cerevisiae* Cyt_cO show high sequence similarity with the bovine subunits. All key residues of the K-pathway and all hydrophilic residues of the D-pathway, with the exception of S101 of bovine Cyt_cO (a mutation of which did not alter Cyt_cO activity in *P. denitrificans*¹³³), are conserved in Cyt_cO from *S. cerevisiae*, suggesting the use of these proton pathways also in this organism. There are, however, significant differences in the region of the H-

pathway, challenging the functional relevance of this pathway in the yeast Cyt_cO¹⁵.

Cox5 is the only subunit of Cyt_cO from *S. cerevisiae* that is expressed in two isoforms. Cox5a is expressed under normoxic conditions, while Cox5b is expressed under hypoxic conditions¹³⁴. It has been suggested that the different isoforms of Cox5 modulate the environment of the catalytic site of Cyt_cO and thus effect electron transfer between heme *a* and heme *a*₃¹³⁵.

Yeast Cyt_cO subunits Cox12 and Cox13 show very high sequence similarity (43% and 33%, respectively) to the equivalent bovine Cyt_cO subunits, suggesting similar roles in these Cyt_cO, i.e. to stabilize a possible dimer interface of the enzyme¹⁶.

Two subunits of bovine Cyt_cO, subunits VIIb and VIII, are not found in *S. cerevisiae* (Figure 6).

The subunit composition of yeast and bovine Cyt_cO is compared in Table 1.

Table 1: Subunit composition of yeast and bovine Cyt_cO.

| Yeast Cyt _c O | Bovine Cyt _c O |
|--------------------------|---------------------------|
| Cox1 | Subunit I |
| Cox2 | Subunit II |
| Cox3 | Subunit III |
| Cox4 | Subunit Vb |
| Cox5a | Subunit IV isoform 1/2 |
| Cox5b | |
| Cox6 | Subunit Va |
| Cox7 | Subunit VIIa isoform 1/2 |
| Cox8 | Subunit VIIc |
| Cox9 | Subunit VIc |
| Cox12 | Subunit VIb isoform 1/2 |
| Cox13 | Subunit VIa isoform 1/2 |
| - | Subunit VIIb |
| - | Subunit VIII |

Assembly of yeast Cyt_cO

The assembly of Cyt_cO in yeast involves more than 30 factors¹³⁶, a selection of which are presented in the following section and shown in Figure 10. The core subunits are individually co-translationally inserted into the membrane by the Oxal complex.

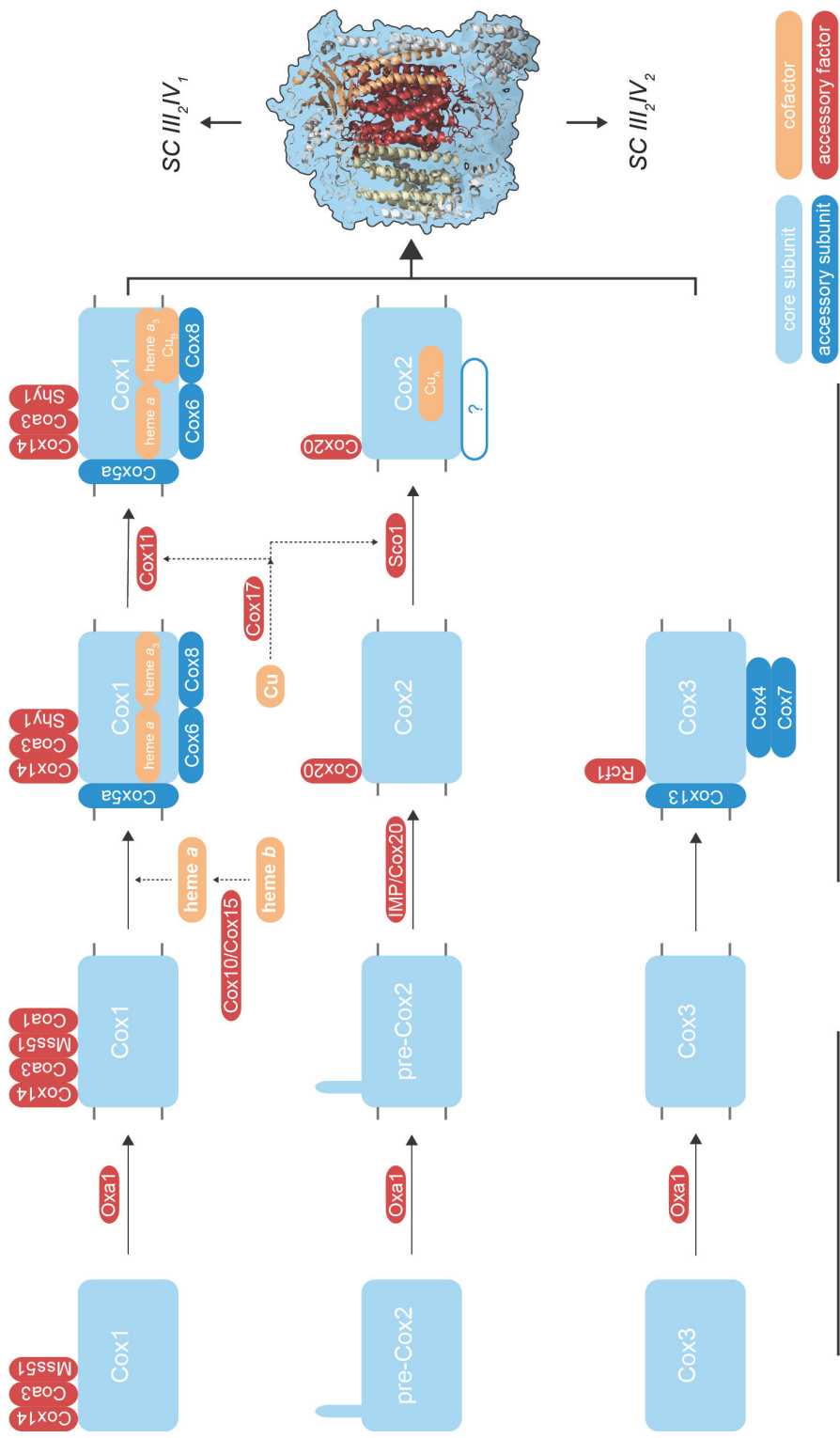
Cox1 is associated with the proteins Cox14, Coa3 and Mss51 during the early assembly process^{137,138}. In a later step the *a* hemes, previously converted from a precursor (heme *b*) by Cox10 and Cox15, are inserted into the Cox1 assembly intermediate¹³⁹⁻¹⁴¹. Cu_B is inserted into the catalytic site with the help of Cox11, to which the copper is delivered by the metallo-chaperone Cox17^{142,143}.

