Membrane effects on proton transfer in cytochrome c oxidase

Linda Näsvik Öjemyr
To David
Abstract

The biological membrane is composed of lipids and proteins that make up dynamic barriers around cells and organelles. Membrane-spanning proteins are involved in many key processes in the cell such as energy conversion, nerve conduction and signal transduction. These proteins interact closely with lipids as well as with other proteins in the membrane, which modulates and affects their structure and function. In the energy-conversion process, membrane-bound proton-transport proteins maintain an electrochemical proton gradient across the mitochondrial inner membrane or the cytoplasmic membrane of bacteria. This gradient is utilized for ATP synthesis or transport of ions and molecules across the membrane. Results from earlier studies have shown that proton transporters are influenced by their environment.

Here, one of these proton transporters, cytochrome c oxidase, was purified and reconstituted into liposomes or nanodiscs and membrane effects on specific proton-transfer processes were studied. In these studies we observed that the membrane accelerated proton transfer to the surface of cytochrome c oxidase and that there was a protonic link, via a Glu residue that mediates proton transfer from the membrane surface to a proton-transfer pathway in this protein. In addition, the membrane was shown to modulate specific internal electron and proton-transfer reactions.

The results from these studies show that the membrane composition influences transmembrane transport. Consequently, our understanding of these processes requires investigation of these transporter proteins in different membrane-mimetic systems of variable and well-defined composition. Furthermore, the data show that membrane surfaces facilitate lateral proton transfer which is presumably essential for maintaining high efficiency in energy conversion. This is particularly important in organisms such as alkaliphilic bacteria where the driving force of the electrochemical proton gradient, between the bulk solution on each side of the membrane is not sufficient for ATP synthesis.
List of publications

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


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Additional publication

Lee, H.J., Öjemyr, L., Vakkasoglu A., Brzezinski, P. and Gennis, R.B. (2009) Properties of Arg481 mutants of the \( a_{3}\)-type cytochrome \( c \) oxidase from *Rhodobacter sphaeroides* suggest that neither R481 nor the nearby D-propionate of heme \( a_{3} \) is likely to be the proton loading site of the proton pump. *Biochemistry 48*, 7123-31.
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Abbreviations and nomenclature

Abbreviations

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<tr>
<td>CL</td>
<td>Cardiolipin</td>
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<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>CytO</td>
<td>Cytochrome ε oxidase</td>
</tr>
<tr>
<td>Cytε</td>
<td>Cytochrome ε</td>
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<tr>
<td>DCMU</td>
<td>3-(3,4-diclorophenyl)-1,1-dimetylurea</td>
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<tr>
<td>DGTS</td>
<td>Diacylglycerl-N,N,N-trimethylhomoserine</td>
</tr>
<tr>
<td>MSP</td>
<td>Membrane scaffold protein</td>
</tr>
<tr>
<td>OL</td>
<td>Ornithine lipid</td>
</tr>
<tr>
<td>QL</td>
<td>Glutamine lipid</td>
</tr>
<tr>
<td>n-side</td>
<td>the more negative side of the membrane</td>
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<tr>
<td>p-side</td>
<td>the more positive side of the membrane</td>
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<tr>
<td>PA</td>
<td>Phosphatic acid</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidyglycerol</td>
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<tr>
<td>pmf</td>
<td>proton motive force</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<tr>
<td>SQDG</td>
<td>Sulfoquinovosyl-diacylglyceride</td>
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Nomenclature

If not otherwise indicated, distances and amino-acid numbering are based on the structure and amino-acid sequence of the aas cytochrome ε oxidase from *Rhodobacter sphaeroides*. Superscripts in connection to amino acids depict subunit location, e.g.

*Asp*<sup>132</sup> denotes aspartate 132 in subunit I
*Asp*<sup>132</sup>Ala denotes a replacement of Asp<sup>132</sup> by alanine
Introduction

Biological membranes make up the boundaries of life, separating the cell interior from its surroundings and in eukaryotic cells compartmentalize specific cellular processes. It is an essential function of the membrane that it is not just an encapsulating permeability barrier, but rather makes up a dynamic boundary through which, e.g. signals can be transmitted, nutrients imported and waste/harmful products exported. Membranes are composed of lipid bilayers, with a hydrophobic core and hydrophilic surface, and proteins, where the latter play important roles in a broad range of processes. There are membrane-spanning proteins that act as channels, transporters or pumps for molecules and ions across the membrane, as well as receptor proteins that can transduce environmental signals into the cell. A number of specific membrane proteins build up ion gradients utilized in nerve conduction or maintain ion gradients for energy-conversion processes.

Lipids and proteins interact in membranes, and the lipids are important for maintaining structural assemblies and function of integral membrane proteins. In energy transducing membranes, some lipids have specific roles for protein dimerization and super-complex formation. In addition, the presence of lipids is needed for functional activity in proton transporters located in these membranes. In the work summarized in this thesis, membrane effects on specific proton-transfer processes were studied. These processes include proton transfer to one of the proton transporters in the respiratory chain (Paper I) and proton uptake into the two proton-transfer pathways in a bacterial cytochrome $c$ oxidase (Cyt$cO$) (Paper II-IV).
The biological membrane

There is a large variation in mixtures of lipids and proteins that make up biological membranes. Some components can be found in all types of membranes and across species, whereas others are specific to certain membranes or patches of membranes. A common theme though is that these components make up dynamic entities where lipid-lipid, lipid-protein and protein-protein interactions take place. The properties of the two main components, lipids and proteins, and their interactions are discussed in a broader perspective in this chapter whereas the following chapters are focused on specific components and interactions in energy-transducing membranes.

Properties of phospholipid bilayers

Membrane lipids are amphipathic molecules with hydrophilic head groups and hydrophobic acyl chains that self-assemble in aqueous environments due to the hydrophobic effect. In bilayers the hydrophilic parts make up an interface towards the aqueous environment, shielding off the hydrophobic part. The thickness of the hydrophobic part of the membrane is usually 25-30 Å. There is a considerable structural variation in the hydrophobic part of bilayers, such as the acyl chain length, degree of saturation, branching of chains and linkage between the chain and head group, features that all affect the fluidity in the bilayer. Membrane fluidity can be divided into two states, either fluid liquid crystalline or frozen gel state, the former being favored at physiological temperatures. The transition between these two states in a membrane occurs at the transition temperature of the lipids. An increased number of unsaturated acyl chains leads to a less ordered membrane and a lowered transition temperature. For saturated chains, an increased chain length promotes a more ordered bilayer and results in an increase in transition temperature. A similar effect is also found when cholesterol is present in the bilayer. In systems of mixed lipid species, as in most biological membranes, phase separation between the lipids occur when the transition temperature of the different lipid components differ by more than 20 °C. In metabolic membranes the transition temperature can approach values almost 60 °C lower than the physiological temperature (f).
It is not only the acyl chain region that influences the bilayer. Lipid head groups have different net charges at physiological pH and different ability to form hydrogen bonds. The size of the head group, in combination with the acyl chains of the lipid, yields a certain shape. Cylindrically-shaped lipids promote bilayer formation and are therefore referred to as bilayer-prone lipids. In addition to these bilayer-prone lipids, lipids with a conical shape are found to high extent in biological membranes. Even though conical lipids, classified as non-bilayer prone lipids, have a preference for spontaneously inducing negative curvature in monolayers that promote hexagonal structures, they do form bilayers when mixed with bilayer-promoting lipids (1, 2). In Escherichia coli membranes the mole fraction of the non-bilayer prone phosphatidyl-ethanolamine (PE) can be as high as 70 % and still bilayers are formed (3, 4). Integral membrane proteins also promote formation of bilayer structures of non-bilayer prone lipids (2, 5).

When non-bilayer prone lipids are forced to adopt bilayer structures, a curvature stress is imposed within the membrane. The resulting pressure profile induces a large negative pressure in the glycerol backbone region and a lower order and packing density in the end of the chains. However, the shape of a lipid is not rigid, but rather influenced by surrounding factors such as pH and salt concentration (1, 6–8). For example, it has been shown, using molecular-dynamic simulations, that the area of 1-palmitoyl-2-oleoyl:PG and 1-palmitoyl-2-oleoyl:PC lipids in a bilayer decreases with increasing salt concentration, giving a closer packing that leads to an increased thickness of the membrane (7).

