Biochimica et Biophysica Acta 1797 (2010) 550–556

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

A pathogenic mutation in cytochrome *c* oxidase results in impaired proton pumping while retaining O_2-reduction activity

Ida Namslauer [a], Hyun Ju Lee [b], Robert B. Gennis [b], Peter Brzezinski [a,*]

[a] Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden
[b] Department of Biochemistry, University of Illinois, Urbana, IL, 16801, USA

ARTICLE INFO

Article history:
Received 22 October 2009
Received in revised form 21 January 2010
Accepted 25 January 2010
Available online 1 February 2010

Keywords:
Respiratory chain
Electron transfer
Mitochondrial disease
Cytochrome aa_3
Mitochondria
Deficiency
mtDNA

ABSTRACT

In this work we have investigated the effect of a pathogenic mitochondrial DNA mutation found in human colon cells, at a functional-molecular level. The mutation results in the amino-acid substitution Tyr19His in subunit I of the human CytcO and it is associated with respiratory deficiency. It was introduced into *Rhodobacter sphaeroides*, which carries a cytochrome *c* oxidase (cytochrome aa_3) that serves as a model of the mitochondrial counterpart. The residue is situated in the middle of a pathway that is used to transfer substrate protons as well as protons that are pumped across the membrane. The Tyr33His (equivalent residue in the bacterial CytcO) structural variant of the enzyme was purified and its function was investigated. The results show that in the structurally altered CytcO the activity decreased due to slowed proton transfer; proton transfer from an internal proton donor, the highly-conserved Glu286, to the catalytic site was slowed by a factor of ~5, while reprotonation of the Glu from solution was slowed by a factor of ~40. In addition, in the structural variant proton pumping was completely impaired. These results are explained in terms of introduction of a barrier for proton transfer through the D pathway and changes in the coordination of water molecules surrounding the Glu286 residue. The study offers an explanation, at the molecular level, to the link between a specific amino-acid substitution and a pathogenic phenotype identified in human colon cells.

1. Introduction

Cellular respiration in aerobic organisms involves the transfer of electrons, coupled to transmembrane proton translocation, through the respiratory chain. This process takes place in the inner mitochondrial membrane of eukaryotes or in the inner cell membrane of prokaryotes. The final electron acceptor is molecular oxygen, which is reduced to water at the catalytic site of cytochrome *c* oxidase (CytcO). Thirteen of the polypeptides of the respiratory chain complexes are encoded by the mitochondrial DNA (mtDNA), including subunits I–III of CytcO. Mutations in the mtDNA have for a long time been known to be linked to various disease states. Typically, mtDNA mutations are recognized as causes of primary, mitochondrial diseases such as mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged red fibers (MERRF) and Kearns–Sayre syndrome (KSS) due to the mutations accumulating to high levels in muscle and CNS tissues (see [1,2] for review). Recent evidence suggest that acquired mtDNA mutations may play a role in the aetiology of various neurodegenerative disorders [3] [4] and cancers [5–10]. Furthermore, somatic mtDNA mutations are likely to appear during the process of normal ageing [2]. Although the list of mtDNA mutations linked to various diseases is constantly growing, the consequences of the mutations at a molecular level are rarely known.

In this work, we have investigated the effects of a pathogenic mtDNA mutation found in mtDNA encoded subunit I of CytcO. Pye et al. identified the mutation in respiratory-deficient *trans*mitochondrial cybrid clones isolated from normal colonic crypt cells [11]. Upon sequencing the DNA, a single base substitution, m.5958 T>C (amino-acid substitution Tyr19His in subunit I, human CytcO amino-acid residue numbering) was detected. The cell line harboring the Tyr19His mutation showed a decrease in CytcO activity and a perturbed complex assembly compared to the controls [11].

To investigate the functional properties of the Tyr19His structural alteration, we introduced it into the CytcO (cytochrome aa_3) of *Rhodobacter sphaeroides*. This CytcO is composed of four subunits [12,13] (Fig. 1) of which subunits I–III (the catalytic core) are nearly identical (sequence and structure) to those of the mammalian CytcO [14] (subunit IV of the bacterial CytcO is composed of one transmembrane

Abbreviations: CytcO, cytochrome *c* oxidase; **R**, CytcO with a two-electron reduced catalytic site; $\mathbf{P_R}$, the "peroxy" intermediate formed at the catalytic site upon reaction of the 4-electron reduced CytcO with O_2; **F**, "oxo-ferryl" intermediate; **O**, fully-oxidized CytcO; *n*-side, negative side of the membrane; *p*-side, positive side of the membrane; time constants are given as (rate constant)$^{-1}$, Amino-acid residues are numbered according to the *R. sphaeroides* cytochrome aa_3 subunit I sequence

* Corresponding author. Tel.: +46 8 163280; fax: +46 8 153679.
E-mail address: peterb@dbb.su.se (P. Brzezinski).

