Stockholm University

Regulation of proton-coupled electron transfer in cytochrome c oxidase

The role of membrane potential, proton pathways and ATP

Markus Björck

Doctoral Thesis in Biochemistry at Stockholm University, Sweden 2019

Regulation of proton-coupled electron transfer in cytochrome *c* oxidase

The role of membrane potential, proton pathways and ATP

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Abstract

Cytochrome *c* oxidase (Cyt*c*O) is the final electron acceptor of the respiratory chain. In this chain a current of electrons, derived from degradation of nutrients, along with protons, are used to reduce oxygen to water. The reaction is exergonic and the excess energy is used to pump protons across the membrane. This proton-coupled electron transfer is regulated, for example, by the membrane potential, the composition of the membrane and the ATP/ADP concentrations.

Here, we have investigated the mechanism of this regulation. Specifically, we investigated ligand binding to Cyt*c*O in mitochondria, which provides mechanistic information about Cyt*c*O in its native environment. In addition to Cyt*c*O, a water soluble protein, flavohemoglobin (yHb) was found to bind CO and we found that it is localized in the intermembrane space (IMS). We also extracted Cyt*c*O from mitochondria without detergent using the styrene maleic acid (SMA) co-polymer. We could show that the SMA-extracted Cyt*c*O behaved similarly in its reaction with O_2 and CO as Cyt*c*O in mitochondria.

In mitochondria and bacterial membranes Cyt*c*O transports charges against a transmembrane electrochemical gradient. We induced a membrane potential across sub-mitochondrial particles (SMPs) by addition of ATP and measured single Cyt*c*O turnover. Our results indicate that proton transfer, but not electron transfer, across the membrane is affected by the membrane potential.

In yeast Cyt*c*O subunit Cox13 has been shown to play a role in ATP/ADP binding to regulate activity. We have solved the structure of Cox13 using NMR and identified the residues that constitute the ATP-binding site, which is located at the C-terminus.

Finally we showed that the main proton-transfer pathways in yeast Cyt*c*O function similarly to their bacterial counterparts and that the proposed H-pathway, absent in bacteria, is not responsible for proton translocation in mitochondrial Cyt*c*O from *S. cerevisiae*.

Keywords: *cytochrome c oxidase, charge transfer, membrane potential, membrane protein, mitochondria, ATP, proton pump.*

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List of publications

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III: Björck ML, Brzezinski P. **Control of transmembrane charge transfer in cytochrome c oxidase by the membrane potential**, Nature Comm. 9 (2018) 3187

IV: Zhou S, Petterson P, **Björck ML**, Brzezinski P, Dawitz H, Mäler L, Ädelroth P. **NMR structural analysis of Cox13 reveals its C-terminus in interaction with ATP**, manuscript.

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Additional publications

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Dierckx A, Miannay F-A, Ben Gaied N, Preus S, **Björck M**, Brown T, Wilhelmsson L.M. **Quadracyclic adenine: A non-perturbing fluorescent adenine analogue**, Chemistry. 18 (2012) 5987-97

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Abbreviations

Cyt*c*O	cytochrome *c* oxidase
cyt. *c*	cytochrome *c*
yHb	yeast flavohemoglobin
OMM	outer mitochondrial membrane
IMS	intermembrane space
IMM	inner mitochondrial membrane
SMA	styrene maleic acid co-polymer
SMPs	submitochondrial particles
Complex I	NADH:ubiquinone oxidoreductase
Complex II	succinate:quinon oxidoreductase
Complex III, bc_1 complex	ubiquinol-cytochrome *c* oxidoreductase
Complex IV	cytochrome *c* oxidase
Complex V	ATP-synthase
FMN	flavin mononucleotide
FAD	flavin adenine dinucleotide
Q	ubiquinone
$Q^{\bullet-}$	semiquinone
QH_2	ubiquinol
PCET	proton-coupled electron transfer
RCR	respiratory control ratio
Δp	proton-motive force

Introduction

There are many aspects of life, philosophical as well as practical. A prerequisite, and necessary component for life is energy. Energy may be acquired from light (photosynthesis) or by degradation of various molecular compounds. A general feature of these processes is that they involve consecutive oxidation and reduction of the substrates and proteins involved in the process. The subfield of Biochemistry that is devoted to studies of these processes is called Bioenergetics.

Early organisms most likely acquired energy by reducing compounds such as sulfur and nitrate using electrons from hydrogen gas (1). During these early stages of life on Earth the oxygen levels were low and organisms lived under highly anaerobic conditions. Eventually the oxygen levels on Earth started to rise as photosynthetic organisms in the oceans produced molecular oxygen. This process lead to an increase in the oxygen concentration in the oceans and on land, which reached the current levels about 600 million years ago (2,3).

Oxygen can be used as an ultimate electron acceptor. When oxygen is reduced to water, in a process called aerobic respiration, energy is released and can be used to drive cellular processes. The reduction of O_2 to water is catalyzed by protein complexes. Many of the proteins involved in this process harbor hemes, iron-containing redox-active co-factors. Heme-containing proteins are called cytochromes. The cytochromes were initially discovered in animal tissues, and noted for their spectral signatures, by Charles Macmunn in the late 19th century and were then named myohaematins and histohaematins (4). Keilin continued the research in the 1920:s, renaming the compounds cytochromes, and categorized the different types based on their main absorption bands, pioneering research in Bioenergetics (5). The identification of the cytochromes and their spectral signatures is of great importance in studies of aerobic respiration.

Aerobic respiration is tightly associated with cellular membranes. In higher organisms and animals this process takes place in mitochondria, a cellular organelle confined by two membranes. A series of respiratory complexes, denoted I-IV, all situated in the inner mitochondrial membrane (IMM), shuttle electrons obtained from metabolic processes, e.g. glycolysis and the citric acid cycle, to O_2. All respiratory complexes are membrane proteins. Electrons are delivered by NADH or succinate and are shuttled between the complexes either by water soluble (cytochrome *c* (cyt. *c*)) or hydrophobic carriers in the

membrane (quinones (Q)/quinoles (QH_2)). This assembly of protein complexes is usually referred to as a respiratory chain. In aerobic respiration protons are transported from the mitochondrial matrix to the intermembrane space (IMS) by complexes I, III and IV. This process results in a charge separation over the IMM, which maintains a transmembrane electrochemical potential. The membrane potential is used by the ATP synthase (Complex V) to produce ATP as the protons are transferred back, across the membrane through the ATP synthase (for a schematic picture of the respiratory chain, see Figure 1).

The terminal electron acceptor of the respiratory chain is Complex IV, also called cytochrome *c* oxidase (Cyt*c*O). This protein belongs to a superfamily called heme-copper oxidases. The heme-copper oxidase superfamily is divided into three classes, A, B and C, based on the composition and architecture of their proton-uptake pathways (6–9). This thesis focuses on the A-type Cyt*c*Os. The reduction of oxygen to water, performed by Cyt*c*O, involves proton-coupled electron transfer (PCET)

The PCET mechanism in Cyt*c*O has been studied extensively. However, most of these studies were performed on detergent-solubilized enzyme under conditions far from the enzyme's native state. Upon extraction of the Cyt*c*O from the membrane the lipids surrounding the enzyme are removed, which may affect its function. Previous results with Cyt*c*O reconstituted in liposomes and in native membranes have shown that there are differences in the reaction rates during single Cyt*c*O turnover (10,11). In addition, under native conditions there is a membrane potential across the membrane. Because Cyt*c*O transfers charges against the gradient, the reaction is slowed in the presence of a membrane potential. This is seen by a stimulation of multiple CytcO turnover activity when the membrane potential is removed (12,13). This phenomenon is referred to as respiratory control ratio (RCR), i.e. the ratio of the activities without and with membrane potential, respectively, which is typically >1. Whereas the observed effect is well known it is not known which part of the reaction mechanism that is affected. Single turnover measurements on Cyt*c*O in the presence of a membrane potential are likely to answer this question.

