Cytochrome c Oxidase dysfunction in cancer
Exploring the molecular mechanisms

Ida Namslauer
List of publications

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


Additional publication

Contents

Introduction .................................................................................................................. 9
  Metabolism and oxidative phosphorylation ......................................................... 9
  Oxidative phosphorylation ................................................................................. 10
  The mitochondrial respiratory chain ................................................................. 12

Cytochrome c Oxidase ......................................................................................... 13
  Structure and function of R. sphaeroides CytcO ........................................ 14
    General structural features ............................................................................. 14
    Function ........................................................................................................... 15
    Proton pumping in CytcO ............................................................................. 17
    Methods for studying CytcO function ........................................................... 18
  Functionally important structural features of CytcO ................................... 19
    The D pathway for proton uptake ................................................................. 19
    Glu286: an internal H⁺ donor/acceptor ......................................................... 20
    Structural changes in the D pathway affect Glu286 ..................................... 21
    The proton exit route beyond Glu286 ............................................................ 22
  The mammalian CytcO ................................................................................... 24
    Similarities between the mammalian and the R. sphaeroides CytcO ....... 24
    The additional subunits of the mammalian CytcO ..................................... 26
    An additional proton transfer pathway .......................................................... 27

The aim of this thesis ............................................................................................ 28

Modeling mtDNA mutations in the R. sphaeroides CytcO ....................... 29
  Mitochondrial DNA mutations ....................................................................... 29
  Model systems of mtDNA mutations ............................................................... 30
    Transmitochondrial hybrid cell lines ............................................................. 31
    Yeast models ................................................................................................ 31
    Mouse models .............................................................................................. 32
    The relevance of the R. sphaeroides CytcO model .................................... 33

CytcO amino-acid substitutions in cancer .................................................... 35
  Reports on CytcO defects in cancer ................................................................. 35
  Functional characterization of the pathogenic substitutions ....................... 37
  Effects on the CytcO catalytic activity ............................................................ 38
Effects on proton pumping ................................................................. 40
The results correlate between mammalian and *R. sphaeroides* CytcO .... 42
Comments on the results ................................................................. 43

The relevance of CytcO substitutions found in cancer ................. 44
Energy metabolism of cancer cells ................................................. 45
Oxidative stress and apoptosis in cancer ........................................ 46
The mitochondrial electrochemical gradient in cancer cells .......... 47

Concluding remarks and future perspectives................................. 49

Sammanfattning på svenska ......................................................... 50

Acknowledgements .................................................................... 52
References ................................................................................. 54
Introduction

Mutations in the mitochondrial DNA (mtDNA) have been found in connection to various types of human cancer. Since the mtDNA encode several polypeptides of the respiratory-chain enzymes, mtDNA mutations often affect the function of oxidative phosphorylation. Some of the identified mutations cause amino-acid substitutions in the enzyme Cytochrome c Oxidase (CytcO). CytcO is an essential enzyme in oxidative phosphorylation and it is found in almost all eukaryotic organisms as well as in many bacterial and archaeal species. Although amino-acid substitutions in CytcO have been found in human cancer cells, the impact of the substitutions on the function of the enzyme has in most cases not been verified.

We have investigated, on a molecular level, the functional effects of substitutions in CytcO related to colon- and prostate cancer. For these studies the well-characterized bacterial CytcO from the model organism Rhodobacter sphaeroides was used. The studies have provided detailed insights into how the function of CytcO is affected by the mtDNA mutations originally reported in connection to cancer.

In this thesis the relevance of using a bacterial model system when investigating molecular mechanisms of diseases found in humans is discussed. Further, possible links between a defectively functioning CytcO and the development of cancer are proposed. First, however, there is some background information on oxidative phosphorylation and on the structure and function of both R. sphaeroides and mammalian CytcO.

Metabolism and oxidative phosphorylation

Every living cell needs energy. The source of energy that is used by a cell varies depending on for example the cell type and its environment. Humans, like all mammals and other animals, use chemical energy derived from food. In every cell the energy needs to be converted in order to be accessible for
energy-requiring processes such as growth, organization, transport and reproduction. Metabolism is a set of chemical reactions involved in the uptake, conversion, digestion and excretion of energy-containing nutrients. The key players of metabolism, catalyzing the chemical reactions, are enzymes. In eukaryotic cells, mitochondria are the cellular compartments (organelles) that play a key role in energy metabolism. Mitochondria contain the enzyme complexes of metabolic processes such as the citric acid cycle, the fatty acid oxidation and oxidative phosphorylation. Therefore, most of the adenosine triphosphate (ATP), which is the molecule essential for many energy-requiring reactions in the cell, is generated in mitochondria. Mitochondria also contain their own genetic material; mitochondrial DNA (mtDNA).

**Oxidative phosphorylation**

The process of producing ATP using energy released by oxidation of nutrient molecules is called oxidative phosphorylation. In this process, electrons (derived from nutrients) are transferred from electron donors to electron acceptors in redox reactions. The reactions are catalyzed by a number of membrane-bound enzyme complexes constituting the respiratory chain (figure 1). The flow of electrons from an electron donor having a low midpoint potential to an electron acceptor with a higher midpoint potential is exergonic, releasing free energy. The energy is used by the enzyme complexes to translocate protons across the inner mitochondrial membrane in eukaryotes or the plasma membrane in bacteria and archaea. Thus, the energy is transiently stored in the electrochemical proton gradient. The exergonic flow of protons down the gradient is allowed through the enzyme ATP synthase in which the proton flow is coupled to the phosphorylation of adenosine diphosphate (ADP), producing ATP. The ATP is then transported out of the mitochondrion to be used in various energy-requiring processes in the cell.

Compared to glycolysis or lactic-acid fermentation, oxidative phosphorylation is a highly efficient way of utilizing chemically stored energy and is a process that is used by almost all aerobic organisms. In mammalian cells with functional mitochondria it has been estimated that approximately 90% of the total cellular ATP is produced by oxidative
phosphorylation [1]. However, oxidative phosphorylation is also involved in the production of potentially harmful reactive oxygen species (ROS). During electron transfer between the complexes of the respiratory chain a small amount of ROS is constantly being formed, mainly in respiratory chain complexes I and III [2-4]. Normally, cells have ways of eliminating ROS. Several enzymes, such as superoxide dismutase, peroxidases and catalase, are involved in ROS detoxification. In addition, small antioxidant molecules such as ascorbic acid and tocopherols are important in reducing the amount of ROS in cells. Although ROS have been suggested to be important in certain cell-signaling processes, an excess production of ROS (oxidative stress) is harmful to cells, leading to damage of DNA, proteins and lipids. Oxidative stress has been linked to the normal aging process as well as to diseases such as cancer and Alzheimer’s disease [5-8].

![Figure 1. The enzyme complexes of the mitochondrial respiratory chain; NADH dehydrogenase (I), succinate dehydrogenase (II), the bc1 complex (III) and CytC oxidase (IV), together with ATP synthase. Electrons are provided through the oxidation of NADH and succinate. Quinone (Q) and cytochrome c (cyt c) are electron carriers. Complexes I, III and IV contribute to the establishment of the electrochemical proton gradient by translocating protons from the negative side of the membrane to the positive. Protons flow down the gradient through ATP synthase, resulting in the production of ATP from ADP and inorganic phosphate (P_i). Electron transfers are indicated by yellow arrows and protons by red arrows. The position of the membrane is indicated in yellow.](image-url)
The mitochondrial respiratory chain

There is a general organization of the mitochondrial respiratory chain in most eukaryotes. The mammalian respiratory chain consists of four enzyme complexes: NADH dehydrogenase, succinate dehydrogenase, the $bc_1$ complex and Cyt$c$O (figure 1). All four enzymes contain redox-active cofactors involved in the transfer of electrons from an electron donor to an acceptor. The electron donors of the respiratory chain are NADH and $\text{FADH}_2$. Quinone and cytochrome $c$ act as electron carriers, transferring electrons from one enzyme complex to another. The final electron acceptor of the mammalian respiratory chain is molecular oxygen, which is reduced to water at the catalytic site of Cyt$c$O. In mammalian cells the protein subunits of NADH dehydrogenase, the $bc_1$ complex, Cyt$c$O and ATP synthase are encoded both in the nuclear genome and in the mtDNA.

Traditionally, it was believed that the enzyme complexes diffuse freely in the membrane and that electron transfer would occur upon random collisions [9]. A more recent suggestion is that the respiratory-chain complexes form large supercomplexes. Such supercomplexes have been found in mammals as well as in yeast and bacteria [10-12] (figure 2). An organization of the enzymes into larger complexes has been suggested to allow greater structural stability of the individual enzymes, efficient substrate channeling and a minimum formation of ROS, as compared to a scenario where the individual enzymes are located at some distance from each other.

