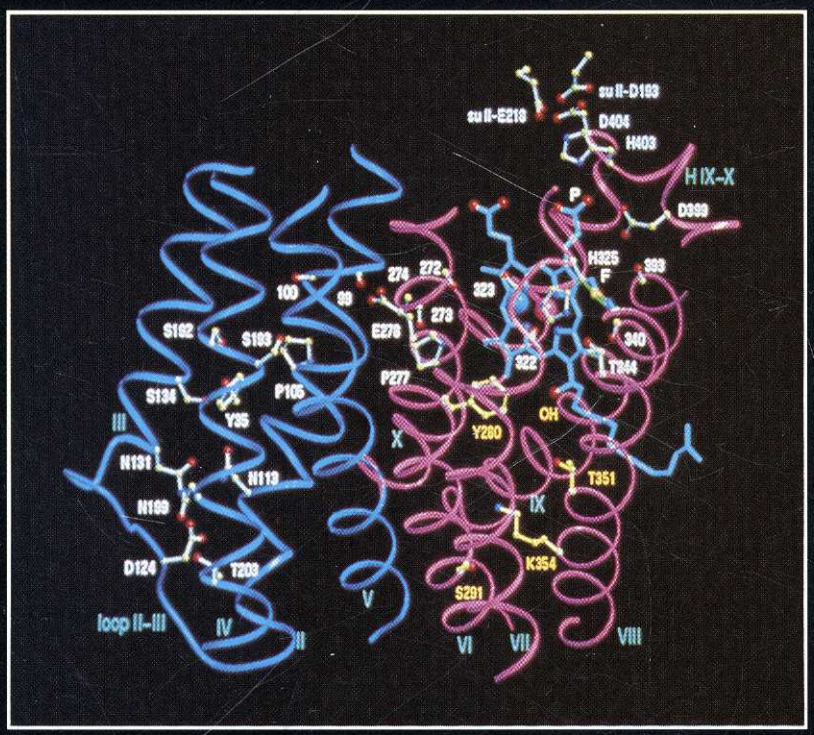
OXYGEN REDUCTION BY TERMINAL OXIDASES

THE COUPLING BETWEEN ELECTRON AND PROTON TRANSFER



Margareta Svensson Ek

Department of Biochemistry and Biophysics
Göteborg University and Chalmers University of Technology
Göteborg 1996

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Dissertation abstract

The final reaction in the respiratory chain of *Escherichia coli* is the oxidation of ubiquinol by dioxygen. This reaction is catalyzed by the membrane-bound enzyme cytochrome bo_3, a heme-copper terminal oxidase. The energy liberated in this reaction is stored in an electrochemical gradient which the enzyme creates by transferring protons across the cell membrane. This proton gradient is then used for the synthesis of ATP which is later used in many energy-demanding reactions within the cell.

This thesis is focused on electron and proton transfer during oxygen reduction by cytochrome bo_3. Also included is a study of a related oxidase from *Rhodobacter sphaeroides*, cytochrome aa_3. Oxygen reduction by these enzymes was investigated using time-resolved optical-absorption spectroscopy. To identify amino-acid residues involved in proton-transfer reactions, the same reactions were studied in mutant enzymes where protonatable amino-acid residues have been modified.

A reaction mechanism for wild-type cytochrome bo_3 is suggested and then discussed in terms of similarities and dissimilarities to the more extensively investigated terminal oxidase from bovine heart.

Some of the amino-acid residues modified are thought to be involved in two proton pathways of the enzymes (Iwata, S., Ostermeier, C., Ludwig, B., & Michel, H. (1995) *Nature 376*, 660-669). One pathway for chemical protons used in oxygen reduction (K362 and T359) and another for protons pumped across the membrane (D135 and E286). The results presented in this thesis clearly show that residues in the transmembrane helix VIII (K362 and T359) are not involved in proton transfer during oxygen reduction when starting from the fully-reduced enzyme. However, the E286 residue in the transmembrane helix VI, is essential for proton uptake during oxygen reduction by the fully-reduced enzyme.

Key words: cytochrome bo_3, *E. coli*, flow-flash, electron transfer, proton transfer, heme-copper, terminal oxidase, mutant enzymes.

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Cover: Part of the structure of cytochrome *c* oxidase from *Paracoccus denitrificans* determined by Iwata et al. (1995) showing two proton pathways. The figure was kindly provided by Prof. H. Michel.

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LIST OF ORIGINAL PUBLICATIONS

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

I Margareta Svensson and Thomas Nilsson
Flow-flash study of the reaction between cytochrome *bo* and oxygen.
(1993) *Biochemistry 32*, 5442-5447.

II Stefan Hallén, Margareta Svensson and Thomas Nilsson
Cytochrome *bo* from *E. coli* does not exhibit the same proton transfer characteristics as the bovine cytochrome *c* oxidase during oxygen reduction.
(1993) *FEBS Lett 325*, 299-302.

III Margareta Svensson, Stefan Hallén, Jeffrey W. Thomas, Laura Lemieux, Robert B. Gennis and Thomas Nilsson
Oxygen reaction and proton uptake in helix VIII mutants of cytochrome bo_3.
(1995) *Biochemistry 34*, 5252-5258.

IV Margareta Svensson-Ek, Jeffrey W. Thomas, Robert B. Gennis, Thomas Nilsson and Peter Brzezinski
Kinetics of electron and proton transfer during the reaction of oxygen with wild-type and helix VI mutants of cytochrome bo_3.
(1996) *Biochemistry 35*, 13673-13680.

V Margareta Svensson-Ek and Peter Brzezinski
Kinetics of electron and proton transfer associated with oxidation of ubiquinol by cytochrome bo_3 from *E. coli*.
(1996) Submitted to *Biochemistry*

VI Pia Ädelroth, Margareta Svensson-Ek, David Mitchell, Robert B. Gennis and Peter Brzezinski
Glutamate 286 in cytochrome aa_3 from *Rhodobacter sphaeroides* is involved in proton uptake during the reaction of the fully reduced enzyme with oxygen.
(1996) Submitted to *Biochemistry*

ABBREVIATIONS

ADP	adenosine 5′-diphosphate
ATP	adenosine 5′-triphosphate
EPR	electron paramagnetic resonance
FTIR	fourier transform infrared spectroscopy
HQNO	2-heptyl-4-hydroxyquinolone N-oxide
NADH	reduced nicotinamide adenine dinucleotide
NAD^+	oxidized nicotinamide adenine dinucleotide

CONTENTS

SUMMARY OF PAPERS

The aim of this study has been to investigate the catalytic reaction of the ubiquinol oxidase cytochrome bo_3 from *Escherichia coli*. In particular, I have focused on electron- and proton-transfer reactions during dioxygen reduction. Time-resolved optical-absorption spectroscopy was used to investigate wild-type and site-directed mutant enzymes.

In **paper I** we describe results from studies of the reaction between the three-electron fully-reduced cytochrome bo_3 and oxygen. Two kinetic phases were observed and interpreted in terms of formation of the peroxy and ferryl intermediates, in analogy with the reactions observed in the mitochondrial cytochrome *c* oxidase. Later work showed this interpretation to be incorrect (paper IV).

In **paper II,** we present a study of proton uptake during oxygen reduction in wild-type cytochrome bo_3. We found that the proton-uptake rate was between the rates of the two electron-transfer phases described in paper I. This was interpreted in terms of proton uptake not rate limited for ferryl-intermediate formation, in contrast to what is found in cytochrome *c* oxidase.

Results from experiments with mutants of cytochrome bo_3, in which amino-acid residues in helix VIII of subunit I were modified, are presented in **paper III**. These residues have been proposed to be important for proton transfer. The mutant enzymes studied were K362L, M, Q; T359A; T352A, N, S; P358A; M353A, where the K362, T359 and T352 mutant enzymes displayed severely reduced overall catalytic activity. During oxygen reduction, the kinetics and extent of electron and proton transfer were found to be the same in the helix-VIII mutant enzymes as in the wild-type enzyme. This indicates that the mutated amino-acid residues are not important for proton uptake during reaction of the fully-reduced enzyme with oxygen, when the end product is a ferryl intermediate.

In **paper IV**, a study of another set of mutant enzymes, modified in helix VI of subunit I, is presented: W280F, P285G, E286A and Y288F. In

these mutant enzymes, the oxygen chemistry appears normal up to the formation of the ferryl intermediate, but the proton uptake, seen in the wild-type enzyme, is impaired. This suggests that E286 and the other amino-acid residues in the neighborhood of E286 are important for proton transfer during oxygen reduction. A study of different wild-type enzymes with variable heme content is also included in paper IV. This study shows that the first and second phases described in paper I are due to two different populations of the enzyme, cytochrome bo_3 and oo_3, respectively. There is only one electron-transfer phase when a pure cytochrome bo_3, without bound quinol, reacts with oxygen. The end product is a ferryl intermediate.

Paper V deals with the oxygen reaction of a five-electron reduced cytochrome bo_3 complex, i. e. where a quinol is bound to the enzyme. Here, the absorbance changes associated with oxygen reduction are similar to those observed in the mitochondrial cytochrome *c* oxidase, with three phases of electron transfer with rates of $3 \cdot 10^4\ s^{-1}$, $1.5 \cdot 10^3\ s^{-1}$ and $250\ s^{-1}$ and two proton-uptake phases with rates of $1 \cdot 10^4\ s^{-1}$ and $250\ s^{-1}$. In addition, a proton release on the same time scale as heme *b* re-reduction (during quinol oxidation) was observed.

In **paper VI**, an investigation of electron transfer and proton uptake in a mutant, E286Q, of cytochrome aa_3 from *Rhodobacter sphaeroides* is presented. The first phase of the oxygen reduction, corresponding to oxidation of both hemes and peroxy-intermediate formation, is similar to that observed in the wild-type enzyme. However, the last two electron-transfer phases associated with the formation of the ferryl intermediate and oxy form of the enzyme, are not observed. In addition, there is no proton uptake in the mutant enzyme, which confirms the observations described in paper IV. This also strengthens the suggestion presented in paper IV, that E286 is essential for proton uptake after formation of the peroxy intermediate, and that the $1 \cdot 10^4\ s^{-1}$ proton uptake is needed for the delivery of the fourth electron to the binuclear center.

1. INTRODUCTION TO THE BIOENERGETICS OF RESPIRATION

In this thesis, an investigation of the catalytic reaction of the ubiquinol oxidase cytochrome bo_3 is presented. This enzyme catalyzes the reaction between reduced ubiquinol (QH_2) and dioxygen:

$$2QH_2 + O_2 + 8H^+_{in} \rightarrow 2Q + 2H_2O + 8H^+_{out}$$

where H^+_{in} and H^+_{out} refer to protons on the inside and outside of a membrane, respectively. This reaction is part of a larger system of reactions in aerobic organisms called *respiration* and *oxidative phoshorylation*. The respiratory chain consists of a number of membrane proteins catalyzing the exergonic oxidation of foodstuff by dioxygen and storing the energy from this electron transfer in an electrochemical proton gradient over a membrane (Mitchell, 1961). The proton gradient, created by the membrane proteins, is then used to convert ADP and phosphate to ATP (figure 1) which is used in energy-demanding reactions in every form of life. These reactions take place in the plasma membrane of prokaryotes and in the inner membrane of mitochondria in eukaryotic cells (figure 2).