With this thesis I hope to shed some light on the importance of studying Cyt*c*O under more native conditions and discuss the type of information that may be obtained to elucidate the reaction mechanism of the Cyt*c*O.

The respiratory chain

This chapter is written with the perspective on mitochondrial respiratory chains if not specified otherwise.

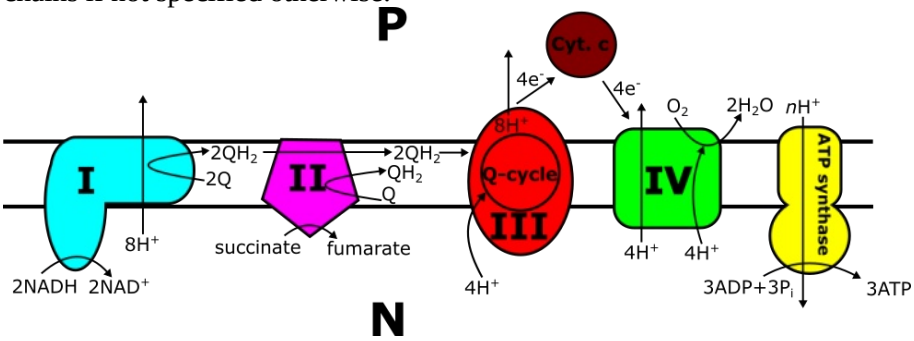

Figure 1. *The respiratory chain. A schematic representation of the components of the respiratory chain. The presence of certain complexes and nomenclature vary depending on the organism, e.g. S. cerevisiae lacks Complex I whereas it is present in B. taurus (14). The positive side of the membrane is marked P and the negative side N. The number of protons released by each complex on the positive side of the membrane is indicated in the figure. This stoichiometry is for the reducing equivalents required for the reduction of one O_2 molecule to water. The number n indicates the number of protons required to synthesize 3 ATP molecules, which is 8 in B. taurus and 10 in S. cerevisiae.*

The organization of the respiratory chain has been discussed over the years. Initially, the complexes were thought to be clustered in larger supramolecular assemblies. This view shifted to a model where each complex is a separate entity freely diffusing in the membrane. The current consensus is that most of the complexes (except Complex II) are organized into supramolecular assemblies referred to as respiratory chain supercomplexes (15–17). The cellular function of this organization is yet to be determined. It was initially thought that the supercomplexes could play a role in substrate channeling i.e. providing a direct route for electron transfer without equilibration of the electron carriers (i.e. Q/QH_2 and cyt. *c*) within the pool of each carrier. However, data from several studies suggest that there is no such substrate channeling (18–20). Among other factors influencing the respiratory chain are a class of proteins, mainly studied in yeast, called respiratory supercomplex factors (Rcf)

that are members of the Hig-family (21–23). Both Rcf1 and Rcf2 have been shown to modulate Cyt*c*O activity in *S. cerevisiae* yeast, where the greatest effects were observed upon removal of Rcf1 (24–27).

Complex I

NADH:ubiquinone oxidoreductase (Complex I) is the first complex of the respiratory chain. It uses NADH to reduce Q to QH_2 and in the process protons are pumped from the negative side to the positive side of the membrane. The net reaction catalyzed is:

$$NADH + Q + H^+ + 4H^+_{\text{negative side}} \rightarrow NAD^+ + 4H^+_{\text{positive side}} + QH_2$$

The protein has 14 core subunits, conserved from bacteria to mammals, and up to 31 supernumerary subunits depending on organism (28). It consists of two main domains, a water soluble domain protruding out of the membrane and a hydrophobic domain in the membrane. The part of the water-soluble domain most distant from the membrane contains a flavin mononucleotide co-factor (FMN) capable of oxidizing NADH. The two electrons from NADH are transferred along a chain of iron-sulfur clusters towards a Q/QH_2-binding pocket located at the interface between the water soluble and hydrophobic parts of the enzyme. In this site Q is reduced to QH_2.

The other part of the enzyme is an elongated hydrophobic domain, which harbors four proton half channels, facing each side of the membrane. These modules most likely transport one proton each per NADH oxidized (29–31).

Complex II

Succinate:quinone oxidoreductase (Complex II) is part of the citric acid cycle and does not transfer protons across the membrane. In the citric acid cycle Complex II oxidizes succinate to fumarate. The electrons are transferred to Q, which is reduced to QH_2. The QH_2 is released into the membrane and eventually reduces Complex III. The net reaction catalyzed by Complex II is:

$$\text{succinate} + Q \rightarrow \text{fumarate} + QH_2$$

The mitochondrial Complex II consists of four subunits, two hydrophilic subunits and two hydrophobic transmembrane subunits. The hydrophilic part harbors a flavine adenine dinucleotide co-factor (FAD). At the FAD site succinate is oxidized to fumarate. The electrons are then transferred along three iron-sulfur clusters to the Q reduction site in the hydrophobic subunits. The

hydrophobic subunits also harbor a *b*-heme whose function is unknown (32–34).

Complex III

Ubiquinol-cytochrome *c* oxidoreductase (Complex III or bc_1 complex) utilizes the QH_2 released by Complexes I and II to reduce cyt. *c* in a mechanism that is called the Q-cycle. The Q-cycle does not involve proton pumping, but protons are taken up from the negative side and released on the positive side of the membrane during turnover, which results in a net charge separation across the membrane.

Complex III is a homodimer under native conditions. The enzyme harbors two Q/QH_2 binding sites (Q_o and Q_i), two *b*-hemes (b_L and b_H), one iron sulfur cluster and a *c*-heme close to the positive side of the membrane (35). The simplified net reaction catalyzed by the enzyme is:

$$QH_2 + 2\text{cyt. } c^{3+} + 2H^+_{\text{negative side}} \rightarrow Q + 2\text{cyt. } c^{2+} + 4H^+_{\text{positive side}}$$

In the Q-cycle QH_2 binds to the Q_o-site, from where it delivers one electron to cyt. c_1 (used to reduce cyt. *c*), and two protons are released on the positive side of the membrane. A semiquinone ($Q^{\bullet-}$) is then formed, which donates its electron to the b_L-heme thus forming Q. This electron is then transferred consecutively to heme b_H and then to another Q that binds in the Q_i-site forming a $Q^{\bullet-}$. The first part of the cycle is repeated (resulting in cyt. *c* reduction and proton release) but in this case the second electron from the Q_o site is used to reduce $Q^{\bullet-}$ to QH_2 in the Q_i site, linked to the uptake of two protons from the negative side of the membrane (36).

Complex IV

Cytochrome *c* oxidase (Complex IV) is the terminal electron acceptor of the respiratory chain. It reduces O_2 to water with electrons transferred from cyt. *c* on the positive side and protons taken up from the negative side of the membrane. The free energy released in the reaction is used to translocate protons across the membrane (37–41). The net reaction catalyzed by Cyt*c*O is:

$$O_2 + 4e^-_{\text{positive side}} + 8H^+_{\text{negative side}} \rightarrow 2H_2O + 4H^+_{\text{positive side}}$$

For a more detailed description of the enzyme and its mechanism, see the chapter on Cyt*c*O.