0005-2728/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2010.01.027

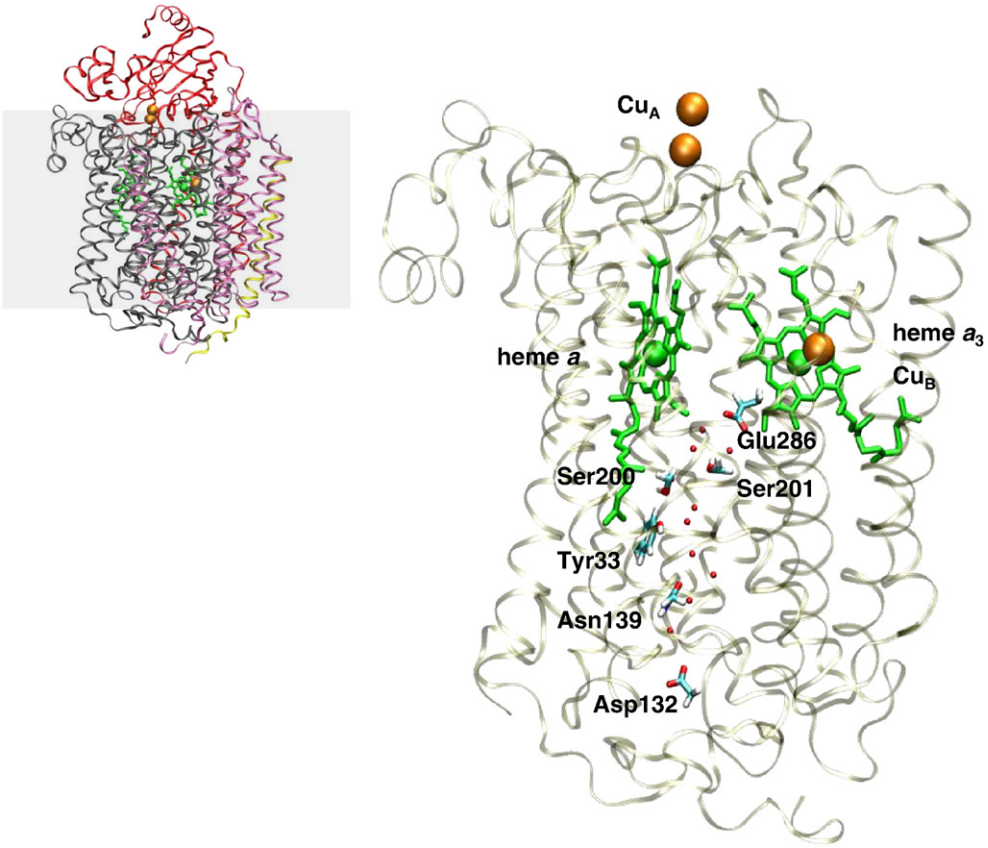

Fig. 1. Overall structure of *R. sphaeroides* CytcO (small picture) and subunit I (large picture). Subunit I is shown in gray, subunit II in red, subunit III in pink and subunit IV in yellow. The gray square illustrates the approximate position of the plasma membrane. The D pathway stretches from Asp132, via protonatable amino-acid residues and water molecules, to Glu286. The hemes and cupper ions are shown in green and orange, respectively.

helix). Accordingly, the bacterial Cyt*c*O is frequently used as a model of the mitochondrial counterpart [15]. In earlier studies, CytcOs from *P. denitrificans*, *R. sphaeroides* and yeast have been used to investigate the effect of other disease-related mutations on the function of Cyt*c*O [16–21].

As indicated above, CytcO catalyzes reduction of O_2 to H_2O:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O.$$

The protons are taken up from the more negative (*n*) side of the membrane, while the electrons are donated at the positive (*p*) side, which results in a transmembrane charge separation conserving part of the free energy available from the O_2-reduction reaction. Furthermore, for each electron transferred to O_2 one additional proton is taken up from the *n*-side and released to the *p*-side (i.e. pumped across the membrane). In the bacterial Cyt*c*O, two proton pathways lead from the *n*-side solution towards the catalytic site; the D- and K pathways, named after conserved residues within these pathways. For each turnover, two of the protons required for O_2 reduction are taken up through the K pathway while the remaining six protons are taken up through the D pathway. In other words, the latter pathway is used for transfer of all four protons that are pumped and the remaining two protons used for O_2 reduction. The D pathway consists of ~10 water molecules, which connect a highly-conserved Asp (132 in the *R. sphaeroides* Cyt*c*O) with a Glu286 (Fig. 1). The latter residue is the branching site within the D pathway from which protons are transferred either to the catalytic site or to an acceptor for pumped protons. The order of electron and proton-transfer events and the kinetics of these reactions are essentially identical in the *R. sphaeroides* and mitochondrial oxidases, and the apparent pK_as of the internal proton donor/acceptor Glu286 are very similar (~9). These results from mechanistic studies further support the assumption that the bacterial oxidase is an adequate model of the mitochondrial counterpart. For recent reviews on the structure and function of Cyt*c*O, see [22–30].

Tyr19 is a highly-conserved amino-acid residue corresponding to Tyr33 in the *R. sphaeroides* Cyt*c*O. It is situated within the D pathway between residues Asp132 and Glu286 (see Fig. 1) and it is hydrogen-bonded to one of the water molecules within the pathway. Results from earlier studies have shown that mutation of residues within the D pathway influence rates of proton transfer through the pathway and, in some cases, also proton pumping is impaired (for review, see [24]). In the present study we isolated the Tyr33His structural variant of Cyt*c*O from *R. sphaeroides* and investigated the effects of the mutation. The results show that the mutant CytcO displayed ~38% activity (O_2 consumption) of that of the wild-type CytcO and that activity was not linked to proton pumping. Furthermore, we also used time-resolved absorption spectroscopy to investigate specific electron and proton-transfer reactions during reaction of CytcO with O_2 in the Tyr33His mutant CytcO and found that all reaction steps being linked to proton uptake were slowed.