Escherichia coli

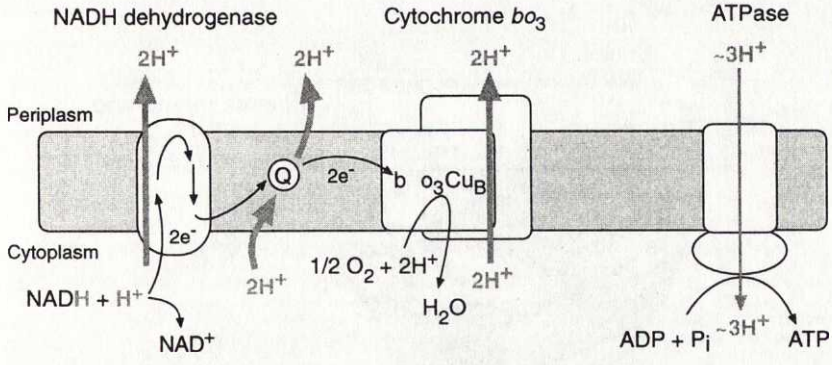


Figure 1a. The electron-transport chain in *Escherichia coli*. The coupling of electron transport (black arrows) to ATP synthesis, by the generation of a proton gradient across the membrane (the chemiosmotic theory). Protons are pumped (thick gray arrows) out of the cytoplasm and their return (thin gray arrow) drives the synthesis of ATP. Depending on environmental conditions, NADH dehydrogenase can be replaced by a large variety of other dehydrogenases.

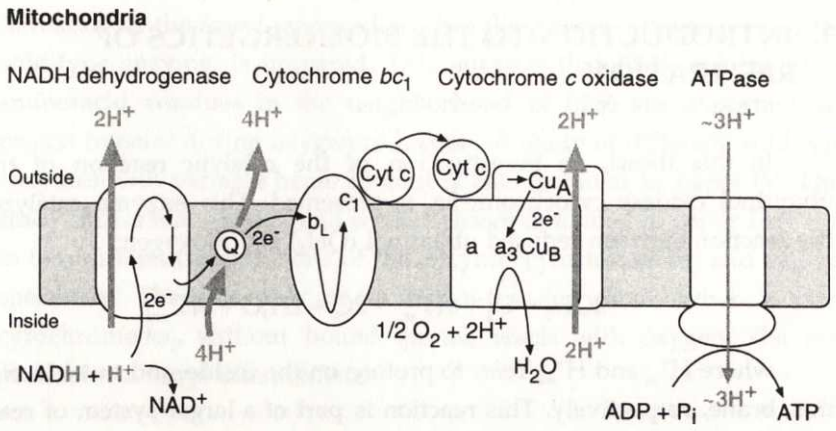


Figure 1b. The mitochondrial respiratory chain. Protons are pumped (thick gray arrows) out of the mitochondria and their return (thin gray arrow) drives the synthesis of ATP as in *E. coli*.

Peter Mitchell was awarded the Nobel Prize in chemistry in 1978 for the formulation of the chemiosmotic theory, which describes the coupling between the energetically "downhill" transfer of electrons from substrates to O_2 and the "uphill" synthesis of ATP from ADP and phosphate. The mitochondrion, as an organelle in eukaryotic cells, is commonly thought to have evolved from an aerobic prokaryote which formed a symbiotic relationship with a primitive, non-respiring eukaryotic cell.

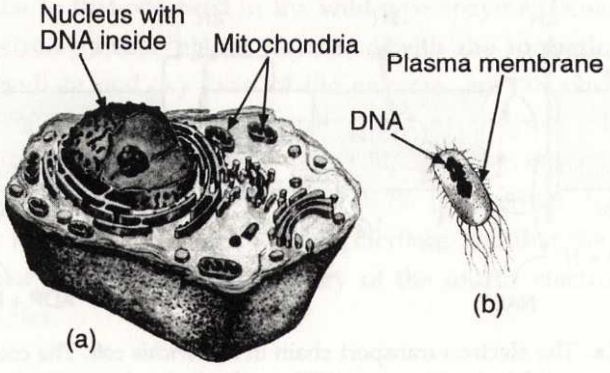


Figure 2. (a) An eukaryotic (animal) cell and (b) a prokaryotic (bacterial) cell. The true size of the bacterium is about the same as that of the mitochondrion.

Aerobic organisms depend in turn on *photosynthesis* in algae, plants and cyanobacteria which use solar light energy to produce food-stuff and oxygen by reducing carbon dioxide (figure 3).

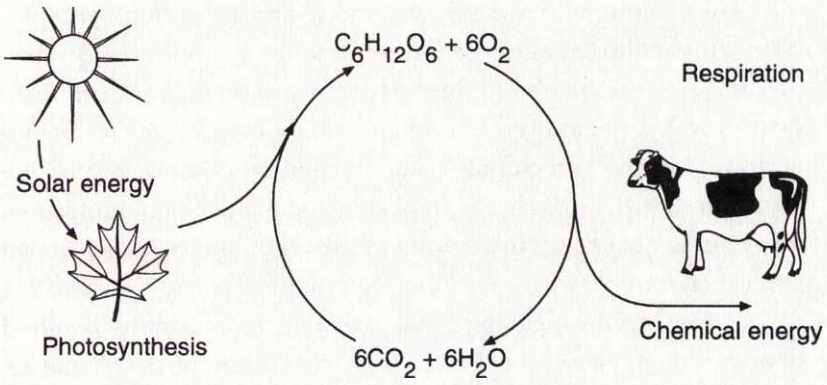


Figure 3. Respiration of eukaryotes and photosynthesis in plants constitute the cyclic energy conversion in the biosphere.

This thesis is focused on one of the terminal oxidases in the respiratory chain of *Escherichia coli*, the ubiquinol oxidase cytochrome bo_3.

2. STRUCTURE AND FUNCTION OF TERMINAL OXIDASES

Most terminal oxidases are redox-driven proton pumps that couple the reduction of oxygen to water to the vectorial translocation of protons across a membrane, generating a transmembrane proton electrochemical gradient. The ubiquinol oxidase cytochrome bo_3 belongs to a large group of structurally and functionally closely related heme-copper terminal oxidases which also includes the bovine mitochondrial cytochrome *c* oxidase, the most extensively investigated terminal oxidase (for a review, see for example Trumpower and Gennis, 1994).

In the heme-copper terminal oxidases, four protons required for oxygen reduction are consumed from the inner (matrix) side of the membrane of mitochondria (Wikström, 1988) or from the cytoplasm in bacteria. Four additional protons per reduced O_2-molecule are pumped from the inside to the outside of the membrane as the electrons pass through the enzyme (Wikström, 1977, 1984; Antonini et al., 1993; figure 4).

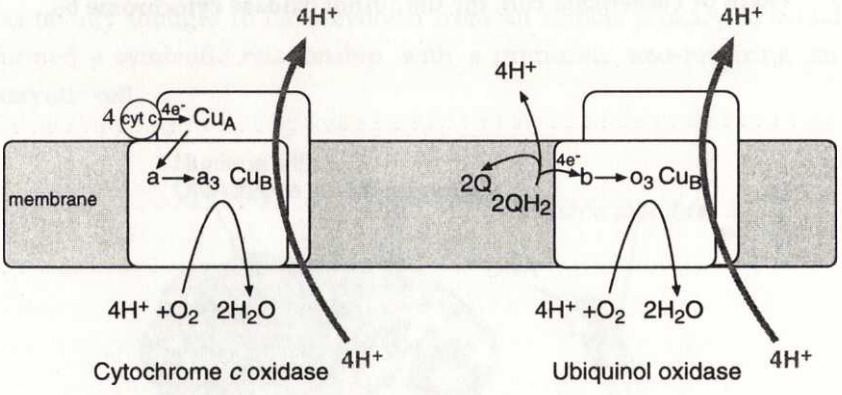


Figure 4. A schematic picture of cytochrome *c* oxidase (cytochrome aa_3) and ubiquinol oxidase (cytochrome bo_3) showing the similarities and dissimilarities of the two oxidases.

A likely common core structure of the heme-copper terminal oxidases has been proposed based on genetic and biophysical studies

(Shapleigh et al., 1992; Hosler et al., 1993) and verified by two recently-determined crystal structures (Iwata et al., 1995; Tsukihara et al., 1995, 1996). They have in common a binuclear center consisting of a heme group, heme a_3 (or o_3 in cytochrome bo_3) and a copper ion, Cu_B, as the active site for O_2 reduction. In addition, a low-spin heme group, heme *a* (or *b* in cytochrome bo_3) serves as the electron mediator (together with a fourth metal center in cytochrome *c* oxidases) between the electron donor and the binuclear center.

Hemes *a* (*b*) and a_3 (o_3), and Cu_B are located near the mitochondrial intermembrane space or the bacterial periplasm. Cytochrome *c* oxidases generally have a fourth metal center, Cu_A, at the outside of the mitochondrial inner membrane or the bacterial cell membrane. This site is absent in ubiquinol oxidase. The electrons from the donor either enter the enzyme at Cu_A and are then transferred to the low-spin heme (cytochrome *c* oxidase), or are transferred directly to the low-spin heme (ubiquinol oxidase), and then to the binuclear site. In addition to oxygen reduction, proton-pumping is also thought to involve the binuclear center (see for example Morgan et al., 1994; Rich, 1995; Iwata et al., 1995).

As indicated above, the main differences between heme-copper oxidases are the types of heme groups, heme A, B or O, and the electron donors, cytochrome *c* or ubiquinol (see figure 4). Heme A differs from heme B in that it contains a formyl group instead of a methyl group in position 8 and a farnesyl side chain of unsaturated isoprenoid groups in position 2 (figure 5). Heme O has the same structure as heme A except for a methyl group in place of the formyl group (Puustinen and Wikström, 1991). The nature of the heme groups in a particular oxidase does not correlate with the substrate oxidized by the enzyme; e. g., cytochrome aa_3 from *Bacillus subtilis* uses quinol as substrate (Lauraeus et al., 1991) and cytochrome ba_3 from *Thermus thermophilus* uses cytochrome *c* (Zimmermann et al., 1988).

All cytochrome *c* oxidases oxidizing cytochrome *c* have a Cu_A center, with one known exception, cbb_3 from *Rhodobacter sphaeroides*, which lacks the Cu_A center and has instead two membrane-bound cytochrome *c* subunits (Garcia-Horsman et al., 1994). It is common among bacteria to have more than one terminal oxidase to be able to adapt to different environmental conditions.

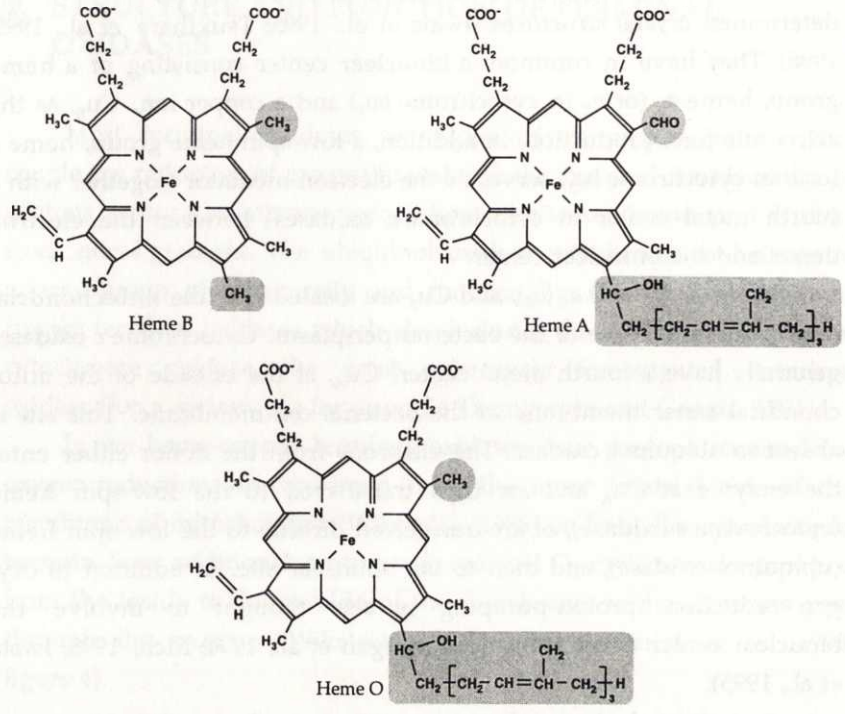


Figure 5. Different heme groups incorporated in heme-copper terminal oxidases. The shaded areas indicate differences of the heme groups.