Figure 2. Cryo-EM 3D map of a purified supercomplex from bovine heart mitochondria, with the docked x-ray crystal structures of complex I (blue), complex III (red) and complex IV (green). The figure is modified from [10] and is reprinted with permission.
Cytochrome c Oxidase

Cytochrome c Oxidase (CytcO) is the terminal oxidase of the respiratory chain in mitochondria and many bacteria and archaea. CytcO was discovered in the 1930’s as a member of the respiratory chain. It was mainly the work of Otto Warburg and David Keilin that established its role in metabolism [13, 14]. In the 1970’s and 1980’s the knowledge on CytcO was greatly increased and in 1977 it was shown to be a proton pump [15].

Figure 3. The structure of the bacterial CytcO from R. sphaeroides (PDB 1M56). The four subunits are shown in different colours: Subunit I in yellow, subunit II in magenta, subunit III in orange and subunit IV in pink. The heme groups; heme a and heme a₃, are shown in green and the copper cofactors as orange spheres. The black line indicate the approximate position of the lipid bilayer. The figure was prepared using the PyMOL software.
About 15 years ago, the first x-ray crystal structures of CytcO were published. These were the mammalian CytcO from bovine heart mitochondria [16, 17] and the bacterial CytcO from Paracoccus denitrificans [18]. A few years later, the R. sphaeroides CytcO x-ray crystal structure was determined [19]. The following chapter describes features of the bacterial CytcO from R. sphaeroides. Then, there is a comparison between the bacterial and the mammalian enzyme.

Structure and function of R. sphaeroides CytcO

General structural features

The available 3D-structures of the R. sphaeroides CytcO have revealed an enzyme complex containing four protein subunits [19] (figure 3). Subunit I harbors three of the four redox-active cofactors; heme a and the heme a\textsubscript{3}-Cu\textsubscript{B} catalytic site. In subunit I, two proton transfer pathways leading from the negatively charged (N) side of the membrane to the vicinity of the catalytic site have been identified [19, 20]. Subunit II contains two transmembrane α-helices and an extramembrane β-barrel domain where the additional copper cofactor; Cu\textsubscript{A} resides. The cytochrome c-binding site is presumably found in subunit II [21, 22]. The interaction between reduced cytochrome c and CytcO occurs through hydrophobic as well as electrostatic interactions between positively charged Lysine residues on cytochrome c and negatively charged residues on CytcO. Subunit III consists of seven membrane-spanning α-helices. Although subunit III does not contain any redox-active cofactors it is essential for the function of the enzyme. Removal of subunit III results in defective proton pumping [23, 24]. There is also experimental evidence showing that removal of subunit III leads to turnover-induced inactivation of the enzyme [25]. Subunit IV of the R. sphaeroides CytcO consists of one single transmembrane α-helix. It is connected to subunits I-III and held in position only via lipid molecules [19].
Figure 4. Electron and proton transfers to the *R. sphaeroides* CytcO catalytic site. The heme and copper cofactors are shown in green and orange, respectively. Electrons are transferred via Cu₄ and heme a to the heme a₃-Cu₃ catalytic site. Oxygen binds to the reduced heme a₃ iron. Protons are transferred via specific pathways to the catalytic site. For the reduction of one oxygen molecule, four protons and four electrons are required. In addition, one proton per electron transferred to the catalytic site is pumped through the enzyme. Subunit I of the CytcO is in yellow and subunit II in pink. The figure was prepared using the PDB entry 1M56 and the PyMOL software.

**Function**

CytcO catalyses the reduction of oxygen to water. For this reaction to take place electrons, protons and oxygen need to reach the catalytic site (figure 4). The electrons are donated one-by-one from reduced cytochrome c and are transferred via the redox-active cofactors Cu₄ and heme a to the CytcO catalytic site [26]. Oxygen binds to the reduced heme a₃ iron at the catalytic site. The electron transfer is coupled to proton uptake to the catalytic site and oxygen is reduced to water [27, 28]. Part of the energy available from the exergonic reduction of oxygen is used by the enzyme to pump four protons per oxygen molecule from the N side of the membrane to the positively charged (P) side, contributing to the formation of the electrochemical gradient [15].

Since the electrons needed for oxygen reduction are transferred one-by-one to CytcO during turnover, the catalytic site cycles through a number of
partly reduced intermediate states, identified spectroscopically by their different redox states and ligand-binding properties [29]. These are illustrated schematically in the CytcO catalytic cycle (figure 5). In brief, oxygen binds to the two-electron reduced (R²) catalytic site. Breakage of the O-O bond forms the P² intermediate. The next intermediate, F³, is formed when an electron and a proton is transferred to the catalytic site. The F³ intermediate decays into the fully oxidized O⁴(0) state when another electron and a proton is transferred to the catalytic site. Subsequent transfer of two protons and two electrons results in formation of water and reduction of the catalytic site. Protons are pumped upon formation of the F³, O⁴(0) and R² intermediate states. If the CytcO is preloaded with four electrons (all cofactors are reduced) the oxidative part of the catalytic cycle (R² to O⁴(0)) can be studied spectroscopically using the flow-flash technique [30]. In that case, there is electron transfer from heme a to the catalytic site upon formation of the P³ intermediate. Thus, the electron and proton transfer to the catalytic site required for formation of the F³ intermediate are separated in time and can be studied individually.
Figure 5. (a). A schematic illustration of the catalytic cycle of O$_2$ reduction in CytcO. The redox intermediate states (reduced; R, perox; P, ferryl; F and oxidized; O) are shown as well as the events taking place in the transitions between the states. The digits in superscript indicate the number of electrons present at the catalytic site. (b). The sequence of events observed when starting with the fully reduced CytcO, as in the flow-flash absorption spectroscopy. The enzyme cofactors are shown as spheres and the colours indicate an oxidized (grey) or a reduced (red) cofactor. The catalytic cycle is further described in the text.

Proton pumping in CytcO

For proton pumping to be achieved, there are some basic requirements that need to be fulfilled. First, there has to be a proton pathway all the way through the enzyme. This pathway can, however, not be open to both sides of the membrane simultaneously since that would result in proton leaks and disruption of the electrochemical gradient. Thus, there has to be a gating mechanism ensuring that the proton transfer is unidirectional. The gating mechanism could, for example, be one or several amino-acid residues alternating between two different positions. There are also suggestions for an acceptor site for pumped protons in CytcO. This acceptor site (proton loading site) is a yet unidentified protonatable group situated above the
hemes. Most probably, there is a connection between the proton gate and the proton loading site, so that the proton loading site changes its protonation state depending on the position of the gate. Such a mechanism implies that the pK_a of the proton loading site is high when there is a connection to the N side and low when there is a connection to the P side [31, 32]. Experimental evidence indicate that the proton loading site is located in the area near the propionates of heme a and heme a_3 [33, 34].

In CytcO, the energy needed for the pumping of protons against the electrochemical gradient is provided by the exergonic reduction of oxygen. As soon as the electrons and protons needed for the reaction reach the catalytic site, the free energy is lost. Therefore, the events that drive proton pumping must occur before that. There are several ways in which this could be accomplished: The proton to be pumped could be transferred to the proton loading site before the electron and substrate proton reach the catalytic site or the transfer of the substrate proton to the catalytic site is coupled to a reaction (e.g. a structural change) that conserves the free energy. Then the transfer of the pumped proton would be triggered by the structural change. The mechanism for proton pumping in CytcO is still not fully revealed, but is reviewed in [31, 35].

Methods for studying CytcO function
The function of CytcO has been studied for several decades. Optical absorption spectroscopy, together with other spectroscopic techniques, such as Fourier Transform Infrared Spectroscopy (FTIR) and Electron Paramagnetic Resonance Spectroscopy (EPR), are valuable tools for functional studies of CytcO [32, 36-38]. Site-directed mutagenesis of the bacterial CytcO allows substitution of specific amino-acid residues. In combination with spectroscopic techniques, mutational studies have offered insights into CytcO function and provided information on what parts of the protein are involved in specific processes. The atomic resolution 3D structures of CytcO are of great importance for the interpretation of results from functional studies since they can be used to link the function of the enzyme to the structure. Lately, computational tools such as electrostatic calculations and molecular-dynamics simulations have been used, especially for the mammalian CytcO, to investigate processes such as shifts in pK_a.
values of titratable groups and small structural changes taking place within the enzyme [39-41]. Quantum chemistry tools are also used and are valuable for evaluating whether a proposed mechanistic event is energetically favorable or even possible [42]. In general, results from computer simulations and calculations agree with experimental findings.