2. Materials and methods

2.1. Site-directed mutagenesis, growth of bacteria and CytcO purification

The Tyr33His mutation was constructed using the Quick-Change mutagenesis kit (Stratagene). The pJS3-SH plasmid [31] was used as the template for the mutation and the pRK415-1 plasmid [32], containing the genes for subunits I–III of CytcO, was used as expression plasmid. The expression plasmid with the mutation was transferred into *E. coli* S-17-1 cells by electroporation. The plasmid was transferred

into *R. sphaeroides* JS100 strain by conjugation. The mutation was verified by sequencing. *R. sphaeroides* cells were grown aerobically in shake incubators. After harvesting, membranes were prepared and the Cyt*c*O was solubilized from the membrane fraction using dodecyl-β-D-maltoside (DDM). Finally, the His-tagged Cyt*c*O was purified using Ni^{2+}-NTA affinity chromatography, essentially as described in [33].

2.2. Steady-state kinetics

The steady-state enzyme kinetics was measured using a Clark-type oxygen electrode (Hansatech Instruments). Briefly, 0.1 nM purified Cyt*c*O, 0.05% DDM, 36 μM reduced cytochrome *c* and 50 mM K^+ phosphate buffer at pH 6.5 were added to the oxygraph chamber to a final volume of 1 ml.

2.3. Oxidation of CytcO by oxygen

Cyt*c*O at a concentration of 7 μM in 0.1 M Hepes–KOH at pH 7.4, 0.1% DDM was transferred to an anaerobic cuvette. The atmosphere in the cuvette was exchanged for N_2 after which the Cyt*c*O molecules were reduced using 1 μM phenazine methosulfate and 2 mM ascorbate. Complete reduction of Cyt*c*O was verified by analysis of the absorption spectrum. The N_2 atmosphere was then exchanged for CO, resulting in formation of the Cyt*c*O–CO complex where CO is bound to heme a_3. The oxygen-reduction reaction was monitored upon rapidly mixing fully reduced CO-bound Cyt*c*O with an O_2-saturated solution of 0.1 M Hepes–KOH at pH 7.4 and 0.1% DDM in a stopped-flow apparatus (Applied Photophysics) (mixing ratio 1:5). Approximately 200 ms after mixing, the CO molecule was dissociated from the catalytic site by means of a short laser flash (Quantel, Brilliant B, ~200 mJ at 532 nm). This allowed O_2 to bind to the reduced catalytic site and the subsequent reaction was monitored in time by recording the absorbance changes at single wavelengths.

2.4. Proton-uptake measurements

A solution of 7 μM Cyt*c*O in 0.1 M KCl at pH 7.8, 0.1% DDM, supplemented with 40 μM phenol red was prepared. It was transferred to an anaerobic cuvette and air was exchanged for CO as described above. The CO-bound Cyt*c*O was mixed with an O_2-saturated solution consisting of 0.1 M KCl, 40 μM phenol red at pH 7.8 and 0.1% DDM after which the CO ligand was dissociated by means of a laser flash (Flow-flash system from Applied Photophysics). Proton uptake from solution results in deprotonation of the dye, which is seen as an increase in absorbance at 560 nm.

2.5. Reconstitution of CytcO into liposomes

Cyt*c*O-containing lipid vesicles were prepared essentially as described in [34]. Briefly, purified Cyt*c*O was diluted to 4 μM in 0.1 M Hepes–KOH at pH 7.4 and 4% sodium cholate. Soybean lecithin at a concentration of 40 mg/ml was dissolved in 0.1 M Hepes–KOH at pH 7.4 and 2% sodium cholate. The lipid solution was sonicated and mixed with the Cyt*c*O solution at a 1:1 ratio. The sodium cholate was gradually removed using BioBeads SM2 Adsorbent (BioRad Laboratories). Finally, the buffer was exchanged for a 0.1 M KCl solution at pH 7.4 using a PD10 column (GE Health Care). Using the above-mentioned lipid-to-Cyt*c*O ratio, each vesicle typically contains at most one Cyt*c*O molecule and in 75% of the vesicles the Cyt*c*Os molecules are oriented with the cytochrome *c*-binding site towards the outside solution [34].

2.6. Proton pumping

Liposome-reconstituted Cyt*c*O at a concentration of 0.5 μM in 50 μM Hepes–KOH, 45 mM KCl, 44 mM sucrose, 1 mM EDTA and 100 μM phenol red at pH 7.6 was mixed at a 1:1 ratio with 16 μM reduced cytochrome *c* (in a solution with the same composition as that in the other syringe) in a stopped-flow spectrophotometer (Applied Photophysics). Absorbance changes of the pH-sensitive dye were detected at 554 nm. The potassium ionophore valinomycin was used to equilibrate the electrical component of the electrochemical gradient. The proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used to detect the net consumption of protons during enzyme turnover.

3. Results

The Tyr33His mutation in subunit I of Cyt*c*O was expressed in *R. sphaeroides* cells and the structural variant ("mutant") of Cyt*c*O was purified using Ni^{2+}-NTA affinity chromatography. The spectral characteristics did not differ from those measured with the wild-type Cyt*c*O, indicating that the metal cofactors and their environments were intact. In addition, we investigated O_2 binding to the reduced catalytic site and found that the time constant was the same as that measured with the wild-type Cyt*c*O (not shown), also indicating that the catalytic site was unperturbed in the structural variant of the Cyt*c*O. The multiple turnover activity of the Tyr33His Cyt*c*O was measured at pH 6.5 and found to be 38 ± 6% of that of the wild-type Cyt*c*O.