The low-spin heme is six-coordinated and the heme in the binuclear center is five-coordinated. Heme a_3 is high-spin both in the resting ferric (Fe^{3+}) state and the reduced ferrous (Fe^{2+}) state. Heme a_3 and Cu_B are spin-coupled, abolishing the EPR signal that would arise from the oxidized form of either isolated component.

2.1 CYTOCHROME *c* OXIDASE

The bovine mitochondrial cytochrome *c* oxidase has thirteen subunits. Ten subunits are encoded by the nuclear genome whereas three subunits are mitochondrially encoded. There are no reports of prokaryotic genes corresponding to any of the nuclear-encoded subunits of eukaryotic oxidases. The minimal unit required for electron transfer linked to proton pumping consists of subunits I and II (Haltia et al., 1991; Hendler et al., 1991). These subunits of the bacterial enzymes show

high amino-acid residue similarities to those of the bovine enzyme. Three of the four redox-active metal centers, heme *a*, heme a_3 and Cu_B are located in subunit I. The fourth metal center, Cu_A, consists of two copper ions and is located in subunit II.

In addition to these four redox centers, the bovine cytochrome *c* oxidase contains a zinc bound by a nuclear-encoded subunit on the matrix side of the membrane and a magnesium ion located at the interface of subunits I and II. It has been suggested that the magnesium ion could be important for regulating proton and/or water exit (Tsukihara et al., 1995; Ferguson-Miller and Babcock, 1996). The function, if any, of the zinc ion is unknown.

To investigate the complicated mechanism of oxygen reduction and proton pumping in cytochrome *c* oxidase it has proven valuable to construct mutants of bacterial terminal oxidases (Calhoun et al., 1994). These oxidases are simpler systems with fewer subunits, and it is much simpler to construct mutants in bacteria. From studies of mutant forms of bacterial enzymes, it is possible to correlate amino-acid residues with specific functions. Cytochrome *c* oxidase from *Rhodobacter sphaeroides* is one of the most studied bacterial oxidases. It has three subunits and 50 % of the amino-acid residues of subunit I are identical to those of the bovine oxidase (Chepuri et al., 1990).

2.2 CYTOCHROME bo_3

Cytochrome bo_3 is one of *Escherichia coli*'s two terminal oxidases. It is the predominant terminal oxidase in the aerobic respiratory chain under conditions of high aeration, and it catalyzes the oxidation of ubiquinol-8 and the reduction of molecular oxygen to water. Cytochrome bo_3 is a proton pump (Puustinen et al. 1989). In addition to the vectorial proton translocation, the two-electron oxidation of quinol is associated with the release of two protons to the periplasmic side of the membrane (Puustinen et al., 1989, 1991). The other terminal oxidase in *E. coli*, cytochrome *bd*, is not a proton pump, and is expressed when the oxygen concentration is low.

Cytochrome bo_3 contains four subunits encoded by the *cyo* operon (Matsushita et al., 1984; Georgiou et al., 1988) with molecular masses of 58, 33, 22 and 17 kDa (Welter et al., 1994). Subunits I-III are homologous to the corresponding subunits of the mitochondrial cytochrome *c*

oxidases. About 40 % of the amino-acid residues of subunit I are identical to those of the bovine cytochrome *c* oxidase (Chepuri et al., 1990). Subunit I consists of 15 transmembrane alpha helices as determined by site-directed mutagenesis in combination with gene-fusion experiments (Chepuri and Gennis, 1990) and it contains all the prosthetic groups. Twelve of these fifteen helices are also found in cytochrome aa_3 from *Rhodobacter sphaeroides* and bovine heart. At the N-terminal in *E. coli* there is one extra transmembrane helix and at the C-terminal there are two extra helices. Fifteen transmembrane helices have also been found in subunit I of other bacterial oxidases (Saraste et al., 1991) and in those cases where there are two extra transmembrane helices at the C-terminal, subunit III has two fewer helices at the N-terminal. Thus the two helices can be associated with either the C-terminal of subunit I or the N-terminal of subunit III, presumably without major structural consequences.

Subunit II from the *E. coli* oxidase cytochrome bo_3 has two transmembrane helices and a large hydrophilic domain, facing the periplasm, that has been suggested to be involved in quinol binding (Welter et al., 1994). Cytochrome bo_3 lacks the Cu_A center bound in subunit II of cytochrome *c* oxidases. However, the Cu_A center can be restored in the ubiquinol oxidase by inserting its missing ligands (van der Oost et al., 1992).

Subunit III has five putative transmembrane spans but, as discussed above, most of the other heme-copper oxidases have two additional transmembrane spans at the N-terminal.

Subunit IV in cytochrome bo_3 appears to be unrelated to the corresponding subunit of mammalian oxidases. It has been suggested that subunit IV assists the Cu_B binding to the binuclear center in subunit I during biosynthesis or assembly of the oxidase complex (Saiki et al., 1996).

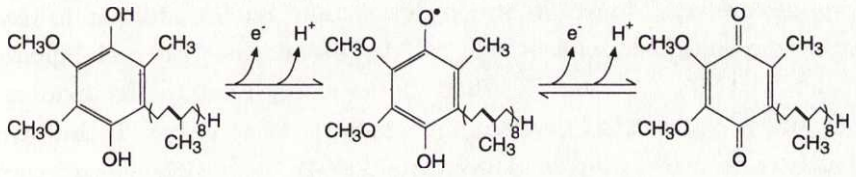
The *cyo* operon contains a fifth gene, *cyoE*, that has been shown to code for a farnesyltransferase, required for the synthesis of heme O (Saiki et al., 1992).

There are no indications of any major structural differences between subunits I of the *E. coli* ubiquinol oxidase and cytochrome *c* oxidase; the ligands and the amino-acid residues in helices around the binuclear center are the same (Lemieux et al., 1992; Minagawa et al., 1992). As pointed out above, cytochrome bo_3 contains three redox-active

groups, a low-spin heme (heme *b*), a high-spin heme (heme o_3), and a copper ion (Cu_B). The high-spin heme and Cu_B form a binuclear center where oxygen binds, and the low-spin heme delivers electrons from ubiquinol to the oxygen-binding binuclear center. *E. coli* cannot synthesize heme A because it lacks the gene for the heme A synthesizing enzyme (*CtaA*; Svensson et al., 1993).

2.2.1 *The ubiquinol-binding site*

The natural substrate for cytochrome bo_3 is ubiquinol-8 (figure 6). The binding site of the electron donor has not been identified yet, but it has been shown by radioactive-labeling studies that subunit II is involved (Welter et al., 1994).



Ubiquinol Ubisemiquinone Ubiquinone

Figure 6. Ubiquinol-8, the natural substrate of cytochrome bo_3 and the oxidized forms ubisemiquinone and ubiquinone.

It has been suggested that there are two binding sites on the protein (Sato-Watanabe et al., 1994). One high- and one low-affinity site, where the high-affinity site mediates electron transfer between the low-affinity (the quinol oxidizing) site and the low-spin heme. Others have suggested that it is very unlikely that there is more than one quinol-reactive site (Meunier et al., 1995). A semiquinone from the substrate bound in the quinol oxidation site has recently been described (Ingledew et al., 1995). It was suggested that the semiquinone is the two-electron to one-electron converter between the quinol pool and the low-spin heme in cytochrome bo_3.

2.2.2 *Two populations of the ubiquinol oxidase due to heterogeneity of the low-spin site*

In some strains of *E. coli* that overproduce the oxidase due to the presence of the *cyo* operon on a multicopy plasmid, heme *o* instead of

heme *b* is incorporated into the low-spin site in a fraction of the enzyme. This results in a mixture of two enzyme populations, cytochromes oo_3 and bo_3. It has been suggested that the farnesyl group in the low-spin site of cytochrome oo_3 is not embedded within the protein matrix as it would disrupt the 3D structure of the enzyme, but rather is located on the surface of the protein in contact with the lipids (Puustinen et al., 1992). The cytochrome oo_3 enzyme has the same catalytic turnover rate as cytochrome bo_3, but the internal electron-transfer rates differ (Morgan et al., 1993).

2.2.3 *The binuclear center*

Ligand-binding studies of cytochrome *c* oxidase and cytochrome bo_3 show that the binuclear center from the different species are very similar in structure and function. In addition to oxygen, the binuclear center is able to bind several other external ligands, such as H_2O_2, CN^- and N_3^-, that all preferably bind to the oxidized binuclear center, and CO which binds to the reduced center. Techniques such as resonance Raman (Hirota et al., 1994), UV/visible spectroscopy (Mitchell et al., 1995; Brown et al., 1994), FTIR (Hill et al., 1992) and EPR (Ingledew et al., 1993; Watmough et al., 1993), have been used to investigate the properties of the binuclear center in cytochrome bo_3. It has been suggested that Cu_B acts as a gate, controlling access of certain ligands, in particular O_2 and CO, to the heme iron in both cytochrome *c* oxidase and cytochrome bo_3 (Oliveberg and Malmström, 1992; Woodruff, 1993; Einarsdóttir et al., 1993).

2.2.4 *Redox properties of the metal centers and of the bound quinol/semiquinone*

Due to the heterogeneity in the low-spin site, the redox potentials have been difficult to determine. The midpoint potentials of hemes *b* and o_3 are both about 200 mV (pH 7) in cytochrome bo_3, i. e. slightly lower than the corresponding sites in cytochrome *c* oxidase. Heme *b* has a redox potential around 20 mV lower than the oxygen-binding heme (Morgan et al., 1993). It has also been shown that the low-spin heme *o* has a 20-30 mV lower potential than low-spin heme *b* (Bolgiano et al., 1991; Morgan et al., 1993). The redox properties of each of the metal centers can be influenced by the redox state of the others. Negative redox cooperativity between the low-spin heme and the binuclear center has

been observed both in mitochondrial cytochrome *c* oxidase and cytochrome bo_3. The negative redox cooperativity means that reduction of one metal center lowers the redox potential of the others. The midpoint potential of heme *b* is decreased by 60 mV when heme o_3 is reduced (Salerno et al., 1990). In bovine oxidase the potential of the low-spin heme is decreased by 35 mV when heme a_3 is reduced (Blair et al., 1986).

The redox potential for quinol has been determined by Ingledew et al. (1995). The quinol/semiquinone couple has a midpoint potential of 115 mV at pH 7 and the potential of the semiquinone/quinone couple is 25 mV at pH 7.

2.2.5 *Spectral properties of cytochrome* bo_3

The absorption spectra of two wild-type ubiquinol oxidases with different ratios of cytochromes bo_3 and oo_3 are shown in figure 7.

As the fraction of heme *o* in a preparation increases, the relative contribution of the absorbance at 557 nm in the reduced-*minus*-oxidized spectrum also increases. The CO-difference spectrum is not affected by the type of heme in the low-spin site. The different absorption contributions of heme *b*, heme *o* and heme o_3, respectively, have been investigated by Morgan et al. (1993) by clamping the redox state of the oxygen-binding heme by an external ligand (Blair et al., 1982).

