Functional Effects of Mutations in Cytochrome *c* Oxidase Related to Prostate Cancer

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Abbreviations: Cyt*c*O, cytochrome *c* oxidase; *N, P*-side, negative and positive sides of the membrane, respectively; SU, subunit; time constants are given as (rate constant)$^{-1}$;

Abstract

A number of missense mutations in subunit I of cytochrome *c* oxidase (Cyt*c*O) have previously been linked to prostate cancer (Petros *et al*. (2005) *PNAS*, 102, 719). To investigate the effects of these mutations at the molecular level, in the present study we prepared four different structural variants of the bacterial *Rhodobacter sphaeroides* Cyt*c*O (cytochrome aa_3), each carrying one amino-acid residue replacement corresponding to the following substitutions identified in the above-mentioned study: Asn11Ser, Ala122Thr, Ala341Ser and Val380Ile (residues Asn25, Ser168, Ala384 and Val423 in the *R. sphaeroides* oxidase). This bacterial Cyt*c*O displays essentially the same structural and functional characteristics as those of the mitochondrial counterpart. We investigated the overall activity, proton pumping and internal electron- and proton- transfer reactions in the structural variants. The results show that the turnover activities of the mutant Cyt*c*Os were reduced by at most a factor of two. All variants pumped protons, but in Ser168Thr, Ala384Ser and Val423Ile we observed slight internal proton leaks. In all structural variants the internal electron equilibrium was slightly shifted away from the catalytic site at high pH (10), resulting in a slower observed ferryl to oxidized transition. Even though the effects of the mutations were relatively modest, the results suggest that they destabilize the proton-gating machinery. Such effects could be manifested in the presence of a transmembrane electrochemical gradient resulting in less efficient energy conservation.

Introduction

The human mitochondrial DNA (mtDNA) encodes 37 genes of which 13 are polypeptides of the respiratory chain complexes. For over 20 years it has been known that mutations in the mtDNA are found in connection to diseases such as the mitochondrial myopathies MELAS, LHON and MERFF (reviewed in [1]). More recently, mutations of the mtDNA have been found in connection to neurodegenerative diseases [2-5], ageing (reviewed in [6]) as well as different types of cancers [7-11]. Often, mtDNA mutations affect the function of one or several complexes of the respiratory chain. However, the link (if any) between tumorigenesis and effects of mtDNA mutations at a molecular/functional level is largely unknown. Most mtDNA mutations related to various cancers or tumors have been identified in genes encoding subunits of complex I, but there are also a great number of reports of mutations in genes encoding the other respiratory complexes, including Cyt*c*O (reviewed in [12]). In 2005, Petros *et al*. sequenced the mtDNA from tumor cells of a number of prostate cancer patients and found that the number of missense mutations in subunit I of Cyt*c*O was significantly increased compared to controls [13]. In total, 21 mutations in Cyt*c*O subunit I were reported. However, the effects of these mutations on the activity of Cyt*c*O at the molecular level were not investigated.

In the present study, four of the missense mutations found in subunit I of Cyt*c*O in prostate cancer cells [13]; Asn11Ser, Ala122Thr, Ala341Ser and Val380Ile, were introduced into the Cyt*c*O of the purple bacterium *Rhodobacter sphaeroides*, which is used as a well-studied model of the mitochondrial counterpart [14-17]. The functional effects of these mutations were investigated in detail by measuring the overall activities, proton pumping and rates of specific electron- and proton-transfer reactions.

The *R. sphaeroides* Cyt*c*O is composed of four subunits where subunits I-III; the catalytic core, are nearly identical (sequence and structure) to those of the mammalian Cyt*c*O [14, 18-20] (in *R. sphaeroides* SU IV is composed of only one transmembrane helix). In previous studies, Cyt*c*Os from yeast, *R. sphaeroides* and *P. denitrificans* have been used to investigate the effect of other disease-related mutations on the function of Cyt*c*O [16, 17, 21-25]. Similar to the mitochondrial Cyt*c*O, in the *R. sphaeroides* Cyt*c*O electrons are initially transferred from cytochrome *c* to Cu_A located in SU II. From there, electrons are transferred consecutively to heme *a* and to the catalytic site, composed of heme a_3 and Cu_B, where O_2 binds and is reduced to water. The O_2 reduction reaction is linked to proton pumping from the more negative (*N*) side of the membrane to the more positive (*P*) side at a stoichiometry of approximately one pumped proton per electron transferred to the catalytic site.