3.1. Reaction of the fully reduced CytcO with O_2

The reaction of the fully reduced Cyt*c*O with O_2 was studied as a function of time using time-resolved absorption spectroscopy. Fully reduced (i.e. with four electrons), CO-bound Cyt*c*O was mixed with an O_2-saturated solution. About 200 ms after mixing, the blocking CO ligand was dissociated by means of a laser flash, which enabled O_2 to bind at the catalytic site thereby initiating the reaction. Fig. 2 shows absorbance changes at 580 nm measured with the wild-type and Tyr33His Cyt*c*Os at pH 7.4. Results from earlier studies with the wild-type *R. sphaeroides* Cyt*c*O have shown that these absorbance changes reflect four distinct transitions ([35,36], for review, see [24,30,37,38]): (*i*) Binding of O_2 to the reduced Cyt*c*O immediately after dissociation of the CO ligand ($\tau \cong 8$ μs at 1 mM O_2), not seen on the time scale shown in Fig. 2. (*ii*) Transfer of an electron from heme *a* to the catalytic site forming a state that is denoted $\mathbf{P_R}$ in which the O-O bond is broken ($\tau \cong 30$ μs at pH 7.4). This reaction is seen as a decrease

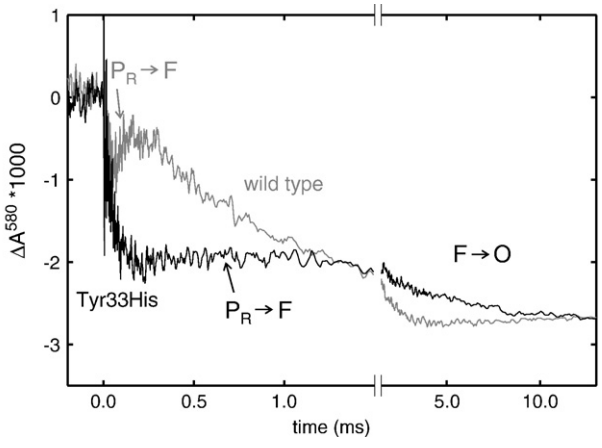

Fig. 2. Absorbance changes as a function of time after flash photolysis of CO from the fully reduced wild-type and Tyr33His Cyt*c*O in the presence of O_2. Formation of the $\mathbf{P_R}$ state ($\tau \cong 30$ μs) is seen as an absorbance decrease at 580 nm. It is followed in time by formation of state **F**, which is seen as an absorbance increase at 580 nm ($\tau \cong 100$ μs and $\tau \cong 570$ μs with the wild-type and Tyr33His Cyt*c*Os, respectively). Formation of the last intermediate, **O**, seen as an absorbance decrease at 580 nm, is slower with the Tyr33His than with the wild-type Cyt*c*O ($\tau \cong 4$ ms and 1.5 ms, respectively). Experimental conditions: 0.1 M Hepes–KOH pH 7.4, 0.1% DDM, 1 mM O_2 and ~1 μM reacting enzyme.

in absorbance at 580 nm. (*iii*) Proton uptake (Fig. 3) through the D pathway to the catalytic site forming a state denoted **F**, fractional electron transfer from Cu_A to heme *a* and proton pumping, all occurring with a time constant of ~100 μs at pH 7.4. This step is most clearly seen as an absorbance increase at 580 nm (Fig. 2). (*iv*) Transfer of the last (fourth) electron and a proton through the D pathway to the catalytic site forming the oxidized CytcO, as well as proton pumping ($\tau \cong 1.5$ ms at pH 7.4). This last transition is seen as a decrease in absorbance at 580 nm.

As shown in Fig. 2, the first two reactions until formation of the $\mathbf{P_R}$ state occurred over the same time scale with the Tyr33His and wild-type CytcOs. However, those transitions that are linked to proton uptake (and pumping in the wild-type CytcO) were slowed in the Tyr33His CytcO; The $\mathbf{P_R}\rightarrow\mathbf{F}$ reaction was slowed from ~100 μs with the wild-type CytcO to ~600 μs with the Tyr33His enzyme, while the next, **F→O** reaction, was slowed from 1.5 ms to 4 ms.

3.2. Dependency of the CytcO oxidation reaction on pH

The rate of the oxygen-reduction reaction was also measured at a number of different pH values from 6.5 to 10 (Fig. 4a). With the Tyr33His mutant CytcO the **F**-formation rate was essentially the same at all pH values (~600 μs). This is in contrast to results with the wild-type CytcO, for which the time constant is essentially constant (~100 μs) up to pH ~9 after which it increases displaying an apparent pK_a of ~9.4 [39]. The time constant for formation of state **O** increased with increasing pH as shown in Fig. 4b, which is qualitatively the same dependence as that found with the wild-type CytcO, although the slowing as a result of the mutation is more pronounced at low than at high pH.

3.3. Proton-uptake measurements

Proton uptake to the catalytic site during oxidation of the reduced CytcO was measured using the pH-sensitive dye phenol red upon flash photolysis of the CO ligand after mixing of the CO-bound CytcO with an O_2-saturated buffer-free solution at pH 7.8 (Fig. 3, an increase in absorbance corresponds to uptake of protons). With the wild-type CytcO two main kinetic components are typically observed with time constants corresponding to those of the $\mathbf{P_R}\rightarrow\mathbf{F}$ and **F→O** transitions, i.e. ~100 μs and ~1.5 ms [35]. With the Tyr33His CytcO only one component, with a time constant of ~3 ms, was observed i.e. it approximately coincided in time with formation of the **O** state.