Functionally important structural features of CytcO

Functional studies, using site-directed mutagenesis, as well as investigations of the x-ray crystal structures have identified specific regions of CytcO that are important for the function of the enzyme. Those are, for example, two proton uptake pathways in subunit I, a highly conserved Glutamate residue (Glu286) close to the catalytic site and a region close to the heme groups, towards the positive side of the membrane, involved in the exit of pumped protons [19, 20, 33, 34, 43-45]. Most of the amino-acid residue substitutions investigated in the studies presented in this thesis are localized to the above-mentioned areas, which are described in more detail below.

The D pathway for proton uptake

Since protons are charged they need specific transport pathways in a protein interior. Such pathways consist of water molecules and polar amino-acid residues, forming a hydrogen-bonded chain along which protons can travel. In the R. sphaeroides CytcO, two input pathways for protons have been identified [19, 20]. One of them is called the D pathway, named after an Aspartic acid residue (Asp132) closest to the entrance. This pathway is used for the transfer of protons needed for the reduction of oxygen as well as for the pumped protons. The D pathway stretches along a distance of ~24 Å from the N side of the membrane to the Glutamate residue (Glu286) near the catalytic site (figure 6). According to the x-ray crystal structure ~10 water molecules are found within the pathway. These molecules are hydrogen bonded to a number of amino-acid residues along the path [19, 46]. Using site-directed mutagenesis, it has been shown that these residues are important for the efficient transfer of protons through the D pathway [20, 47-50]. In addition to the D pathway, there is a second proton-uptake pathway
called the K pathway, which is suggested to be used for proton uptake during reduction of the catalytic site [30].

**Figure 6.** The D pathway is used for delivery of protons to the catalytic site and for the uptake of pumped protons. It consists of approximately 10 water molecules (shown as red spheres) and several polar amino-acid residues forming a hydrogen-bonded chain from the N side of the membrane to the Glutamic acid residue (Glu286) near the catalytic site. The cluster of water molecules and the two Arginine residues (Arg481 and Arg482), located above the heme groups, are involved in the transfer of pumped protons. The heme groups are in green and the copper cofactors as orange spheres. The CytcO subunit I is shown in yellow and subunit II in pink. The figure was prepared using the 1M56 PDB entry and the PymOL software.

**Glu286: an internal H⁺ donor/acceptor**

The Glu286 residue is situated close to the catalytic site (figure 6). In wild-type CytcO, proton pumping and proton transfer to the catalytic site takes place upon formation of the F and O intermediates, as well as upon reduction.
of the oxidized Cyt cO [32] (figure 5). Site-directed mutagenesis studies of the R. sphaeroides Cyt cO in combination with spectroscopic techniques have shown that the substitution of Glu286 to Glutamine, Alanine or Histidine impairs proton transfer to the catalytic site as well as proton pumping [43, 51] (paper I). The Glu286 residue is considered to be a branching point, from where protons can be transferred either to the catalytic site or to a proton loading site. In addition, Glu286 (or water molecules around this residue) is suggested to function as a transient donor/acceptor for protons. It is believed that protons can be transferred from Glu286 to the catalytic site and that Glu286 is then quickly reprotonated from the N side. Results from investigations of the internal proton transfer from Glu286 to the catalytic site indicate that the pKₐ of Glu286 is 9.4 [52].

There are suggestions that the Glu286 residue can adopt two different conformations and that the structural change of Glu286 is involved in directing protons to the catalytic site and to the proton loading site [53]. This mechanism was based on information obtained from Cyt cO x-ray crystal structures. A comparison of the wild-type and the Glu286Gln Cyt cO crystal structures showed that the Glu286 side chain adopted different conformations in the two structures [19]. This conformational change of Glu286 also conferred structural changes in the region around the hemes and two conserved Arginine residues. A proton pumping mechanism involving the conformational change of Glu286 was proposed [53]. More recently, a conformational change of the equivalent Glutamate residue was observed in the crystal structure of P. denitrificans Cyt cO harboring an amino-acid substitution in the D pathway [54]. In this case, the proton pumping was abolished and it was suggested that the reason for uncoupling was the altered conformation of the Glutamate side chain resulting from the amino-acid substitution in the D pathway.

**Structural changes in the D pathway affect Glu286**

Experimental evidence show that substitutions of specific amino-acid residues in the D pathway result in both uncoupling of proton pumping and a changed apparent pKₐ of the Glu286 residue [48, 50], but with retained oxygen reduction activity. From these results, it was suggested that the amino-acid substitutions change the hydrogen-bonded network around the
Glu286 residue. As a result, protons would still be transferred to the catalytic site but not to the proton loading site. In addition, molecular-dynamics studies based on x-ray crystal structures of CytcO have identified a cluster of water molecules hydrogen-bonded to two Serine residues (Ser200 and Ser201) in the D pathway [40, 55]. This region was suggested to be a “proton trap” allowing fast protonation of Glu286, which is consistent with the high \( pK_a \) of Glu286. To further investigate the interactions between amino-acid residues and water molecules in the D pathway and their role in the proton-pumping mechanism, the Serine residues were substituted for Valines. Absorption spectroscopy studies of the CytcO variants showed that the substitutions resulted in a decreased steady-state activity, lowered stoichiometry of proton pumping and slowed proton transfer from Glu286 to the catalytic site [47]. The rate of the internal proton transfer to the catalytic site was independent on the pH and the \( pK_a \) of Glu286 was suggested to increase to >12 in the Ser200Val/Ser201Val CytcO.

The proton exit route beyond Glu286

A specific pathway for pumped protons from Glu286 towards the P side of the membrane has not been identified. However, there is a hydrogen-bonded network observed in the area above the heme groups [19, 33, 34, 56] (figure 6). This region contains water molecules and two Arginine residues (Arg481 and Arg482) that interact electrostatically with the D-propionates of heme \( a \) and heme \( a_3 \). Spectroscopic studies have shown that the Arg481 and 482 residues are important for the function of CytcO [33, 34, 56]. Based on the experimental data on CytcO in which the Arg481 was exchanged for a Lysine, it was suggested that the proton loading site is the heme \( a_3 \) D-propionate [33]. In another study, though, the identity of the proton loading site was suggested to be the heme \( a \) A-propionate [34]. Although a specific group acting as a proton loading site has not been identified, the area around the heme propionates and the Arginines are likely to be involved in the proton pumping activity.

In paper I, structural elements involved in proton pumping were investigated using amide hydrogen/deuterium exchange followed by mass spectrometry. The study lead to the suggestion that a region of CytcO involved in gating of pumped protons resides in a loop consisting of amino-
acid residues 169-175 (the gating loop) [57]. With the *R. sphaeroides* wild-type CytcO, the gating loop showed a different amide H/D exchange pattern in the F intermediate of the CytcO catalytic cycle compared to the P and O intermediates, indicating that a conformational change takes place upon formation of the F intermediate. Since proton pumping occurs before as well as after formation of the F intermediate, a conformational change of the 169-175 loop was suggested to be involved in proton gating, ensuring unidirectional proton transfer. With the CytcO in which the Glu286 was substituted for a Histidine, the amide H/D exchange pattern of the gating loop was similar in all intermediate states of the catalytic cycle (paper I). In addition, a peptide of residues 320-340, suggested to be part of a proton exit pathway, showed dramatically decreased deuterium incorporation with the Glu286His CytcO compared to the wild-type enzyme. The Glu286His CytcO is unable to pump protons. Presumably, the Glu286His substitution prevents conformational changes required for proton pumping. Since the substitution of the Glu286 residue leads to conformational changes in the fairly distant proton gating region of the enzyme, the results from paper I indicate that there is a connection, probably formed by hydrogen bonds, between Glu286 and the suggested proton gating region.
The mammalian CytcO

Now, the structure and function of the bacterial CytcO from *R. sphaeroides* have been described. Since in these studies, the *R. sphaeroides* CytcO has been used as a model system for investigations of amino-acid residue substitutions originally identified in the human enzyme, the mammalian CytcO from bovine heart mitochondria (figure 7) will now be described in terms of structure and function. Overall, the mammalian and the *R. sphaeroides* CytcOs are functionally as well as structurally similar.