Cytochrome *c*

Cytochrome *c* is a small and water soluble protein with a molecular weight of 12-13 kDa that harbors a single heme *c*. It is reduced by Complex III and delivers a single electron at a time to Cyt*c*O, hence mediating the electron transfer between Complexes III and IV. In *S. cerevisiae* yeast there are two cyt. *c* isoforms, 1 and 2, that are expressed under normoxic and hypoxic conditions, respectively. Under normoxia isoform 1 constitutes about 95 % of the total cyt. *c* content (42).

ATP synthase

ATP synthase (Complex V) utilizes the proton-motive force generated by the respiratory complexes to produce ATP. Briefly, Complex V has three main structural features. A membrane domain called the C-ring composed of several identical subunits. As protons enter from the positive side of the membrane and exit on the negative side the C-ring rotates in the membrane. The second part is the central stalk. This is an elongated domain located in the middle of the C-ring. It rotates along with the C-ring and is the connecting domain between the membrane and water soluble domains of Complex V. The third part is the hydrophilic head of Complex V. This domain harbors three nucleotide-binding domains. One domain binds ADP and phosphate tightly, another binds ATP tightly and the third one binds ATP loosely. As the central stalk rotates along with the C-ring the three domains shift between each of these states, consecutively synthesizing ATP. This process is reversible, and if an excess of ATP is present, Complex V instead hydrolyzes ATP to ADP. In this process protons are pumped in the opposite direction compared to the proton flow during synthesis (43–45).

Charge transfer in Bioenergetics

Electron transfer

Electron transfer in biological systems occurs by tunneling, i.e. the electron is transferred over long distances through bonds and space between the donor and acceptor. The rate of electron transfer can be described by the Marcus theory; it decreases exponentially as the distance between donor and acceptor increases (46,47). The maximum distance for electron tunneling in proteins at rates comparable to turnover of most enzymes has been estimated to be ~25 Å, which sets the maximum distance between the donor and acceptor (48). For transfer exceeding the limit a series of donor and acceptors is typically required. The sequence of an electron flux may also be determined by the difference in redox potentials of the involved redox sites. One example where electron tunneling takes place over distances of ~20 Å is in Cyt*c*O (49–52).

Proton transfer

Protons can also tunnel, but due to their larger mass compared to that of electrons the tunneling distances are less than 1 Å, which is shorter than a covalent bond (53).

Protons may be transferred over longer distances in proteins utilizing a mechanism that is called the Grotthuss mechanism. In the Grotthuss mechanism the proton-transfer pathway consists of a chain of hydrogen bonded water molecules or side chains of protonable residues. The proton entering at the start of the chain forms a covalent bond to the water molecule, which in turn transfers a proton to the next water in the chain. Eventually a proton is released at the other end of the chain. In this mechanism the proton initially taken up is not the one being expelled at the other side of the chain (54,55). One example of such a chain that conducts protons is the D-pathway in Cyt*c*O (56,57).

Membranes

Organization of mitochondrial membranes

The mitochondrial membranes are densely packed with proteins. Compared to other cellular membranes, the protein-to-lipid ratio is 2-8 times higher, depending on organism (58). The membranes themselves display an intricate ultrastructure. This is seen in the IMM, where multiple invaginations called cristae, greatly enhance the surface area. This membrane curvature is formed by long rows of ATP-synthase dimers that presumably bend the membrane (59,60).

The IMM and OMM are connected via a protein complex, called MICOS, located at the edge of the cristae invaginations. It extends to the OMM thereby acting as an anchor that also facilitates transport between the two membranes (61–63). As opposed to the IMM, the OMM is permeable to ions and smaller molecules, which means that the ionic strength and composition of the IMS is similar to that of the cytosol (64).

The main lipids present in mitochondria are phosphatidylcholine and phosphatidylethanolamine, which constitute ~70 % of the total lipid content. There is also an enrichment of cardiolipin in mitochondria. The fraction is 10-15 % of the total lipid content (depending on organism) as opposed to ~1% in other cellular membranes. Most of the cardiolipin found in mitochondria is located in the IMM (58,65).

Proton motive force

The proton motive force (Δp) is the driving force for ATP synthesis as postulated by Mitchell in the chemiosmotic theory (66). This transmembrane electrochemical gradient consists of two components and is expressed as:

$$\Delta p = \Delta\psi + \Delta pH$$

where $\Delta\psi$ is the contribution of the electrical component (the charge difference between the positive and negative side of the membrane) and ΔpH is the difference in proton concentrations. In isolated rat mitochondria the Δp was estimated to be ~180 mV with a contribution from $\Delta\psi$ of 80 % (67), while in rat cells it has been estimated to be ~140 mV (68).

Cytochrome *c* oxidase

The composition of the core subunits I-III, as well as the general function of Cyt*c*Os from different organisms is conserved. Yet, the composition of the other subunits of Cyt*c*O varies depending on the organism. These supernumerary subunits are presumably involved in regulation of the enzyme. The *R. sphaeroides* Cyt*c*O is composed of four subunits (57) whereas the *B. taurus* Cyt*c*O consist of a total of 13 (69) and *S. cerevisiae* of 12 (70–72). It has been suggested that *B. taurus* Cyt*c*O contains a fourteenth subunit (73–75), but this has been disputed (76). The three conserved core subunits all display a similar architecture as described in the following chapter.

Structure

X-ray crystallography and cryogenic electron microscopy have provided structural information about Cyt*c*O (56,57,71,72,77).

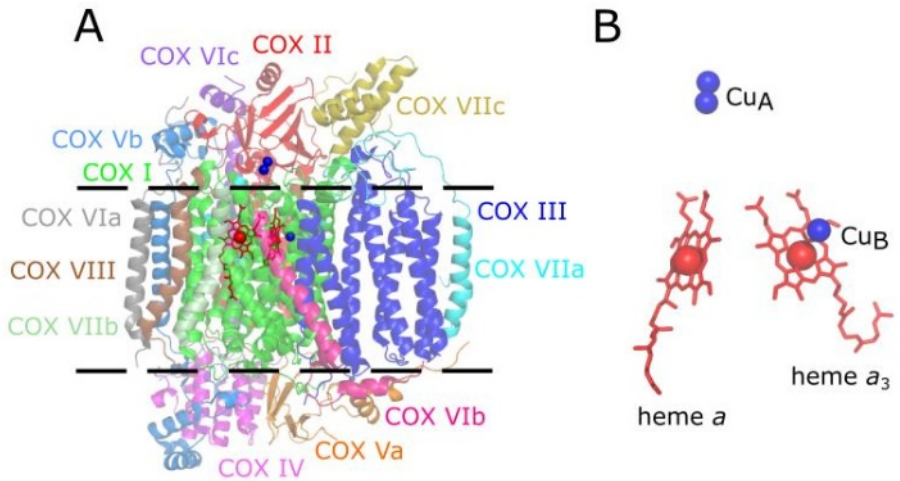

Figure 2. *The structure of B. taurus CytcO.* ***A.*** *All 13 subunits of B. taurus CytcO. The position of the membrane is marked with a dashed line. Hemes are colored in red and copper ions in blue. The structure also contain other non-redox active metals ions that are not shown in the figure.* ***B.*** *The orientation of the redox co-factors in CytcO. The structure is based on PDB entry 5B1A* (78).