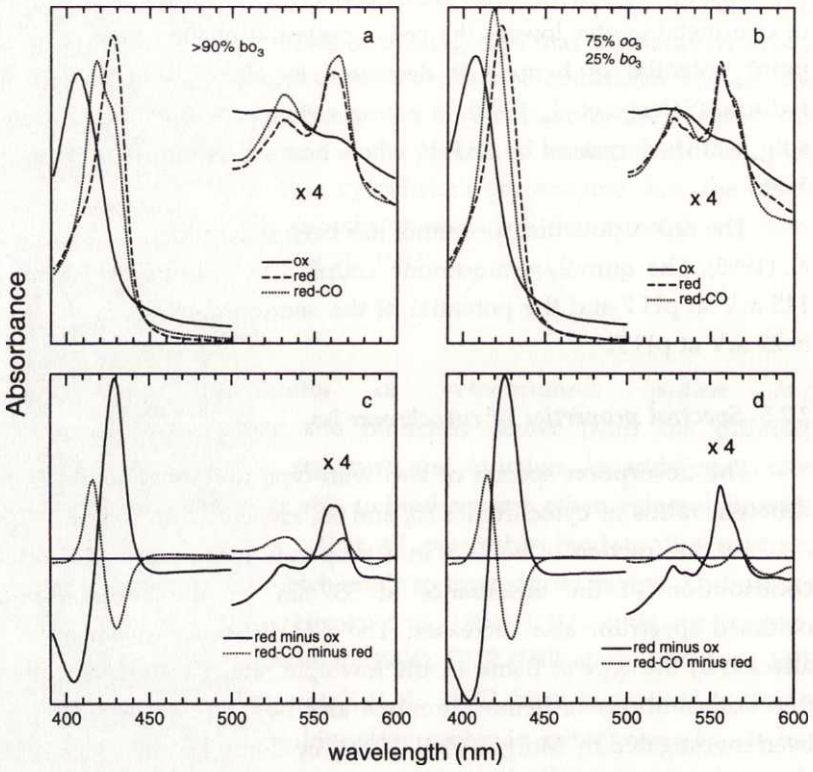


Figure 7. Absorption spectra of a histidine-tagged wild-type enzyme containing > 90 % cytochrome bo_3 (a and c) and of an enzyme preparation from the strain RG145 containing 75 % cytochrome oo_3 and 25 % cytochrome bo_3 (b and d). The oxidized form of a pure cytochrome bo_3 (a) has peaks at 407 nm, 531 nm, and 565 nm and in the reduced form the peaks are 427 nm and 560 nm. In the reduced-*minus*-oxidized spectrum of the pure cytochrome bo_3 (c), there is a single peak at 560 nm. The heterogeneity of the low-spin site (b and d) is observed as an additional peak at 557 nm in both the reduced and the reduced-*minus*-oxidized spectrum. The spectra of the differences between the fully-reduced CO-bound and reduced enzymes are shown in c and d, with a trough at 430 nm and a peak at 416 nm.

2.3 THE CRYSTAL STRUCTURES OF TWO TERMINAL OXIDASES

The crystal structures of terminal oxidases from *Paracoccus denitrificans* and bovine heart have recently been solved to 2.8 Å resolution (Iwata et al., 1995; Tsukihara et al., 1995, 1996). The environment around the metal centers of the two oxidases is highly conserved.

The *P. denitrificans* enzyme is much smaller than the bovine enzyme and consists of only four subunits. The model for subunit I, based on mutagenesis studies on cytochrome aa_3 from *Rhodobacter sphaeroides* and cytochrome bo_3 from *E. coli* (Hosler et al., 1993), which has been our working-model, is in good agreement with the crystal structures. However, the helices are longer than predicted because they are tilted in the membrane.

The three redox centers in subunit I of *P. denitrificans* are positioned at the same depth in the membrane. The distance from edge to edge between the heme groups is 4.7 Å and the center-to-center distance is 13.2 Å. Heme *a* has two axial histidine ligands, H106 and H421 (*E. coli* numbering, for interconversion between the numbering of different species see appendix) and heme a_3 is five coordinated, with H419 as an axial ligand. The heme a_3 iron is 0.7 Å out of the heme plane towards the histidine. The distance from heme a_3 to Cu_B is 5.2 Å. The copper ion has three histidine ligands, H284, H333 and H334. There is no electron density found in the crystal structure for the H333 side chain which indicates some disorder or possibly multiple conformations for this residue. This has tempted Iwata et al. (1995) to propose a proton-pumping mechanism where H333 acts as a switching ligand (see A proton-pump model).

Two possible proton pathways were found in the *P. denitrificans* structure (Iwata et al., 1995). One pathway, suggested for protons consumed in oxygen reduction (chemical protons), involves amino-acid residues of helices VI and VIII. Another pathway, lined with residues from helices II, III, IV and VI, was suggested for pumped protons. The same pathway for chemical protons is seen in the bovine structure, but the pathways suggested for pumped protons in this enzyme are different from the pathway observed in the *P. denitrificans* structure (Tsukihara et al., 1996).

Subunit II consists of three segments; an N-terminal loop, two transmembrane helices and a C-terminal globular domain which contains the fourth redox center, Cu_A. The C-terminal domain of subunit II has a fold similar to the so-called class I copper proteins, such as azurin and plastocyanin. However, the Cu_A center in the oxidases is a dinuclear center with two copper ions situated at the interface of subunits I and II. A mixed-valence form of this complex [Cu(1.5)-Cu(1.5)] has been suggested (Malmström and Aasa, 1993; von

Wachenfeldt et al., 1994) and showed by Fee et al. (1995) and Karpefors et al. (1996). The distance between the copper ions is 2.6 Å and they are bridged by two cysteines, C216 and C220 (*P. denitrificans* numbering). In addition, four other ligands to the two different copper ions, H181, M227, E218 and H224 (*P. denitrificans* numbering) have been identified. The ubiquinol oxidase from *E. coli* lacks the Cu_A center found in cytochrome aa_3 and its ligands.

2.4 THE CATALYTIC CYCLE OF OXYGEN REDUCTION

Reduction of dioxygen to water requires four electrons and four protons and takes place in a stepwise manner. The ground state of oxygen is a triplet electronic state, where two electrons are unpaired and can bind to metal centers that also have unpaired electrons, like the high-spin heme. Electrons are transferred one at a time from the reducing substrate to the binuclear center where O_2 is bound. The enzyme stabilizes the oxygen intermediates without the release of highly reactive and toxic species such as superoxide, hydrogen peroxide or hydroxyl radical. Oxygen reduction in the bovine cytochrome *c* oxidase has been studied using techniques such as resonance Raman and UV/visible-absorption spectroscopies. These techniques have provided information about different intermediates during oxygen reduction. In resonance Raman, the vibration characteristics of different metal-ligand bond strengths are monitored and the oxidation- and spin-states, and ligand type can be determined for the metal centers. In UV/visible absorption spectroscopy, the redox states of the metal centers are monitored.

During the catalytic cycle, electrons from cytochrome *c* are transferred via Cu_A and the low-spin heme to the binuclear center. Since it is difficult to resolve in time the partial reactions during turnover, the enzyme is often clamped in the fully-reduced state by binding of CO before mixing with oxygen (see the flow-flash technique). Since each step in the oxygen reduction by the fully-reduced enzyme is slower than the preceding step, partially reduced intermediates accumulate to detectable levels (Varotsis et al., 1993).

During a single turnover, starting from the fully-reduced enzyme, the following sequence of reactions has been suggested (figure 8, for a review see for example Babcock and Wikström, 1992; Einarsdóttir, 1995; Ferguson-Miller and Babcock, 1996; Morgan et al., 1996). After flash-photolysis of CO, oxygen binds to the binuclear center. It has been suggested that oxygen binds to Cu_B before being transferred to heme a_3 (Oliveberg and Malmström, 1992; Woodruff, 1993; Verkhovsky et al., 1994).

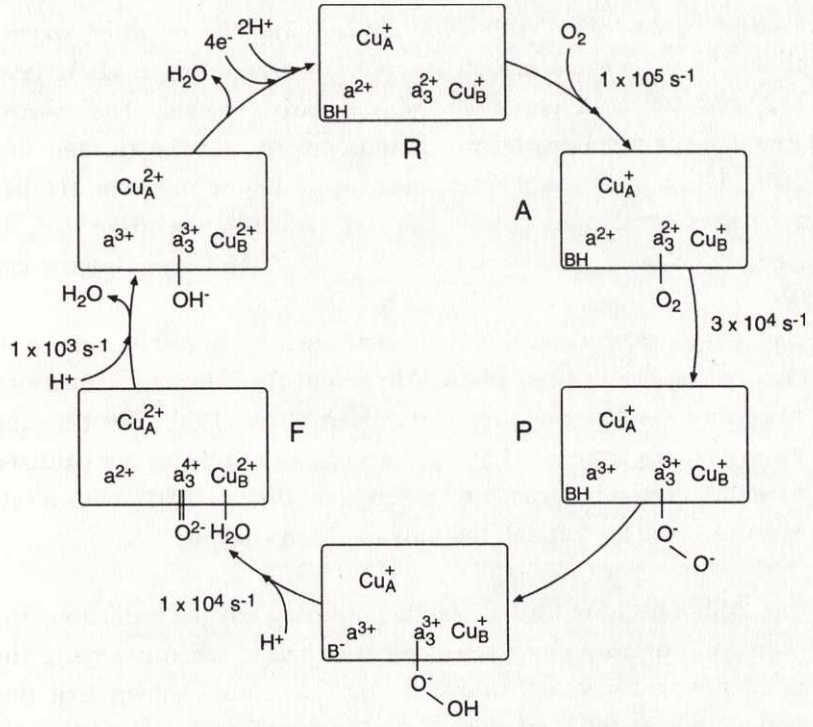


Figure 8. The catalytic cycle of solubilized fully-reduced cytochrome *c* oxidase. Two protons are taken up during reduction of the enzyme and another two protons are taken up during oxygen reduction. Since the sum of the scalar and the vectorial proton transfer of the solubilized enzyme is observed, the protons indicated in the figure can be involved either in oxygen reduction or proton pumping. R, A, P and F refer to fully-reduced enzyme, oxygen bound to the fully-reduced enzyme, peroxy intermediate and ferryl intermediate, respectively. The base B is not indicated in the ferryl and ferric-hydroxy intermediates, since it is not known whether it is reprotonated.

However, the first well-defined product is an oxygen-ferrous ($Fe^{2+} - O_2$) species (A) similar to oxymyoglobin (Chance et al., 1975; Verkhovsky et al., 1994). It is formed with a rate constant of $1 \cdot 10^5\ s^{-1}$ at 1 mM O_2 and room temperature (Oliveberg et al., 1989; Verkhovsky et al., 1994). This oxy intermediate has been identified in time-resolved resonance Raman measurements (Varotsis et al., 1989; Ogura et al., 1990; Han et al., 1990; Blackmore et al., 1991; Varotsis and Babcock, 1995). The oxygen binding is followed by oxidation of both hemes and electron transfer to the bound oxygen with a rate constant of $3 \cdot 10^4\ s^{-1}$ (Oliveberg et al., 1989; Verkhovsky et al., 1994). The resulting oxygen intermediate (P) is characterized by a 607 nm peak (Morgan et al., 1996) and it has been proposed to be a peroxy species. The peroxy intermediate is stabilized by an internal proton transfer (Hallén and Nilsson, 1992). A proton is then taken up from the medium (Hallén and Nilsson, 1992) and partial electron redistribution from Cu_A to heme *a* occurs with a rate constant of $1 \cdot 10^4\ s^{-1}$ (Hill and Greenwood, 1984).

A ferryl intermediate (F), characterized by a 580 nm peak, is formed during the $1 \cdot 10^4\ s^{-1}$ phase (Morgan et al., 1996), also confirmed by resonance Raman measurements (Han et al., 1990; Varotsis and Babcock, 1990; Ogura et al., 1993). Finally, heme *a* and Cu_A are oxidized and another proton is taken up (Hallén and Nilsson, 1992), with a rate constant of $1 \cdot 10^3\ s^{-1}$, forming the fully-oxidized enzyme.

In addition to the protons taken up during oxygen reduction, the enzyme also pumps protons across the membrane. When reversing the catalytic cycle in energized mitochondria, it has been shown that this takes place during the formation of the ferryl intermediate and the oxy form of the enzyme (Wikström, 1989).

Although the reaction of the fully-reduced enzyme and oxygen is probably not the physiological reaction (Babcock and Wikström, 1992), it is a good model for the oxygen-reduction mechanism. During catalytic turnover, oxygen will probably be reduced to peroxy by the binuclear center before new electrons enter the enzyme.