*Figure 7. The structure of the mammalian CytcO from bovine heart mitochondria (PDB entry 10CC). The mammalian CytcO consists of 13 subunits illustrated in different colours. The figure was prepared using the PyMOL software.*

**Similarities between the mammalian and the *R. sphaeroides* CytcO**

The amino-acid sequence identities of the *R. sphaeroides* CytcO compared to the mammalian enzyme are 52 % (subunit I), 39 % (subunit II) and 49 % (subunit III) [58], which is considered a high sequence identity. These numbers are comparable to subunit I of *P. denitrificans* NADH
dehydrogenase, having a 64 % sequence identity to the mammalian counterpart [59]. The ATP synthase from *E. coli* is, with a 70 % amino-acid sequence identity, even slightly more similar to the mammalian enzyme [60]. The structures of subunits I, II and III of the mammalian CytcO, which are considered to be the catalytically active core of the enzyme, are very similar to the equivalent subunits of *R. sphaeroides* CytcO [61]. A structural alignment of subunits I and II of *R. sphaeroides* and mammalian CytcO is shown in figure 8. The only clear differences between the structures are at the N- and C-termini and in a loop consisting of residues 64-89 (*R. sphaeroides* amino-acid numbering). The functionally important parts of the *R. sphaeroides* CytcO discussed previously are also found in the mammalian enzyme. The D pathway is present in the mammalian CytcO and is believed to be used for transfer of protons from the N side to the catalytic site [17]. The Glu286 residue and the two Arginines (Arg481 and 481) close to heme a are conserved between the *R. sphaeroides* and the mammalian CytcO. Due to the difficulties of using site-directed mutagenesis to introduce amino-acid substitutions in the mammalian CytcO, functional mechanisms are often investigated using computational methods. Results from several in silico studies, including molecular dynamics simulations and electrostatic calculations, suggest a crucial role of Glu242 (Glu286 in the *R. sphaeroides* enzyme) in the mammalian CytcO [39, 40, 62]. The area above the heme groups, important for gating and exit of pumped protons, has also been investigated using computational studies [41, 63]. Based on simulations showing dissociation of a hydrogen-bonded network near the heme a propionate upon reduction of heme a, it was suggested that the proton loading site is localized to the area of the heme a propionates.

Experimental studies indicate that neither the spectral properties nor the function of the mammalian CytcO differ considerably from the *R. sphaeroides* enzyme [58]. Electron transfer that takes place during the single turnover reaction of fully reduced to oxidized CytcO from *R. sphaeroides* and bovine heart mitochondria have been evaluated and compared using absorption spectroscopy techniques [64]. The rates of the electron-transfer events in the bacterial and the mammalian CytcO are comparable and the
same intermediates of the catalytic cycle (figure 5) are formed with similar rates [65].

Figure 8. An alignment of subunits I and II of the bovine (PDB 10CC) and the R. sphaeroides (PDB 1M56) CytcO. The R. sphaeroides enzyme is shown in magenta and the mammalian CytcO in blue. The figure was prepared using the PyMOL software.

The additional subunits of the mammalian CytcO
The mammalian CytcO is a large enzyme complex consisting of 13 subunits (figure 7). The nuclear-encoded subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc and VIII) are translated in the cytosol and transported into the mitochondrial inner membrane. These are not present in the bacterial CytcO. Considering the functional similarities between the mammalian CytcO and
the four-subunit *R. sphaeroides* CytcO, the function of the additional 10 subunits is elusive. It has been suggested that these subunits stabilize and/or regulate the mitochondrial CytcO. In a recent study, small interfering RNA (siRNA) depletion of subunit Vb resulted in decreased activity and assembly of CytcO as well as in a decreased membrane potential and increased ROS formation [66]. Other studies have focused on the regulatory roles of the nuclear encoded subunits and suggest that there are several ATP/ADP binding sites in the mammalian CytcO, allowing allosteric regulation of the enzymatic activity [67].

**An additional proton transfer pathway**

Apart from the two proton-conducting D and K pathways found in both the bacterial and the mammalian CytcO, in the mitochondrial protein an additional proton pathway, the H pathway, has been identified based on the existence of a hydrogen-bonded network observed in the x-ray crystal structure [17]. Mutational studies of the bovine CytcO confirmed the importance of the H pathway for maintaining proton pumping [68, 69]. The mutations were made using an expression system constructed in HeLa cells. The system produces a bovine-human hybrid enzyme, which could complicate the interpretation of the results. Moreover, there is no evidence for an H pathway in the bacterial CytcO. Several of the amino-acid residues found in the mitochondrial CytcO H pathway are not conserved in the *R. sphaeroides* enzyme and substituting the corresponding H pathway amino-acid residues in the bacterial CytcO had no effect on the proton pumping activity [70, 71]. In the yeast (*Saccharomyces cerevisiae*) CytcO structure, which is a homology model based on the bovine x-ray crystal structure, there are hydrophilic amino-acid residues identified in the area of the H pathway. However, amino-acid residues of the D and the K pathway are conserved to a higher extent than residues of the H pathway [72].
The aim of this thesis

Despite the large number of reports on mtDNA mutations associated with cancer, the functional significance of the amino-acid substitutions caused by the mutations remains elusive in the majority of cases. In order to understand disease mechanisms and develop therapeutic approaches it is important to uncover the functional characteristics resulting from potentially pathogenic mtDNA mutations. The aim of my studies has been to characterize, on a molecular level, a number of phenotypes of CytcO found in connection to cancer. On the basis of the results, the remaining part of this thesis addresses the following issues:

1. The relevance of using CytcO from the bacterial model *R. sphaeroides* for functional studies on amino-acid residue substitutions found in human disease.

2. The possible consequences of the substitutions for the development or progression of cancer.
Mitochondrial DNA mutations

The human mtDNA is folded into a double-stranded, circular molecule of approximately 16600 basepairs and contains 37 genes. 13 genes encode polypeptides of the electron transport chain of which seven are subunits for NADH dehydrogenase, one for the bc₁ complex, three for CytcO and two for ATP synthase. The other genes encode 22 tRNA molecules and two ribosomal RNA molecules (figure 9).

Figure 9. A schematic illustration of the human mtDNA; a circular molecule of approximately 16600 basepairs. The mtDNA encodes mRNA for cyt b, NADH dehydrogenase, CytcO and ATP synthase as well as tRNA and rRNA. The D loop (displacement loop) is a non-coding segment and is part of the control region of the mtDNA molecule.
The mammalian mtDNA molecule lacks introns and thus has a high proportion of coding DNA. Moreover, the mtDNA has a higher mutation rate compared to the nuclear genome [73, 74]. This is, at least partly, probably due to its close proximity to the generation sites of ROS. Many mtDNA alterations are neutral polymorphisms [75]. However, in the late 1980’s the first pathogenic mtDNA mutations were identified [76, 77]. Since then, there has been extensive research on mtDNA and several hundreds of potentially pathogenic mutations have been identified [78].

Mutations in the mtDNA have been found in a large number of different diseases. The so-called mitochondrial diseases such as Leigh syndrome, LHON (Leber’s hereditary optical neuropathy) and mitochondrial myopathy are all severe disorders affecting the function of oxidative phosphorylation, resulting in insufficient synthesis of ATP [78, 79]. More recently, mtDNA mutations have been found in cancer, neurodegenerative diseases and normal aging [5-8, 79, 80]. One of the first reports on mtDNA mutations in connection to cancer is from 1998, when Polyak et al identified a number of mtDNA mutations in human colorectal tumors [81]. Since then, there have been more than 300 reports on human cancers containing mutations in coding parts of the mtDNA [82].

Model systems of mtDNA mutations

Although several mtDNA mutations are observed in cancer cells, there is a lack of experimental data, investigating the phenotype resulting from the mutations and how it could be linked to the disease. This has resulted in discussions on the actual functional relevance of the mtDNA mutations [83-85]. To determine the effects of a mutation and understand how the amino-acid residue substitution resulting from a mutation could be involved in disease mechanisms, functional studies have to be made. This requires some kind of model system. There are various models available, enabling observations of how different features and cellular processes are affected by an amino-acid substitution. Three model systems that are frequently used to investigate the functional effects of mtDNA mutations are transmitochondrial hybrid cell lines, yeast cells and mouse models. I will describe these briefly and discuss what information can be obtained using
the different systems. For my studies, I chose to model mtDNA mutations in CytcO from the bacterium *R. sphaeroides*. The relevance of using this bacterial model to investigate mtDNA mutations found in humans will also be discussed.

A summary of some advantages and shortcomings of the different models used for studies on mtDNA mutations are listed in Table 1.

**Transmitochondrial hybrid cell lines**

Transmitochondrial hybrid cell lines or cybrids, are obtained by fusing enucleated cytoplasts, derived from patient cell lines, with immortalized human cell lines devoid of mtDNA. The first transmitochondrial cybrids were described in 1989 [86]. Since then, cybrids have been used for studies of potentially pathogenic human mtDNA point mutations [87, 88]. Transmitochondrial cybrids is an *in vitro* system with the advantage that the phenotype resulting from a mtDNA mutation can be studied against a fixed nuclear background. The model allows a certain phenotype to be assayed regarding for example oxygen consumption rates, activities of the respiratory-chain enzymes, ROS production and apoptosis. Further, there are studies where the transmitochondrial cybrids containing a mtDNA mutation have been transferred to mice in order to investigate the impact of the mutation on tumor development or metastasis [89, 90]. However, the use of cybrids is not uncontroversial. There are several cases where *in vivo* phenotypes could not be replicated in an *in vitro* cybrid system [91, 92].