Subunit I contains three out of four redox-active co-factors of Cyt*c*O, including the two redox centers that form the catalytic site of the enzyme, i.e. Cu_B and heme a_3. The third cofactor is a hexa-ligated, low-spin heme *a*, capable of holding one electron. The catalytic site is composed of a high-spin penta-ligated heme a_3 and a copper ion denoted Cu_B. The two hemes are located at the same distance from the membrane surface, with their porphyrin rings in a perpendicular orientation to each other. Hence, electron transfer between the hemes is parallel to the membrane surface. Most of the residues that define the proton-transfer pathways are also located in subunit I (for the structure of *B. taurus* Cyt*c*O and the orientation of the redox co-factors see Figure 2).

Subunit II contains the fourth co-factor, denoted Cu_A, which consists of two copper ions. These copper ions can hold one electron, evenly distributed between the two ions. Cu_A is localized close to the positive side of the membrane, above heme *a*. The site is the primary electron acceptor from cyt. *c*.

Subunit III does not contain any co-factors or proton pathways. Studies with the *R. sphaeroides* Cyt*c*O have shown that removal of subunit III, either by mutation or treatment with detergent, results in a "suicidal" Cyt*c*O capable of performing a limited number of turnovers before becoming inactivated (79,80). When subunit III was removed in *B. taurus* Cyt*c*O the stoichiometry of pumped protons per electron decreased from 1 to 0.5 (81). The "suicide" effect is thought to originate from destabilization of the catalytic site and slower proton uptake when subunit III is lost (82,83).

Not all functions of the supernumerary subunits are known, but some are likely to be involved in regulation of the Cyt*c*O activity (see chapter on regulation of Cyt*c*O by ATP).

Proton-transfer pathways

Wikström demonstrated in 1977 that Cyt*c*O is a proton pump (84). Proton uptake in Cyt*c*O occurs through defined pathways. There are at least two such pathways (denoted by letters D and K), and a third,H-pathway, has been suggested to be operational in mitochondrial Cyt*c*Os. While the proton-uptake pathways have been characterized and studied in detail (see section below) there is at present little experimental support for a defined proton-exit pathway, even though attempts, mainly computational, have been made to identify such a pathway (85,86).

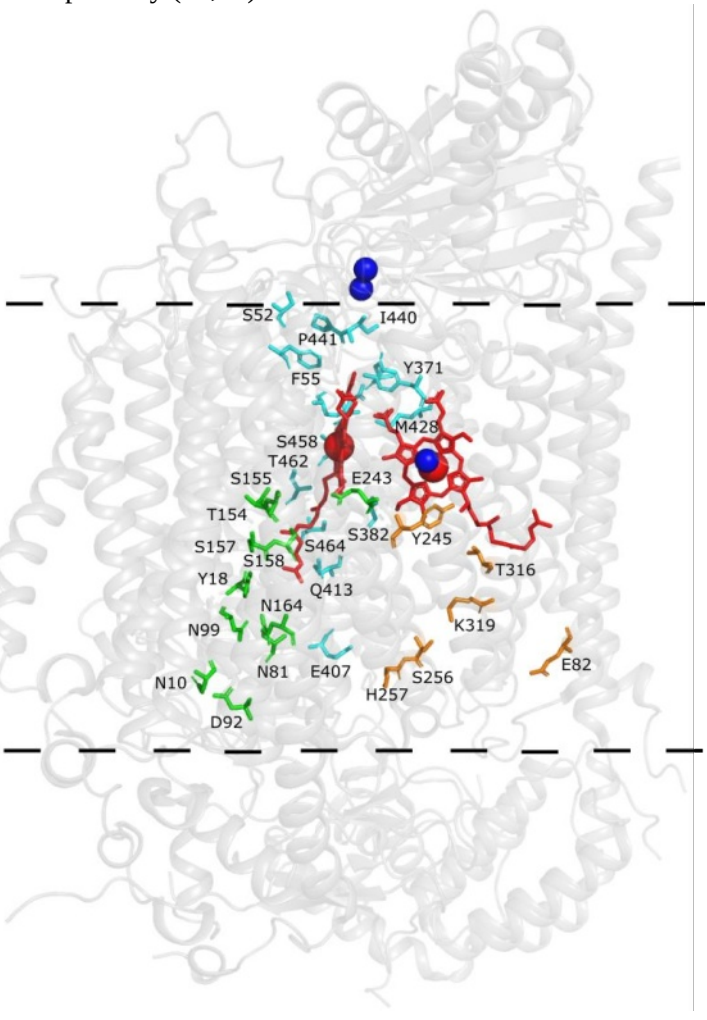

Figure 3. *Proton-transfer pathways in CytcO. The D-pathway residues are marked in green, K-pathway residues in orange and H-pathway residues in cyan. Hemes are colored red and copper ions in blue. The membrane is marked with a dashed line. The structure is based on S. cerevisiae CytcO structure, PDB entry 6HU9* (72).

D-pathway

The entrance of the D-pathway is a conserved aspartate residue (D92) located at the negative side of the membrane (Figure 3). The pathway continues through ~10 residues, and a series of water molecules (not shown in the figure) connected in a chain of hydrogen bonds, and ends at a glutamate (E243), located below the two hemes. This pathway has a dual function and transports both protons that are pumped and those that are used for water formation (39,87–89). Mutations of residues in the pathway result in impaired proton transfer or uncoupling, i.e. O_2 is reduced, but the reaction is not linked to proton pumping (90–93).

The glutamate at the end of the pathway is suggested to be the branching point for pumped protons and those used to form water, which is based on data from studies of structural variants of the D-pathway (93–95) as well as theoretical studies (96–98).

K-pathway

The K-pathway extends from the negative side of the membrane from a conserved glutamate residue (E82) through six residues including a lysine (K319) in the middle of the pathway, after which it is named, and ends at a tyrosine (Y245) located close to Cu_B (39,99) (Figure 3). The glutamate residue at the entrance is localized in subunit II, as opposed to all other residues of the pathway, which are localized in subunit I. The pathway is used for the uptake of protons involved in water formation only during the reductive phase of the catalytic cycle (87,100,101). It has also been suggested to play a role in charge compensation during the oxidative part of the catalytic cycle (102,103).

H-pathway

The presence of a third proton pathway in Cyt*c*O, referred to as the H-pathway, has been suggested, but only for the mitochondrial Cyt*c*Os (38,39). It is named after a histidine residue (H413 in *B. taurus*) at its entrance, located at the negative side of the membrane. The histidine residue is not conserved in *S. cerevisiae* (Q413, Figure 3). As opposed to the other two pathways, the H-pathway spans across the entire membrane from residue E407 to S52 (Figure 3) (39,104,105). An aspartate residue, which in the *B. taurus* Cyt*c*O is located at the top of the pathway (D51), (S52 in *S. cerevisiae*) was shown to undergo structural changes during reduction of CytcO. From these observations it was speculated that it has a role in proton pumping (104). Mutations of the D51 residue in *B. taurus* Cyt*c*O were reported to abolish proton pumping while retaining the ability to reduce O_2 (106,107). These results led the authors to conclude that the H-pathway is involved in proton pumping in *B.*

taurus Cyt*c*O, which would indicate a completely different pumping mechanism compared to the bacterial counterparts. The conclusion is considered controversial, partly due to the extensive work on the D-pathway in bacterial Cyt*c*Os indicating its role in proton pumping (91,92). In addition, it should be noted that the D-pathway is present also in the *B. taurus* Cyt*c*O (56). An alternative function for the H-pathway has been suggested where it is not directly involved in proton transfer, but acts as a dielectric well that compensates for charge changes during Cyt*c*O turnover (39,108,109).