In the *R. sphaeroides* Cyt*c*O the protons needed for O_2 reduction and those that are pumped are taken up from the *N* side of the membrane through two proton pathways. One of these pathways is called the K-pathway as it holds a highly conserved Lys residue (Lys362). The other pathway is called the D-pathway. It is defined by an Asp residue (Asp132) near the protein surface on the *N* side, which is connected by ~10 water molecules to the highly conserved Glu286 residue located between the two heme groups at a distance of ~10 Å from the catalytic site. From Glu286 protons are transferred either towards the catalytic site (substrate protons used in O_2 reduction) or towards the heme propionates, which are found on the trajectory of the pumped protons.

The four mutations; Asn11Ser, Ala122Thr, Ala341Ser and Val380Ile found in Cyt*c*O in human prostate cancer cells were chosen because they are highly conserved among species. These four residues correspond to Asn25, Ser168, Ala384 and Val423 in

R. sphaeroides Cyt*c*O. As seen in **Figure 1**, the locations of the mutated amino-acid residues are almost identical in the *R. sphaeroides* and the mitochondrial Cyt*c*O structures.

Materials and Methods

Site-directed mutagenesis, cell growth and CytcO purification

The mutations were constructed using the Quick-Change site-directed mutagenesis kit (Stratagene). They were introduced into subunit I of Cyt*c*O encoded on the pJS3-SH plasmid [26]. The mutated fragment was transferred to the expression plasmid pRK415-1 [27] containing the genes for subunits I-III of *R. sphaeroides* Cyt*c*O, with a 6His-tag attached to SU I and also containing a gene for tetracycline resistance. The mutated pRK415-1 plasmid was transferred into cells of the *E. coli* S-17 strain by electroporation and then into the *R. sphaeroides* JS100 strain by conjugation. The mutations were verified by sequencing. To express the Cyt*c*O, *R. sphaeroides* cells were grown aerobically in the dark in a SIS minimal medium and in the presence of tetracycline. Cyt*c*O was solubilized from the cell membrane fraction using dodecyl-β-D-maltoside (DDM) and the His-tagged Cyt*c*O was purified using Ni^{2+}-NTA affinity chromatography, as described previously [28].

Steady-state kinetics

The steady-state enzyme kinetics was measured using a Clark-type O_2 electrode (Hansatech instruments). In this assay, 50 mM K^+-phosphate buffer at pH 6.5, supplemented with 0.05% DDM was added to the sample chamber to a final volume of 1 ml. Then, 36 μM reduced cytochrome *c* was added. The reaction was initiated by addition of 0.1 nM purified Cyt*c*O and the O_2 consumption was monitored. The Cyt*c*O activity, defined as the number of electrons transferred from reduced cytochrome *c* to Cyt*c*O per second per enzyme complex, was calculated.

Proton-pumping measurements

Liposomes containing Cyt*c*O were prepared as described [29]. Each proteoliposome typically contained at most one enzyme molecule and in 75% of the vesicles the Cyt*c*O molecules were oriented with the cytochrome *c*-binding site towards the outside solution [30]. To measure the proton-pumping stoichiometry, liposome-reconstituted Cyt*c*O at a concentration of 0.5 μM in the presence of 5 μM valinomycin was mixed at a 1:1 ratio with 16 μM reduced cytochrome *c* using a stopped-flow spectrophotometer (Applied Photophysics). The Cyt*c*O as well as the cytochrome *c* were diluted in a solution containing 50 μM Hepes-KOH, 45 mM KCl, 44 mM sucrose, 1 mM EDTA and 0.1 mM of the pH-sensitive dye phenol red at pH 7.3-7.6. Absorbance changes of phenol red were detected at 554 nm. Then, 5 μM of the proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was added to the proteoliposome sample and the measurement was repeated with the uncoupled system, detecting the net consumption of protons during enzyme turnover.