**Membrane proteins**

Biological membranes are not only composed of lipids, but also of membrane proteins, both peripheral and integral ones. In contrast to peripheral membrane proteins, whose electrostatic interactions with the membrane can be disrupted, integral membrane proteins are embedded in the lipid bilayer. There are two classes of integral membrane proteins, β-barrel proteins are found in the outer membrane of gram negative bacteria, mitochondria and chloroplasts, and α-helical proteins are situated in the inner membrane of these bacteria and organelles as well as in membranes of gram positive bacteria, archaea and all other membranes in eukaryotic cells.

The proteins discussed further in this thesis are integral α-helical membrane proteins. They have a membrane-spanning part in form of α-helixes with hydrophobic amino acids making up the surface of the membrane-spanning part of the protein. At the membrane-water interface region there is a
preference for aromatic residues, foremost Trp. A higher degree of charged residues can be found in loop regions that face towards aqueous environments.

Lipid-protein interactions in the membrane

Proteins and lipids in the membrane do not act as separate entities; instead they interact and influence one another. When it comes to lipid-protein interactions, lipids are classified according to their interaction pattern with integral membrane proteins. Annular lipids surround integral membrane proteins and form a solvation shell. It is suggested that as long as the number of lipids around a given protein is above or the same as the number of annular lipids, the activity can remain unaltered (6).

Non-annular lipids associate within proteins as cofactors or can be found between subunits in protein complexes, and between monomers in larger assemblies. Disruption of these specific interactions can lead to dissociation of protein complexes or inactivation of their enzymatic function. The third class is referred to as bulk lipids, and these are the lipids that at a given time do not interact with a protein. However, bulk lipids are not in isolated patches, but they are rather in rapid exchange with the annular lipids (reviewed in (2, 9)).

Lipids influence protein function via specific interactions, for example via head group motifs or sterol binding sites on protein surfaces (10). In addition, hydrophobic matching between the bilayer and hydrophobic parts of transmembrane segments has been shown to affect the activity of channels, pumps and transporters. To prevent hydrophobic mismatch the lipids can be distorted, either stretched or compressed, to match the hydrophobic transmembrane segment. Furthermore, transmembrane segments can both tilt and change packing mode (2, 11).

In addition to hydrophobic mismatch, some membrane proteins are shown to be affected by changes in membrane tension and lateral pressure (2, 12). For other proteins, no functional effects have been detected upon changes of the lateral pressure in the membrane (2). Furthermore, membrane properties can affect protein topology and function (4, 13). In the following chapters the properties of membrane surfaces are discussed in more detail and how these properties affect proton transfer processes.
Energy-transducing membranes

One class of membrane proteins that is of great interest includes those that are involved in energy-transduction processes, which comprise conversion of chemical or light energy to ATP synthesis e.g. during oxidative-phosphorylation and photo-phosphorylation. Enzymes involved in these processes have been under investigation for a long time and for many of them high resolution x-ray crystal structures are available, often revealing specific interactions between proteins and lipids.

Eukaryotic membranes that harbor protein components involved in oxidative phosphorylation and photo-phosphorylation, have their own specific lipid composition. The mitochondrial cristae membrane, where oxidative phosphorylation takes place, is composed of approximately 20% cardiolipin (CL). Several roles have been suggested for the high content of CL in the cristae membrane. For example, invaginations similar to those seen in the cristae membrane were observed upon increasing the proton concentration by acid injections at the surface of CL-doped giant liposomes (14). This observation, in combination with a bilayer asymmetry of the non-bilayer prone CL (the concentration of CL is higher in the outer than in the inner leaflet (15)) suggests that CL might promote invaginations of the cristae membranes. Another role proposed for CL is to act as an interaction partner in dimer formation of respiratory-chain complexes as well as a “molecular glue” between different complexes in super-complex assemblies (16, 17). Removal of CL from yeast complex III led to inactivation and destabilization of the protein, which was reversed by addition of CL (16).

For non-photosynthetic eukaryotes, phosphatidyglycerol (PG) is only found in mitochondrial membranes and its presence is suggested mainly to depend on its role as precursor in CL synthesis (18). In thylakoid membranes of chloroplasts, where complexes involved in photo-phosphorylation are situated, the most common lipids are glycolipids and the major phospholipid present is PG (18). This lipids is also found in x-ray crystal structures of photosystem I and II reaction centers (19). The effect of PG depletion was more pronounced in photosystem II reaction centers where electron transport between Q$_A$ and Q$_B$ sites was inhibited (in the absence of PG) in the same way as seen when the conventional photosystem II inhibitor, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), is used (18).
The membrane composition in the purple non-sulfur bacterium *Rhodobacter sphaeroides*, capable of both photo-phosphorylation and oxidative phosphorylation, changes with growth conditions and metabolic process utilized (20–23). During phototrophic growth or phosphate-starving conditions glycolipids are predominant just as in thylakoid membranes, whereas during aerobic growth PE, PG and phosphatidylecholine (PC) are the major components. Interestingly though, during aerobic growth under low phosphate conditions both CL and PG are almost as abundant as during regular phosphate conditions, whereas PE and PC were not detected in the membrane. This indicates that for synthesis of phospholipids, CL, and its precursor PG, are prioritized, whereas the other phospholipids readily can be interchanged for non-phospholipids (23).

To investigate the role of CL during oxidative growth, a CL-synthase deletion strain of *R. sphaeroides* was engineered in the laboratories of Bening and Fergusson-Miller (22). When grown in the presence of phosphate, the amount of PE increased and the amount of PG more than doubled in the deletion strain. Furthermore, CL was still detected although in reduced amounts. Taken together, it was suggested that in the CL deletion strain under low phosphate conditions, negatively charged sulfoquinovosyl-diacylglyceride (SQDG) and glutamine lipids (QL) could replace PG and CL, while ornithine lipids (OL) and diacylglyceril-N, N-trimethylhomoserine (DGTS) could replace the neutral PE and PC, respectively (23). Still, these changes in the lipid profile did not influence the growth of *R. sphaeroides*, or the expression and stability of respiratory-chain complexes (22, 23). Di-oleoyl (18:1\(\Delta^9\)) chains were the most dominant species in the membrane at growth at sufficient phosphate levels (23).

**Aerobic respiratory chain**

In catabolic reactions carbohydrates, proteins and fats are degraded and the energy released is conserved by reduction of electron carriers, NAD\(^+\) to NADH and FAD\(^+\) to FADH\(_2\), e.g. in complex II. These electron carriers in turn donate electrons to the respiratory chain located in the mitochondrial cristae membrane of eukaryotes or inner membrane of aerobic bacteria. In mammalian mitochondria (figure 1), upon oxidation of NADH at complex I, two electrons are transferred via Fe-S clusters to a quinone-binding site. During reduction of quinone to quinol two protons are taken up from the negative (n) side of the membrane. Similarly, FADH\(_2\) in complex II is oxidized and electrons are used for reduction of quinone to quinol. Quinols diffuse in the membrane to complex III, and electrons are donated via the Q-cycle one-by-one to water soluble cytochrome c (Cyt\(c\)) and protons are released at the
positive (p) side of the membrane. Reduced Cyt c docks at the p-side surface of complex IV and the electrons are transferred via red-ox active co-factors to the catalytic site where oxygen is reduced to water.

**Figure 1.** Respiratory chain complexes and ATP synthase. Electron transfer through the respiratory chain complexes is coupled to proton uptake from the n-side and proton release on the p-side. This proton translocation maintains the proton electrochemical gradient that is utilized for synthesis of ATP from ADP and inorganic phosphate (Pi). Electron and proton transfer are indicated with black dashed or blue arrows, respectively.

This transfer of electrons is an energetically downhill process that is coupled to proton translocation from the n-side to the p-side of the membrane, for maintaining a proton electrochemical gradient that drives ATP synthesis and transport of ions and molecules across the membrane. In complex I, the reduction of quinone in the peripheral part induces conformational changes in the membrane-embedded part where protons are pumped. The energy released in the exergonic reduction of oxygen to water in complex IV is utilized to pump protons from the n-side to the p-side (24). In addition, electron transfer from the p-side and proton transfer from the n-side to the catalytic site for oxygen reduction to water in complex IV is electrogenic and contributes to the proton electrochemical gradient. Current estimations indicate that a total of 10 protons is transferred across the membrane for every two electrons released from a NADH molecule oxidized at complex I. However, a recent re-examination of the previously estimated pumping stoichiometry for complex I suggested that this complex pumps three rather than four protons per two electrons, which would yield a total of 9 protons translocated across the membrane for each NADH oxidized (25).
Coupling of electron transfer in the respiratory chain to ATP synthesis

The exergonic electron-transfer reactions in the respiratory chain are coupled to the endergonic phosphorylation of ADP to ATP, via the proton electrochemical gradient across the membrane. According to the chemiosmotic theory, as postulated by Mitchell, the energy stored in the proton electrochemical gradient, the proton motive force \( \text{pmf} \), is composed of an electrical component \( \Delta \psi \) and the pH difference across the membrane \( \Delta \text{pH} \):

\[
\text{pmf} = \Delta \psi - 2.3 \frac{RT}{F} \Delta \text{pH}
\]

(1)

Here, the membrane acts as a permeability barrier where proton transporters and consumers are situated. Proton transporters translocate protons from the bulk solution on the \( n \)-side to the bulk solution on the \( p \)-side, which results in a pH and charge difference between the two solutions. The \( \text{pmf} \) drives transport of protons by proton consumers in the opposite direction (figure 2A) (26).