In this thesis I present results from investigations on the reduction of oxygen by cytochrome bo_3.

2.5 A PROTON-PUMP MODEL

Several proton-pump models have been proposed during the last decade. Each of the four redox centers in cytochrome *c* oxidase have been suggested to be the linkage site for proton translocation. The more recent models share a common theme in that a relatively small movement of ligands linked to the oxygen-reduction chemistry can result in the vectorial movement of protons.

It has been suggested that the hydrogen bond from the formyl group of heme *a* to a protein residue is involved in proton-pumping (Babcock and Callahan, 1983). Ligands of Cu_A have also been proposed to facilitate redox-linked proton movements (Gelles et al., 1986; Musser et al., 1995).

Since proton pumping has also been observed in quinol oxidases (Puustinen et al., 1989) which lack the Cu_A center and the formyl group of heme *a*, the coupling site of proton transfer has lately been suggested to be localized at the binuclear center.

The model described by Iwata et al. (1995) derived from the crystal structure is essentially identical to the model proposed by Morgan et al. (1994). This model describes a directly coupled proton-pump mechanism where a redox-linked ligand exchange at Cu_B controls both the binding of protons at the pumping site (a histidine) and the directionality of the uptake and release (figure 9).

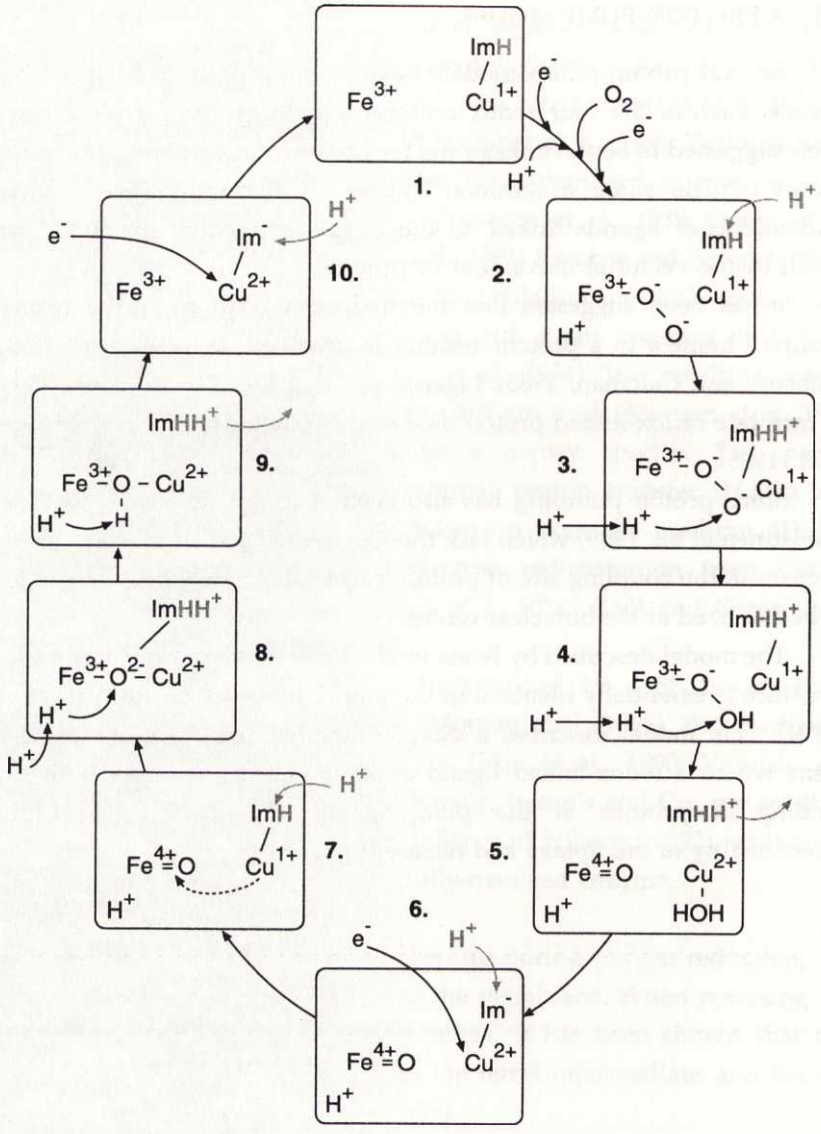


Figure 9. The histidine cycle reproduced after Morgan et al. (1994). The pumped protons are shown in gray. Oxygen binds to the reduced binuclear center and a peroxy intermediate (without a proton) is formed. The negative peroxy intermediate promotes the uptake of one proton at one of the histidine ligands (H284 (Morgan et al., 1994) or H333 (Iwata et al., 1995)) and the ligand dissociates from Cu_B. The oxygen intermediate then takes up a scalar proton and the negative charge is neutralized. The doubly protonated histidine is electrostatically destabilized and two protons are released on the outer side of the membrane. This cycle is repeated for the conversion of the ferryl intermediate to the fully-oxidized state. Here another two protons are pumped.

2.6 PROTON-TRANSFER PATHWAYS

A pathway for protons used in the reduction of oxygen to water is identified both in the crystal structure from *Paracoccus denitrificans* and that from bovine heart. The residues (*E. coli* numbering) S299, K362 and T359, the hydroxyl group of heme a_3 and Y288 line this pathway ending at the binuclear center (figure 10). The residues are connected by hydrogen bonds except for the K362 residue. The environment of K362 is hydrophobic and the lysine could therefore be deprotonated (Iwata et al., 1995). The residue Y288 might donate the proton to dioxygen (Iwata et al., 1995).

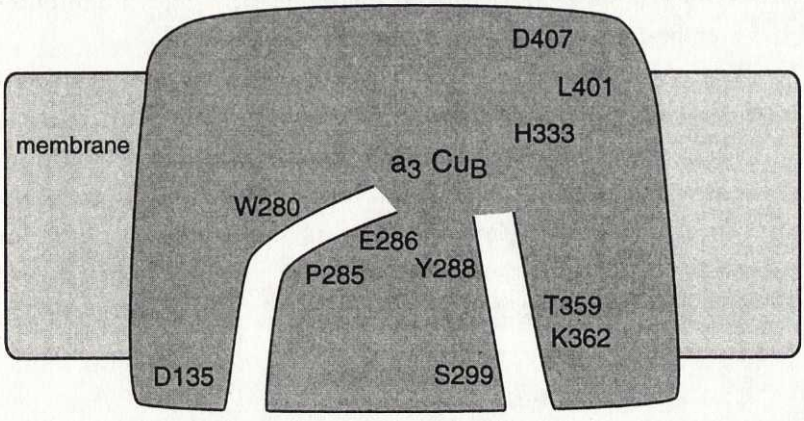


Figure 10. A schematic picture of the two proposed proton pathways based on the crystal structure of *P. denitrificans* (Iwata et al., 1995). The left pathway is proposed for "pumped" protons. *E. coli* numbering of the amino-acid residues is used. For interconversion between the numbering of *E. coli* and *P. denitrificans* see appendix.

The proton pathway for pumped protons found in the *P. denitrificans* crystal structure starts with an aspartate residue D135 (*E. coli* numbering, D132 in *R. sphaeroides*) situated in the loop between helices II and III. Beyond this residue there is a possible hydrogen-bonded pathway with several hydrophilic amino-acid residues and solvent molecules leading to E286 in helix VI (see figure 10). After E286 the pathway is less clear. Iwata et al. (1995) suggest two alternatives; (1) A direct proton transfer from E286 to the heme a_3 propionate, which with certain conformational changes could move close enough to E286 to allow proton transfer. (2) A transfer from E286

to H333 (the proposed pumping site). The regular hydrogen-bonding pattern in this area is interrupted by P285, the back-bone carbonyl atom of which could play an important role in proton conductance by binding solvent molecules. The switch of H333 could allow further conduction of protons to the carbonyl oxygen of L401 and D407 mediated by the formyl group of heme a_3. Since heme o_3 in cytochrome bo_3 has no formyl group, the protons must be transferred directly to the carbonyl oxygen of L401.

In addition, a region of hydrophilic amino-acid residues in a cleft between subunits I and II and a cluster of negatively charged residues nearby have been found in the crystal structures. These residues might be involved in proton transfer from the binuclear center to the outside of the membrane.

The pathway found in the *P. denitrificans* structure is not seen in the bovine-enzyme crystal structure (Tsukihara et al., 1996). Tsukihara et al. (1996) suggest an indirect conformationally-coupled proton pumping mechanism which means that proton translocation occurs at a site distant from the redox element. This is in contrast to the direct coupling to the oxygen chemistry suggested by many other research groups (for example Morgan et al., 1994; Rich, 1995; Iwata et al., 1995).

3. THE PRESENT STUDY

3.1 AIM OF THE PRESENT STUDY

The aim of the present study is to illuminate the reaction mechanism of terminal oxidases, how electron transfer and proton translocation are coupled, and to identify amino-acid residues involved in these processes. The oxygen reaction of wild-type and mutant forms of cytochrome bo_3 from *Escherichia coli* and cytochrome aa_3 from *Rhodobacter sphaeroides* has been studied using time-resolved optical absorption spectroscopy. The mutant enzymes were used to investigate which amino-acid residues are involved in proton-transfer reactions during oxygen reduction. The mutations, where completely or partially conserved protonatable amino-acid residues have been modified, are mainly located in two transmembrane helices; VI and VIII. These helices were chosen before the crystal structures of cytochrome *c* oxidases were reported. They were selected because they had been predicted to be near the binuclear center and because these two helices contain the only conserved protonatable amino-acid residues in the predicted transmembrane region. They were therefore assigned a role in proton translocation.

3.2 EXPERIMENTAL APPROACHES

3.2.1 *Flash photolysis of fully-reduced or mixed-valence CO-bound enzymes*

Carbon monoxide can bind to the reduced binuclear center where the oxygen chemistry normally takes place. The bond is photolabile and CO can be photodissociated from the enzyme with a short laser flash. In the absence of oxygen, CO recombines with the enzyme in about 15 ms (at 1 mM CO).

The photolability of CO can be used to study internal electron transfer in the so-called mixed-valence enzyme. The mixed-valence complex is formed by incubation of the oxidized enzyme in a CO atmosphere which reduces the binuclear center. In this state, heme *b* is

oxidized and heme o_3 and Cu_B are reduced. Since CO stabilizes the ferrous form of heme o_3, the apparent redox potential of the heme increases. After photodissociation of CO from the mixed-valence complex, the apparent potential of heme o_3 drops, and electrons redistribute within the enzyme.

3.2.2 *The flow-flash technique*

The reaction between the fully-reduced enzyme and oxygen is very fast, it is complete within milliseconds, and it cannot be studied using conventional mixing techniques. To overcome this problem, the flow-flash technique was developed by Gibson and Greenwood (1963). It is a combination of the stopped-flow and flash-photolysis methods (figure 11). Two solutions, the fully-reduced enzyme-CO complex and an oxygenated buffer are rapidly mixed. After mixing, CO is photodissociated by a laser flash and the following reactions of oxygen reduction are studied. It is important that the mixing time in the flow-flash experiment is shorter than it takes for CO to be replaced by O_2 in the dark ($t_{1/2}$=7 s for cytochrome bo_3). Takahashi et al. (1995) have shown that the photodissociation of CO does not interfere with the oxygen reaction.

In this work, the flow-flash technique has been used in combination with optical absorption spectroscopy. The electron-transfer reactions were followed by monitoring absorbance changes of the metal centers at different wavelengths (see figure 7). To identify electron-transfer steps coupled to protonation reactions, the effect of pH and the effect of replacing H_2O with D_2O in the solvent during the oxygen reduction were also investigated. Reactions limited by proton transfer usually display an isotope effect. The kinetics of proton uptake and release during O_2 reduction were monitored using pH-indicator dyes. The dyes have different absorption properties in their protonated and deprotonated states and serve as optical pH meters. Proton transfer has been studied in the solubilized enzyme, where only the net proton uptake (or release), i. e., the sum of scalar and vectorial protons, can be followed.