Oxygen reduction kinetics

Cyt*c*O at a concentration of ~7 μM in 100 mM KCl, 200 μM Hepes-KOH at pH 7.4, 0.1 % DDM was reduced with 2 mM ascorbate and 1 μM Phenazine methosulfate (PMS) under N_2 atmosphere. After complete reduction of Cyt*c*O, verified by analysis of the absorption spectrum, the N_2 atmosphere was exchanged for CO, which binds to reduced heme a_3. The reduced Cyt*c*O-CO complex was mixed at a ratio of 1:5 with an O_2-saturated buffer solution in a stopped-flow apparatus (Applied Photophysics). Approximately 200 ms after mixing, the CO molecule was dissociated by means of a short laser flash (Quantel Brilliant B, ~200 mJ, 532 nm) enabling O_2 to bind to the reduced catalytic site, and the reaction to proceed. Absorbance changes at single wavelengths were recorded as a function of time. The rate constants and amplitudes of the transient

absorbance changes during the reaction were analyzed by fitting the data to multi-exponential functions using the ProK software (Applied Photophysics).

Results

The *R. sphaeroides* Cyt*c*Os containing the four mutations; Asn25Ser, Ser168Thr, Ala384Ser and Val423Ile (*R. sphaeroides* numbering) were all expressed and purified. The spectral characteristics of the purified mutated Cyt*c*Os did not differ from those of the wild-type enzyme (not shown), which indicates that the metal cofactors, including those constituting the catalytic site, were intact. The turnover activities of the mutant Cyt*c*Os were measured as a function of pH by measuring the O_2-consumption rates and found to be at most a factor of two lower than the corresponding activity of the wild-type Cyt*c*O (**Figure 2**).

Proton pumping

To measure the proton-pumping stoichiometry, the vesicle-reconstituted Cyt*c*O was mixed with an excess of reduced cytochrome *c* in the presence of O_2 and the pH indicator phenol red in a stopped-flow apparatus. The used cytochrome *c*:Cyt*c*O ratio allowed a total of eight turnovers (i.e. 32 electrons from cytochrome *c*). Absorbance changes of the pH-sensitive dye were monitored at 554 nm, which is an isosbestic point for oxidation-reduction of cytochrome *c*. The potassium ionophore valinomycin was added to prevent the buildup of an electrical gradient that could inhibit the proton-pumping activity. **Figure 3** shows the traces obtained from the proton-pumping experiments with all four mutant Cyt*c*Os as well as the wild-type Cyt*c*O. The protons being pumped out of the vesicles acidify the outside solution, which results in a decrease in absorbance at 554 nm of the pH-sensitive dye. Addition of the proton ionophore CCCP, allows the pumped protons to equilibrate across the membrane during turnover and only a net proton uptake,

associated with the O_2-reduction, is seen as an increase in absorbance (alkalinization of the medium). The data show that all four mutant Cyt*c*Os pumped protons, although the stoichiometry was slightly lower with the Asn25Ser mutant Cyt*c*O. Furthermore, the Ser168Thr, Ala384Ser and Val423Ile mutant Cyt*c*Os displayed a slight increase in absorbance after the initial decrease associated with proton-pumping. Such an increase is indicative of a proton leak into the vesicles.

The O_2 reduction reaction

The Cyt*c*O was fully reduced using ascorbate and PMS after which the sample was incubated under CO atmosphere. The CO ligand binds to the reduced heme a_3 forming a heme a_3-CO complex. The kinetics of the reaction of the reduced Cyt*c*O with O_2 was investigated by mixing the four-electron reduced, CO-bound Cyt*c*O with O_2 in a stopped-flow apparatus, followed by flash photolysis of the blocking CO ligand (see e.g. [31]). Upon CO dissociation, O_2 binds to heme a_3, which initiates the reaction. Oxidation of the heme groups was monitored time-resolved at a number of different wavelengths. **Figure 4** shows the absorbance changes at 445 nm and 605 nm at pH 7.4. Earlier studies with wild-type *R. sphaeroides* Cyt*c*O have shown that the absorbance changes reflect distinct transitions of the Cyt*c*O catalytic cycle [31]. The initial instant increase in absorbance, most clearly seen at 445 nm, is due to dissociation of the CO ligand. Binding of O_2 results in a decrease in absorbance with a time constant of ~10 μs (at 1 mM O_2). The subsequent transfer of an electron from heme *a* to the catalytic site is seen at 605 nm (and also to some extent at 445 nm) as an absorbance decrease with a time constant of ~40 μs. This electron transfer is associated with breaking of the O-O bond and formation of a state called "peroxy", denoted $\mathbf{P_R}$. Next, a proton is transferred from solution to the catalytic site, which results in formation of a state called "ferryl", denoted **F**. The $\mathbf{P_R} \rightarrow \mathbf{F}$ reaction