![Figure 2](image)

**Figure 2.** Proton transfer in oxidative phosphorylation. A) Mitchell’s chemiosmotic model for proton transfer from bulk to bulk solution yielding a \( \text{pmf} \) across the membrane B) Williams’ localized model, where protons ejected from the surface of proton transporters diffuse laterally along the membrane surface to a proton consumer. Protons are shown as blue spheres and proton transfer directions are indicate with blue dashed arrows.

A slightly different model of coupling in oxidative phosphorylation was proposed by Williams (27). In this model, protons ejected from proton transporters would instead of equilibrating with the bulk solution remain at the membrane surface and diffuse laterally along the surface to proton consumers. This scenario would yield a localized coupling between the respiratory chain and ATP synthase (figure 2B) that will provide a more energy-efficient oxidative phosphorylation where protons translocated across the membrane are not lost to the surrounding solution. Lateral proton transfer along membrane surfaces is discussed below.
Proton transfer along membrane surfaces

Proton diffusion along surfaces has been investigated in theoretical as well as in experimental studies. The results from theoretical studies showed that the properties of the surface water differ from those of bulk water, due to the presence of polar lipid head groups (28, 29). This difference in properties was suggested to create a potential barrier that led to retention of protons on the membrane surface, which in turn promoted lateral proton diffusion on a faster time scale than equilibration of the protons between the membrane surface and the bulk solution (30–33). Proton transfer to the bulk solution was accelerated by addition of mobile buffers, which diminished the energy barrier (32, 34). With increasing negative charge of the mobile buffer, higher concentrations were needed for acceleration of proton transfer from the membrane surface to the bulk solution, which was interpreted as that the barrier increases with an increased net charge of the ion that passes the barrier (32, 35). Furthermore, in membrane-embedded enzymes where highly charged ions, such as ATP, that are used in catalysis, the catalytic site is located at a distance from the membrane-water interface, above the barrier. In this way highly charged ions do not need to pass through the barrier, and this provides a more efficient/rapid catalysis (29). When it comes to protons, it was suggested from simulations under non-equilibrium conditions, that the barrier is higher for proton transfer in the direction from the membrane surface to the bulk solution, than in the opposite direction (28).

Membrane surface properties that affect lateral proton diffusion

The membrane-surface composition determines the kinetics of lateral proton transfer, which is affected by the pKₐs of the lipid head groups and concentration of buffering groups (i.e. groups that have a pKₐ close to the pH of the solution). Negatively charged lipids, such as PG (36) and phosphatidylserine (PS) (33) promoted proton diffusion along the surface and the rate increased upon introduction of buffering groups, e.g. phosphatic acid (PA) (36). When no high-pKₐ or negatively charge groups were present, as in the case of pure PC liposomes, direct proton transfer to bulk solution was detected (33, 37). However, in other studies, lateral proton diffusion was also detected on the surface of PC liposomes, although with a lower rate compared to the one observed when PG liposomes were used (36, 38). This effect was suggested to be due to the negative charge of the PG lipids on the membrane surface. With increased salt concentration in solution the rate of lateral proton diffusion approached that obtained with PC liposomes (38). In a recent study, it was observed that varying the lipid head-group properties did not have any influence on proton diffusion along the membrane surface, as seen in earlier studies (39). However, it was pointed out that in these experiments the surface...
may be saturated with protons, which would yield the same results independently of lipids used (40).

In addition to the effect of lipids, membrane proteins also contribute to the properties of the membrane surface. Introduction of membrane proteins with surface-exposed carboxyl groups resulted in retention of protons at the surface, and may promote lateral proton transfer (34). It was observed that the average distances between acidic residues on the p-side surface of proton transporters are shorter than on the surface of other membrane proteins involved in oxidative phosphorylation and photo-phosphorylation. This shorter distance was proposed to promote lateral proton diffusion along surfaces of proton transporters (35).

Relevance in biological systems

From experiments with mitochondria it has been suggested that the proton coupling may alternate between chemiosmotic and localized modes, depending on cellular conditions (41). It has been proposed that the key to this change in coupling mode would be the size of the p-side surface. When the p-side surface is small, as is the case on the inside of liposomes, the pumped protons would quickly saturate the surface and protons would equilibrate rapidly between the surface and the bulk solution. On the other hand, if the p-side surface is large it would not promptly be saturated with protons, which would lead to retention of protons at the surface, rendering a lower surface pH compared to the pH in the bulk solution. This phenomenon has been observed in experiments with energized sub-bacterial E. coli particles, which had a right-side out orientation and thereby a large p-side surface. In experiment with these particles the n-side pH was kept at 7.5, while varying the p-side pH and the ΔΨ was -80 mV, independent of p-side pH. Upon diminishing the ΔpH component by increasing the p-side pH from 5.5 to 7.5 will, according to equation 1, give a pmf of -80 mV. Nevertheless, the effective pmf, calculated from the level of substrate import into the particles corresponded roughly to -150-160 mV, which gave an estimated surface pH of 6.2-6.3 (29, 42, 43). This discrepancy in the calculated and effective pmf values can provide an explanation for an efficient energy conversion in alkaliphilic bacteria, as describe below.

For alkaliphilic bacteria, which have a large p-side surface, a pure chemiosmotic coupling would not be favorable for an efficient energy conversion. These bacteria are found in high-pH environments and their cytoplasmic pH can be 2-3 pH units lower than the exterior pH, which give a large ΔpH component with the opposite sign compared to the one found across the mitochondrial membrane. Some of these bacteria utilize sodium ions instead of protons for transport across the membrane (44). However, these species, including Bacillus firmus, do use protons as the coupling ion in energy transduction. This
facultative alkaliphile can grow in the pH range 7.5-11. When grown at pH 10.5 the cytoplasmic pH was kept at values of 8.3, i.e. ΔpH = 2.2. The ΔΨ increases with increased p-side pH, but without fully compensating for the smaller ΔpH component. With these values pmf would be ~ -50 mV, compared to ~ -140 mV, observed when the p-side pH was 7.5 (45, 46). The phosphorylation potential, (ΔGₚ, which reflects the [ATP]/[ADP][Pi]) at both pHs is in the same range (47) and the relationship between ΔGₚ and pmf, which reflects the number of protons per ATP molecule synthesized, indicates that ~3 and ~10 protons are needed per ATP synthesized at pH 7.5 and 10.5, respectively (46, 47). A change in proton per ATP stoichiometry seems unlikely since that indicate a changed e-ring stoichiometry in the ATP synthase complex with growth conditions, such a change has not been observed in B. firmus (48).

From these observations it was suggested that another mechanism than the conventional chemosmotic coupling model would be involved. There were several possible explanations for this apparent discrepancy that were ruled out, such as trapping of protons between the membrane and a cell wall or compartmentalization (45). In alkaliphilic bacteria, proteins involved in energy transduction have more basic residues on their p-side surfaces compared to their homologs in neutrophiles. In high pH environments these residues provide a better buffer capacity at the surface in order to retain protons ejected by proton transporters and to promote a lateral diffusion along the membrane surface to ATP synthase at high pH. Furthermore, in B. firmus grown at pH 10.5 the expression of a specific oxidase, caa₃, was three times higher than at pH 7.5, which would increase the probability for a short distance between the oxidase and ATP synthase. In addition, it was suggested, based on the inability to induce ATP synthesis, using a K⁺-valinomycin diffusion potential in cells that had been grown at high pH, that at high pH there would be a switch on the ATP synthase complex facilitating direct delivery of protons from the caa₃ oxidase instead of proton uptake from the surrounding solution (45–47). Based on the above-described observations, it is most probable that in alkaliphilic bacteria alternative modes of coupling in oxidative phosphorylation co-exist.