Cox2 is synthesized as a precursor protein (pre-Cox2) with a prolonged amino acid sequence¹⁴⁴. The precursor form is, as Cox1, co-translationally inserted in the membrane by the Oxal complex^{145,146} before it is processed by the inner membrane peptidase (IMP) complex in association with Cox20^{147,148}. After cleavage of the pre-Cox2 leader sequence, two copper ions are inserted into the Cu_A site of Cox2 by the metallo-chaperone Sco1¹⁴⁹. As in the case of Cox11, copper ions are delivered to Sco1 by Cox17^{150,151}.

Cox3 associates with the accessory subunits Cox4, Cox7 and Cox13 during assembly. It was furthermore shown that Rcf1 interacts with the Cox3 assembly module during Cyt_cO assembly¹⁵².

The combination of the different assembly modules for the formation of the holoenzyme remains unclear. To avoid accumulation of unassembled subunits in the mitochondria, they are degraded by AAA-proteases^{153,154}. The entire assembly of Cyt_cO is highly regulated^{155,156}.

Figure 10: Assembly of yeast Cyt_cO. Schematic representation of the proposed modular assembly of yeast Cyt_cO. In short, the core subunits are assembled separately in three modules, which are then combined to the holoenzyme¹³⁶. The metal cofactors are inserted in the course of the assembly of the core-modules with the assistance of different factors^{157,158}. More than 30 factors are involved in the assembly process of Cyt_cO.



Methods

Spectral features of Cyt c O

One of the main advantages in the study of heme-copper oxidases is their absorption of visible light. Heme proteins absorb light at specific wavelengths, which are influenced by the structure and oxidation state of the heme as well as its (protein-) environment¹⁵⁹. This feature can be made use of in the study of Cyt c O, because changes in the surrounding of the hemes can alter the spectral properties of the enzyme. With the knowledge of the spectra of specific intermediates of the catalytic cycle, the reaction of Cyt c O can be followed in time.

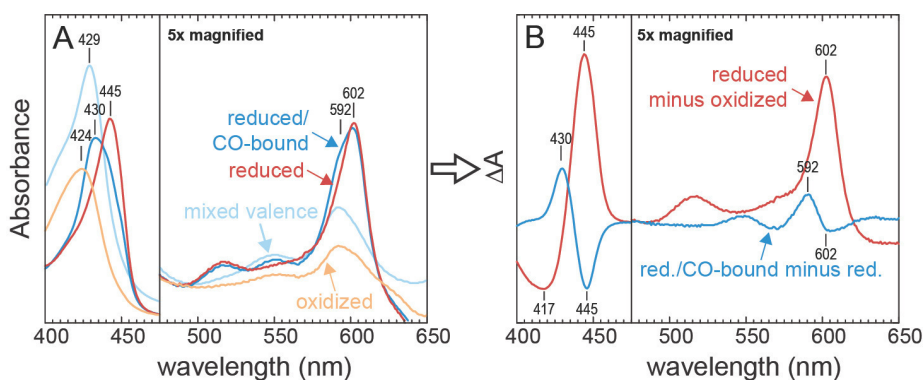


Figure 11: Absorbance spectra of yeast Cyt c O. (A) Absorbance spectra of yeast Cyt c O. Oxidized (light orange), reduced (red), reduced/CO-bound (dark blue) and mixed valence state (light blue) (B) Reduced minus oxidized difference spectrum (red) and reduced/CO-bound minus reduced difference spectrum (blue). Positions of the major peaks are indicated in the figure.