Catalytic cycle

Under native conditions CytcO receives its electrons, one at a time, from cyt. *c*. As soon as two electrons have reached the catalytic site the enzyme binds O_2 and the reaction is initiated. It should be noted that my experiments on single Cyt*c*O turnover were performed with the four-electron reduced enzyme. There are some differences in the intermediate states formed in this experiment as compared to turnover where electrons are delivered to the Cyt*c*O one-by-one.

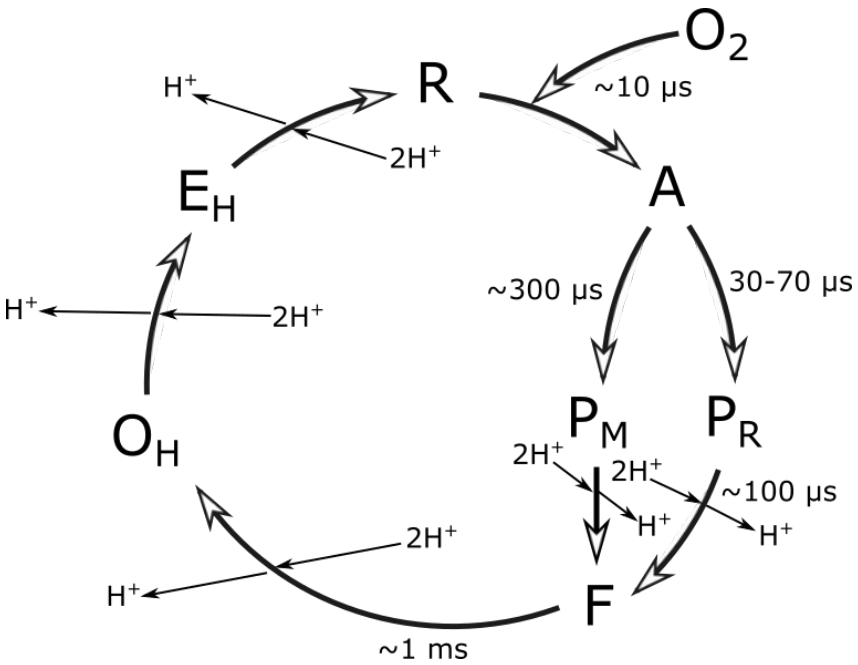

Figure 4. *The catalytic cycle of CytcO. The steps that involve proton uptake to the catalytic site and pumping are marked with arrows. In each of these steps two protons are taken up from the negative side, one for water formation and the other is pumped*

*across the membrane. The transition from **R** to **O_H** and **O_H** to **R** are termed the oxidative and reductive parts of the cycle, respectively*.

In state **R** [Fe^{2+}] [Cu_B^+] the catalytic site is in a reduced state with one electron at heme *a* and Cu_B, respectively, enabling ligand binding to the active site.

When oxygen binds to the catalytic site state **A** [Fe^{2+}-O_2] [Cu_B^+] is formed (110). The initial O_2 binding occurs with a time constant of ~10 µs at 1 mM O_2 (111,112).

After oxygen is bound, state **P** [Fe^{4+}=O^{2-}] [Cu_B^{2+}-OH^-] is formed. During formation of **P** the O-O-bond is broken and, an electron and an internal proton are transferred to the catalytic site. For the mixed-valence state, i.e. only the catalytic site is reduced at initiation of the experiment, an electron and a proton are donated from a tyrosine residue (Y245, see Figure 3) in the proximity of Cu_B and state **P_M** is formed (see Figure 4). When the fully reduced Cyt*c*O reacts with O_2 the electron is donated from heme *a* and the proton from the tyrosine mentioned above, resulting in the formation of the **P_R** intermediate. The time constants for formation of **P_R** and **P_M** are 30-70 µs and ~300 µs, respectively (112–115). The two states are spectrally indistinguishable from each other (116).

Formation of state **F** [Fe^{4+}=O^{2-}] [Cu_B^{2+}-OH_2] is associated with uptake of one proton to the catalytic site and the pumping of a proton across the membrane. Over the same time scale the electron at Cu_A equilibrates between Cu_A and heme *a*. The time constant for **F** formation is ~100 µs at physiological pH (93,112).

A reduced variant of **F**, called **F_R** [Fe^{4+}=O^{2-}] [Cu_B^+-OH_2] has been predicted by computational studies and verified by experimental studies (117–120). This intermediate is formed when the fourth electron reaches the catalytic site prior to the proton uptake required to form the oxidized species **O**. For detergent-solubilized enzyme this intermediate is expected to decay faster than the next step of the reaction, i.e. it cannot be observed. In **paper III** we show that **F_R** is formed with a time constant of ~5 ms in the presence of a membrane potential.

When the fourth electron reaches the active site a proton is taken up forming the oxidized state called **O** [Fe^{3+}-OH_2] [Cu_B^{2+}-OH^-]. This step is associated with proton pumping across the membrane and occurs in ~1 ms for detergent solubilized enzyme (95,112) and ~5 ms for membrane-reconstituted Cyt*c*O (10,11). In this step the proton uptake and pumping occur simultaneously (121,122).

The **O** state has been suggested two exist in two forms, a non-activated species **O,** called “resting” and an activated variant **O_H** termed “pulsed”. The

pulsed state is obtained after the resting state has gone through at least one turnover. In the resting state the energy coupled to the reductive part of the catalytic cycle is not sufficient to pump two protons across the membrane against Δp. It is currently not known what the structural difference between states **O** and $\mathbf{O_H}$ is. It was suggested that the redox potential of Cu_B may be elevated in state $\mathbf{O_H}$ as compared to **O** (123–125). However, results from a recent study have shown that this is not the case; the fifth electron entering Cyt*c*O after prior oxidation of the enzyme (forming the pulsed state) mainly ends up at Cu_A and heme *a* (126), i.e. not at Cu_B.

Transfer of one electron to the catalytic site of the oxidized Cyt*c*O renders the **E** state. The electron transfer is accompanied by proton uptake to the catalytic site and the pumping of a proton across the membrane. The same process, electron transfer accompanied by proton uptake and pumping, is repeated again and state **R** is formed.

pH-dependence of **F** and **O** formation

The reduction of O_2 to water by Cyt*c*O is a pH dependent process. For example, with the *R. sphaeroides* and *B. taurus* Cyt*c*Os the rate constants for **F** and **O** formation are both pH dependent. At more alkaline pH-values the reaction is slowed, which is expected because these steps are linked to proton uptake and pumping. The protonation state of the glutamate (E243 in *S. cerevisiae*, see Figure 3, corresponding numbering in *B. taurus* and *R. sphaeroides* is E242 and E286 respectively) at the end of the D-pathway determines the rate of the rate limiting step for proton transfer through the D pathway. This finding is based on studies of D pathway mutants in bacterial Cyt*c*Os (93,95). The apparent pK_a values of this glutamate was found to be 9.4 (93,127) and 8.3 (111) for Cyt*c*Os from *R. sphaeroides* and *B. taurus* respectively.