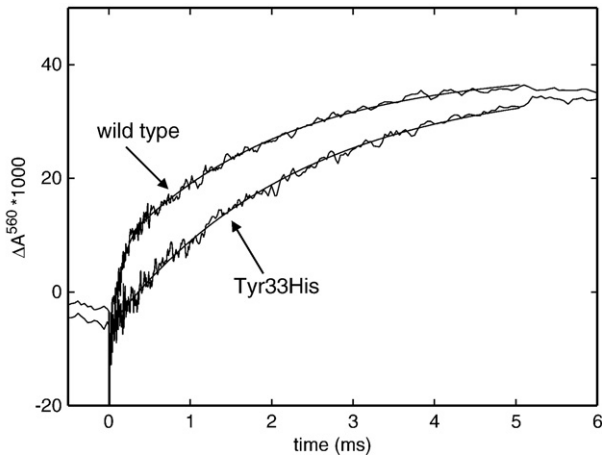

Fig. 3. Absorbance changes of the dye phenol red at 560 nm associated with changes in proton concentration of the bulk solution upon reaction of the fully reduced wild-type and Tyr33His CytcO with O_2. An increase in absorbance indicates alkalinization of the surrounding solution. The solid lines superimposed on the traces are fits of the data. With the wild-type enzyme the fit is a sum of two exponentials with time constants of ~100 μs ($\mathbf{P_R}$ to **F**) and ~1.7 ms (**F** to **O**), respectively. With the Tyr33His CytcO, the absorbance increase was fitted to a single exponential with a time constant of ~3 ms. Experimental conditions: 0.1 M KCl, pH ~7.8, 0.1% DDM, 40 μM phenol red, 1 mM O_2.

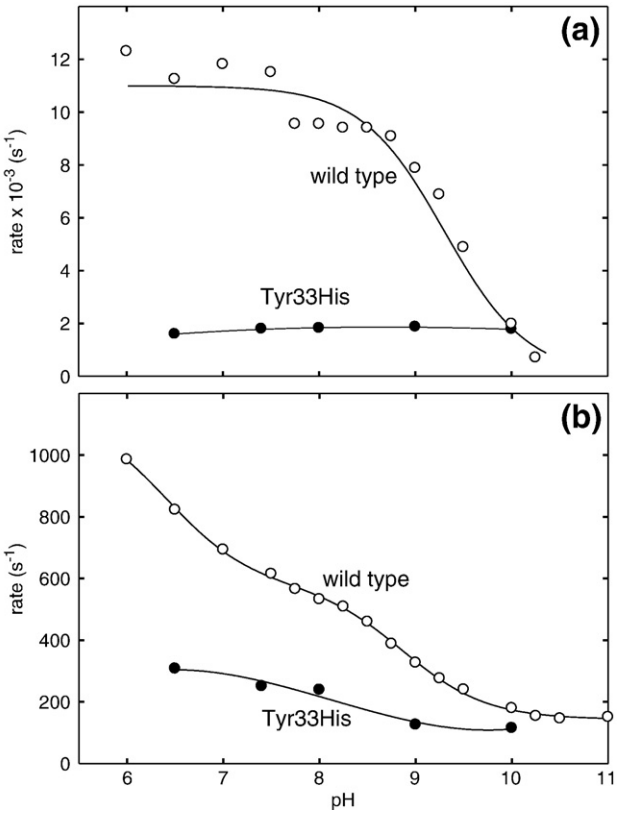

Fig. 4. The pH dependence of the (a) $\mathbf{P_R}$ to **F** and (b) **F** to **O** rates during reaction of the fully reduced wild-type and Tyr33His CytcO with O_2. The rates were extracted from data obtained at 580 nm and 445 nm. Filled and open circles show data for the Tyr33His and wild-type CytcOs, respectively. The data obtained with the wild-type CytcO are from [30,39]. Experimental conditions: 0.1 M MES (pH 6.5), 0.1 M Hepes–KOH (pH 7.4 and 8.0), 0.1 M Tris (pH 9.0) and 0.1 M CAPS (pH 10.0), 0.1% DDM, 1 mM O_2 and ~1 μM reacting enzyme.

3.4. Proton-pumping activity

Proton pumping was investigated upon mixing a solution of CytcO, reconstituted in liposomes, with reduced cytochrome *c* in the presence of O_2. The pH-sensitive dye phenol red was added on the outside of the vesicles and changes in the dye absorbance were detected at 554 nm (Fig. 5), which is an isosbestic point for the reduced and oxidized forms of cytochrome *c* (yet it is close to the absorbance maximum for protonation changes of the pH dye). First, the potassium ionophore valinomycin was added to equilibrate the electrical component of the electrochemical gradient formed across the vesicle membrane. With the wild-type CytcO, there is a decrease in absorbance, consistent with acidification of the solution outside of the vesicles. Then, upon addition of the proton ionophore CCCP, protons equilibrate across the membrane and an increase in the dye absorbance is seen, consistent with a net uptake of protons (the "pumped protons" equilibrate across the membrane and only the net uptake of substrate protons is observed). Because four protons are consumed for each turnover, the absorbance increase with CCCP can be used to calibrate the signals. As seen in Fig. 5, no proton pumping was detected with the Tyr33His CytcO.