In this work, we exploited the spectral signature of mainly four different states of Cyt c O (Figure 11A): the oxidized form, in which the enzyme is isolated, the reduced form and two forms in which the ligand CO is bound to heme a_3 of either

the two- (“mixed valence”) or four-electron reduced (“reduced/CO-bound”) enzyme. In these two complexes, heme a_3 is in its ferrous state ($\text{Fe } a_3^{2+}$)^{160,161}. The complex can be dissociated by means of a laser flash¹⁶².

In the reduced minus oxidized difference spectrum (Figure 11B, red spectrum), absorption of heme a and a_3 contributes approximately equally to a peak in the Soret-region, at 445 nm (55% heme a_3), while a peak at 602 nm is dominated by heme a (approx. 80%)¹⁶³.

Upon binding of the CO ligand to heme a_3 , the Soret-peak shifts from 445 to 430 nm. The heme a_3 -CO complex also shows a peak at 592 nm, while the absorbance at 602 nm is decreased compared to that of the reduced state¹⁶⁴ (Figure 11B, blue spectrum).

Flash photolysis

The heme a_3 -CO bond is sensitive to light and can be broken by means of a laser flash. In a flash photolysis experiment, Cyt c O is prepared in its two- or four-electron reduced and CO-bound forms under an atmosphere of CO. After laser flash-induced dissociation from heme a_3 , CO binds to Cu_B over a picosecond time scale before equilibrating with the solvent. Recombination of CO with Cyt c O occurs via equilibration of the CO ligand with Cu_B and yields the stable Fe a_3 -CO complex within milliseconds¹⁶⁵. The time-dependent dissociation and recombination of the heme a_3 -CO complex can be followed spectrophotometrically^{165–167} (Figure 12).

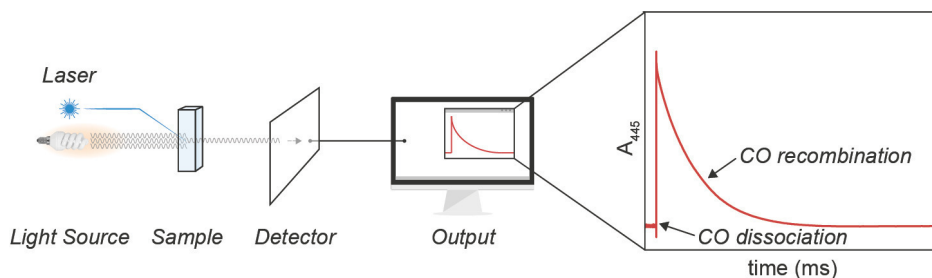


Figure 12: Schematic illustration of a flash photolysis experiment. Cyt c O with bound CO is illuminated by a laser flash, which dissociates the CO bond (initial step on a ns time scale). The dissociation and recombination of CO can be followed in time spectrophotometrically.

To further analyze the time-dependent CO-recombination after laser flash-induced dissociation, the amplitudes of the CO-dissociation steps can be combined at different wavelengths in the construction of a kinetic difference spectrum for the different kinetic components (Figure 13). A kinetic difference spectrum carries information about the structural environment of the catalytic site, including e.g. solvent polarity or coordination of the heme iron. In an experiment in which a sample contains multiple populations of Cyt c O, construction of kinetic difference spectra for each population may reveal differences in the structure of the catalytic site in the different sub-populations.