Regulation of CytcO by ATP

The ultimate function of cellular respiration is the production of ATP, which occurs continuously to drive cellular processes. The steady-state ratio of ATP/ADP in rat hearts is roughly 100 (i.e., in the 5 mM and 50 µM range, respectively (128,129)). Being the terminal oxidase in the respiratory chain, Cyt*c*O is a viable target for regulation of respiratory activity. One mode of regulation is by binding of ATP, which has been shown to regulate Cyt*c*O activity in organisms such as yeast (130) and *B. taurus* (131,132), whereas this effect is not seen in prokaryotes (133). In mitochondrial Cyt*c*Os ATP has an inhibitory effect while ADP has a stimulatory effect. None of these effects is influenced by the membrane potential (134,135).

Nucleotide-binding sites have been identified in Cyt*c*O in different subunits in *B. taurus* and *S. cerevisiae* (see Figure 5). In *B. taurus* subunits IV, VIa and VIII have been identified while in *S. cerevisiae* Cox13 binds the nucleotide (equivalent of VIa in *B. taurus* numbering) (136–138).

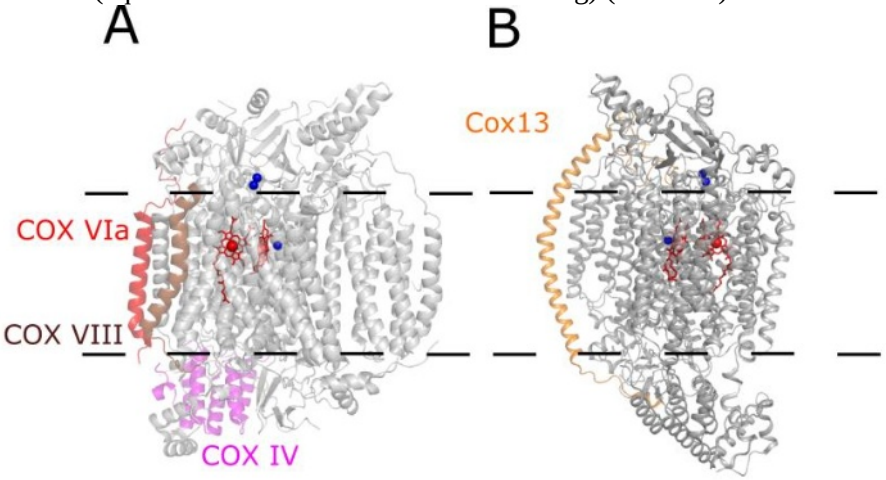

Figure 5. *ATP binding subunits in CytcO. Subunits reported to bind ATP/ADP are colored, hemes are colored red and copper ions blue. The membrane is marked by a dashed line.* ***A.*** *The subunits reported to bind ATP/ADP in B. taurus CytcO. The structure is based on PDB entry 5B1A* (78). ***B.*** *The subunit reported to bind ATP/ADP in S. cerevisiae CytcO. The structure is based on PDB entry 6HU9* (72).

Yeast flavohemoglobin

Yeast flavohemoglobin (yHb) is a 46 kDa water-soluble protein present in *S. cerevisiae*. The protein consists of a single subunit that has three distinct domains: a globin domain that harbors a *b*-heme, a flavin domain that contains a flavin adenine dinucleotide (FAD) co-factor and a NADH-binding domain (139). The protein has previously been reported to reside in the cytosol and mitochondrial matrix (140), but in **paper I** we show that it is also found in the IMS. The *b*-heme in yHb has an open ligand space enabling the binding of O_2, NO or CO. The time constants for recombination with CO are different from those typically observed with Cyt*c*O (141), which enables its identification in mitochondrial samples. Identification of yHb in mitochondria by monitoring CO recombination is more feasible compared to identification by its spectral signature since it is complicated to separate the *b*-heme signature from yHb compared to those in bc_1 complex and Complex II. The role of the enzyme is not known, but it can function as a nitric oxide oxidoreductase and it has been speculated that it has a role in protection against nitrosative and oxidative stress (140–144).

Methods

An important property of Cyt*c*O is the absorbance by its metal co-factors. The redox state of three of the co-factors can be monitored spectroscopically (heme *a*, heme a_3 and Cu_A). The absorbance signature of the metal co-factors are different depending on whether they are oxidized and reduced or upon ligand binding to heme a_3. Hence the reduction of O_2 to water, catalyzed by the Cyt*c*O can be followed in time. Also the kinetics of ligand binding to Cyt*c*O can be studied by following in time absorbance changes. These properties are utilized in two of the techniques presented below used to probe the active site and to study single turnover of Cyt*c*O. Multiple turnover is most easily investigated by polarographic measurements that monitor oxygen reduction over time.

When investigating Cyt*c*O using spectroscopic techniques in native membranes light scattering may pose a problem. There are solutions to this problem as described below.

Flash photolysis

Flash photolysis can be used to monitor the state of the catalytic site of Cyt*c*O. The technique utilizes the spectral changes upon binding of inert ligands, such as CO, to the enzyme. The CO ligand binds tightly to the catalytic site of Cyt*c*O when heme a_3 and Cu_B are reduced. Upon ligand binding the spectral properties of heme a_3 change. An anaerobic sample of Cyt*c*O is prepared. The sample is either completely reduced (all four co-factors) or two-electron reduced (mixed valence, only catalytic site reduced). The sample is then incubated with CO, which binds to the active site resulting in a spectral shift. The absorbance is monitored at a single wavelength that is characteristic for ligand binding. A laser flash is used to dissociate the CO from the active site. The CO ligand then re-binds to the enzyme, with a typical time constant of ~10 ms for Cyt*c*O (at 1 atm CO). The CO recombination kinetics is monitored at different wavelengths and the time constant can be fitted. These measurements can be used to probe the catalytic site, e.g. when comparing Cyt*c*O from wild-type and mutants, respectively. See Figure 6 for an example of a typical flash photolysis experiment.

Flow-flash

The flow-flash technique is used to study single Cyt*c*O turnover. Binding of O_2 to Cyt*c*O is fast (~10 µs at 1 atm O_2) and the enzyme is oxidized within about 1 ms. These rapid reaction rates present a challenge when studying time resolved kinetics. The dead-time of a conventional stopped-flow set-up is ~1 ms, i.e. the reaction cannot be resolved in time. The problem can be circumvented by using the flow-flash technique. When using this approach the fully reduced anaerobic Cyt*c*O with CO bound to its catalytic site is loaded into one of the syringes of a stopped-flow apparatus. The other syringe is loaded with an oxygen-saturated buffer (~1.2 mM). These two solutions are then mixed. Since CO is bound to the catalytic site, O_2 cannot react with Cyt*c*O. After mixing, the sample is subjected to a short laser flash (~10 ns), which dissociates the CO ligand from the catalytic site. Since CO rebinds in ~10 ms and O_2 binds to Cyt*c*O in ~10 µs the reaction with O_2 is initiated. The absorbance changes associated with the different redox states of the co-factors are then followed over time. For a schematic picture of the experimental setup and a typical flow-flash trace, see Figure 6.