**Yeast models**

Yeast models have been used in several cases to study the effects of disease-related mtDNA mutations [93-95]. Introduction of mutations into the mtDNA of yeast (such as *S. cerevisiae*) is possible [72]. The respiratory-chain complexes of yeast show a high similarity in both structure and function to the mammalian counterparts. The use of yeast cells enables investigations of the impact of a mutation on for example cellular growth rate, respiratory function, activities of the individual respiratory chain enzymes and ROS production. In addition, the protein with the amino-acid substitution resulting from the mutation can be purified. In that way a more
detailed analysis of the function of the protein can be obtained. The ability of *S. cerevisiae* to survive without a functional respiratory chain makes it possible to study the effects of mtDNA mutations that result in severe respiratory defects. As *S. cerevisiae* lacks NADH dehydrogenase, studies on mutations affecting complex I have been made using the obligate aerobic yeast *Yarrowia lipolytica*, that contain a respiratory chain including NADH dehydrogenase [96].

**Mouse models**

Using mouse models for studies on mtDNA mutations allows genetic manipulations such as depletion or deletion of genes. Introduction of specific point mutations in the mouse mtDNA is not easily accomplished. However, the development of *trans*mitochondrial mice models has enabled *in vivo* studies of the impact of mtDNA point mutations [97]. Mammalian *in vivo* models is the only alternative when studying certain aspects of mtDNA mutation pathogenesis, such as how one specific mutation can result in multiple different phenotypes, why a deficiency of a specific enzyme is observed in several different diseases or why some tissues are more affected by a mutation than others. Using mice it is also possible to address questions concerning the onset and severity of a disease.
Table 1. Some advantages and shortcomings of the different model systems that can be used when studying functional effects of mtDNA mutations.

### Advantages Shortcomings

<table>
<thead>
<tr>
<th>Purified bacterial enzyme</th>
<th>• Genetic modifications easily accomplished</th>
<th>• Prokaryotic system lacking several subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>• Well-defined system</td>
<td>• No cellular context</td>
</tr>
<tr>
<td></td>
<td>• Relatively cheap &amp; quick</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Molecular level studies</td>
<td></td>
</tr>
<tr>
<td>Yeast cells</td>
<td>• Eukaryotic system</td>
<td>• Genetic modifications more difficult compared to bacteria</td>
</tr>
<tr>
<td></td>
<td>• Genetic modifications possible</td>
<td></td>
</tr>
<tr>
<td>Cybrids</td>
<td>• Allows comparisons of phenotypes against a fixed nuclear background</td>
<td>• Experimentally difficult</td>
</tr>
<tr>
<td></td>
<td>• Mammalian system</td>
<td></td>
</tr>
<tr>
<td>Animals</td>
<td>• Mammalian system</td>
<td>• Genetic modifications difficult</td>
</tr>
<tr>
<td></td>
<td>• Cellular context</td>
<td>• Expensive</td>
</tr>
<tr>
<td></td>
<td>• Organismal level</td>
<td>• Ethical issues</td>
</tr>
</tbody>
</table>

The relevance of the *R. sphaeroides* CytcO model

Traditionally, CytcO has been purified from bovine heart mitochondria, *S. cerevisiae* and the bacterial species *P. denitrificans* and *R. sphaeroides*. Since the beginning of the 1990’s the *R. sphaeroides* CytcO has been used in a large number of both structural and functional studies. Several investigations have thoroughly evaluated the *R. sphaeroides* CytcO as a model for the eukaryotic enzyme and found that it is functionally homologous to the mammalian CytcO [58, 64, 65]. Functional studies using *R. sphaeroides* CytcO have been of great importance for the elucidation of for example the proton-transfer pathways and the proton-pumping mechanism. Although some pieces of information are still missing, today we have a good picture of the functional mechanisms of *R. sphaeroides* CytcO. The increasing number of x-ray crystal structures, provide valuable information for the interpretations of experimental results. Bacterial CytcO has previously been used for studies on functional effects of pathogenic mutations [93, 98]. These investigations provided detailed analyses of the effects of mtDNA mutations found in mitochondrial diseases.
Similar to other bacterial models, *R. sphaeroides* is experimentally easy and fairly cheap to work with. Methods for both expression and purification of *R. sphaeroides* CytcO have been developed and work satisfactory. Further, the use of *R. sphaeroides* CytcO enables site-directed mutagenesis studies. As mentioned previously, *S. cerevisiae* CytcO has also been used to study effects of pathogenic mtDNA mutations. However, genetic modifications of *S. cerevisiae* are more difficult and time-consuming compared to a bacterial organism. In spite of the fact that yeast is a eukaryotic organism, the amino-acid sequence of subunits I-III of *S. cerevisiae* and *R. sphaeroides* CytcO has in fact a similar degree of homology to the mammalian enzyme [19, 99]. Further, the number of functional studies made with the *R. sphaeroides* CytcO greatly outnumber the ones where yeast has been used. Naturally, a highly validated model is more attractive to use. Experimental results are easier to interpret and evaluate if there are already a large number of studies made using that specific model.

The various models for studying mtDNA mutations enable investigation of different functional characteristics of the phenotype. *In vivo* and also *in vitro* models, such as purified mitochondria or trans-mitochondrial cybrids, provide a cellular context that is absent when using purified proteins. On the other hand, the use of purified proteins enables molecular-level studies and can provide information that is impossible to obtain using other model systems. Detailed information on the functional effects of amino-acid substitutions found in pathogenic conditions is valuable since it leads to elucidation of possible disease mechanisms.
CytcO amino-acid substitutions in cancer

**Reports on CytcO defects in cancer**

Amino-acid substitutions in CytcO have been found for example in prostate cancer, pancreatic cancer and ovarian cancer [90, 100-102]. In addition, substitutions in CytcO have been reported in normal colonic crypt stem cells in colon cancer patients [103, 104]. Greaves et al identified the mutation 6277A>G in the CytcO subunit I gene. The mutation, resulting in the amino-acid substitution Gly125Asp, was found in colonic crypt stem cells showing a CytcO deficiency. The authors suggested that the substitution was highly likely to be the cause for the CytcO deficiency, but no further studies were made to confirm this. In another study, Pye et al identified a missense mutation in the gene encoding subunit I of CytcO in respiratory-deficient transmitochondrial cybrids [104]. The mutation resulted in a Tyr19His amino-acid substitution. The transmitochondrial cybrids were produced using normal human colon cells from patients treated for adenocarcinoma. Studies on mitochondria isolated from the cybrids showed a decrease in the CytcO activity. In 2005, there was a report on a number of mutations in the gene encoding subunit I of CytcO found in prostate cancer cells [90]. The amino-acid substitutions resulting from the mutations were located to different regions of subunit I. Some of the substitutions affected highly conserved amino-acid residues. The functional effects of the substitutions were, however, not investigated.

The background to my studies is the reports on CytcO substitutions found in patients with colon cancer and prostate cancer [90, 103, 104] and the aim of the work was to elucidate if and/or how the functional mechanisms of the enzyme was affected by the substitutions. All substitutions were found in subunit I of CytcO (figure 10). The equivalent mutations were produced in the subunit I gene of *R. sphaeroides* CytcO and the enzyme containing the
corresponding amino-acid substitutions was purified. The Gly125 and the 
Tyr19 residues that were affected in the colon cancer patients, correspond to 
Gly171 and Tyr33, respectively in the R. sphaeroides CytcO. Among the 
amino-acid substitutions found in CytcO of prostate cancer cells, Asn11Ser, 
Ala122Thr, Ala341Ser and Val380Ile were chosen for the study. In R. 
sphaeroides CytcO these residues correspond to Asn25, Ser168, Ala384 and 
Val423. For the functional studies, various spectroscopic techniques were 
used. Thereby we could examine, at a molecular level, how the amino-acid 
substitutions affected the individual electron- and proton transfer events of 
the CytcO catalytic cycle as well as the proton pumping activity of the 
enzyme.
Figure 10. Subunit I (yellow) and II (pink) of R. sphaeroides CytcO. The amino-acid residues that were substituted in colon- and prostate cancer are indicated in blue. The red spheres are the oxygen atoms of the water molecules in the D pathway. The Arginine residues Arg481 and Arg482 are shown in cyan. The arrows indicate the proton-transfer routes. The figure was prepared using the 1M56 PDB entry and the PyMOL software