Proton transfer to proton-uptake pathways

Protons that are transferred across the membrane are taken up via specific pathways. These proton pathways are lined with polar amino-acid residues and water molecules that form hydrogen-bonded chains through which protons are conducted utilizing a Grotthuss mechanism (49). Efficient proton uptake to a proton pathway is often dependent on carboxyl, e.g. Asp and Glu, and imidazole groups, e.g. His, at the entrance of the pathway. Substitution of these entry-point residues to non-polar amino acids results in decreased proton-uptake rates. In CytcO the decreased activity upon substitutions of a carboxyl
groups could partially be restored by addition of chemicals that contain carboxyl groups, such as fatty acids and cholic acid derivatives (10, 50–52). This phenomenon was also observed for proton uptake in bacterial photosynthetic reaction centers, where two His residues were replaced by Ala residues, and additions of imidazole restored the proton uptake rate (53).

The rates of proton uptake in CytcO and reaction centers can exceed that of proton diffusion in water (54, 55). This observation is attributed to so-called proton-carrying antennae on the surface of proton-transport proteins. These antennae are composed of low-pK~a~ groups, Asp and Glu, attracting protons to the surface, and other protonatable groups, His, with pK~a~s of 6-7, which serve as local buffers. Together, these groups act to increase the local proton concentration on the protein surface and funnel protons to residues that function as entry points to proton-uptake pathways (54, 56).

Accordingly, membrane surfaces was proposed to mimic the properties of proton-carrying antennae. Introduction of protonatable lipids, e.g PA with a pK~a~ around 7-8, in liposomes composed of low-pK~a~ lipids, e.g PG or PC, resulted in acceleration of lateral proton transfer along the membrane surface (36). The concept of the membrane acting as a proton-carrying antenna is thus consistent with a localized proton transfer, where protons can diffuse laterally between the surface of proton transporters and membrane surfaces (Paper I and ref (30, 31)).

Proton transfer to the surface of proton transporters

In the work described in Paper I proton transfer to the surface of CytcO was probed under equilibrium conditions using fluorescence correlation spectroscopy. A pH-sensitive fluorophore, fluorescein (Flu) was covalently attached to the n-side surface of CytcO. The results from these studies showed that the rate of proton transfer to the Flu molecule was increased upon reconstitution of CytcO into PG liposomes, by a factor of approximately 400, from 7.1±0.8 x 10^{10} \text{s}^{-1} \text{M}^{-1} to 3.1±0.4 x 10^{13} \text{s}^{-1} \text{M}^{-1} (figure 3A). This indicates that the membrane surface can act to accelerate proton transfer to the surface of CytcO.

A contact between the protein and membrane surface was previously reported from studies on bacteriorhodopsin in purple membranes. Upon photo-excitation of the retinal in bacteriorhodopsin a proton is ejected on the p-side and a proton is taken up from the n-side. Proton release was monitored by two pH-sensitive dyes, pyranine in the bulk solution and a Flu molecule attached to the n-side protein surface. The protonation rate of the Flu molecule was reported to be between 6 (30) and 12 (31) times higher than the protonation rate of the pyranine in solution (figure 3B). Furthermore, the protonation rate
of pyranine increased when bacteriorhodopsin was found in micelles compared to purple membranes (37).

Figure 3. Proton transfer between membrane and protein surfaces. A) Membrane accelerated proton transfer to a Flu molecule (green hexagon) attached to the surface of CytcO. B) Upon proton release from bacteriorhodopsin on the p-side the protonation rate of a Flu molecule attached to the n-side protein surface was higher than that of pyranine (brown hexagon) in solution.

Taken together, the experiments with CytcO and bacteriorhodopsin in purple membranes indicate the possibility for lateral proton transfer along the membrane surface between proton transporters and proton consumers. This might be important for an efficient energy conversion during oxidative phosphorylation, especially for alkaliophilic bacteria. The contribution of the membrane in accelerating proton transfer is further discussed below with specific focus on CytcO.
Membrane-mimetic systems

Membrane proteins can be studied in whole cells or membrane preparations. However, a common approach in studies of membrane protein function and structure is to solubilize the membrane with detergents and isolate the protein of interest. There is a broad range of detergents available with different properties, such as chain length, head group moiety, critical micelle concentration (CMC) and size of detergent micelles (for a summary of detergent properties see (57)). A detergent that efficiently solubilizes membranes can have negative effects on specific membrane proteins, which can lead to destabilization, dis-assembly of protein complexes and/or functional inactivation. When it comes to function, detergent inhibition can for some proteins be reversible and the activity can be restored by changing the detergent environment or reconstituting the protein back into a lipid environment.

Artificial membrane environments used for studies of membrane protein structure and function is often adjusted depending on the application or method used. For example, bicelles or mixed micelles are common in NMR studies (58), whereas lipid cubic phases are becoming a popular tool for crystallization and structure determination using x-ray diffraction (59). Functional studies of membrane embedded transporters traditionally involve reconstitution of the protein into liposomes or planar surfaces, such as black lipid membranes (60, 61). More recently, a new type of membrane-mimetic system composed of small discs has become more widespread. The two membrane-mimetic systems used for the studies on membrane effects on proton transfer processes (Papers I-IV) are further described below.

Liposomes

Although there are often technical advantages to study membrane-protein function in detergent solution, this approach cannot be used for membrane proteins that inactivate or become destabilized in detergent solution. Furthermore, in studies of ion or molecule transport across membranes by transport proteins, the uptake and release sides of the membrane must be separated. For these purposes liposomes can be used. Liposomes are unilamellar bilayer assemblies classified according to diameter into small (25-50
nm), large (50-500 nm) or giant ( > 1 µm). They can be composed of either pure synthetic lipids in different mixtures or extracts from natural sources; the two most common used extracts are from soybean and *E. coli*.

The number of parameters that can be varied when reconstituting a membrane protein into liposomes is very high. Several different approaches can be used where the starting point can be either pre-formed liposomes partly doped with different detergents or mixed micelles. The choice of detergent affects both the solubilization properties of pre-formed liposomes, and the method for detergent removal. Most commonly applied methods for detergent removal are dialysis or the use of hydrophobic beads that absorb detergent molecules. However, protein incorporation into membranes has also been successful using dilution, where the reconstitution mixture is diluted so that the detergent concentration reaches a level below its CMC, or gel-filtration, where the detergent molecules are retained on the gel-filtration column. For a more detailed description on protein-reconstitution methods see (57, 60–62).

Depending on the combination of the detergent-removal approach and the detergent used, the protein orientation in liposomes can differ. There are reports on how charges, either on the protein surface or of the lipid head groups in the liposome, affect protein orientation (63). Uniformly oriented transport proteins are important when transmembrane transport of ions or molecules is under investigation or interactions with externally added proteins or molecules are investigated. Nevertheless, some functional processes in membrane proteins are independent of protein orientation, for example internal electron and proton transfer in Cyt*O*. Reconstitution of Cyt*O* from bovine heart mitochondria with dialysis was shown to yield an orientation with mostly the Cyt* binding site oriented outwards (right-side out orientation), while sonication gave a mixed orientation (63). The two approaches used for reconstitution of *R. sphaeroides* Cyt*O* into small liposomes (Papers I-IV), bio-bead and gel filtration, gave a protein orientation of approximately 80 % right-side out.

Although liposomes supply a more native-like environment for membrane proteins, there are some disadvantages to use them in functional studies. As mentioned above, one drawback is non-uniform protein orientation where different protein molecules might sense different solvent phases. When optical spectroscopy techniques are applied light scattering from liposomes is a disadvantage, resulting in a decreased signal-to-noise ratio. Figure 4 shows optical spectra of Cyt*O* in detergent solution, liposomes or nanodiscs. A comparison of these spectra demonstrates the disadvantage of using liposomes in optical spectroscopy.
Nanodiscs

A different membrane mimetic, so called nanodiscs, was recently developed and added to the tool-box for membrane-protein studies. In the case where proteins are either non-stable or non-functional in detergent solution and the use of liposomes is not necessary (or a problem), this new mimetic system is an alternative. Nanodiscs are planar lipid-bilayer assemblies that are held together by two membrane-scaffold proteins (MSPs), which form a belt around the lipid interior. Depending on the MSP used, the diameter of the disc can be varied in the range of 10-17 nm (64). The MSP is based on a part of the human serum apo-lipoprotein AI that has been modified in different versions to yield stable discs of various sizes (64–66). The number of lipids in nanodiscs varies between 120 and 330 lipids, depending on the MSP and lipid molecules used (64), which can be compared to liposomes with a diameter of 30-200 nm that contain 4-180 x10^3 lipids. Characterization of 9-10 nm discs with small angle neutron and x-ray scattering has shown that these discs are rather elliptical than circular in shape (67).