4. Discussion

In this work we have investigated, at a molecular level, the effect of the pathogenic Tyr33His mutation in subunit I of CytcO, originally

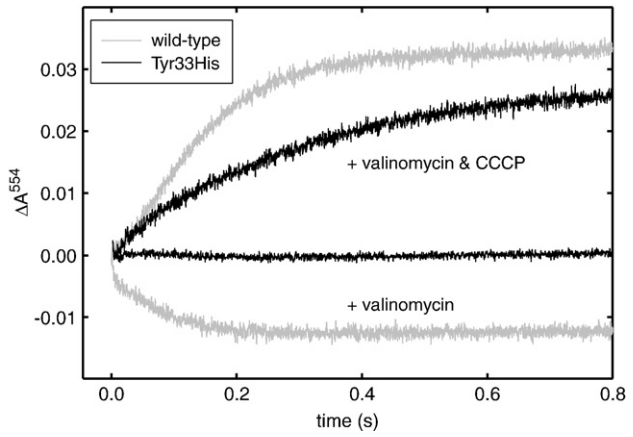

Fig. 5. Absorbance changes of the dye phenol red at 554 nm associated with changes in proton concentration upon mixing vesicle-reconstituted wild-type and Tyr33His Cyt*c*Os with reduced cytochrome *c*. The potassium ionophore valinomycin was added to equilibrate the electrical component of the electrochemical gradient formed across the vesicle membrane (lower two traces). With the wild-type CytcO, there is a decrease in absorbance due to acidification of the solution outside the vesicles. With the Tyr33His Cyt*c*O, there is no absorbance decrease, indicating that no protons are released to the outside of the vesicles. Addition of the proton ionophore CCCP results in proton equilibration across the vesicle membrane and an increase in the phenol red absorbance is seen, consistent with a net uptake of protons. Experimental conditions after mixing: 0.25 μM liposome-reconstituted Cyt*c*O, 50 μM Hepes–KOH, pH 7.6, 45 mM KCl, 44 mM sucrose, 1 mM EDTA, 100 μM phenol red, 8 μM reduced cytochrome *c*, 1 μM valinomycin, 2.5 μM CCCP.

identified in human colon cells. The structural variant of the Cyt*c*O was successfully expressed in *R. sphaeroides* cells and could be purified using Ni^{2+}-NTA affinity chromatography. The Tyr33 residue is found in the D pathway, which is used to transfer protons to the catalytic site during oxygen reduction as well as protons that are pumped across the membrane (see Fig. 1).

The purified Tyr33His Cyt*c*O showed a number of differences compared to the wild-type enzyme: (*i*) the steady-state turnover rate (i.e. oxygen reduction) was decreased to ~38% of that of the wild-type Cyt*c*O; (*ii*) the $\mathbf{P_R}\rightarrow\mathbf{F}$ and $\mathbf{F}\rightarrow\mathbf{O}$ rates in the reaction of the reduced Cyt*c*O with O_2 were decreased; (*iii*) no proton uptake to the catalytic site from solution was observed on the time scale of **F** formation; (*iv*) the proton-pumping activity of the Cyt*c*O was abolished.

The lower steady-state activity observed with the Tyr33His Cyt*c*O is consistent with the results of Pye et al. [11], which showed that the Cyt*c*O activity decreased in the *trans*mitochondrial cybrids harboring the equivalent mutation. Also results from other studies have shown that mutation of a number of specific amino-acid residues lining the D pathway result in a decrease in the steady-state activity of Cyt*c*O, presumably due to slowed proton transfer through the pathway (see for example [40,41]). Pfitzner et al. previously investigated the Tyr35Phe mutation in Cyt*c*O from *P. denitrificans* (corresponds to Tyr33Phe in the *R. sphaeroides* Cyt*c*O) [40] and found that the mutant displayed lower steady-state activity compared to the wild-type Cyt*c*O. However, the Tyr35Phe mutation had no effect on proton pumping.

To investigate which specific reaction steps that were slowed as a result of the Tyr33His mutation, we investigated the reaction of the reduced Cyt*c*O with O_2 as a function of time. In this reaction the initial binding of O_2 to the reduced catalytic site and splitting of the O-O bond are not associated with any proton uptake from solution; these reactions only involve internal electron transfer from heme *a* to heme a_3 and an internal redistribution of protons. Accordingly, the rates of these reactions were not affected by the Tyr33His mutation and formation of the $\mathbf{P_R}$ state occurred over the same time scale as in the wild-type Cyt*c*O, which indicates that the mutation did not affect the inter-heme electron transfer.

The first transition that involves proton uptake from solution and pumping (in the wild-type Cyt*c*O) is the $\mathbf{P_R}\rightarrow\mathbf{F}$ transition [42,43], where formation of the **F** state requires protonation of a group at the catalytic site. Also the next transition, $\mathbf{F}\rightarrow\mathbf{O}$, involves proton uptake from solution and pumping, and all these proton transfers take place through the D pathway.