Functional characterization of the pathogenic substitutions

None of the amino-acid substitutions in subunit I of CytcO that were studied resulted in a complete loss of enzymatic activity. However, with the Gly171Asp and the Tyr33His CytcO, a decreased catalytic activity was observed. Moreover, in these two cases specific steps of the catalytic cycle were slowed compared to the wild-type enzyme. Investigations of the
proton-pumping activity showed that in four out of six of the CytcO variants the proton pumping was lost or clearly defective. The results from papers I-IV are summarized in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>Steady-state activity (% of wt)</th>
<th>Proton pumping (number of H⁺/e⁻)</th>
<th>Proton leak</th>
<th>Rate of O₂ binding (µs)</th>
<th>Rate of P formation (µs)</th>
<th>Rate of F formation (µs)</th>
<th>Rate of O formation (ms)</th>
</tr>
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<tr>
<td>Wild-type</td>
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<td>No</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Glu286His</td>
<td>1</td>
<td>0</td>
<td>No</td>
<td>10</td>
<td>80</td>
<td>10000</td>
<td>1200</td>
</tr>
<tr>
<td>Gly171Asp</td>
<td>30</td>
<td>0.35</td>
<td>Yes</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Tyr33His</td>
<td>40</td>
<td>0.3</td>
<td>Yes</td>
<td>10</td>
<td>50</td>
<td>600</td>
<td>4</td>
</tr>
<tr>
<td>Asn25Ser</td>
<td>80</td>
<td>0.25</td>
<td>No</td>
<td>10</td>
<td>50</td>
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<tr>
<td>Ser168Thr</td>
<td>50</td>
<td>0.4</td>
<td>Slight</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>1</td>
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<tr>
<td>Ala384Ser</td>
<td>90</td>
<td>0.6</td>
<td>Slight</td>
<td>10</td>
<td>50</td>
<td>100</td>
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<tr>
<td>Val423Ile</td>
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<td>0.6</td>
<td>Slight</td>
<td>10</td>
<td>50</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

*Table 2. Summary of the results from papers I-IV. For details on the different catalytic intermediates (P, F and O) see figure 5 and page 16.*

**Effects on the CytcO catalytic activity**

The Tyr33 residue is situated in the D pathway. Substitution of the Tyr33 for a Histidine residue resulted in slowed proton uptake through the D pathway ([paper II](#)) and a steady-state activity of approximately 40 % of that of the wild-type CytcO. The rate of internal proton transfer from the Glu286 residue to the catalytic site was also slowed with the Tyr33His CytcO compared to the wild-type enzyme. We suggest that the Tyr33His amino-acid substitution results in a structural change of the hydrogen-bonded network around the Glu286 residue and that this structural change affects the proton delivery to the catalytic site as well as to the pump site. A similar effect was observed with the CytcO in which the D pathway residues Ser200 and Ser201 were substituted to Valines [47]. The Tyr33 residue is located next to the Ser200 and Ser201 residues (figure 11).

The Gly171 residue is situated close to heme a. It is part of the putative proton gating loop above heme a (figure 11). With the Gly171Asp CytcO the reduction of heme a was significantly slowed compared to the wild-type enzyme, which caused a decrease of the steady-state catalytic activity to
around 30% of the wild-type activity (paper III). Probably, the Gly171Asp substitution results in a lowered midpoint potential of heme $a$. This effect has been observed before upon the insertion of a negatively charged amino-acid residue close to heme $a$ [38].

The four amino-acid substitutions that were originally found in human prostate cancer cells, showed steady-state turnover activities between 50 and 80% of the wild-type (paper IV). At neutral pH, no effects on the rate of heme oxidation were observed. However, when the heme oxidation was monitored at pH 10, there was a decrease in the rate of the slowest phase of the heme oxidation in all Cyt$\alpha$O variants. Since the substituted amino acids are located at different positions in subunit I of Cyt$\alpha$O, we explain the slower rate of heme oxidation as originating from a structural destabilization of the protein, occurring only at high pH. Most likely there are small changes in the electron equilibrium of the redox cofactors in the structurally modified Cyt$\alpha$Os at pH 10.
Effects on proton pumping

Most of the disease-related substitutions in CytcO induced changes in the proton-pumping activity. With the Tyr33His CytcO, there was a complete loss of the proton-pumping activity (paper II). This could in part be due to the slowed proton-transfer rate through the D pathway, as has been observed before with the Asp132Asn, Asp132Ala, Ser197Asp and Ser200Val/Ser201Val CytcOs [20, 47, 49]. There are, however, examples of amino-acid residue substitutions in the D pathway that result in lost proton pumping but a normal proton-transfer rate through the D pathway [48, 52, 105]. In these cases, changes in the structure around the Glu286 and associated shifts in the pKₐ of the Glu286 side chain have been proposed to
lead to uncoupling of proton pumping. With the Tyr33His CytcO, there is indeed a shift in the $pK_a$ of the Glu286 residue compared to the wild-type CytcO. Presumably, also in this case, the amino-acid substitution results in a change in the structure of water molecules around the Glu286 residue, leading to loss of proton pumping.

With the Gly171Asp CytcO, there was a putative proton leak through the enzyme (paper III). The residue is part of a peptide, consisting of residues 169-175, suggested to be involved in the gating of pumped protons [57]. A suggested conformational change of the gating loop could control the formation of a putative proton-exit path. Exchange of the Gly171 to an Aspartic acid-residue could probably result in a structural change of the gating loop allowing a proton back leak. Preliminary results from H/D exchange studies indicate that with the Gly171Asp CytcO the loop consisting of residues 158-172 is highly accessible to deuterium exchange at all catalytic intermediate states (Busenlehner, Namslauer et al, unpublished). This is different from the results obtained with the wild-type CytcO, with which there was decreased deuterium incorporation in the F intermediate of the catalytic cycle. Moreover, the Gly171 residue is situated close to the Arg481 residue, that is suggested to be involved in the exit of pumped protons [33, 56]. Taken together, data from paper III and from the unpublished study by Busenlehner, Namslauer et al. indicate that the structure and the function of the proton gating region is indeed affected by the Gly171Asp substitution.

With the Asn25Ser, Ser168Thr, Ala384Ser and Val423Ile CytcOs proton pumping was detected in all cases (paper IV), although the Asn25Ser CytcO showed somewhat less proton pumping compared to the wild-type CytcO. With the other three there were indications of a slight proton leak, although not at all as prominent as with the Gly171Asp CytcO. As the Ser168 is part of the putative gating loop and also close to Arg481 and Arg482, the effect on proton pumping could in this case be explained in terms of a slight structural change in the proton-gating region. Ala384 and Val423 are both found in the interior of subunit I (figure 10). There were indications of a slight proton leak also with these structurally modified CytcOs. The Val423 residue is close to heme $a_3$; with a distance of about 7 Å to the heme $a_3$ iron. Ala384 is further away from both the catalytic site and Glu286.
Nevertheless, as evident from the slight tendency of a proton leak, both Val423 and Ala384 are probably close enough to the structural parts of the enzyme involved in gating of the pumped protons.

The results correlate between eukaryotic and *R. sphaeroides* CytC

With the Gly171Asp and the Tyr33His CytC, found in colon cancer patients, cellular and *trans*mitochondrial cybrid studies suggested that the amino-acid substitutions caused a CytC deficiency and a lowered respiratory function, respectively [103, 104]. My studies provided detailed information on how the substitutions affected the CytC functional mechanisms (*paper II, paper III*). The observed effects confirmed the pathogenic properties of the substitutions and lead to a deeper understanding of the phenotypes.

In an unpublished study, we investigated the effects of a mutation in the CytC subunit I gene that was previously linked to a mitochondrial disease. In the mammalian CytC the mutation resulted in the substitution of a Leucine residue to an Isoleucine (Leu240Ile) [106]. In the *R. sphaeroides* CytC Leu240 corresponds to Leu196, which is situated next to Ser197 in the D pathway (figure 6). Previously published and unpublished cellular studies indicated ambiguities concerning the relevance of the substitution for the respiratory deficiency. [106](Taylor R W, personal communication). Our studies showed that the Leu196Ile substitution had no effects on the CytC function (Namslauer I et al, unpublished). These results, using the *R. sphaeroides* CytC, correlated well with the results from previous investigation of the equivalent substitution in *S. cerevisiae* CytC [93]. In that study, Bratton et al. investigated the functional properties of several CytC substitutions in yeast and bacteria. In the cases where the same substitution was studied both in yeast and in *R. sphaeroides*, the results were similar.