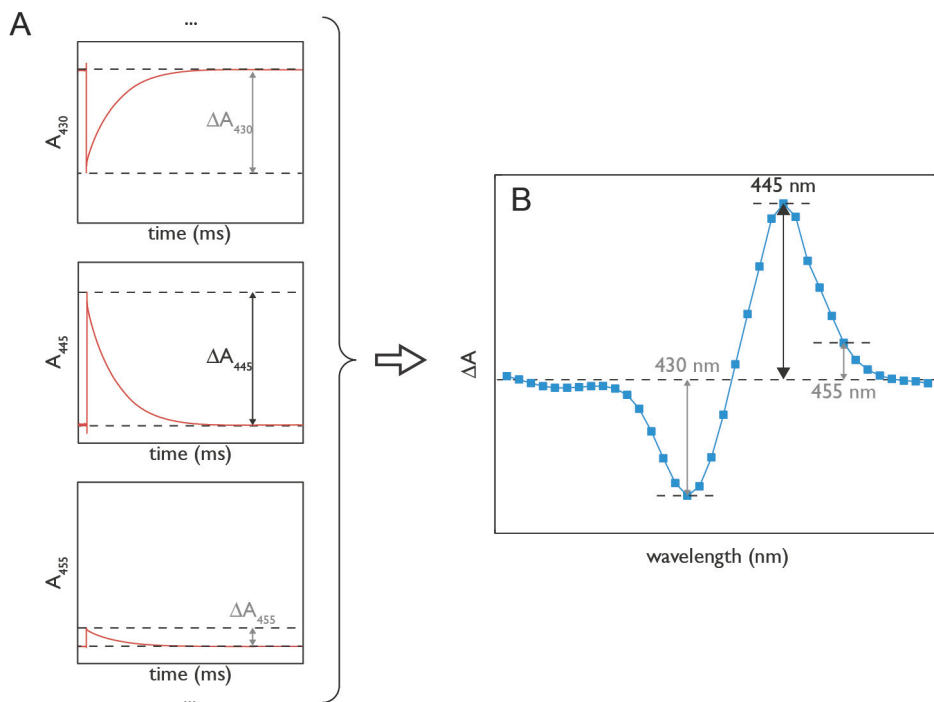


Figure 13: Construction of a kinetic difference spectrum. (A) CO-recombination is recorded on a set of different wavelengths. (B) The amplitudes of each CO-recombination component can be combined to a kinetic different spectrum, which can be used to characterize a CO-recombination component.

CytcO activity

To assess the steady state activity of CytcO, changes of oxygen concentration in a solution are measured using a Clark-type oxygen electrode (Figure 14A). The use of different substrates in combination with specific inhibitors of respiratory complexes allows the detailed determination of the electron flux through the respiratory chain.

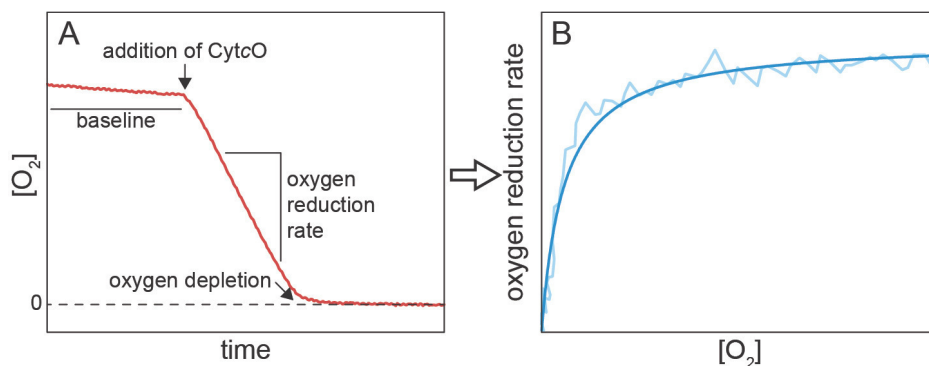


Figure 14: Oxygen reduction. (A) Using a Clark-type electrode, the change of oxygen concentration in a solution is monitored. Given a known concentration of CytcO, this rate can be converted into a steady-state activity of the enzyme. (B) The oxygen reduction rate is plotted against the concentration of oxygen in solution. By fitting a Michaelis-Menten curve (dark blue) to the data, kinetic parameters of the oxygen binding to the catalytic site can be determined.

When using quinol as a substrate, electron flow via the bc_1 complex and CytcO can be investigated. Using specific inhibitors, the electron flow can be blocked at different sites, allowing for a detailed analysis of the separate components of the respiratory chain. By varying the concentration of cyt. *c*, the binding kinetics of cyt. *c* to CytcO can be determined. Alternatively, plotting the oxygen-reduction rate of CytcO against the oxygen concentration in solution offers information on the substrate-dependent kinetics of oxygen binding to the catalytic site (Figure 14B).

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) in combination with confocal microscopy (Figure 15A) provides information about physical, chemical or biological changes in systems containing one or two fluorophores¹⁶⁸. The parameter of interest in FCS is the fluctuation of fluorescence intensity over time. This fluctuation contains information about different properties of the investigated system, such as equilibria, diffusion or molecular interactions.