In the wild-type Cyt*c*O, during the $\mathbf{P_R}\rightarrow\mathbf{F}$ transition, initially a proton is transferred from Glu286 to the catalytic site with a time constant of ~100 μs and then the residue is rapidly ($\ll$100 μs) reprotonated from solution (see Fig. 6a), i.e. the $\mathbf{P_R}\rightarrow\mathbf{F}$ reaction is linked to proton uptake *from solution* with the same rate as that of the $\mathbf{P_R}\rightarrow\mathbf{F}$ reaction itself. The reaction is pH dependent titrating with an apparent pK_a of 9.4, where this pK_a reflects the protonation state of the Glu286 residue [39]. Observation of an apparent pK_a in the pH dependence of the rate requires that Glu286 is in rapid ($\ll$100 μs) equilibrium with solution [39]. If this is not the case and the equilibrium occurs over time scales $\gg$100 μs, a pH-independent $\mathbf{P_R}\rightarrow\mathbf{F}$ rate would be observed. This pH-independent rate would be observed because in this case the reaction rate is only determined by proton transfer from Glu286 to the catalytic site [39] and Glu286 is reprotonated after the internal proton transfer (i.e. proton uptake from solution would follow in time after the $\mathbf{P_R}\rightarrow\mathbf{F}$ transition itself). In the Tyr33His Cyt*c*O the $\mathbf{P_R}\rightarrow\mathbf{F}$ rate was approximately the same in the pH range from 6.5 to 10.0 ($\tau\cong$600 μs) (Fig. 4a) and there was no proton uptake associated with formation of state **F** (Fig. 3). Thus, these results indicate that in the Tyr33His mutant Cyt*c*O the $\mathbf{P_R}\rightarrow\mathbf{F}$ transition rate is determined only by internal proton transfer from Glu286 to the catalytic site ($\tau\cong$600 μs). Observation of the $\mathbf{P_R}\rightarrow\mathbf{F}$ transition at pH 10.0 indicates that there is a proton available at Glu286 at this pH, which would indicate that the pK_a of the Glu is elevated as compared to the wild-type Cyt*c*O. Reprotonation of Glu286 occurred over a longer time scale as it coincided with the next transition, $\mathbf{F}\rightarrow\mathbf{O}$ (Fig. 3). In other words, the results suggest that reprotonation of the Glu286 residue through the D pathway was slowed by a factor of >30 because proton uptake from solution was observed only with a time constant of ~3 ms (pH 7.4) compared to $\ll$100 μs in the wild-type Cyt*c*O (see Fig. 6).

A number of studies with bacterial Cyt*c*Os have shown that mutations of residues in the D pathway result in either slowed proton uptake to the catalytic site or impaired proton pumping, or both [26,40,44–50] (see also [51–58] for relevant results from theoretical studies). The residue investigated in the present study is located very close to a water cluster within the D pathway, located 6–7 Å from (below) Glu286. This cluster, stabilized by residues Ser200 and Ser201 has been proposed to be important for proton conduction to Glu286 as well as for the proton-pumping function [53,59,60]. Indeed, mutations of residues Ser200 and Ser201 result in lower Cyt*c*O activity [40] and proton transfer from Glu286 is significantly slowed, presumably due to changes in the water structure around the Glu (Lee et al., unpublished data). As Tyr33 is located just below these Ser residues, we speculate that the Tyr33His mutation may also result in altering the structure of this water cluster and thereby the hydrogen-bonding pattern around Glu286. The slowed reprotonation of the Glu286 residue in the mutant Cyt*c*O is most likely due to introduction of a barrier around the His33 residue (in the mutant Cyt*c*O), which is located in between the entrance of the D pathway and Glu286.

In the Tyr33His mutant Cyt*c*O proton pumping was impaired (see Fig. 5). As discussed above, uncoupling of proton pumping from O_2 reduction has been observed previously upon mutation of other residues in the D pathways of the *P. denitrificans* and *R. sphaeroides* Cyt*c*Os. In some cases, such as the Asp132Asn mutant Cyt*c*O, the mutations result in slowed proton transfer through the D pathway [41]. In other cases, such as e.g. Asn139Asp or Asn139Thr, the proton-transfer rate through the D pathway was not affected [45,46]. In other words, a decrease in (or absence of) the pumping stoichiometry is not necessarily linked to a decrease in the proton-transfer rate through the D pathway. On the other hand, even though proton transfer through the D pathway may be dramatically decreased, the mutant Cyt*c*O may still pump protons. For example, in the Glu286Ala/Ile112Glu [61] and