When the phenotypes of the mtDNA mutations found in prostate cancer cells were characterized, in most cases no functional studies had been made previously [90]. Thus, it is not possible to correlate the results to any functional properties observed in mammalian cells. It should be mentioned, though, that the Ser168Thr substitution (corresponding to the Ala122Thr
substitution in mammalian CytcO has, apart from prostate cancer cells, also been identified in breast cancer [100]. In this study, a 50 % decrease in the CytcO steady-state activity was observed due to the substitution. This corresponds well to the slight reduction in enzymatic activity that was observed with the R. sphaeroides Ser168Thr CytcO (paper IV).

**Comments on the results**

To conclude, these studies have lead to a deep understanding of how the cancer-related substitutions in CytcO affect the function of the enzyme on a molecular level. This was accomplished using CytcO from the highly validated model organism R. sphaeroides and various absorption spectroscopy techniques. In several cases, the substitutions had large effects on the enzymatic function. Considering the similarities between the bacterial and the mammalian enzyme, the effects would most probably be similar in the human CytcO. In cases where the phenotype resulting from a mtDNA mutation has been investigated not only in R. sphaeroides but in CytcO from other species as well, the results are similar.

In addition to elucidating possible disease mechanisms, this work has contributed to the general knowledge of the function of CytcO, especially concerning the D pathway and the putative gating loop. The results with the Tyr33His CytcO agree with the observation that D pathway substitutions can lead to hydrogen-bond reorganizations around Glu286. Further, the study supports the suggestions that Glu286 is involved in directing protons to different sites and that changes in the structure around Glu286 result in uncoupling of proton pumping. The investigations of the Gly171Asp CytcO propose that there is a proton-gating mechanism also in the vicinity of that residue. Perhaps a conformational change in this region takes place at certain steps of the CytcO catalytic cycle, making sure that protons can only be transferred in one direction.

The rest of this thesis deals with the question whether the functional impairments of CytcO that were observed could be involved in the development or progression of cancer.
The relevance of CytcO substitutions found in cancer

As numerous mtDNA mutations have been observed in cancer, the effects of the mutations might somehow be involved in disease mechanisms. By modeling the mtDNA mutations found in colon and prostate cancer in the *R. sphaeroides* CytcO and using spectroscopy techniques to study the enzymatic activity, the effects of the amino-acid substitutions on the molecular function of CytcO have been ascertained. In several cases, the substitutions lead to a decreased catalytic activity and a defective proton-pumping activity. Now; could these functional defects be involved in the onset or progress of the disease in which they were detected? And in that case, what could be the connection between the defects in CytcO and cancer?

Analysis of tumor cells has revealed that mitochondrial functions, including the respiratory chain activity, are impaired in cancer cells as compared to normal cells [107-111]. Respiratory chain dysfunction could be involved in many other abnormalities, that are frequently observed in cancer cells, such as an increased dependency on glycolysis for ATP synthesis, decreased apoptosis and increased ROS production [112, 113]. Some examples of these and how they could be linked to each other are illustrated in figure 12.
Energy metabolism of cancer cells

Cancer cells, to a much higher extent than normal cells, rely on glycolysis for production of ATP [107]. This phenomenon is called “aerobic glycolysis”, or the Warburg effect, and has been known for over 50 years to be a common feature of tumor cells [118]. The mechanisms of metabolic reprogramming are complex, involving activation or deactivation of a large number of genes [108]. Even today the reason for the upregulation of glycolysis is not fully understood and it remains controversial if the metabolic shift is a cause or a consequence of cancer. Nevertheless, by relying on glycolysis instead of oxidative phosphorylation, cancer cells are able to survive in conditions of fluctuating oxygen tension. Although the increased glycolytic activity is not necessarily accompanied by a decreased respiratory chain activity, mtDNA mutations causing defects in oxidative phosphorylation could contribute to the increased glycolysis observed in cancer cells. Such a link has been observed for example in [116], where inhibition of respiratory-chain activity by oligomycin triggered an increase in glycolysis in cancer cells.

The amino-acid substitutions found in colon cancer resulted in a lowered CytcO activity as compared to the wild-type enzyme (paper II and III). A lowered CytcO catalytic activity could lead to a decreased rate of electron
flux through the entire respiratory chain and thus insufficient amounts of ATP being produced. In addition, with the Tyr33His CytcO the oxygen reduction was uncoupled from proton pumping (paper II). Thus, the energy from the chemical reaction at the catalytic site is not conserved to the same extent as in the wild-type CytcO, resulting in an even less energy-efficient respiratory chain. Also in the Gly171Asp CytcO, the putative proton leak (paper III) could dissipate the electrochemical gradient and thereby reduce the energy-efficiency of the respiratory chain. A decreased synthesis of ATP through oxidative phosphorylation might however not be as devastating for a cancer cell as for a normal cell, due to the metabolic shift occurring in tumors. In fact, a defective respiratory-chain activity could contribute to initiating the metabolic reprogramming that occurs in cancer cells. If the metabolic shift is a prerequisite for a normal cell to develop into a cancer cell, then a decreased respiratory-chain activity could be involved in the onset of the disease.

**Oxidative stress and apoptosis in cancer**

Oxidative stress has been one of the suggestions for how respiratory-chain defects contribute to the development of cancer as well as to aging and neurodegeneration. This was first explained in the so-called mitochondrial free radical theory, proposing that mitochondria-derived ROS cause aging due to oxidative damage to macromolecules [119]. This theory has been further developed to suggest a ROS vicious cycle, where oxidative damage to mitochondria themselves results in even faster rates of ROS production [120]. Today, it is well known that ROS could have harmful effects on DNA, lipids and amino acids. In addition, ROS can activate apoptosis [121]. An increased ROS production has been shown to result from decreased respiratory-chain activity as well as increased mitochondrial membrane potential [113, 115, 122].

There are several studies where ROS production, caused by mtDNA mutations in cancer, has been investigated [89, 90, 117]. Petros et al., found that an amino-acid substitution in ATP synthase enhanced cancer cell growth. The same substitution caused a decreased ATP-synthase activity and an increased ROS production [114]. Further, mutations in the NADH dehydrogenase subunit 6 gene result in decreased complex I activity and
overproduction of ROS. These functional effects were indeed coupled to
tumor cell metastasis by Ishikawa et al [89]. An additional study on the role
of complex I substitutions in cancer showed that increased tumor growth was
linked to changes in ROS generation and apoptosis [117].

Normally, ROS are not formed from the reduction of oxygen at the
catalytic site of CytcO [123]. However, several studies have shown that
chemical inhibition of CytcO activity could in fact lead to increased ROS
formation from other respiratory-chain complexes. An increased ROS
production due to CytcO inhibition by azide has been observed in both
mammalian cells and submitochondrial particles [2, 124, 125]. Use of
cyanide and NO to inhibit CytcO also results in an increased ROS formation
[126, 127]. In these cases the suggested mechanism is that the inhibition of
CytcO causes an increased leak of electrons, and hence ROS formation, from
complexes I and/or III of the respiratory chain. Taking this into account, it is
possible that the decreased CytcO activity resulting from the Gly171Asp and
Tyr33His substitutions (paper II and III) could lead to an increased
mitochondrial ROS production. Moreover, the slowed rate of intramolecular
electron transfer observed in both the Gly171Asp and the Tyr33His CytcO,
result in accumulation of partly reduced intermediate states of the enzyme.
This could increase the probability of electron leakage and hence ROS
production also from CytcO itself.

**The mitochondrial electrochemical gradient in cancer cells**

The mitochondrial electrochemical gradient formed by the chemiosmotic
coupling is essential, not only for ATP synthesis, but also for other processes
such as ion, metabolite and protein trafficking. It constitutes an electrical as
well as a pH difference across the membrane. The electrical component (the
mitochondrial membrane potential) can be monitored in absolute scale and
has a value around 200 mV measured in isolated mitochondria [128, 129].
However, the value of the membrane potential varies depending on for
example cell type, metabolic state and age of the organism [130, 131].
Alterations of the mitochondrial membrane potential have been observed in
cancer cells [132, 133]. Heerdt et al found that an increased mitochondrial
membrane potential of mammalian cells was beneficial to tumor progression
as well as to the initiation of angiogenesis and escape from apoptosis occurring in cancer cells.

Interestingly, mitochondrial uncoupling has recently been observed in cancer cells [134]. In this case, an increase in expression of the mitochondrial uncoupling protein-2 (UCP-2) in cancer cells resulted in a decreased membrane potential. Further, overexpression of UCP-2 in cancer cells results in a lowered production of ROS, inhibition of apoptosis and an onset of the metabolic reprogramming [135-139]. Using the chemical mitochondrial uncoupler FCCP, a similar effect on ROS production was observed. Thus, it is suggested that a benefit of mitochondrial uncoupling in cancer cells is a minimal production of ROS [140, 141].