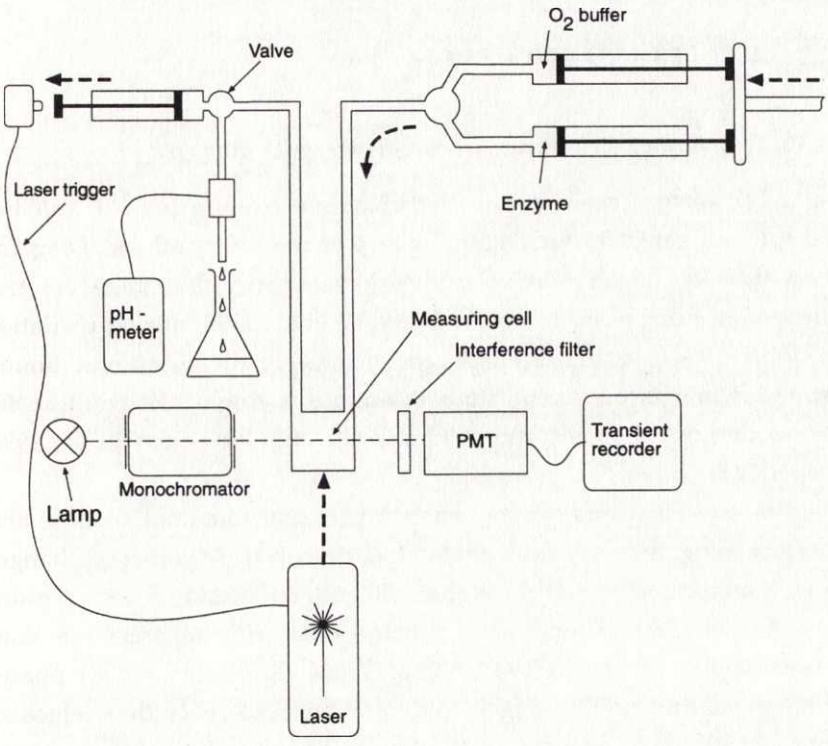


Figure 11. A schematic drawing of the flow-flash apparatus. Two solutions, the fully-reduced enzyme with bound CO and an oxygenated solution, are mixed in a stopped-flow mixing device. The stop syringe triggers a laser flash which initiates, by photodissociation of CO, the reaction between the fully-reduced enzyme and dioxygen. The measuring light passes through the cuvette and into a photomultiplier tube (PMT). The output signal from the PMT is monitored using a transient recorder.

3.3 RESULTS AND DISCUSSION

3.3.1 *Reaction of wild-type cytochrome bo_3 with dioxygen*

The oxygen chemistry in the ubiquinol oxidase from *E. coli* has been investigated by our research group as well as by others using the flow-flash technique. Earlier studies have shown that there are two electron-transfer phases (Paper I, III; Wang et al., 1995) during oxidation of the three-electron (fully-reduced) enzyme in the absence of bound quinol while more recent studies indicate a single electron-transfer phase during the same process (Orii et al., 1995; Puustinen et al., 1996; paper IV).

In paper I, a study of the reaction between ubiquinol oxidase and oxygen using the flow-flash method is described. Absorbance changes were monitored in the alpha (500-600 nm) and Soret regions (400-450 nm). Two kinetic phases were found with apparent rate constants of $3.2 \cdot 10^4\ s^{-1}$ and $3.4 \cdot 10^3\ s^{-1}$ at 1 mM O_2. Rates of both phases were dependent on the oxygen concentration. Since only three electron equivalents are available in the fully-reduced state, a ferryl intermediate should be the stable end product of the oxygen reaction in the absence of additional electron donors. The first phase was interpreted in terms of the formation of a ferric-cuprous peroxide (P). The second phase was assigned to the formation of a ferryl intermediate (F), but the observed spectral changes also contained contributions from low-spin heme oxidation.

The electron-transfer kinetics were re-investigated after it was shown that the low-spin site in the ubiquinol oxidase from *E. coli* could contain either heme *b* or heme *o*, giving rise to two different populations of the enzyme, cytochrome bo_3 and cytochrome oo_3 (Puustinen et al., 1992). Preparations with different ratios of heme *b* and heme *o* were used in the studies presented in paper III and IV. In paper III, we suggest that the heme *b*/*o* ratio is the main determinant of the relative contributions of the first and second phases. In the study described in paper IV, the heme *b*/*o* ratio was determined for different wild-type preparations and the heme content was correlated with the electron-transfer kinetics. It was found that the cytochrome bo_3 content correlated well with the amplitude of the fast phase ($3 \cdot 10^4\ s^{-1}$) and the content of

cytochrome oo_3 with the slower phase ($3 \cdot 10^3\ s^{-1}$). We concluded from this study that there is only one kinetic phase in a preparation with pure cytochrome bo_3 or pure cytochrome oo_3 as suggested by Verkhovsky et al. (1994). This single phase corresponds to oxidation of both hemes and probably to peroxy intermediate formation. The peroxy-to-ferryl transition that follows is not associated with any significant absorbance changes in the Soret region of the spectrum.

One exception to the relation between the heme *b*/*o* ratio and the fraction of the fast and second phases was found. The relative contribution of the second phase in enzyme from the strain GO103 was not consistent with the fraction of heme *o* (papers I and III). We showed in paper IV that enzyme prepared from this strain contains excess heme *b* bound unspecifically, which makes it impossible to estimate the populations of cytochrome bo_3 and oo_3, respectively. Thus, enzyme prepared from the GO103 strain most probably contains a small amount of cytochrome oo_3 in addition to the major cytochrome bo_3 population.

It is not known why heme *o* is incorporated in the low-spin site instead of heme *b* or why excess heme *b* can be found in some preparations.

The ferryl species is also obtained when cytochrome bo_3 reacts with hydrogen peroxide (Moody and Rich, 1994; Cheesman et al., 1994; Watmough et al., 1994). This ferryl form was spectroscopically identical to the product of the reaction between two-electron reduced cytochrome bo_3 and oxygen (Watmough et al., 1994), which in the bovine-heart enzyme is the peroxy intermediate (P). This observation was interpreted in terms of a protein radical in cytochrome bo_3 donating the third electron (Watmough et al., 1994). However, no clear evidence for the presence of a radical was presented. A radical involved in the reaction between the fully-reduced enzyme and oxygen has also been suggested because a ferryl species with heme *b* still reduced was observed as the end product of the three-electron reduced enzyme (Wang et al., 1995). The above results are not consistent with our multi-wavelength stopped-flow investigation of the reoxidation of cytochrome bo_3 (not published) which indicated that the end product is indeed a ferryl intermediate with heme *b* oxidized. It is formed after about 2 ms and decays in about 1 s. A fraction reduced heme *b* was only observed in enzyme prepared from the GO103 strain (discussed above)

where the heme *b* / *o* ratio is larger than one, i.e., the preparation contained excess heme *b* that cannot react with oxygen. This excess of heme *b* could be the reason why Wang et al. (1995) observe a large amount of reduced heme *b* after oxygen reduction.

The question was raised whether P and F have the same spectra or if there is a radical mechanism in cytochrome bo_3. In the latter case, the conversion of the peroxy intermediate into the ferryl species would be mediated by an amino-acid residue. The peroxy intermediate would then immediately be converted to a ferryl intermediate and the former would not be detected. In cytochrome *c* oxidase, the peroxy intermediate builds up during the reaction between the fully-reduced enzyme and dioxygen. In this enzyme, this intermediate has a spectrum similar to that of the ferryl intermediate in the Soret region, but they are different in the alpha region.

Recently, a peroxy species with an alpha-region spectrum different from that of the ferryl intermediate was identified in the reaction of cytochrome bo_3 with hydrogen peroxide, and in the reaction of oxygen with the CO-mixed valence form of the enzyme (Morgan et al., 1995). Decay of the peroxy intermediate and slow formation of the oxy-ferryl intermediate were also observed in the reaction between oxidized cytochrome bo_3 and hydrogen peroxide. Since the transition from P to F was much slower than the enzyme turnover, an additional electron could come from an amino-acid in the protein (Morgan et al., 1995), but it is more likely that the observed decay was due to electron exchange between different enzyme molecules. A peroxy species has yet to be clearly identified during the reaction of fully-reduced cytochrome bo_3 with dioxygen.

Most flow-flash investigations have been performed using optical detection in the Soret region. For cytochrome bo_3, as for cytochrome *c* oxidase, the spectra of the peroxy and ferryl intermediates are very similar in this region (Morgan et al., 1995). In addition, because heme *o* and heme *b* have large and different contributions in the Soret region (Morgan et al., 1993), and since the absorbance changes associated with the conversion of the fully-reduced enzyme to the oxygen intermediates are small, oxidation of the heme groups in the low-spin sites obscures the formation of peroxy and ferryl intermediates. Even in the alpha region where the spectrum of the peroxy species is more distinct,

the oxidation of the heterogeneous low-spin heme probably obscures the formation of the peroxy intermediate.

Taken together, these results do not imply different mechanisms in cytochrome bo_3 and cytochrome *c* oxidase. Most probably, both the peroxy and the ferryl intermediate accumulate during oxygen reduction by cytochrome bo_3 and are formed with the same mechanism as in cytochrome *c* oxidase.

In paper V, the reduction of oxygen by a pure cytochrome bo_3 enzyme preparation with bound quinol is described. This system contains five electrons. The profile of the absorbance changes measured in the flow-flash experiment of the oxygen reaction at 430 nm is similar to that of the bovine enzyme at 445 nm. The reaction was studied at different wavelengths in the range 410-440 nm; three electron-transfer phases were observed at 1 mM O_2 with rate constants of $2.2 \cdot 10^4$ s^{-1}, $1.5 \cdot 10^3$ s^{-1} and 250 s^{-1}. These rates were assigned to oxidation of hemes *b* and o_3, electron transfer from QH_2 to heme *b* and electron transfer from heme *b* to the binuclear center, respectively. The second and third phases were not observed after the addition of the herbicide HQNO, which is structurally similar to ubiquinol-8 (figure 12) and can therefore displace the ubiquinol and inhibit electron transfer from the substrate to the enzyme.

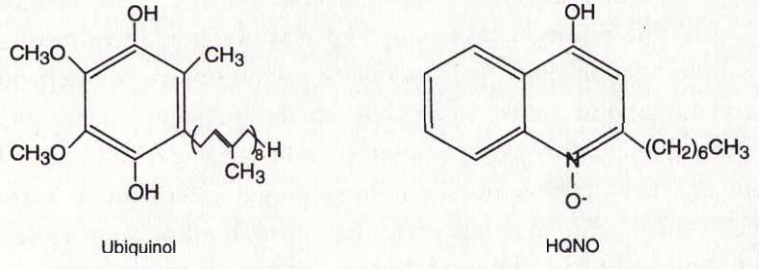


Figure 12. The substrate ubiquinol-8 and the herbicide HQNO.

The results from this study suggest that cytochrome bo_3 and bovine cytochrome *c* oxidase have the same reaction mechanisms but the rates of the re-reduction and re-oxidation of the low-spin heme differ due to the different substrates used.