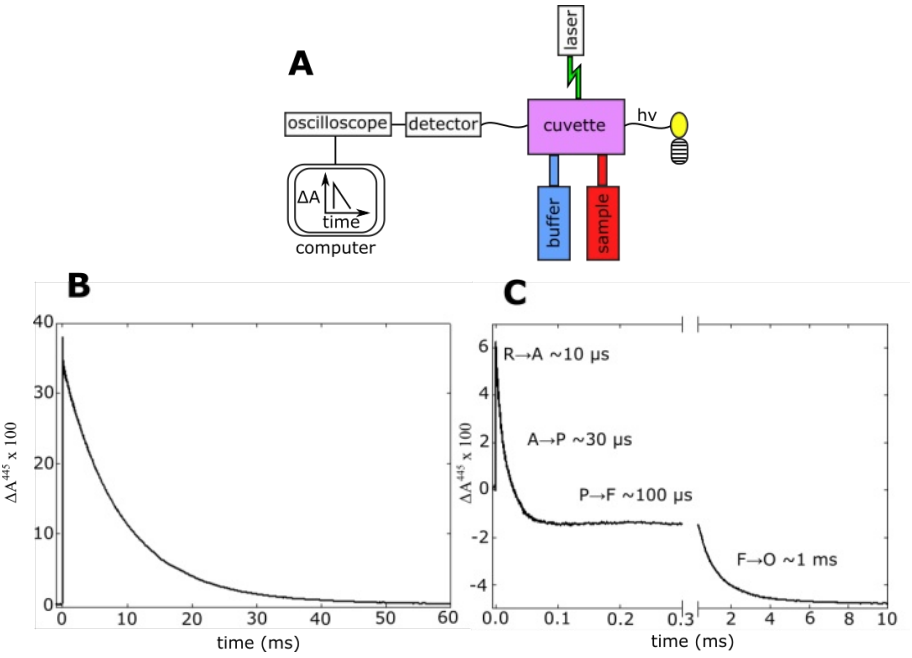

Figure 6. *Flash photolysis and flow-flash.* ***A.*** *A schematic view of the flow-flash technique.* ***B.*** *CO-rebinding to detergent solubilized CytcO from B. taurus.* ***C.*** *The reaction of detergent-solubilized CytcO from B. taurus with O_2, monitored using the flow-flash technique. Absorbance changes that correspond to transitions between the different intermediates, and their time constants, are marked in the figure.*

Multiple turnover

Multiple Cyt*c*O turnover can be investigated by measuring the rate of oxygen reduction i.e. the decrease in oxygen concentration. Typically, the oxygen reduction is measured over time in the presence of an excess of electron donors using a Clark-type electrode. The electron donor typically consists of ascorbate and a mediator, e.g. TMPD, ensuring that the delivery of electrons is not rate limiting. The reaction rate of Cyt*c*O can then be calculated.

Reducing light scattering of mitochondrial membranes

Light scattering from membranes may pose a problem when performing spectroscopic measurements. Dilution of the samples results in a decrease of light scattering, but also a decrease in the absorbance due to the lower concentration. A highly concentrated sample would yield a larger signal, but may result in drift due to aggregation, i.e. increased scattering, which reduces the signal-to-noise ratio. There are methods to reduce light scattering in native membranes.

The approximate size of mitochondria is 1 μm (145). One approach to form smaller particles that scatter less light is by sonication of mitochondria, which results in formation of sub-mitochondrial particles (SMPs) with a typical diameter of ~40 nm (146). The SMPs contain all the components of the inner mitochondrial membrane. For objects where the diameter is smaller than the wavelength of light, the intensity of the scattered light is proportional to the diameter of the object, i.e. a larger object scatters more light.

Another approach is to remove the OMM by decreasing the osmolarity of the buffer of the sample containing mitochondria. The ruptured OMM can be removed by centrifugation. There is a correlation between the absorbance of mitochondria and osmolarity where the absorbance decreases as the osmolarity decreases (147).

Main findings

In **paper I** we have studied Cyt*c*O from *S. cerevisiae* mitochondria with and without the outer membrane. Binding of the CO ligand to the active site as well as the reaction of Cyt*c*O with O_2 was studied. Studies of CO binding to the active site can provide information about structural perturbations of the active site, e.g. induced by a mutation. When studying CO-recombination in intact mitochondria we found that the CO-recombination was biphasic. One time constant (~12 ms) was assigned to CO binding to Cyt*c*O while the other (~270 ms) was suggested to be associated with CO binding to yHb, a small water soluble heme-containing protein. When the OMM was removed, monophasic CO-recombination was observed, attributed only to Cyt*c*O. These data suggested that yHb is localized to the intermembrane space in addition to the cytosol and matrix, which contrasts earlier reports. The yHb fraction in the matrix, which remains after OMM removal, is not observed to bind CO. This is most likely due to its inability to be reduced by the electron donors used, as opposed to the IMS fraction. The non-reduced yHb does not bind CO and, hence, no light-induced signal is observed. We also studied the reaction of Cyt*c*O with O_2 in two of the yeast strains used in our laboratory, W303 and BY474. A previous study had indicated differences in their reaction with O_2 (18). In contrast, we found that their reaction with O_2 is the same in a single Cyt*c*O turnover. With these results we have characterized the mitochondria to develop tools for studies of Cyt*c*O in native mitochondrial membranes.

In **paper II** we extracted Cyt*c*O from *S. cerevisiae* mitochondria without the use of detergents. Typically, purification of membrane proteins involves detergent solubilization of the membranes to extract the protein of interest. The protein is extracted into detergent micelles and most of the native lipids are lost in this process. Extracting Cyt*c*O from its native membrane has been known to alter its activity and it is hence desirable to use methods that retain the native lipid environment of the enzyme. In this study, we extracted histidine-tagged Cyt*c*O from *S. cerevisiae* mitochondria using the styrene maleic acid co-polymer (SMA). The SMA-extracted Cyt*c*O displayed the same behavior as Cyt*c*O in mitochondrial membranes in ligand binding to the active site and in its reaction with O_2. In addition, the native lipids found in *S. cerevisiae* mitochondria were also present in the lipids surrounding the extracted enzyme. With this method it is possible to study Cyt*c*O in a more native-like environment.

In **paper III** the effect of membrane potential on proton and electron transfer in Cyt*c*O were investigated. Under native conditions Cyt*c*O operates in the presence of a transmembrane potential that is generated by all proton-pumping complexes of the respiratory chain. Since Cyt*c*O transfers charges, i.e. electrons and protons, perpendicular to the membrane surface, it may influence the kinetics of these reactions. However, most studies on the reaction mechanism have been performed with detergent-solubilized enzyme in the absence of a membrane potential. We studied the effect of a membrane potential on Cyt*c*O function in SMPs. A transmembrane charge gradient was generated by addition of ATP to the SMPs, resulting in proton pumping to the interior of the SMPs by ATP-hydrolysis. The single-turnover reaction of the fully reduced Cyt*c*O with O_2 was investigated under these conditions in the presence and absence of uncouplers (valinomycin and FCCP). We found that proton transfer, but not electron transfer was affected by the membrane potential. Furthermore, we found that in the presence of a membrane potential a reduced ferryl intermediate, $\mathbf{F_R}$, was formed with a time constant of ~5 ms before the final oxidation occurred. The formation of $\mathbf{F_R}$ has previously been proposed, but without a membrane potential it decays faster than formation of the oxidized state, **O**. Hence, it could not be observed experimentally. With these results we have unraveled the Cyt*c*O reaction mechanism in the presence of a membrane potential.