displays a time constant of ~100 μs at pH 7.4 and it is also linked to fractional electron transfer from Cu_A to heme *a* and proton-pumping, both observed as an increase in absorbance at 580 nm (not shown, c.f. data at pH 10 in **Figure 5**). Finally, the last electron is transferred from the Cu_A-heme *a* equilibrium to the catalytic site, linked to proton-uptake from solution. The electron-transfer is seen as a decrease in absorbance at 445 nm and 605 nm with a time constant of ~1.2 ms at pH 7.4. As seen in **Figure 4**, all reaction steps upon oxidation of the four-electron reduced Cyt*c*O were unaffected by the mutations at pH 7.4. This is also true for the absorbance changes at 580 nm associated with the $\mathbf{P_R \rightarrow F}$ reaction (not shown). Furthermore, because the Ser168 corresponds to an Ala (Ala122) in the human mitochondrial Cyt*c*O, in addition to investigating the Ser168Thr mutation, we tested the effect of a Ser168Ala mutation in the *R. sphaeroides* Cyt*c*O. The behavior of this mutant Cyt*c*O was similar to that of the Ser168Thr structural variant (data not shown).

Figure 5 shows absorbance changes associated with the reaction of the reduced Cyt*c*O with O_2 at pH 10. At this pH there was no significant difference in the kinetics associated with the $\mathbf{P_R \rightarrow F}$ reaction (**Figure 5a**). However, the $\mathbf{F \rightarrow O}$ transition with all four mutant Cyt*c*Os was a factor of ~3 slower (~50 s^{-1}) than with the wild-type Cyt*c*O (~180 s^{-1}) (**Figure 5b**). **Figure 6** shows the pH dependence of the $\mathbf{P_R \rightarrow F}$ and $\mathbf{F \rightarrow O}$ rates. As seen in the figure, the difference in the $\mathbf{F \rightarrow O}$ rate appears only at high pH. The small difference seen in the $\mathbf{P_R \rightarrow F}$ rates at pH 10 in **Figure 6a** is not significant (see, the increase in absorbance, associated with the $\mathbf{P_R \rightarrow F}$ reaction, in **Figure 5b**).

Discussion

In this work, we have investigated the effects of four mutations, Asn25Ser, Ser168Thr, Ala384Ser and Val423Ile, on the function of Cyt*c*O. The mutations were originally identified in tumor cells of prostate cancer patients [13]. We introduced the equivalent amino-acid residue substitutions into the Cyt*c*O of the bacterial model system *R sphaeroides*, which is structurally and functionally closely related to the mammalian counterpart (**Figure 1**). Although the substituted amino-acid residues studied in this work are conserved, they are not located within segments of subunit I in Cyt*c*O that have previously been shown to be directly involved in the function of the enzyme.

The Val423 residue is positioned ~7 Å away from the heme a_3 iron, but on the "back side" of the catalytic site such that the distance to Cu_B is 11 Å. The relative proximity to the heme a_3 iron could result in changes in ligand binding or the redox potential of this site, but this was not observed experimentally at neutral pH. This is not unexpected given that the substitution of a Val to an Ile is fairly conservative, where the latter is larger by only one carbon atom.

The Ala384 residue, which was mutated to a Ser, is located in between the two identified proton conducting pathways in SU I of Cyt*c*O at a distance of 12 Å from Lys362 and 20 Å from Glu286, i.e. most likely too far to directly influence the structural properties of these pathways. The distance to the heme groups is ~20 Å.