3.3.2 *Proton transfer during oxygen reduction by wild-type cytochrome bo_3*

Proton uptake during oxygen reduction by the three-electron fully-reduced wild-type enzyme is described in papers II and IV. The proton-transfer reactions were monitored at 580 nm by following the absorbance changes of the pH-indicator dye cresol red. A monophasic proton uptake with a rate constant of about $1 \cdot 10^4\ s^{-1}$ and a stoichiometry of 0.8 H^+ per enzyme was observed. In the study described in paper II, we found that the proton-uptake rate lay between the two rates of electron transfer ($3 \cdot 10^4\ s^{-1}$ and $3 \cdot 10^3\ s^{-1}$) seen in ubiquinol oxidase (paper I). In the mammalian cytochrome *c* oxidase, the first proton uptake ($1 \cdot 10^4\ s^{-1}$) has been suggested to be rate limiting for the ferryl formation (Hallén & Nilsson, 1992). Therefore, since in paper I we interpreted the two electron-transfer phases in terms of formation of peroxy- and ferryl-intermediates, our results from paper II were interpreted in terms of differences in the reaction mechanisms of the two oxidases. In view of our later findings, that the two phases are associated with low-spin oxidation in cytochrome bo_3 and oo_3 (paper IV), this interpretation has to be revised. It is more likely that the two oxidases have the same mechanisms of peroxy- and ferryl-intermediate formation and proton uptake.

In the study presented in paper IV, different wild-type preparations with different ratios of cytochrome bo_3/oo_3 were investigated. In all cases one phase of proton uptake was observed. The results from this investigation show that the rate of proton uptake is independent of the heme ratio in the enzyme. Based on these results, a mechanism for oxygen reduction by cytochromes bo_3 and oo_3 is suggested (paper IV). In both enzymes, proton uptake follows peroxy-intermediate formation. In cytochrome bo_3, the electron transfer from the low-spin heme occurs simultaneously with the formation of the peroxy intermediate whereas in cytochrome oo_3, electron transfer from the low-spin heme *o* to the binuclear center is slower than peroxy-intermediate formation and proton uptake.

In paper V, a study of the reaction between dioxygen and reduced cytochrome bo_3 with bound ubiquinol is described. The first electron-transfer phase was followed by a slower proton uptake ($1 \cdot 10^4\ s^{-1}$) (see also above). The second electron transfer was accompanied by a net release of 1.1 H^+ per enzyme with a rate constant of about $1.5 \cdot 10^3\ s^{-1}$.

We assigned this phase to electron transfer from ubiquinol to heme *b* and release of a proton during oxidation of QH_2. This was followed by a slower proton uptake of about 1.2 H^+ per enzyme, with a rate constant ($250\ s^{-1}$) similar to that of the third electron-transfer phase. Consequently, this proton uptake is most likely associated with transfer of the fourth electron to the binuclear center. A scheme for electron and proton transfer in cytochrome bo_3 with bound ubiquinol is presented in figure 13.

3.3.3 *Formation of the peroxy intermediate and oxidation of the low-spin heme*

As in the mitochondrial cytochrome *c* oxidase, there is a $3 \cdot 10^4\ s^{-1}$ kinetic component in the reaction between fully-reduced cytochrome bo_3 and dioxygen. In both enzymes, this phase corresponds to oxidation of both hemes and probably to peroxy-intermediate formation. In bovine oxidase, absorbance changes associated with heme *a* oxidation dominate those associated with peroxy-intermediate formation (Morgan et al., 1996). This is evident from a comparison between the reactions of dioxygen with the mixed-valence and the fully-reduced enzyme (Morgan et al., 1996). In the mixed-valence enzyme, the peroxy intermediate is formed by electron transfer from the binuclear center. In this reaction there are no redox changes of the low-spin heme which interfere with the spectrum. The $3 \cdot 10^4\ s^{-1}$ phase observed in the reduction of oxygen by the fully-reduced cytochrome *c* oxidase contains, in the Soret region, mainly contributions from heme *a* oxidation (Morgan et al., 1996).

We suggest that the peroxy intermediate is formed with a rate of $3 \cdot 10^4\ s^{-1}$ in both cytochrome bo_3 and cytochrome oo_3. In the cytochrome bo_3 population, electron transfer from the low-spin heme *b* to the binuclear center occurs with the same rate as the formation of the peroxy intermediate. However, in the cytochrome oo_3 population the electron transfer from the low-spin heme to the binuclear center is slower ($3 \cdot 10^3\ s^{-1}$). This means that in cytochrome oo_3 the peroxy intermediate is formed with electrons from the binuclear center. A proton is taken up with a rate constant of $1 \cdot 10^4\ s^{-1}$ in both cytochromes bo_3 and oo_3.

In the reaction between mixed-valence cytochrome *c* oxidase and oxygen, a peroxy intermediate is formed with electrons from the

binuclear center as in the cytochrome oo_3 population. However, in the mixed-valence cytochrome *c* oxidase there is no proton uptake observed after the peroxy-intermediate formation (Oliveberg et al., 1991). The major difference between the cytochrome oo_3 population and the mixed-valence cytochrome *c* oxidase is the redox state of the low-spin heme. The reduced low-spin heme may induce proton uptake after peroxy-intermediate formation by shifting the pK_a values of the groups involved. The model of peroxy-intermediate formation in cytochrome oo_3 is consistent with the oxygen concentration dependence of the $3 \cdot 10^3$ s^{-1} heme *o* oxidation phase (paper I) if all intermediates are in equilibrium until the electron has been transferred.

It has been shown that the intrinsic electron transfer between hemes *a* and a_3 is very fast (Oliveberg and Malmström, 1991) and that the rate of peroxy-intermediate formation in cytochrome *c* oxidase is limited by factors other than heme-heme electron transfer. Thus, the peroxy intermediate could be formed with two electrons from the binuclear site even in the reaction of fully-reduced cytochrome *c* oxidase with oxygen (Oliveberg and Malmström, 1992). The peroxy intermediate would then be stabilized by transfer of an internal proton followed by very fast electron transfer from heme *a* to the binuclear center as suggested by Varotsis and Babcock (1995). The electron in the binuclear center would be localized on Cu_B until ferryl-intermediate formation.

3.3.4 *Formation of the ferryl intermediate and the associated electron redistribution*

The formation of the ferryl intermediate in cytochrome *c* oxidase has been detected by resonance Raman, and found to have about the same rate (Varotsis et al., 1993) as the first proton uptake phase. On the same time scale, there is an electron redistribution between Cu_A and heme *a* within the enzyme. The proton-uptake reaction was suggested to be rate limiting for the electron redistribution and for the formation of the ferryl intermediate (Hallén and Nilsson, 1992).

The formation of the ferryl intermediate in cytochrome bo_3 is also probably limited by the $1 \cdot 10^4$ s^{-1} proton uptake phase. However, the re-reduction of the low-spin heme in cytochrome bo_3 is not only limited by formation of the ferryl intermediate but also by the quinol oxidation and the accompanying proton release. This is probably the only step in

the oxygen reduction that differs for cytochrome bo_3 and cytochrome *c* oxidase. Considering the different substrates of the oxidases, this difference seems reasonable.

3.3.5 *Electron and proton transfer during reduction of oxygen in mutant enzymes where protonatable amino-acid residues have been modified*

Paper III, IV and VI describe investigations of oxygen reduction by mutant enzymes. Paper III deals with mutations made in the transmembrane helix VIII and paper IV and VI with mutations made in the transmembrane helix VI. Electron and proton transfer were studied using the flow-flash method. The mutant-enzyme preparations have no or little bound ubiquinone, so the fully-reduced enzyme contains three electrons.

The mutants investigated in helix VIII (paper III) are K362L, -M, and Q; T359A and -S; P358A; M353A; and T352S, -N, and -A. The K362, T359A, and T352A and -N mutant enzymes are inactive *in vivo* (Thomas et al., 1993a). As pointed out above, the residues K362 and T359 have been proposed by Iwata et al. (1995) to be part of a proton pathway used for transport of protons consumed in the oxygen reduction (see Proton-transfer pathways).

The electron transfer during oxygen reduction is not affected in the helix VIII mutant enzymes. A monophasic proton uptake was observed, with a rate constant at most a factor of two larger than that in wild-type oxidase. This higher rate of proton uptake was not considered to be significant and was observed in both active and inactive mutant enzymes. From the results described in paper III, we suggested that none of the polar residues in helix VIII are necessary for the proton uptake that accompanies oxygen reduction in the three-electron reduced enzyme.

An investigation of the following mutant enzymes in helix VI is presented in paper IV: W280F, P285G, E286A and Y288F. According to the model based on the crystal structure of *Paracoccus denitrificans*, Y288 lies on the proton pathway used for chemical protons, whereas W280, P285 and E286 line the pathway for pumped protons. The amino-acid residue Y288 has also been proposed to form a hydrogen bond with

one of the Cu_B-histidine ligands. The steady-state activities of the mutant enzymes were found to be very low. Electron-transfer associated with oxygen reduction was not affected in the helix VI mutant enzymes except in E286A. In this mutant enzyme, the amplitude of the first phase was larger than in the wild-type enzyme, and the second phase, associated with electron transfer in cytochrome oo_3, was faster than in the wild-type enzyme. Qualitatively, this behavior could be due to a change in the driving force for electron transfer between the low-spin heme and the binuclear center caused by the mutation. Proton uptake was impaired in all studied helix VI mutants. The conclusion from the results in paper IV is that E286 is involved in proton uptake after formation of the peroxy intermediate. The W280 and P285 residues are also important for this proton uptake but their role may be more for maintaining the structural integrity of the proton-transfer pathway.

We suggested that the end products of the oxygen reaction were the same for the mutant and wild-type enzymes except for the Y288F mutant enzyme. This mutant enzyme displays an altered absorption spectrum, probably due to the lack of the strong interaction between Y288 and one of the Cu_B ligands. Since the conversion of the peroxy species to the ferryl intermediate is associated with very small absorbance changes in the Soret region (Morgan et al., 1995), it has not been possible to determine whether the end product is the peroxy or the ferryl intermediate for the mutant enzymes. A ferryl intermediate with OH^- instead of H_2O bound to Cu_B could be formed when proton uptake is impaired.

In paper VI, a flow-flash investigation of the oxygen reduction by the E286Q mutant of cytochrome aa_3 from *Rhodobacter sphaeroides* is described. Since this enzyme contains four electrons in the fully-reduced state, it can complete the reduction of oxygen to water. Both electron and proton transfer associated with O_2 reduction were investigated. The electron transfer in wild-type cytochrome aa_3 has been studied by Ädelroth et al. (1996) and will not be discussed in detail in this thesis. In the *Rhodobacter sphaeroides* wild-type enzyme, three phases are observed at neutral pH with rate constants of $4 \cdot 10^4\ s^{-1}$, $1 \cdot 10^4\ s^{-1}$ and $1 \cdot 10^3\ s^{-1}$, similar to those observed in the bovine cytochrome *c* oxidase. The first phase is associated with oxidation of the heme groups and peroxy formation, the second phase contains contri-

bution from electron redistribution in the enzyme in addition to the formation of the ferryl intermediate, and the last phase is associated with formation of the oxy form of the enzyme. In the mutant enzyme, the first phase is observed whereas the two latter phases are absent.

As in the bovine cytochrome *c* oxidase, two proton-uptake phases are detected in wild-type *R. sphaeroides* cytochrome aa_3, with rate constants of $7 \cdot 10^3$ s^{-1} and 650 s^{-1} at pH 8. In the E286Q mutant enzyme, both these proton-uptake phases are absent.

These results are consistent with the suggestion made in paper IV, that E286 is essential for proton uptake after formation of the peroxy intermediate that initiates the transfer of the fourth electron to the binuclear center.