Paper IV addresses the structure of subunit Cox13 from *S. cerevisiae* Cyt*c*O and its interaction with ATP. The production of ATP is the ultimate function of the respiratory chain. Being the terminal electron acceptor, Cyt*c*O plays an important role in the regulation of ATP-production in cells. Previous studies have shown that Cyt*c*O is regulated by binding of ATP and ADP. In *S. cerevisiae* subunit Cox13 of Cyt*c*O has been shown to interact with ATP. We have solved the structure of subunit Cox13 using NMR and identified its ATP-binding sites. We found that these sites are located in the C-terminus part of the subunit. In addition, we also showed that ATP-binding to Cox13 has an inhibitory effect. If Cox13 is removed from Cyt*c*O, addition of ATP can stimulate the activity by a factor of 2.5, which is consistent with earlier observations (138).

In **paper V** we have investigated proton-transfer pathways in Cyt*c*O from *S. cerevisiae*. The core part of Cyt*c*O is highly conserved from bacteria to yeast and mammals with the main difference being the additional supernumerary subunits in the higher organisms. Presumably the mechanism of O_2 reduction is also conserved. The composition of the yeast Cyt*c*O resembles that of higher organisms and it is possible to mutate residues that have been investigated previously in bacterial model systems. There are two proton pathways in Cyt*c*O used for uptake during the reaction (the D- and K-pathway). A third proton pathway, the H-pathway, has been proposed to be functional in eukaryotes and it has been suggested that all pumped protons are taken up through

this pathway in *B. taurus* Cyt*c*O. If true, these data would indicate a completely different proton-uptake mechanism in bacteria and mitochondria, respectively. *S. cerevisiae* Cyt*c*O also harbors an H-channel, and to address the role of this H-pathway in mitochondrial Cyt*c*O we have studied four mutants in *S. cerevisiae* Cyt*c*O (two D-pathway mutants, N99D and I67N, and two H-pathway mutants S458A and S358A) and one D-pathway mutant in *R. sphaeroides* (M107C). Our main findings indicate that the yeast mutants behave similarly to their bacterial counterparts. We showed that mutations in the D-pathway of *S. cerevisiae* Cyt*c*O impair reaction steps linked to proton uptake and pumping, whereas mutations in the H-pathway have no effect. This suggests that the H-pathway is not directly responsible for proton uptake and pumping in *S. cerevisiae* Cyt*c*O. These results indicate that the oxygen-reduction mechanism with accompanying proton uptake and pumping is indeed highly conserved from bacteria to yeast.

Populärvetenskaplig sammanfattning

Alla levande organismer är beroende av energi för sin fortlevnad. Energi kan fås från solen via fotosyntes som används av bl.a. växter och alger eller genom att elektroner extraheras ur kemiska föreningar, t.ex. kolhydrater och fett. Elektronerna levereras till en slutlig elektronacceptor som reduceras (tar emot en elektron). En vanlig elektronacceptor är syre. Det syre vi andas reduceras till vatten. För att reducera syre till vatten måste bindningen mellan de två syremolekylerna i syrgas brytas. När detta sker frigörs energi som kan driva andra reaktioner. Denna process kallas oxidativ fosforylering och sker hos både bakterier, jäst och människor.

Hos högre organismer och varelser som jäst och människor sker denna process i en särskild del av cellen som kallas för mitokondrien. Mitokondrien är en egen mikromiljö inuti cellen som omgärdas av två membran (ett skal av fettmolekyler som selektivt släpper igenom molekyler). I det innersta av de två mitokondriemembranen sitter 3-4 proteinkomplex (molekylära maskiner som utför arbete i celler) numrerade I-IV. Elektroner rör sig från ett komplex till ett annat i en specificerad ordning tills de når det sista komplexet, komplex IV. Dessa komplex kallas elektrontransportkedjan. I denna process transporteras plusladdade vätejoner från insidan av det inre mitokondriemembranet till utrymmet mellan de två membranen. Detta laddar upp det inre mitokondriemembranet som ett batteri då utrymmet mellan membranen blir mer positivt laddat. Då universum strävar efter maximalt kaos så vill det gärna jämna ut laddningsskillnaden över det inre mitokondriemembranet. Vätejoner är plusladdade och kan därför inte röra sig fritt över cell membran. Enda vägen tillbaka över membranet går igenom ett protein som heter ATP-syntas. När vätejonerna rör sig tillbaka genom ATP-syntaset roterar de likt ett vattenhjul. Denna rotation driver tillverkningen av en molekyl som heter ATP. ATP kan användas av cellen för att driva energikrävande processer. Denna avhandling behandlar regleringen av komplex IV även kallat cytokrom *c* oxidas (Cyt*c*O).

Cyt*c*O reducerar syre till vatten. I processen pumpas även fyra vätejoner över mitokondriens innermembran. Många studier av Cyt*c*O reaktion med syre har utförts på det extraherade proteinet, d.v.s. det har isolerats från mitokondrien och tagits ut ur sin naturliga miljö. Det är därför av intresse att studera Cyt*c*O i en mer naturlig miljö.

I det första arbetet har vi undersökt inbindning av kolmonoxid till Cyt*c*O i jästmitokondrier med eller utan yttermembran. Kolmonoxid kan ge oss information om hur inbindingen av molekyler, t.ex. syre, går till utan att någon reaktion sker. Detta ger oss en möjlighet att undersöka hur Cyt*c*O beter sig i olika miljöer. Vi fann att inte bara Cyt*c*O band kolmonoxid i jästmitokondrierna utan även ett ytterligare, flavohemoglobin. Om yttermembranet tas bort från mitokondrierna så kan flavohemoglobinet tvättas bort och enbart kolmonoxidbindning till Cyt*c*O kan studeras.

I det andra arbetet har vi extraherat Cyt*c*O från mitokondrier med en förening som kallas SMA. Med SMA kan vi extrahera Cyt*c*O omringat av sin naturliga membranmiljö. Vi har visat att SMA extraherat Cyt*c*O beter sig likadant som Cyt*c*O i mitokondrier med avseende på kolmonoxidinbindning och vid reduktion av syre till vatten.

I det tredje arbetet undersöker vi hur Cyt*c*O reaktion med syre påverkas av laddningsskillnaden över membranet på detaljerad nivå. Vi studerade reaktionen med och utan en laddningsskillnad över membranet. Vi fann att de steg som inbegrep elektrontransport inte påverkades men ett utav de senare reaktionsstegen blev markant långsammare. Detta steg inbegriper vätejonspumpning över membranet och vi drar därför slutsatsen att vätejonspumpningen, men inte elektrontransporten, påverkades av laddningsskillnaden.

I det fjärde arbetet är vi intresserade av hur ATP påverkar Cyt*c*O reaktion med syre och specifikt i en särskild del utav Cyt*c*O från jäst som kallas för Cox13. Vi har renat fram Cox13 och bestämt dess tredimensionella struktur. Vi har även identifierat de delar av Cox13 där ATP binder in.

I det femte arbetet har vi studerat protonpumpningsmeaknismen i Cyt*c*O från jäst. Vi studerat fyra varianter av Cyt*c*O från jäst samt en bakteriell variant och deras reaktion med syre vid olika vätejonskoncentrationer. I två av varianterna har vi undersökt en föreslagen protonpumpningskanal, kallad H-kanalen, som föreslås vara viktig i Cyt*c*O från mitokondrier men inte spela någon roll i bakterier. Vi fann att Cyt*c*O från jäst beter sig liknande som sin bakteriella motsvarighet vad det gäller vätejonspumpning och att H-kanalen inte verkar fylla någon direkt roll i transporten av vätejoner i jäst.

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