With the Gly171Asp and also to some extent with the Ser168Thr CytcO, a proton leak was observed (paper III and IV). This would diminish the energy efficiency of the respiratory chain, but could also influence other mitochondrial processes such as ion transport. To keep the electrochemical gradient unchanged upon the introduction of a specific proton leak, the relative fraction of the membrane potential would presumably have to increase. An increased membrane potential has been linked to increased ROS production [115]. On the other hand, as pointed out above, mitochondrial uncoupling has also been observed in cancer and is believed to be beneficial for the tumor cells.
Concluding remarks and future perspectives

By using the *R. sphaeroides* bacterial model system, amino-acid substitutions in CytC-O originally found in cancer, have been investigated. The studies have provided molecular insights into how the substitutions affected the function of the enzyme. The results revealed functional characteristics that could be involved in cancer mechanisms. However, since cancer is caused by a number of different mutations, often affecting tumor suppressor genes or oncogenes, it is unlikely that the mtDNA mutations investigated here are the sole cause of the disease. It could be, though, that the mtDNA mutations contribute to the carcinogenic process by impairing the function of the respiratory chain. Respiratory chain dysfunction has previously been observed in cancer cells and it is a feature also found in aging and neurodegenerative disorders. Since both cancer and neurodegenerative diseases are much more prevalent at a higher age, it is possible that there are common mechanisms involved. On the basis of what has been observed concerning cancer and mitochondria, it is possible that the functional properties of the CytC-O variants found in colon and prostate cancer provide the conditions required for the development of a normal cell into a cancer cell.

However, much is still uncertain regarding mtDNA mutations and the metabolic changes occurring in cancer cells. To further investigate the relevance of specific amino-acid substitutions for the development of a disease, a combination of studies using various model systems would be ideal. Studies on yeast cells or trans-mitochondrial cybrids would provide information regarding certain parameters such as ROS formation and apoptosis. By combining several different model systems a clear picture of the phenotype of a specific pathogenic mutation could be obtained. This would be useful information not only for the identification of possible disease mechanisms, but also for the development of future alternative therapeutic treatments.
Sammanfattning på svenska

Genom vår ämnesomsättning (metabolism), bryts maten vi äter ned i allt mindre delar. Maten används dels till att bygga upp våra kroppar (muskler, fettreserver och s.v.), men även till att ge oss den energi som behövs för att driva alla processer som sker i kroppen, inte minst muskelarbete och tankeverksamhet. De delar av maten som omsätts till energi är elektronerna i näringsämnenas molekyler. Efter att maten har passerat ett antal metabola processer hamnar elektronerna i ett protein som heter Cytokrom c Oxidas (CytcO). CytcO är ett enzym, alltså ett protein som katalyserar en kemisk reaktion. Den reaktion som CytcO katalyserar, där elektronerna behövs, är omvandlingen av syre till vatten. Denna reaktion sker konstant i våra celler och är en förutsättning för att vi ska kunna tillgodogöra oss energin i maten. I de allra flesta av oss, fungerar CytcO som det ska. Men förändringar i strukturen av CytcO har hittats i bland annat coloncancer och prostatacancer. Vad som däremot inte har studerats är om, och i så fall hur, dessa förändringar påverkar funktionen av CytcO. Detta är viktigt att veta för att kunna utreda om förändringarna i CytcO är inblandade i uppkomsten av cancer.

I vårt arbete har vi använt CytcO från en bakterie för att studera effekterna av de förändringar i enzymet som hittats i colon- och prostatacancer. Bakterierna har tillverkat det förändrade enzymet, som sedan renats fram från bakterieceller. En biokemisk metod som kallas optisk spektroskopi har sedan använts för att studera enzymets funktion på molekylär nivå, vilket betyder att man kan följa till exempel hur elektroner och protoner rör sig i enzymet.

Resultaten av dessa studier har publicerats i artiklarna II-IV som ligger till grund för denna avhandling. Sex olika förändringar i CytcO har studerats och resultaten visar att i två av fallen har förändringarna stora effekter på CytcO’s funktion. I de andra fallen har endast små effekter kunnat
upptäckas. Artikel I handlar även den om effekterna av en förändring i cytO. I detta fall har däremot inte förändringen hittats i relation till någon sjukdom, utan har konstruerats i syftet att studera den normala funktionen av enzymet. Två viktiga aspekter av mitt arbete, som diskuteras i denna avhandling, är:

1. Relevansen av att använda ett bakteriellt modellsystem för att studera effekterna av förändringar i cytO som hittats i mänskliga celler.
2. Om, och i så fall hur, de funktionella förändringarna i cytO skulle kunna leda till cancer.

Slutsatserna som dras är att det bakteriella cytO vi använt för att studera förändringar i enzymet är ett relevant modellsystem. Anledningarna till det är att både strukturen och funktionen av det bakteriella cytO i hög grad stämmer överens med det mänskliga. Det bakteriella enzymet har använts i snart 20 år i olika studier och är alltså ett mycket validerat system. Vidare kan förändringar i cytO produceras relativt enkelt i bakterieceller och genom att isolera enzymet från bakterierna kan funktionen studeras på detaljnivå. I några fall har de förändringar vi undersökt med hjälp av bakteriellt cytO även studerats i andra system, till exempel jästceller och mänskliga celler och i dessa fall stämmer resultaten bra överens med våra.

Vad gäller kopplingarna mellan förändringarna i cytO och cancer, kan jag bara spekulera och jämföra resultaten av mina studier med vad som tidigare visats. Jämfört med normala celler, har cancerceller en förändrad metabolism. Ett sämre fungerande cytO leder till en förändrad metabolism, vilket skulle kunna vara en möjlig koppling till cancer. Jag hoppas och tror att resultaten som presenteras i denna avhandling, kan bidra till att vi på längre sikt får en tydligare bild av mekanismerna bakom uppkomsten av cancer.
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References


24 Wilson, K. S. and Prochaska, L. J. (1990) Phospholipid vesicles containing bovine heart mitochondrial cytochrome c oxidase and subunit III deficient enzyme - analysis of respiratory control and
proton translocating activities. Archives of Biochemistry and Biophysics 282, 413-420
34 Lee, H. J., Ojemyr, L., Vakkasoglu, A., Brzezinski, P. and Gennis, R. B. (2009) Properties of Arg481 mutants of the aa3-type cytochrome c oxidase from Rhodobacter sphaeroides suggest that neither R481 nor the nearby D-propionate of heme a3 is likely to be the proton loading site of the proton pump. Biochemistry 48, 7123-7131
42 Blomberg, M. and Siegbahn, P. (2011) The mechanism for proton pumping in cytochrome c oxidase from an electrostatic and quantum chemical perspective. Biochimica et Biophysica Acta Sep 28
43 Ådelroth, P., Ek, M. S., Mitchell, D. M., Gennis, R. B. and Brzezinski, P. (1997) Glutamate 286 in cytochrome aa3 from Rhodobacter sphaeroides is involved in proton uptake during the reaction of the fully-reduced enzyme with dioxygen. Biochemistry 36, 13824-13829
structural alteration of the D pathway. Biochimica et Biophysica Acta 1777, 897-903


rification, kinetics, proton pumping, and spectral analysis. Journal of Biological Chemistry 267, 24264-24272


64 Ädelroth, P., Brzezinski, P. and Malmstrom, B. G. (1995) Internal electron transfer in cytochrome c oxidase from Rhodobacter sphaeroides. Biochemistry 34, 2844-2849


channel in the cytochrome c oxidase from *Rhodobacter sphaeroides* show that this channel is not important for proton conduction but reveal modulation of the properties of heme a. Biochemistry 39, 2989-2996


Kucharczyk, R., Salin, B. and DiRago, J. P. (2009) Introducing the human Leigh syndrome mutation T9176G into Saccharomyces cerevisiae mitochondrial DNA leads to severe defects in the incorporation of Atp6p into the ATP synthase and in the mitochondrial morphology. Human Molecular Genetics 18, 2889-2898

neurogenic ataxia retinitis pigmentosa (NARP) T8993G mutation in the mitochondrial ATP synthase-6 gene. Journal of Biological Chemistry 282, 34039-34047


Asn207 by aspartate at the neck of the D channel in the aa3-type cytochrome c oxidase from Rhodobacter sphaeroides results in de-coupling the proton pump. Biochemistry 45, 14064-14074


Hüttemann, M., Lee, I., Pecinova, A., Pecina, P., Przyklenk, K. and Doan, J. W. (2008) Regulation of oxidative phosphorylation, the mi-
tochondrial membrane potential, and their role in human disease. Journal of Bioenergetics and Biomembranes 40, 445-456