Proteins such as bacteriorhodopsin (65), G-protein coupled receptors (68, 69), ABC transporters (70) and ATP synthase (71) have successfully been incorporated into these discs and assayed using NMR, optical spectroscopy or EPR. In addition, different lipids and methods have been used for nanodisc formation and protein reconstitution. This membrane-mimetic system can be

Figure 4. Absorbance spectra of fully reduced \( \Delta^{1} \) oxidase. The spectra show reduced \( \Delta^{1} \) oxidase in detergent solution (blue), liposomes (red) and nanodiscs (green). For experimental conditions, see Paper IV. The picture is modified from figure 2 in Paper IV.
used for investigation of lipid-lipid, lipid-protein interactions and if or how a specific membrane environment affect protein function.

As mentioned above, nanodiscs have some advantages over liposomes. On the other hand, transport processes such as proton pumping cannot be investigated in nanodiscs because the two sides of the bilayer are not separated. In the example of Cyt cO only the net proton uptake is possible to detect, which is also the situation when the protein is in detergent solution. Nevertheless, membrane effects on internal electron and proton-transfer processes can be explored using nanodiscs. Therefore, we have developed a protocol for reconstitution of Cyt cO into soybean nanodiscs (for a detailed description see Paper IV).
Cytochrome c oxidase - a membrane-bound proton transporter

The terminal oxidase in the aerobic respiratory chain, CytO, belongs to the super family of heme-copper oxidases. Oxidases that belong to this family are classified and divided into subfamilies according to the sequence of the core subunit (subunit I) and the presence of six invariant His residues, which are all ligands to three metal cofactors (72).

The mitochondrial enzyme is an aa₃ oxidase, which together with the aa₃ oxidases from Paracoccus denitrificans and R. sphaeroides, belongs to the A-family. These two bacterial enzymes have been used extensively as model systems for the mitochondrial enzyme in functional studies. There are two additional major families among the oxygen-reducing heme-copper oxidases, the B and C family (72, 73). Additional families have been proposed based on DNA sequences from archaea (74). Bacteria can have several different oxidases in parallel, of which some are expressed under different oxygen tensions, ensuring effective energy transduction regardless of oxygen level. This chapter describes in more detail the two oxidases used in the studies described in Paper I-IV, the aa₃ oxidase (A-family) and the ba₃ oxidase (B family).

Structure and function of the aa₃ oxidases

The number of subunits in the aa₃ oxidases varies from four in P. denitrificans and R. sphaeroides to 13 in bovine heart mitochondria. The core subunits (I-III) that are essential for maintaining functional activity, have a high structure and sequence similarity with all known key residues conserved between these species. High resolution crystal structures are available for CytO from all three species (75–77). Figure 5A shows the three core subunits of the R. sphaeroides CytO with red-ox active cofactors and key residues marked (75).

Subunit I is composed of 12 transmembrane helices and contains three of the four red-ox active cofactors, a low spin heme a, a high spin heme a₃ and a copper site, Cu₈, where the latter two make up the catalytic site. The fourth red-ox active cofactor is a di-nuclear copper site, Cu₄₈, situated in the soluble domain of subunit II, which is anchored by two transmembrane helices. The
seven transmembrane helices of subunit III form a cleft that contains several conserved lipid molecules (75–78). Even though subunit III does not contain any red-ox cofactors it is important for stability of the enzyme, especially for subunit I and the catalytic site, as well as for proton uptake and proton pumping. Loss of subunit III has been shown to lead to a decreased catalytic life span, i.e. the number of turnovers before functional inactivation occurs, and loss of CuB (79–82).

The enzymatic function of CytcO involves electron and proton transfer to the catalytic site (figure 5B). Electrons for the reduction reaction are first donated from reduced soluble Cytc to CuA, from where they are transferred to the catalytic site via heme a. Each reduced Cytc supplies one electron; accordingly, four reduced Cytc molecules are required for complete reduction of oxygen to water. For each electron transferred to the catalytic site, one proton is taken up from the n-side for water formation. In addition, the energy released in the oxygen reduction reaction is used to pump protons from the n-side to the p-
side (as indicated with subscripts in equation 2) of the membrane with a stoichiometry of four protons per reduced oxygen molecule:

\[
\text{O}_2 + 4\text{Cytc}^{z+} + 8\text{H}_n^{+} \rightarrow 2\text{H}_2\text{O} + 4\text{Cytc}^{x+} + 4\text{H}_p^{+}
\] .(2)

Catalytic cycle
During the reaction cycle several intermediate states are formed (figure 6A). Here, the historical nomenclature is used, with superscripts indicating number of electrons transferred to the catalytic site. Recently, two new sets of nomenclature for the intermediate states in the catalytic cycle were proposed (83, 84).

In brief, during the reductive phase two electrons are transferred to the fully oxidized catalytic site, O⁰, and two protons are taken up to form R² (85, 86). When the catalytic site is reduced, oxygen binds to heme α₁ forming state A². Binding of O₂ is followed in time by breakage of the O-O bond, which results in formation of the P² intermediate. Simultaneous transfer of one electron and one proton occurs twice to form the F³ and O⁴(0) intermediates, respectively. Four protons are pumped during one reaction cycle, two in the reductive phase (O⁰→R² (87)) and two in the oxidative phase, one in each of P²→F³ and F³→O⁴(0) reaction steps (88).

Functional studies of the CytcO can be performed by first reducing the enzyme with four electrons (figure 6B), i.e. one electron is pre-loaded into each red-ox site. Then oxygen is added and after oxygen binding to the reduced heme α₁, one electron is transferred from heme α to the catalytic site to form a P³ state. Formation of the P³ intermediate is subsequently followed by a proton-transfer step forming F³. Next, the fourth electron transferred (from Cu₅ via heme α) to the catalytic site is accompanied by proton uptake during the F³→O⁴ transition. In this reaction where the fully reduced CytcO reacts with oxygen, electron and proton transfer are separated in time from each other during the R²→P³ and P³→F³ reaction steps, allowing individual studies of the two processes.
Figure 6. Illustration of the catalytic cycle in the oxygen-reduction reaction in Cyt cO, during turnover (A) or with the four electron pre-reduced enzyme (B). Redox-active sites are shown as circles, the catalytic site (CS) comprise heme $a_1$ and CuB. Red and white circles denote reduced and oxidized redox-active sites, respectively. Intermediate states are denoted by their one-letter codes with superscripts indicating the number of electrons at the catalytic site. These reactions are further described in the text.
Proton-transfer pathways

Both protons used for water formation and those that are pumped across the membrane are taken up from $n$-side of the membrane, via specific proton-uptake pathways. In the *R. sphaeroides a$_3$* oxidase, two pathways, D and K, named after the conserved residues Asp132 and Lys362, respectively, are used. These pathways are lined with polar amino-acid residues and water molecules (75–77, 89, 90) that form hydrogen-bonded chains where proton transfer is conducted using the Grotthuss mechanism (49). The D pathway stretches over a distance of ~24 Å from Asp132 to Glu286 with several water molecules resolved in crystal structures. In the K pathway, leading from the proposed entry point residue GluII101 in subunit II (91, 92) to the catalytic site only two water molecules have been identified (50, 93, 94). Lys362 is located in the middle of the pathway and is hydrogen bonded to one of the two water molecules. An additional pathway for pumped protons, the H pathway, has been proposed in the mammalian CytO based on the structure of the bovine-heart mitochondrial enzyme and mutagenesis data (90, 95, 96). This pathway does however not exist in the bacterial enzymes (97, 98).

Alternating access to the D and K pathways

During the reductive phase of the catalytic cycle, when reduction of heme $a_3$ and Cu$\mathrm{b}$ occurs, two protons are taken up via the K pathway to the catalytic site for water formation (85, 86, 99). The remaining two protons needed for water formation are taken up via the D pathway. In addition, all four protons that are pumped across the membrane are taken up via the D pathway (86, 100–102).

Proton-deuteron exchange experiments were conducted to probe solvent accessibility of the two proton pathways during the different catalytic steps. The K pathway was only accessible to deuterons in the R$^2$ state. This alteration in accessibility for protons indicated that there are structural changes in the protein between the oxidized and reduced states (103). Indeed, in the reduced structure of CytO from *R. sphaeroides* the lower part of helix VIII, where Lys362 is situated, was slightly repositioned compared to its location in the oxidized structure. Upon re-oxidation of the reduced crystals this helix shifts back into the position seen in the oxidized structure. Movement of this helix upon reduction of CytO opened up a proton access path in the K pathway between Tyr288-OH and the heme $a$ farnesyl-OH; the distance between these two groups changed from 2.6 Å to 4.1 Å upon reduction (89, 94).