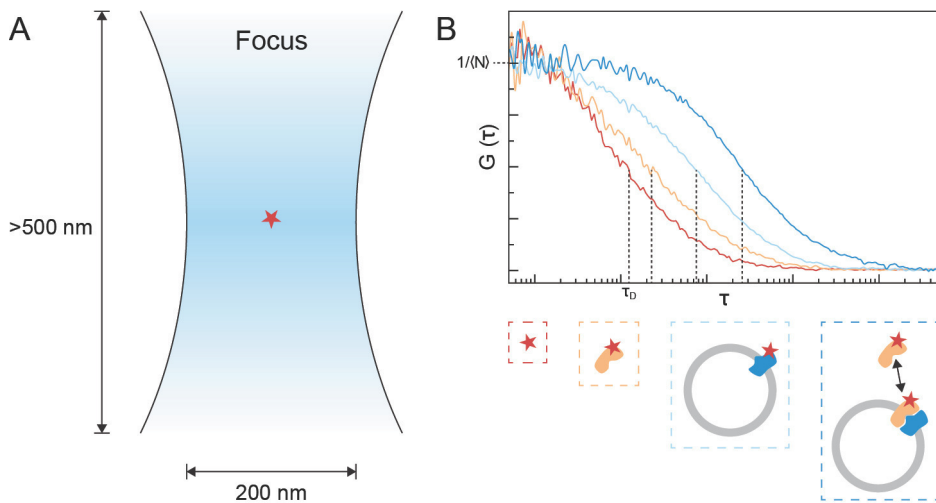


Figure 15: Fluorescence correlation spectroscopy. (A) Schematic of the focus volume of a confocal microscope with a fluorescent probe. (B) Representative autocorrelation curves of four different systems: free fluorophore (red); fluorescently labelled protein in solution (orange), reconstituted in a vesicle (light blue) and interacting with another reconstituted protein (dark blue).

The typical output of an FCS experiment is a so-called autocorrelation curve. The autocorrelation signal $G(\tau)$ is shown as a function of the lag time τ and, when fitted with an appropriate model, can give information about the number of molecules in the probe volume ($1/N$ = amplitude of the curve), diffusion time ($\tau_D = \tau$ at inflection point of the curve) and more (Figure 15B).

By using two fluorophores with sufficiently different excitation wavelengths, interactions between two species can be investigated (fluorescence cross-correlation spectroscopy, FCCS).

Conclusion

While the respiratory chain has been studied for many decades, the idea of an organization of the respiratory chain into supercomplexes is still young. Factors involved in assembly and maintenance of these supercomplexes have only been described in the last few years, leaving many open questions about their molecular interactions and mechanisms.

In this work, the effect of the respiratory supercomplex factor 1 (Rcf1) on the respiratory chain, particularly on Cyt c O, was investigated. In addition to the previously proposed function of Rcf1 in the assembly of respiratory supercomplexes^{43–45}, the protein influences the assembly and activity of Cyt c O^{44,152}.

Cyt c O exists in different populations in the IMM of *S. cerevisiae* (for the detailed study see **Paper I**). Three populations were identified in this work: (i) Intact Cyt c O is fully active and having an unaltered catalytic site, as evident from studies of the ligand-binding kinetics and kinetic difference spectra (for background see page 46). This population largely dominates Cyt c O isolated from the wild-type strain and can be purified using Protein C-affinity chromatography. (ii) In a second population of Cyt c O, the catalytic site of the enzyme is structurally altered, resulting in changes in ligand binding and the kinetic difference spectra. In this population, heme a_3 of Cyt c O has a lower midpoint potential than in the intact Cyt c O (for the detailed study see **Paper II**). This population is present upon genetic removal of Rcf1, indicating a regulatory role of the protein. We propose a mechanism in which, upon removal of Rcf1, the catalytic site of Cyt c O is altered in a way that electron transfer between hemes a and a_3 is slowed. The slowed electron transfer would be compensated for by a tighter binding of oxygen to heme a_3 to yield an unaltered apparent K_M value (see page 38). As result the O $_2$ -ligand binding would be more exergonic, which would result in an increase in heat production on the expense of the proton electrochemical gradient. (iii) A third population of Cyt c O was detected in the flow-through after affinity purification, indicating severe structural changes in

this Cyt_cO that prevent binding to the affinity column. Ligand binding and the resulting kinetic difference spectra of this population are significantly altered, indicating severe changes in the surrounding of the catalytic site. In mitochondria, these populations were observed before⁷⁶, however, due to similar ligand binding kinetics, populations (ii) and (iii) could not be distinguished from one another. Population (i) largely dominates wild-type Cyt_cO in mitochondria, while populations (ii) and (iii) add up to approximately 60% in mitochondria from *rcf1*Δ strains. The partially severe changes in the structure of Cyt_cO dominating the enzyme populations in *rcf1*Δ strains support the role of Rcf1 as a factor also involved in assembly of Cyt_cO (see page 42 and Figure 10).

In addition to its function in the modulation and assembly of Cyt_cO, Rcf1 was shown to interact with the electron carrier cyt. *c* (for detailed study see **Paper III**). Rcf1 was proposed to bind cyt. *c* to Cyt_cO and so facilitate electron transfer from the *bc*₁ complex to Cyt_cO in a supercomplex⁷⁶.

Attaching cyt. *c* to a membrane anchor as part of a respiratory complex is not only seen in the case of yeast, but also in some bacteria, e.g. *Mycobacterium smegmatis*. This bacterium does not have a water-soluble cyt. *c*, but instead a membrane-anchored subunit of the *bc*₁ complex (here *bcc*₁ complex) mediates electron transfer from heme *c*₁ to Cu_A. Here, the supercomplex of *M. smegmatis* was purified and fully characterized (for detailed study see **Paper IV**). The supercomplex contains active *bcc*₁ complex and Cyt_cO. The arrangement of the complexes was solved by cryo-EM to be symmetrical with two copies of the *bcc*₁ complex in the center and one copy of Cyt_cO on each side. Surprisingly, a superoxide dismutase (SOD) was identified as part of the respiratory supercomplex from *M. smegmatis*. The presence of this SOD in purified supercomplexes was confirmed by both activity measurements and unassigned density of the right size in the cryo-EM map.