The Ser168 residue is located at approximately the same depth into the membrane as the heme propionates, but towards SUIII, i.e. closer to heme *a* (~15 Å) than to heme a_3. Furthermore, in the mitochondrial Cyt*c*O the corresponding residue is located ~8 Å from Asp51, a residue that has been implicated to be part of the proton-gating machinery of that Cyt*c*O [32]. Results from an investigation of effects of mutations at another amino-acid

residue in this area; Gly171 [16], indicated that the substitution resulted in a proton leak through the Cyt*c*O. Gly171 is located near Arg481, which has been suggested to be involved in proton transfer to a "proton-loading site" where protons that are pumped across the membrane are temporarily stored. The effects of the Gly171Asp mutation were attributed to its proximity to the Arg481 residue. Residue Ser168 is only three residues away from the Gly171 at a distance of 5-6 Å, but it is further away from the arginines compared to the Gly171. Interestingly, also the results with the Ser168Thr mutant Cyt*c*O indicate that after the initial proton release to the outside of the vesicles there is a slower, small proton back leak (**Figure 3**), not observed with the wild-type Cyt*c*O. This putative leak is not as obvious as with the Gly171Asp mutants Cyt*c*O, which is consistent with the difference in distances to the Arg481 residue. It is also interesting to note that a similar behavior was observed with the Val423Ile and Ala384Ser mutant Cyt*c*Os, although the leak is more pronounced with the Ser168Thr mutant Cyt*c*O.

Residue Asn25 is situated about 4-5 Å away from Asp132, which is close to the entry point of the D proton-transfer pathway [18-20, 33]. Even though removal of the carboxyl group at the position of the Asp132 residue results in slowed proton-uptake rates [34], changes in nearby residues do not change the proton-uptake rates dramatically (unpublished data). Consequently, it is not surprising that essentially no effects on the kinetics of proton transfer through the D-pathway were observed with the Asn25Ser mutation, which would at most result in a minor re-arrangement of the position of the carboxylate.

The steady-state turnover activities of the mutant Cyt*c*Os investigated in this study were at most a factor of two slower than that of the wild-type Cyt*c*O (see **Figure 2**). This activity is sensitive to e.g. internal electron equilibria or pK_as of internal proton acceptors

or donors. A change in activity by a factor of two may be a manifestation of minute changes in these parameters. Furthermore, since the *in vivo* turnover activity of the respiratory chain is much lower than the maximum activity of Cyt*c*O, a decrease in the Cyt*c*O activity by a factor of two is unlikely to affect the overall electron/proton flux of the respiratory chain. Furthermore, we did not observe any effects on the kinetics of specific electron- and proton-transfer reactions during O_2 reduction in the pH range 6-9 (see **Figure 4**). The only significant difference in the reaction kinetics was a factor of ~3 decrease in the **F** $\rightarrow$ **O** rate with the mutant Cyt*c*Os at high pH (~10). Notably, the same effect was observed with all four mutant Cyt*c*Os. In view of the distinctly different location of the mutated residues in different parts of the Cyt*c*O (see **Figure 1**), the effects on the **F** $\rightarrow$ **O** transition at pH 10 are likely to originate from structural destabilization of the protein that occurs only at the highest measured pH values. The rate constant of the **F** $\rightarrow$ **O** reaction is determined by the electron equilibrium among the redox-active cofactors of the Cyt*c*O as well as the proton-transfer rate through the D-pathway. As the proton-transfer rate was not affected (see above), the slowed **F** $\rightarrow$ **O** reaction at high pH is attributed to changes in electron equilibrium among the redox sites, Cu_A, heme *a* and the catalytic site.

As already mentioned above, the Asn25Ser, Ser168Thr, Ala384Ser and Val423Ile mutations are all found at highly conserved sites. However, the substitutions are relatively conservative and neutral, and found in parts of the Cyt*c*O that have been shown not to be directly involved in specific function. Even though it is not surprising that neither of the mutations was functionally relevant for the activity of the Cyt*c*O, one property that is worth noting is the small proton leak observed with the Ser168Thr, Ala384Ser and Val423Ile mutant Cyt*c*Os, but not with the Asn25Ser mutant Cyt*c*O. The three former

mutations are all found within the membrane-spanning part of the Cyt*c*O and 10-20 Å from the region involved in proton gating. Consequently, if these mutations result in small structural destabilization as discussed above, they may also affect structural changes that are involved in the proton gating process. The Asn25Ser mutation, on the other hand, is located at the protein surface, ~30 Å away from the catalytic site.