Proton pumping

For proton pumping to take place some general criteria need to be fulfilled. First, proton pathways must exist where protons are taken up from the $n$-side and released to the $p$-side. Secondly, if these pathways are to be directly linked to each other, protons would leak back from $p$-side to $n$-side and thereby
dissipate the proton electrochemical gradient. Consequently, a gating mechanism must exist that provides an alternating access to the n-side and p-side (104, 105).

In Cyt cO protons are taken up from the n-side through the D-pathway via Glu286 to a proton-loading site, from where the proton is released to the p-side. The proton-loading site has not yet been identified, but most suggestions point towards one of the two heme aa3 propionates or one of the ligands to CuB, His1334 (104, 106–108). It is assumed that when connected to the n- or p-sides, the proton-loading site has a high or low pKa, respectively, such that a proton is taken up from the n-side and released to the p-side (109). The gating mechanism that prevents proton transfer in the direction from the p-side to the n-side is proposed to involve either the redox reactions of heme a, or conformational changes of amino-acid residues (e.g. Glu286) and rearrangement of water molecules (reviewed in (106)).

The proton exit pathway to the p-side has not yet been identified. Results from proton-deuteron exchange experiments with the Glu286His Cyt cO variant, performed during different catalytic steps of the reaction cycle suggested that residues 320-340 in subunit I may be a part of a proton-exit pathway (103, 110).

The aa3 oxidases and lipids

With the advances in x-ray crystallography, high resolution structures of the aa3 oxidase revealed lipid and detergent molecules associated with the protein on its surface or in-between subunits (75–77, 111). Superposition of structures of aa3 oxidases from bovine heart mitochondria, P. denitrificans and R. sphaeroides point towards a number of conserved lipid-binding sites (78, 112). One of these sites is located in a cleft in subunit III, where two lipid molecules are resolved in structures of all three species with their head groups facing the n-side. These two lipids were found to be two PG molecules in the aa3 oxidase from bovine heart mitochondria (77, 111), two PE molecules in the R. sphaeroides enzyme (75) and one PG and one PC in the P. denitrificans enzyme (76). In all cases these lipids interact both via hydrogen bonds and electrostatically with amino-acid residues in subunit I and III. Disruption of these interactions, using site-directed mutagenesis, in the R. sphaeroides enzyme led to partial dissociation of subunit III from the rest of the core complex. Furthermore, incorporation of these Cyt cO variants into soybean liposomes resulted in exchange of lipids associated with the protein and a drastic decrease in catalytic life span of the enzyme. This effect was suggested to be a consequence of an altered acyl-chain composition from 18:1 found in the native membranes of R. sphaeroides, to 18:2, which is the most common one in soybean-lipid extracts (113).
Bovine-heart mitochondrial CytO

The structure of the bovine heart mitochondrial enzyme reveals that the acyl chain ends of the lipids in the subunit III cleft are located near the oxygen pathway. It thus suggested that the acyl chain composition is important for oxygen access to the catalytic site. Moreover, a comparison of the acyl chain content in mitochondrial membranes with the content associated with the protein, showed an enrichment of cis-vaccenate (18:1Δ11) tails, which has also been observed for the *P. denitrificans* membrane and enzyme (111). Vaccenate was also found in an analysis of lipids associated with the *R. sphaeroides* CytO (20, 21). The enrichment of this particular acyl chain in association with the protein, even though the more common oleate-chain (18:1Δ9) is highly abundant in these membranes, led to the suggestion that cis-vaccenate might have a biological function although yet unidentified.

In the bovine-heart mitochondrial enzyme, changes in both acyl chain composition and the head group have been shown to affect the steady-state activity. In a comparison of different mono-unsaturated PC lipids it was shown that di-18:1:PC promoted the highest activity and that 90 % of this activity was retained when the chain length was increased to di-20:1, whereas for other chain lengths the activity was less than 60 % of the one obtained with di-18:1 (114). Lipid-head group dependencies were investigated with CytO solubilized in either Tween or lysolipids. Addition of PC, PE and CL increased the activity, with the highest one obtained with CL (115). In addition, CL (115) as well as to some extent PC and PE (116) were retained upon delipidation. Retention of CL was also seen in complex III, where CL is important for dimerization, stability and function (16). In liposomes composed of different PC:PE mixtures the CytO activity was unchanged. However, mixtures of the two lipids gave higher coupling ratios than when only PC or PE was used (116).

*R. sphaeroides* CytO

When *R. sphaeroides* was grown under low phosphate conditions, the PE and PC content in the membrane was below the detection limit for quantification, while still 73 % of CL and 50 % of PG were present. The PG content increased when CL levels were lowered in a CL-synthase deletion strain. Analysis of lipids associated with the purified CytO from the deletion strain and from the wild-type strain, grown at different phosphate levels, revealed that PC was absent during low phosphate conditions and instead DGTS was more abundant. The same trend was detected for PG that was exchanged for SQDG. In contrast, under normal phosphate conditions the level of CL associated with CytO in the CL synthase deletion strain was 27 % of the amount detected in the wild type strain. In the membrane however, this fraction decreased to 12 % (22, 23), which might indicate that the tightly associated CL have some specific function.
Although some of these phospholipids were detected in the x-ray crystal structure (75) or in analyzes of isolated Cyt cO (113), and therefore might be attributed to be important for stability and function, the decrease in the amounts of phospholipids affected neither the expression levels nor the function of Cyt cO (23).

The steady state activity of the R. sphaeroides Cyt cO increased by 20-30% in the presence of lipids compared to when Cyt cO was only in detergent solution. This effect was independent of lipid type (23, 51, 112). In addition to lipids, detergent molecules have been shown to bind close to the entrance of the K pathway (10, 78, 117) and that different detergents and detergent concentrations affected the steady-state activity of Cyt cO variants (10, 117, 118).

Effects of the membrane on proton-transfer processes in the \textit{aa}_3 oxidase from \textit{R. sphaeroides}

The membrane surrounding an integral membrane protein, can affect its function such as proton conductivity in proton pathways. Effects can be due to either direct interactions between annular or non-annular lipids and the protein, or the collective properties of the bilayer in form of hydrophobic matching or lateral pressure in the membrane. In line with a localized model of oxidative phosphorylation, the membrane surface is further proposed to be important for facilitating proton transfer between the respiratory-chain complexes and ATP synthase (see above).

In the following sections, membrane effects on specific proton-transfer processes in the \textit{aa}_3 oxidase are discussed. The discussion is divided into two parts: proton transfer through the K and D proton-uptake pathways, respectively, because these pathways are used during different parts of the catalytic cycle.

Proton transfer through the K pathway

The proposed entry point of the K pathway in \textit{R. sphaeroides} Cyt cO, Glu\textsuperscript{II101} in subunit II, is close to the membrane interface. There is no cluster of amino acids with proton collecting antenna properties surrounding the K pathway (75, 78). However, the membrane surface is also proposed to act as a proton collecting antenna, when phospholipids with head groups with low and neutral pK\textsubscript{s}s such as PG, PC and PA, respectively, are present (36). Thus, the membrane surface might act as proton collecting antenna for this proton pathway.
The K pathway is used for proton transfer during the reductive phase when the catalytic site becomes reduced with two electrons (85, 86, 99). Proton transfer through this pathway is coupled to electron transfer between the two heme groups. This proton-coupled electron transfer (PCET) can be studied in an experiment where the catalytic site, heme $a_3$ and Cu$_B$, is reduced and a CO ligand is bound to heme $a_3$, while heme $a$ and Cu$_A$ are kept in the oxidized state. The CO ligand is dissociated with a short, intense light pulse triggering electron transfer from heme $a_3$ to heme $a$ over two time scales. First there is a pH independent electron transfer observed on the $\mu$s time scale. This reaction is followed in time on a ms time scale by a pH dependent electron transfer that is coupled to proton release through the K pathway in the detergent-soluble Cyt$c$O (119). The proton originates from a water molecule nearby the catalytic site, which upon deprotonation forms a hydroxide ion that binds to the oxidized heme $a_3$ (120, 121).