To summarize this work, Rcf1 was found to function as a modulator as well as an assembly factor of Cyt_cO and to further facilitate interaction between Cyt_cO and its electron donor cyt. *c*. Although a ground was laid for the understanding of the effect of Rcf1 on the respiratory chain, further experiments are required to investigate detailed mechanisms for these interactions.

Supercomplexes from *M. smegmatis*, a close relative of the pathogenic organism *Mycobacterium tuberculosis*, were characterized. The discovery of a SOD within the supercomplex opens new possibilities for drug development.

Future work and speculation

A major problem that is still unresolved is the location of Rcf1 relative to the bc_1 complex or Cyt c O, and thus the identity of interaction sites with Cyt c O and the bc_1 complex. Although several subunits of Cyt c O have been reported to interact with Rcf1, a detailed knowledge about the interaction is still lacking. However, based on the reported interacting subunits^{43–45} and the proposed structure of Rcf1⁴³, speculations about the possible location of Rcf1 can be made (Figure 16).

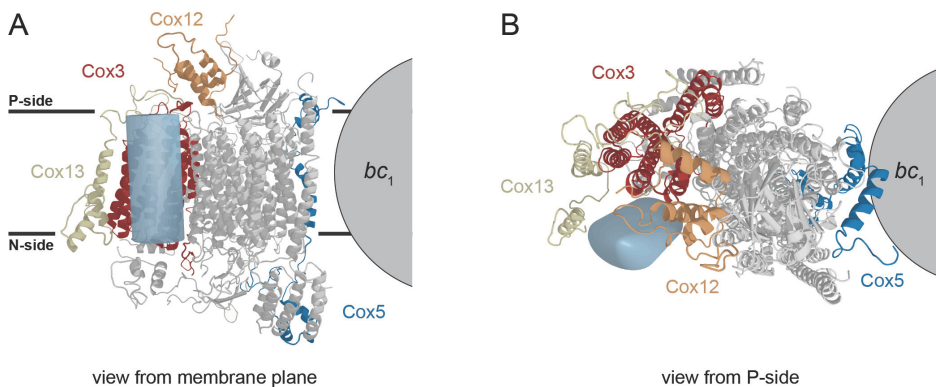


Figure 16: Possible location of Rcf1 relative to Cyt c O. A possible location of Rcf1 (represented as a light blue shape) relative to Cyt c O is shown from the membrane plane (A) or the intermembrane space (B). The location is based on major interactions with subunit Cox3 (red) and minor interactions with subunits Cox12 (orange) and Cox13 (light green). Subunit Cox5, which interacts with the bc_1 complex in a supercomplex (grey shape), is marked in blue.

Rcf1 has been shown to physically interact with Cyt c O core subunit Cox3 already during assembly⁴³. Loss of Rcf1 has been shown to further affect incorporation of the supernumerary subunits Cox12 and Cox13 into Cyt c O⁴⁴, however, evidence for direct physical contact with these subunits has not yet been demonstrated. The structure of Cyt c O allows only one position of Rcf1, assuming

that all the above interactions, including interactions with Cox12 and Cox13, are indeed direct physical interactions and occur simultaneously. This position would be far from the interface between Cyt_cO and the *bc*₁ complex observed in the structural model of the supercomplex⁵⁴, questioning the direct and simultaneous interaction of Rcf1 with both complexes. It is, however, possible that Rcf1 has multiple possible positions and interaction sites relative to Cyt_cO, as it appears to have multiple functions within the respiratory chain. High-resolution cryo-EM structures of the Cyt_cO-Rcf1 complex could help resolving this problem. However, the low abundance of the Rcf1 – Cyt_cO complex⁷⁵ and the possible existence of different forms of the complex with different subunit compositions are major obstacles to be overcome on a way to accomplishing this goal.

As described on page 30, the predicted structure of Rcf1 and the structure determined by solution NMR differ significantly in both the C- and N-terminal regions. The NMR structure of Rcf1 in DPC micelles shows that both regions form TMHs with the C- and N-termini located on opposite sides of the IMM. On the other hand, Strogolova *et al.* showed that in the native membrane, both termini are accessible to proteases after removal of the outer mitochondrial membrane and thus located in the IMS⁴³ (Figure 5). This finding of seemingly conflicting structures might indicate the co-existence of different forms of the protein. In its free form, Rcf1 might exist as a dimer, with the C-terminal region organized in helices spanning the membrane, while contact with the supercomplex might be facilitated by TMHs 4 and 5 flipping out of the membrane and engaging in interactions with cyt. *c*, the *bc*₁ complex and Cyt_cO⁷⁴.