In summary, the bacterial model system used in the present studies allowed us to investigate in detail the effects of four subunit I Cyt*c*O mutations identified in tumor cells of prostate cancer patients. The data show that at neutral pH the turnover activity was reduced by a factor of <2 and there were no effects on internal electron- or proton-transfer. Three of the mutations resulted in minor proton leaks, presumably due to structural destabilization of the gating region of the pump. Furthermore, we observed a significant slowing of internal electron-transfer to the catalytic site (**F** $\rightarrow$ **O** reaction) at pH 10, which is also presumably due to destabilization of the protein structure. Even though these effects are relatively minor, the observation of proton leaks at neutral pH in three of the mutant Cyt*c*Os indicates that the mutations result in a less robust proton pump. In the presence of a transmembrane electrochemical potential such changes may be more significantly manifested and could result in a less efficient energy conversion of the entire respiratory chain.

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Figure Legends

Figure 1. Structural alignment of subunit I of cytochrome *c* oxidase from *Rhodobacter sphaeroides* (green) (PDB entry 1M56) and bovine heart (red) (PDB entry 1OCC). The location of the four mutated amino acids are shown in blue (*R. sphaeroides*) and grey (bovine heart).

Figure 2 The pH dependence of the steady-state turnover activity in electrons per second of the wild-type Cyt*c*O and the four mutated variants. Each point is an average of two measurements. Experimental conditions: 38 μM reduced cytochrome *c*, 0.1 nM cytochrome *c* oxidase, 0.05% DDM, buffers as follows; 0.05 M K^+-phosphate (pH 6.5 & 7.5), 0.05 M Tris-HCl (pH 8.0), 0.05 M CHES (pH 9.0 & 9.5), 0.05 M CAPS (pH 10.0 & 10.5).

Figure 3 Absorbance changes of the pH dye phenol red associated with proton uptake and release by Cyt*c*O. Liposome-reconstituted Cyt*c*O was mixed at a 1:1 ratio with 16 μM reduced cytochrome *c* in a stopped-flow spectrophotometer. Absorbance changes of phenol red were detected at 554 nm. A decrease in absorbance is associated with release of (pumped) protons to the outside of the vesicles. After addition of 5 μM of the proton ionophore CCCP the net consumption of protons was measured (increase in absorbance). Experimental conditions after mixing: 0.25 μM Cyt*c*O, 2.5 μM valinomycin, 8 μM reduced cytochrome *c*, 50 μM Hepes-KOH, 45 mM KCl, 44 mM sucrose, 1 mM EDTA and 100 μM phenol red at pH 7.3-7.6.

Figure 4 Absorbance changes at pH 7.4 associated with the reaction of the four-electron reduced Cyt*c*O with O_2. The absorbance increase at t=0 is associated with dissociation of the CO ligand. The decrease in absorbance following in time is associated with oxidation of the Cyt*c*O. Experimental conditions: ~ 1μM reacting enzyme, 0.1 M HEPES-KOH pH 7.4, 0.1% DDM and 1 mM O_2 at ~ 22°C.

Figure 5 Absorbance changes associated with the reaction of the four-electron reduced Cyt*c*O with O_2 at pH 10. At 580 nm, the increase in absorbance in the time range ~100 μs - 500 μs is associated with the $\mathbf{P_R \rightarrow F}$ reaction. Experimental conditions: ~ 1μM reacting enzyme, 0.1 M CAPS pH 10, 0.1% DDM and 1 mM O_2 at ~ 22°C.

Figure 6 The pH dependence of the $\mathbf{P_R \rightarrow F}$ and $\mathbf{F \rightarrow O}$ reaction rates. The $\mathbf{P_R \rightarrow F}$ rate constants were determined from the data at 580 nm (see **Figure 5**), while the $\mathbf{F \rightarrow O}$ reaction rates were determined from data at 445 nm. Experimental conditions: ~ 1μM reacting enzyme, 0.1% DDM and 1 mM O_2 at ~ 22°C, buffers as follows; 0.1 M MES (pH 6.0-6.5), 0.1 M HEPES (pH 7.0-7.5), 0.1 M Tris-HCl (pH 8.0-9.0), 0.1 M CHES

(pH 9.5-10.0).