This experiment was first conducted in detergent solution and proton release through the K pathway was detected with pH sensitive dyes (119). In combination with site directed mutagenesis important residues in the K pathway were identified (figure 5B) (91, 99, 122). If the enzyme is reconstituted into liposomes the same proton transfer can be monitored electrically because a charge is transferred perpendicular to the membrane surface. In these experiments, the rate of the phase associated with proton release was increased by a factor of 7-8. The difference was attributed to the presence of a membrane around Cyt$c$O in the electrical measurements. This conclusion was verified using optical measurements with Cyt$c$O reconstituted into liposomes (123).

From the above described experiments with the P. denitrificans Cyt$c$O (123) and the corresponding experiments with the R. sphaeroides enzyme (Paper II) we concluded that the membrane environment around Cyt$c$O affect proton transfer through the K-pathway. The same effect was also observed when nanodiscs were used as a membrane mimetic (Paper IV, see also figure 7 for a comparison of the PCET in R. sphaeroides Cyt$c$O at pH 9 in detergent solution, liposomes and nanodiscs).
The membrane can affect the pathway conductivity or the membrane surface can accelerate the proton transfer rate (Paper I, \((30, 31, 123)\)). In the K pathway Ser\(^{299}\) interacts, via a water molecule, with the highly conserved and functionally important Lys\(^{362}\) \((75, 78, 99)\). Replacement of this Ser by either a Gly or a Glu resulted in changes of the pathway conductivity, which led to a slowed PCET rate \((122)\). Incorporation of these Cyt\(\text{c}\)O variants into liposomes led to an increased rate of the PCET to the same extent as seen for the wild-type Cyt\(\text{c}\)O. This indicates that the membrane does not change the pathway conductivity (Paper II).

For the \(R. \text{sphaeroides}\) Cyt\(\text{c}\)O, Glu\(^{101}\), in close proximity of the protein-membrane interface, is proposed as the entry point to the K pathway. Substitution of Glu\(^{101}\) for Ala resulted in a slowed PCET to the same extent as seen for Ser\(^{299}\)Glu in detergent solution (Paper II, \((91)\)). However, in the Glu\(^{101}\)Ala Cyt\(\text{c}\)O variant the membrane did not accelerate the PCET rate (Paper II), which indicates that this Glu residue might be involved in surface-mediated proton uptake to the K pathway.

The low steady-state activity of Glu\(^{101}\)Ala (~5 % of wild type, \((92)\)) was partially restored when deoxy-cholate \((10)\), cholate \((10, 50)\) or arachidonic acid \((50)\) was added. A deoxy-cholate molecule was also detected in the x-ray crystal structure of Cyt\(\text{c}\)O (PDB 3DTU) with its carboxyl group pointing towards the position of Glu\(^{101}\) \((78)\). This suggests that a carboxyl group in this region is

![Figure 7](image_url).

**Figure 7.** Proton-coupled electron transfer in Cyt\(\text{c}\)O in detergent solution (blue), liposomes (red) or nanodiscs (green) at pH 9. The absorbance increase at 598 nm, upon CO dissociation from the two-electron reduced Cyt\(\text{c}\)O, represents electron transfer from heme \(a_3\) to heme \(a_1\), which is coupled to proton release though the K pathway. The picture is modified from figure 3 in Paper IV.
functionally important for proton uptake into the K pathway. Indeed, adding back a carboxyl group in the form of an Asp at this position partially restored the effect of the membrane, i.e. the PCET was accelerated upon reconstitution of this CytO variant into liposomes (Paper II). Taken together, the results of these studies suggest that the membrane surface acts as an extended proton-collecting antenna funneling protons to the protein surface and that GluI101 acts as a protonic link between the membrane surface and the K pathway.

Proton transfer through the D pathway

In contrast to the K pathway, an enrichment of residues with proton-collecting antenna properties (Asp, Glu and His) is found close to the entrance of the D pathway. The rate of proton uptake to this pathway exceeds the rate of proton diffusion in water, which has been attributed to these proposed antenna residues (54, 56, 75, 80, 82). A large number of these residues are located on the n-side surface of subunit III. Accordingly, removal of subunit III led to slowed proton uptake to the D pathway (75, 80, 82). In the presence of subunit III, a slight increase in the maximal proton uptake rate was detected when the aa3 oxidase was reconstituted into liposomes (figure 3c in Paper III), but not to the same extent as seen for proton uptake to the K pathway (Paper II).

Protons are transferred through the D pathway to the Glu286 residue located at the upper part of the pathway close to the catalytic site. From this residue protons are either transferred to the catalytic site or to a proton-loading site for release to the p-side. After proton transfer from Glu286, fast re-protonation is facilitated through the D pathway. Substitution of Glu286 for Gln (100) or His (110) resulted in impaired proton transfer both to the catalytic site and the proton-loading site. Replacement of amino-acid residues further down in the D pathway also affected these processes and decoupled them in such a way that proton pumping was abolished, while proton transfer to the catalytic site remained unaltered (109).

In the reaction of the fully reduced CytO with oxygen, proton uptake through the D pathway for rapid re-protonation of Glu286, occurs in two steps. These two proton uptake steps have time constants of 100 µs and 1 ms, and take place during the P3→F3 and F3→O4 transitions, respectively (124). The first proton uptake step is coupled to equilibration of an electron between CuA and heme a, and the rate of this electron transfer reflects the rate of the proton uptake.

In general, the rate of proton uptake through the D pathway is determined by the fraction of protonated Glu286 and the rate of proton transfer from Glu286 to the catalytic site. The pH-dependence of the observed proton-uptake rate titrates with one pK, assigned as an apparent pK, of Glu286 (109,
For the CytcO from *R. sphaeroides* in detergent solution, the reported pKₐ is 9.4 (125).

When CytcO was reconstituted into liposomes the proton-uptake rate at pH 7.5 was two-three times lower than that determined in detergent solution ((88), Paper III). The lower proton-uptake rate, at neutral pH, was attributed to a shift of the apparent pKₐ of Glu1286 to 6.8 upon reconstitution of CytcO into liposomes (figure 8, Paper III). Changes in the Glu1286 pKₐ was previously observed in variants of CytcO where substitutions were made in the D pathway (summarized in (109)).

![Figure 8](image-url)

**Figure 8.** pH dependence of the rate of the first proton-uptake step during the reaction of the fully reduced CytcO with oxygen. The rate of proton uptake, through the D pathway is determined from kinetic traces recorded at 580 nm for detergent soluble CytcO (black dots) or at 830 nm for CytcO in liposomes (grey squares). The solid lines are fits of data with pKₐ values of 9.4 (black line) or 6.8 (grey line). For experimental conditions see Paper III. The picture is modified from figure 3A in Paper III.

An explanation for changes of the apparent pKₐ of Glu1286, was previously discussed based on the x-ray crystal structures of the Glu1286Gln CytcO variant (75). In this structure, the Gln1286 residue had a different conformation than the one seen in structures for Glu1286 (75–77), which led to the suggestion that Glu1286 can shift between two conformations (126). These two conformations were suggested to have different pKₐs and the apparent pKₐ obtained from the proton-uptake kinetics would then be dependent on the equilibrium constant between these two conformations (109).
In molecular-dynamics simulations, a downward conformation of Glu'286 is generally stabilized (127, 128). However, in a recent study it was shown that the environment of the protein significantly affected the conformation of Glu'286, such that a downward conformation was only promoted when simulations were performed in vacuum (128). In addition, the number of water molecules in the D pathway during simulation also influenced the Glu'286 conformation (128) and it was suggested that the downward conformation observed in most crystal structures (75–77, 89), except for the Asni39Asp CytO variant (129), is due to dehydration of the D pathway during crystallization (128).

Although it might be reasonable to attribute the modified pKa of Glu'286 upon reconstitution of the CytO into liposomes to a shift in the equilibrium constant between the two conformations of Glu'286, the membrane might also affect the true pKa values in these two conformations. Based on the study described in Paper III we cannot attribute the observed membrane effect explicitly to a specific membrane property. To address this question additional experiments in which the properties of the membrane are modulated are needed (see below for effects on changing the lipid head groups).