Results from recent experiments show that the modulation of Cyt_cO activity, caused by deletion of Rcf1, can be reversed by co-reconstitution with recombinantly expressed, purified Rcf1 (Figure 17). Sub-mitochondrial particles (SMPs) obtained from *rcf1Δ* mitochondria showed wild-type Cyt_cO activity after reconstitution with purified Rcf1, but only in the presence of guanidinium hydrochloride (GuHCl).

Since Rcf1 was expressed in *E. coli* and purified from inclusion bodies, the presence of the chaotropic reagent GuHCl was necessary in the experiments to ensure unfolding and correct refolding of Rcf1 in the presence of Cyt_cO. This finding further supports the scenario suggested above, i.e. that the structure determined by NMR might not be the same as that of Rcf1 in complex with Cyt_cO. It is possible that the NMR structure shows the conformation of Rcf1 as a dimer when not bound to Cyt_cO, while when bound to the enzyme, a different, active conformation is adapted.

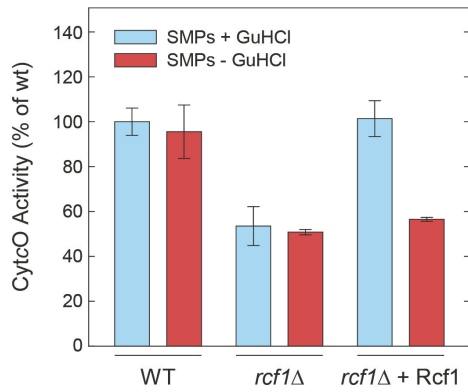


Figure 17: Reconstitution of Rcf1 protein into *rcf1*Δ SMPs in the presence of GuHCl restores wild type activity. Rcf1 was reconstituted into SMPs in the presence or absence of GuHCl. GuHCl is required for restoration of the wild type activity.

Populärwissenschaftliche Zusammenfassung

Sauerstoff, der beim Atmen vom Körper aufgenommen wird, gelangt durch die Blutbahn zu jeder Zelle des Körpers. Gleichzeitig wird die aufgenommene Nahrung umgewandelt und die dabei gewonnene Energie in chemischen Verbindungen gespeichert. In den Mitochondrien, den Kraftwerken der Zelle, wird diese Energie in der Form von Elektronen in einer Kette von Enzymen („biologische Maschinen“) weitergegeben. Diese Enzyme bilden die Atmungskette. Einige der Enzyme der Atmungskette nutzen die Energie der Elektronen, um Protonen (geladene Elementarteilchen) über eine biologische Membran zu bewegen. Dieser Prozess kann mit dem Füllen eines Stausees verglichen werden (siehe Titelbild). Im letzten Enzym der Atmungskette treffen die Elektronen nun auf den Sauerstoff, welcher, zusammen mit Protonen, zu Wasser reagiert. Auch in dieser Reaktion wird Energie freigesetzt, welche das Enzym, die sogenannte Cytochrom *c* Oxidase, auch für das Pumpen von Protonen von einer Seite der Membran auf die andere nutzt. Das Ergebnis ist ein Unterschied in Konzentration und Ladung zwischen beiden Seiten der Membran. Dieser Unterschied kann von einem weiteren Protein, der ATP Synthase, genutzt werden, um den universellen Energieträger der Zelle, ATP, zu erzeugen. Die ATP Synthase funktioniert dabei genau wie ein Wasserkraftwerk, das den Höhenunterschied des Wassers nutzt, um elektrische Energie zu erzeugen.

Der Prozess der Atmung, mitsamt der involvierten Enzyme, existiert nicht nur in Menschen und anderen Tieren, sondern in sehr ähnlicher Weise auch in einfacheren Organismen wie Bakterien oder Hefepilzen. Während sich die Atmungskette von Bakterien in einigen Aspekten, wie dem Aufbau der Enzyme, deutlich von der in Säugetieren unterscheidet, ist diejenige in gewöhnlicher Back- oder Brauhefe der des Menschen sehr ähnlich. Diese Ähnlichkeit kann ausgenutzt werden um die Mechanismen der Atmungskette zu studieren, da sich mit Hefe viel einfacher arbeiten lässt als mit der menschlichen Atmungskette.

In dieser Arbeit wurde die Funktion der Cytochrom *c* Oxidase in Hefe sowie die Interaktion zwischen dem Enzym und anderen Komponenten der Atmungskette untersucht.

In den Publikationen I und II wurde gezeigt, dass Cytochrom *c* Oxidase in mehreren verschiedenen Formen existiert, die sich in ihrem Aufbau und der Funktionsweise ihres aktiven Zentrums, sozusagen des Motors der Maschine, unterscheiden. Der relative Anteil dieser verschiedenen Formen wird verändert, wenn man das kleine Protein Rcf1, welches mit Komponenten der Atmungskette interagiert, entfernt. In Publikation II wurde eine dieser veränderten Formen genauer charakterisiert, wobei sich Unterschiede beim Binden von Sauerstoff zeigten. Hefezellen könnten diese Unterschiede nutzen, um sich den wechselnden Wachstumsbedingungen der Umgebung anzupassen.