3.3.6 *Discussion of the results from mutant-enzyme studies in terms of the proposed proton pathways*

As indicated above, electron transfer and proton uptake during the reaction between the fully-reduced helix VIII mutant enzymes from *E. coli* and oxygen are similar to those in the wild-type enzyme. In the corresponding mutant enzymes in cytochrome aa_3 from *Rhodobacter sphaeroides*, these reactions are also similar to those in the wild-type enzyme except for the T352A mutant, where an incomplete oxygen reduction is observed (Ädelroth, P., Mitchell, D. M., Gennis, R. B., and Brzezinski, P., unpublished results). The results for the T352A mutant enzyme are not conclusive since it has a perturbed binuclear center in both *E. coli* and *R. sphaeroides* as evidenced by FTIR analysis (Thomas et al., 1993a; Hosler et al., 1996). The internal electron transfer characteristics in the absence of O_2 in the helix VIII mutant enzymes (Ädelroth, P., Mitchell, D. M., Gennis, R. B., and Brzezinski, P., unpublished results) is essentially the same as in wild-type *R. sphaeroides* enzyme (Ädelroth et al., 1995). The T359A mutant enzyme from *E. coli* displays an internal electron transfer similar to wild-type enzyme. Electron transfer in the K362M mutant of cytochrome bo_3 has been more difficult to interpret. In paper III we explained our results in terms of a different behavior in the mutant than in wild-type enzyme based on difference traces between mixed-valence and fully-reduced CO-bound enzymes. However, it was difficult to find a wavelength without contributions from CO dissociation, and there were some fast reactions seen after photolysis in the reduced enzyme. The

mixed-valence traces itself of this mutant and of the wild-type enzyme have the same appearance. Consequently, the results should rather be interpreted in terms of an unaffected internal electron transfer in the K362M mutant of cytochrome bo_3.

Thus, since both internal electron transfer and oxygen reduction are not affected, the reduced activity of the helix VIII mutant enzymes must be explained in terms of impaired input of electrons or uptake of protons associated with reduction (Verkhovsky et al., 1995; Ädelroth et al., 1996). This is consistent with the observation that the K362 mutant enzymes are difficult to reduce.

Based on the crystal structures, the investigated helix-VIII residues have been proposed to line a pathway used for "chemical protons" (Iwata et al., 1995; Tsukihara et al., 1995). In the bovine structure, this pathway has been proposed for "chemical protons" since it is the only pathway that ends at the binuclear center, but Tsukihara et al. (1995) point out that this network is not excluded from being involved in proton pumping.

It has been demonstrated that bovine cytochrome *c* oxidase takes up 2.5 protons during reduction of the enzyme (Mitchell and Rich, 1994). It has also been shown that during the reduction of the fully-oxidized enzyme, the observed reduction rate of heme a_3 is not determined by the intrinsic heme *a* to heme a_3 electron-transfer rate, but rather by proton uptake associated with the electron transfer to heme a_3 (Verkhovsky et al., 1995). Iwata et al. (1995) propose a model where the "pumped" protons are loaded, via the pathway lined with D135 and E286, during reduction. The results obtained with the helix VIII mutant enzymes, and in particular with the K362 mutant, are not consistent with this suggestion. The investigated helix VIII amino-acid residues are clearly not important for the scalar proton-transfer reactions, i. e., for protons needed during oxygen reduction when starting from the fully-reduced enzyme. Since the residue K362 is probably involved in uptake of protons during reduction of the enzyme and should be located in a "pumping" proton pathway according to the model suggested by Iwata et al. (1995), either the model or the assignment of the proposed pathways has to be wrong. The protons loaded during reduction could also be involved in oxygen reduction. After the K362 mutant enzyme has been reduced (a slower process than for wild-type enzyme), it reacts normally with oxygen.

The identification of the pathway for pumped protons in the *P. denitrificans* enzyme is consistent with the fact that the mutant D135N of cytochrome bo_3 from *E. coli* has been shown not to pump protons (Thomas et al., 1993b). Proton pumping in this mutant enzyme is decoupled from electron transfer and the mutant has a catalytic activity of 45 % of that in wild-type enzyme. In addition, the three-electron reduced form of D135N in *E. coli* takes up fewer protons than the wild-type enzyme during oxygen reduction (own result, not published). It has also been shown that proton pumping and steady-state activity in D135N can be restored to the level of wild-type enzyme by insertion of an acidic amino-acid residue in the neighborhood of D135N (Garcia-Horsman et al., 1995). In the D132N mutant of cytochrome aa_3 from *R. sphaeroides* (D135 in *E. coli*), an incomplete oxygen reduction is observed (Ädelroth, P. and Brzezinski, P., not published). The mutant enzyme E286Q in *R. sphaeroides* also shows incomplete oxygen reduction (paper VI). The glutamate to alanine (E286A) mutant in cytochrome bo_3 and the glutamate to glutamine (E286Q) mutant in cytochrome aa_3 from *R. sphaeroides* do not take up any protons during oxygen reduction by the fully-reduced enzymes.

From the mutant-enzyme studies made so far it is not possible to uniquely assign the amino-acid residues D135 (D132 in *R. sphaeroides*) and E286 to be involved in scalar or vectorial proton transfer.

3.3.7 *A proposed catalytic cycle for cytochrome bo_3*

A proposed reaction scheme for solubilized fully-reduced cytochrome bo_3 and dioxygen is shown in figure 13. Only the net proton uptake and release can be followed and it is therefore not possible to discriminate between protons used in oxygen chemistry or protons that are pumped. The steps where the mutated amino-acid residues affect the oxygen reduction are indicated. Initially, the ferrous-oxy intermediate is formed with a rate constant of $1 \cdot 10^5$ s^{-1} at 1 mM O_2 (Verkhovsky et al., 1996). This species (Fe^{2+}-O_2) has also been observed in flow-flash resonance Raman measurements (Hirota et al., 1994).

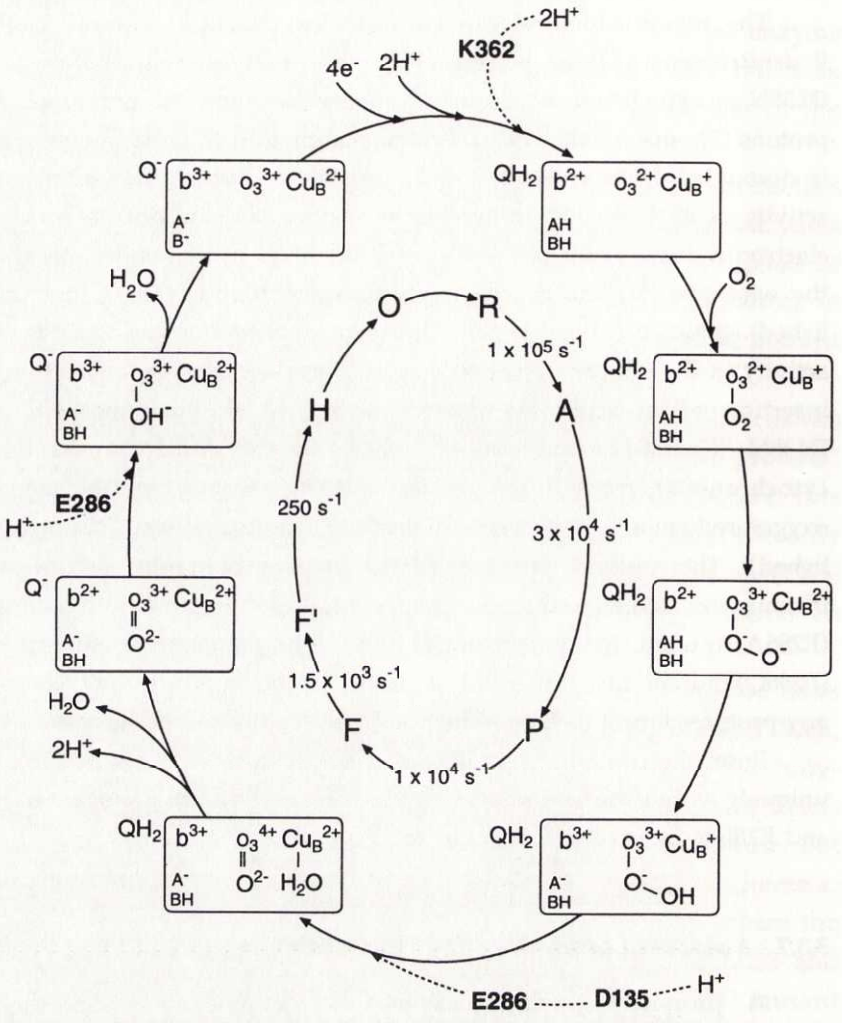


Figure 13. A proposed catalytic cycle for solubilized cytochrome bo_3. The one letter abbreviations for the intermediates represent; R, fully-reduced enzyme; A, oxygen bound to fully-reduced enzyme; P, peroxy intermediate; F, ferryl intermediate; F', a ferryl intermediate with the low-spin heme *b* reduced; H, ferric-hydroxy intermediate; O, oxidized enzyme. Two amino-acid residues, A and B, are protonated during reduction. It has been proposed that one of these protons is involved in an internal proton transfer which stabilizes the peroxy intermediate (Hallén and Nilsson, 1992). Since the net sum of the scalar and the vectorial proton uptake and release is observed in the solubilized enzyme, the protons indicated in the figure can be involved either in oxygen reduction or proton pumping.

The next component seen in the oxygen reduction corresponds to low-spin heme *b* oxidation with a rate constant of about $3 \cdot 10^4\ s^{-1}$. This phase is accompanied by the formation of the peroxy intermediate. An internal proton transfer stabilizes the peroxy intermediate. A proton is taken up with a rate constant of $1 \cdot 10^4\ s^{-1}$ concomitant with the formation of the ferryl intermediate. The bound quinol donates one electron to heme *b* with a rate constant of $1.5 \cdot 10^3\ s^{-1}$. During heme *b* re-reduction, protons are released due to the quinol oxidation. The fourth electron (third phase) is transferred to the binuclear center with a rate constant of $250\ s^{-1}$; this electron-transfer phase, accompanied by a proton uptake, involves conversion from the ferryl to the oxy form of the enzyme. Two protons are taken up during reduction of the enzyme.

The third electron- and proton-transfer phase is slower than the catalytic turnover rate (about $500\ s^{-1}$ for cytochrome bo_3). The discrepancy in these rates can be explained in terms of different driving forces for electron transfer from low-spin heme *b* to the binuclear center. In the flow-flash experiment of the fully-reduced enzyme with bound quinol, there are only five electrons available for reducing oxygen and the enzyme makes a single turnover. In steady-state experiments and *in vivo*, a large excess of ubiquinol drives the reaction towards water formation.

3.4 CONCLUDING REMARKS

The question of how terminal oxidases couple the electron and proton transfer during oxygen reduction still remains to be solved. How the proton pump works and which residues are actually involved in proton transfer, both in the scalar oxygen reduction and the vectorial proton translocation, are not known yet. This thesis demonstrates that a further understanding of these processes can be achieved by comparing the reaction mechanisms of wild-type and mutant enzymes. It has been shown that ubiquinol oxidase displays the same reaction mechanism during the reduction of oxygen as cytochrome *c* oxidase. Amino-acid residues that are important for proton-transfer reactions during oxygen reduction by terminal oxidases have been identified. We have shown that a bound quinol donates an electron to the low-spin heme during oxygen reduction as quinol protons are released. This makes it possible to investigate the input of electrons to the enzyme,

and in combination with mutagenesis studies, to identify the quinol-binding site. Future work on the ubiquinol oxidase from *E. coli* requires the preparation of a pure cytochrome bo_3 with bound ubiquinol for both mutant and wild-type enzymes. This would enable studies of electron transfer within the enzyme to be performed without interference from the low-spin heme *o*, and would greatly facilitate functional comparison to other oxidases.

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6. APPENDIX

The interconversion between the numbering of some important amino-acid residues of subunit I in different species.

Function	*E. coli*	*R. sphaeroides*	*P. denitrificans*	bovine heart
heme *a*/heme *b* ligands	H106 421	H102 421	H94 413	H61 378
heme a_3/o_3 ligand	419	419	411	376
Cu_B ligands	H284 H333 H334	H284 H333 H334	H276 H325 H326	H240 H290 H291
Amino-acid residues in proton pathways	D135 E286 L401 (C=O) D407 K362 T359 Y288	D132 E286 L401 (C=O) D407 K362 T359 Y288	D124 E278 L393 (C=O) D399 K354 T351 Y280	D91 E242 L358 (C=O) D364 K319 T316 Y244

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