Modulation of electron transfer

The membrane does not only affect proton uptake by shifting the apparent pKa of Glu'286. Incorporation of CytO into liposomes composed of negatively charged PG lipids or zwitterionic PC lipids also resulted in an altered electron distribution between CuA and heme a. In both detergent solution and soybean lipid liposomes the extent of oxidation of CuA during the first proton uptake (during the P3→F3 reaction) was roughly 50 %. However, when the net charge of the membrane surface was varied, the extent of CuA oxidation was modulated. In liposomes composed of PG lipids the extent was 41 % compared to 64 % in PC lipids (figure 9, Paper III). The proton uptake rate for all three liposome compositions displayed a time constant of 170-200 µs, indicating that the lipid head-group charge did not alter the proton uptake rate. This effect cannot be attributed to electrostatic interactions between lipid head groups and CuA because such an effect would result in a larger extent of CuA oxidation in PG liposomes compared to PC liposomes. Instead, we suggested that the net charge of the lipids may affect the electrical field within the membrane such that the mid-point potential of heme a was altered. The electrical field within the membrane can be modulated using dipole modifiers. In bacterial reaction centers it was shown that both rates and relative amplitudes of the first biphasic electron-transfer reaction were affected when the electrical field in the membrane was modulated (130).
Structure and function of the \( \text{ba}_3 \) oxidases

The \( \text{ba}_3 \) oxidase from \textit{Thermus thermophilus} is a member of the B-family of heme-copper oxidase superfamily. It contains three subunits, I, II and IIa (figure 10), which structurally overlap with subunits I and II in the \( \text{aa}_3 \) oxidases (131, 132). Three out of four redox-active cofactors are located in subunit I, a low spin heme \( \text{b} \), and a catalytic site composed of a high spin heme \( \text{a}_3 \) and a CuB site. In the soluble domain of subunit II, anchored with one transmembrane helix, the fourth red-ox active site, a di-nuclear CuA site, is located. Distances between the different redox centers are very similar to those found in the \( \text{aa}_3 \) of \textit{R. sphaeroides}.

The \( \text{ba}_3 \) oxidase catalyzes the same reaction as the \( \text{aa}_3 \) oxidase, i.e. oxygen reduction to water. Four electrons are donated sequentially one-by-one from water soluble Cyt\( \text{c} \), via CuA and heme \( \text{b} \) to the catalytic site. In addition to the four protons taken up from the \( n \)-side for water formation, two additional protons are pumped across the membrane from the \( n \)-side to the \( p \)-side (equation 3). As seen for the \( \text{aa}_3 \) oxidases, with the oxygen-reduction reaction follows sequential electron and proton transfers to the catalytic site, rendering partly reduced oxygen-intermediate states. When the four-electron reduced \( \text{ba}_3 \) enzyme reacts with oxygen, protons are taken up on two time scales, with time constants of \( \sim 80 \) µs and \( \sim 1 \) ms (133, 134).

\[
O_2 + 4\text{Cyt}c^{2+} + 6H^+_n \rightarrow 2\text{H}_2O + 4\text{Cyt}c^{3+} + 2H^+_p
\]

(3)
A single proton-transfer pathway

Three proton pathways, leading from the n-side to the catalytic site were proposed from an analysis of the crystal structure of the ba₃ oxidase (131). However, only one of these three putative pathways was found to be functional based on sequence alignments and site-directed mutagenesis (135). A structural comparison of the ba₃ and aa₃ oxidases showed that this proton-uptake pathway overlaps in space with the K pathway in the aa₃ oxidases, and it is therefore referred to as the K-pathway analogue, although it shows no sequential similarity. All protons, both those used for water formation and those that are pumped are taken up via this proton pathway (133–135).

As seen for the K pathway in the aa₃ oxidase from R. sphaeroides, a Glu residue (Glu15 T. thermophilus numbering) is situated at the entry point of the proposed proton-uptake pathway, near the membrane surface. The surface of the membrane acts to accelerate proton transfer to the K pathway in the aa₃ oxidase (see above, Paper II, IV and ref (123)). Similarly, during the oxygen-reduction reaction with the fully reduced ba₃ oxidase the rate of the first proton uptake increased by a factor of two when the ba₃ enzyme was reconstituted into

Figure 10. Structure of the ba₃ oxidase from T. thermophilus (PDB ID 3S8F). A) Subunits I, II and IIa are shown in green, blue and cyan, respectively. B) Redox-active sites are marked as follow heme b and a₃ in orange and copper centers in blue. The electron-transfer pathway is presented by red arrows and the proton-transfer pathway with a blue arrow. The picture was prepared using PyMOL software.
nanodiscs (Paper III and IV). The kinetics of the second proton-uptake phase was unaltered (Paper IV). A net of two protons was taken up during these two phases, both for the enzyme in detergent solution and in nanodiscs (Paper III and IV).
Concluding remarks

This thesis is focused on effects of the phospholipid bilayer on proton-transfer processes. For studies of these processes, a well-characterized proton transporter, CytcO, has been used as a model system. We showed that the rate of proton transfer to the surface of CytcO was accelerated by the membrane surface when CytcO was reconstituted in liposomes. The presence of the membrane also influenced proton-uptake rates through one of the proton-transfer pathways in CytcO and a specific amino-acid residue was shown to act as a protonic link between the membrane surface and the proton pathway. These observations support a localized coupling model in oxidative phosphorylation, where protons ejected by proton transporters diffuse laterally along the membrane surface to proton consumers, which might be crucial for energy transduction, for example in alkaliphilic bacteria.

Electron and proton-transfer processes in CytcO have earlier mainly been studied with the protein in detergent solution, which has provided relative detailed functional insights. However, the results described in this thesis show that upon reconstitution of CytcO into model membranes, internal electron and proton-transfer reactions are modulated, which shows that it is important to study membrane-bound transporters also in a membrane environment. To further investigate membrane effects on proton-transfer processes in CytcO, in the future, additional experiments should be performed where the membrane surface charge is varied, the electrical field in the membrane is modulated or the lateral pressure in the membrane is altered by addition of non-bilayer prone lipids. In addition, these results should be compared with results from studies of CytcO from different species in their native lipid environment. For these future studies the nanodisc technology might serve as a powerful experimental tool.

I denna avhandling beskriver hur protontransport kan påverkas av membranet. Protontransportprocesser i cytokromoxidas har studerats när proteinet är frilagt från membranet och när det frilagda proteinet har introducerats i olika membran-modellsystem. Vi har observerat hur protontransport till ytan på cytokromoxidas accelereras då proteinet förflytts in i ett membran. Dessutom har vi funnit att en specifik del av proteinet utgör en länk mellan membranytan och en av protonkanalerna. Vidare har vi visat att protontransporten i cytokromoxidas påverkas när proteinet introduceras i en membranmiljö och att nettoladdningen på membranytan påverkar transport-processerna. Slutligen, för att underlätta studier av hur membranet växelverkar med och påverkar funktionen av protontransportörer har vi utvecklat en strategi för att introducera cytokromoxidas i ett nytt sorts membranmodellsystem, så kallade nanodiskar.

Sammantaget har de studier som presenteras i denna avhandling visat på att membranbundna protontransportörer så som cytokromoxidas påverkas av det omgivande membranet. Detta visar på att det är viktigt att studera dessa proteiner i olika membranmodellsystem som ett komplement till de studier som
görs med proteinet frilagt från membranet. Dessutom visar dessa studier på att membranet har en funktion i cellens energimetabolism, vilket kan vara avgörande för organismer som lever i extrema miljöer.
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References

mobile pH buffers, on the size and form of membrane particles, and on the 
in favor of the existence of a kinetic barrier for proton transfer from a surface 
of bilayer phospholipid membrane to bulk water. *Biochim. Biophys. Acta* 1130, 
45-50.
microfluorimetry to study proton diffusion along phospholipid membranes. 
*Eur. Biophys. J.* 37, 865-70.
36. Brändén, M., Sandén, T., Brzezinski, P., Widengren, J., Branden, M., and 
Sandén, T. (2006) Localized proton microcircuits at the biological membrane-
in the regimes of fast and slow exchange between the membrane surface and 
38. Sandén, T., Salomonsson, L., Brzezinski, P., and Widengren, J. (2010) Surface-
Acad. Sci. U. S. A.* 107, 4129-34.
(2011) Protons migrate along interfacial water without significant contributions 
localized coupling of respiration and phosphorylation in mitochondria. *Biochim. 
*Escherichia coli* membrane vesicles. *Biochemistry* 16, 848-54.
proton gradient and active transport in *Escherichia coli* membrane vesicles. 
parameters of extremely alkaliphilic *Bacillus* species in batch cultures, and 
abundance of atp gene transcript and of the membrane F1F0-ATPase as a 
function of the growth pH of alkaliphilic *Bacillus firmus* OF4. *J. Bacteriol.* 176, 
5167-70.
*Biochim. Biophys. Acta* 1757, 867-70.


121. Hallen, S., Brzezinski, P., and Malmström, B. G. (1994) Internal electron transfer in cytochrome c oxidase is coupled to the protonation of a group close to the bimetallic site. Biochemistry 33, 1467-72.