In Publikation III wurde gezeigt, dass das oben genannte Protein Rcf1 nicht nur den Motor der Cytochrom *c* Oxidase verändert, sondern auch die Interaktion mit dem Elektronenträger Cytochrom *c* steigert. Dies könnte eine direkte Übertragung von Elektronen von dem vorherigen Glied der Atmungskette zur Cytochrom *c* Oxidase ermöglichen.

In Publikation IV wurden Komponenten der Atmungskette in dem Bakterium *M. smegmatis*, einem Verwandten des Krankheitserregers *M. tuberculosis*, untersucht. In vielen Organismen, wie auch in dem hier untersuchten, bilden die Enzyme der Atmungskette sogenannte Superkomplexe. In dieser Arbeit wurde die Zusammensetzung der Superkomplexe des Bakteriums untersucht. Es wurde gezeigt, dass die Superkomplexe nicht nur Komponenten der Atmungskette, sondern auch ein weiteres Enzym, die sogenannte Superoxid-Dismutase enthalten. Dieses Enzym schützt die Zelle vor Sauerstoffradikalen, welche von befallenen Organismen genutzt werden, um Zellen des Krankheitserregers abzutöten. Das Bakterium wiederum nutzt die Superoxid-Dismutase, um sich vor diesen Angriffen zu schützen.

Populärvetenskaplig sammanfattning

Syre, som tas upp i kroppen medan du andas, passerar genom blodbanan till varje cell i kroppen. Samtidigt bryter vi ner den föda som vi får i oss och lagrar den frigjorda energin i form av kemiska föreningar. I mitokondrier, cellens kraftstation, överförs denna energi i form av elektroner, i en kedja av enzymer ("biologiska maskiner"). Dessa enzymer utgör andningskedjan. Några av andningskedjeproteinerna använder elektronernas energi för att flytta protoner (laddade elementarpartiklar) över ett biologiskt membran. Denna process kan jämföras med att fylla en reservoar vid en vattendamm (se omslaget). I det sista enzymet i andningskedjan hamnar elektronerna på syret, vilket tillsammans med protoner reagerar till att bilda vatten. Också i denna reaktion frigörs energi som används av enzymet cytokrom *c* oxidas för att pumpa protoner från den ena sidan av ett membran till den andra. Resultatet är en skillnad i koncentration och laddning mellan membranets båda sidor. Denna skillnad utnyttjas av ett annat protein, ATP-syntas, för att tillverka cellens universella energibärare, ATP. ATP-synthas fungerar precis som ett vattenkraftverk, som använder skillnaden i vattennivå för att generera elektrisk energi.

Cellandningen och de enzymer som deltar i processen finns inte bara hos människor och andra djur, utan även i enklare organismer såsom bakterier eller jäst. Både proteiner och reaktionsvägar har stora likheter i aeroba bakterier och i däggdjur. I vanligt jäst är de dessutom mycket lika dem som finns hos t.ex. människor. Denna likhet kan utnyttjas för att studera mekanismer för energiomsättningen eftersom det är mycket lättare att arbeta med jäst än med den mänskliga andningskedjan.

I detta avhandlingsarbete undersöktes funktionen för cytokrom *c* oxidas från jäst samt för hur detta komplicerade proteins regleras via bindning av andra komponenter i andningskedjan.

I publikationerna I och II visar vi att cytokrom *c* oxidas finns i olika former vilka skiljer sig åt i sin struktur och funktion. Bland annat visar vi att deras aktiva

centrum, vilket kan liknas vid en maskins motor, har olika egenskaper. Den relativa andelen av dessa olika former förändras genom avlägsnande av det lilla proteinet Rcf1, som binder till komponenter i andningskedjan. I publikation II karakteriserades en av dessa förändrade former mer noggrant och vi kunde observera skillnader i syrebindning. Jästceller kan använda dessa skillnader för att anpassa sig till förändrade förhållandena i miljön.

Resultaten i publikation III har visat att det ovannämnda protein Rcf1 inte bara ändrar motorn av cytokrom *c* oxidase, men ökar även interaktionen med den lilla elektronbäraren cytokrom *c*. Detta skulle kunna möjliggöra direkt överföring av elektroner från den tidigare komponenten i andningskedjan till cytokrom *c* oxidase.

I publikation IV undersökte vi komponenter i andningskedjan i bakterien *M. smegmatis*, en släkting till sjukdomsbakterien *M. tuberculosis*. I många organismer bildar andningskedjans enzymer så kallade superkomplex. I detta arbete undersöktes sammansättningen av bakteriens superkomplex. Vi visade att superkomplexet inte bara innehåller komponenter i andningskedjan, utan också ett annat enzym, det så kallade superoxid-dismutasen. Detta enzym skyddar cellen från syreradikalervilka används av den infekterade organismen för att döda patogener. Bakterien använder i sin tur superoxid-dismutas för att skydda sig från dessa attacker.

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