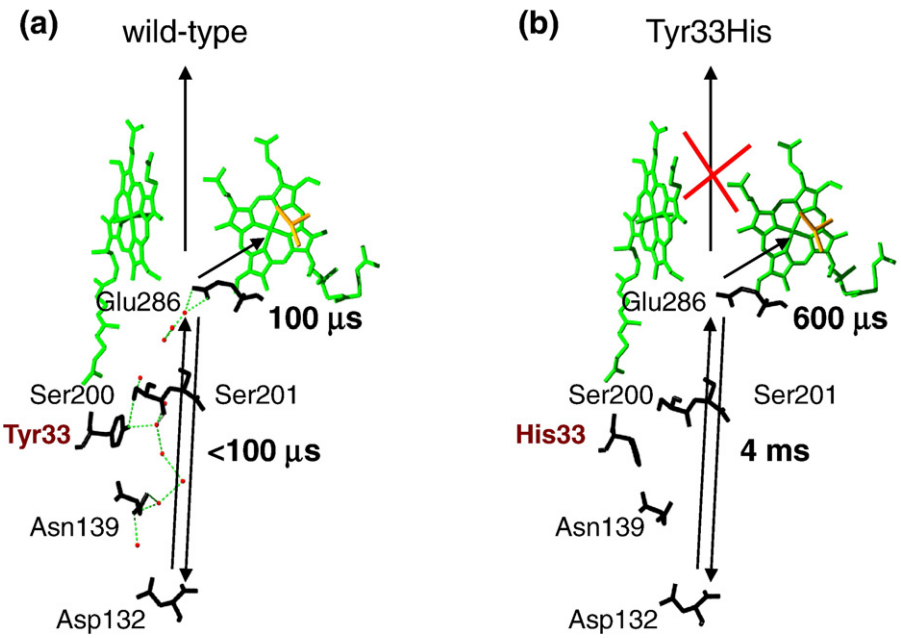

Fig. 6. Models showing the rates of proton transfer from Glu286 to the catalytic site and from solution to Glu286 in (a) wild-type and (b) Tyr33His CytcOs. Amino-acid residues of the D pathway are shown in black and the oxygen atoms of the water molecules lining the pathway are colored red. Hydrogen bonds are shown in green. With the wild-type CytcO a proton is transferred from Glu286 to the catalytic site upon formation of state **F** ($\tau \cong 100$ µs at pH 7.4) and Glu286 is rapidly ($\tau \ll 100$ µs) reprotonated from solution. In addition, a proton is pumped across the membrane. With the Tyr33His enzyme, proton transfer from Glu286 to the catalytic site is slowed ($\tau \cong 570$ µs at pH 7.4) and reprotonation of Glu286 occurs over the same time scale as formation of state **O** ($\tau \cong 4$ ms at pH 7.4). No protons are pumped.

Ser200Val/Ser201Val double mutant (Lee et al., unpublished data) Cyt*c*Os the proton-transfer rates at pH 7 were decreased by a factor of ~50, but these structural variants pumped protons, although with a lowered stoichiometry. Common to non-pumping structural variants where the proton-transfer rate is unperturbed and pumping variants where the rate is slowed is that the apparent pK_a of Glu286 is shifted up or down from the value of 9.4 in the wild-type Cyt*c*O [45,46,48]. Such pK_a shifts most likely reflect changes in structure around the Glu286 site upon mutation of residues within the D pathway. Indeed, small alterations around the Glu286 site have been observed in the structure of the Asn139Asp mutant Cyt*c*O [62] and also in the FTIR spectra of the mutant Cyt*c*O [63]. Furthermore, results from theoretical studies indicate that Glu286 can adopt different conformations with different proton connectivity to solution or the pumping site and that these structural rearrangements are important for controlling proton delivery to the pump element maintaining a high pumping stoichiometry [55]. Changes in the equilibrium constant between the different positions of the Glu286 residue are expected to result in proton leaks (slip of the pump) decreasing the pumping stoichiometry [55]. In a recent study, Qin et al. found that redox-dependent changes in water structure around Glu286 are likely to modulate proton transfer from the residue to the catalytic site and the acceptor for pumped protons. We speculate that in the case of the Tyr33His mutant Cyt*c*O proton pumping is impaired due to changes in the water structure around Glu286 (see above). Another possibility is that the absence of proton pumping is due to slowed proton transfer through the D pathway (c.f. barrier introduced within the D pathway). However, the latter is less likely as there are examples of mutant Cyt*c*Os in which proton uptake is slowed but proton pumping is maintained (see above).

5. Conclusions

In this paper, we report results from studies of the Tyr33His mutation in subunit I of *R. sphaeroides* Cyt*c*O. The corresponding mutation (Tyr19His) originally identified in normal, human colonic crypt cells caused a severe mitochondrial respiratory deficiency, but the basis for the deficiency at a molecular level has not previously been investigated. Using purified Cyt*c*O from a model bacterial system we have shown that in the Tyr33His mutant Cyt*c*O the activity is decreased due to dramatically slowed proton transfer through the D pathway. Furthermore, in the structural variant O_2 reduction was not linked to proton pumping. We speculate that the reason for the uncoupling of the pump is a change in the structure of water molecules surrounding the highly-conserved Glu286 residue, which is responsible for proton delivery to the catalytic site and to the pump element of the Cyt*c*O. In conclusion, the results from this study provide insights into the link between minor protein structural changes caused by mutation of a single residue and a pathogenic phenotype identified in human colonic crypt cells.

Acknowledgements

We would like to thank Rob Taylor (Mitochondrial Research Group, Newcastle University) for valuable discussions and comments on the manuscript. This study was supported by grants from the Swedish Cancer Society.

References

[1] H.A.L. Tuppen, E.L. Blakely, D.M. Turnbull, R.W. Taylor, Mitochondrial DNA mutations and human disease, Biochim. Biophys. Acta, Bioenerg. 1797 (2010) 113–128.
[2] R.W. Taylor, D.M. Turnbull, Mitochondrial DNA mutations in human disease, Nat. rev. 6 (2005) 389–402.
[3] M.T. Lin, D.K. Simon, C.H. Ahn, L.M. Kim, M.F. Beal, High aggregate burden of somatic mtDNA point mutations in aging Alzheimer's disease brain, Hum. Mol. Genet. 11 (2002) 133–145.
[4] P.H. Reddy, M.F. Beal, Are mitochondria critical in the pathogenesis of Alzheimer's disease? Brain Res. Rev. 49 (2005) 618–632.
[5] K. Polyak, Y. Li, H. Zhu, C. Lengauer, J.K.V. Willson, S.D. Markowitz, M.A. Trush, K.W. Kinzler, Somatic mutations of the mitochondrial genome in human colorectal tumours, Nat. Genet. 20 (1998) 291–293.
[6] C. Jerónimo, S. Nomoto, O.L. Caballero, H. Usadel, R. Henrique, G. Varzim, J. Oliveira, C. Lopes, M.S. Fliss, D. Sidransky, Mitochondrial mutations in early stage prostate cancer and bodily fluids, Oncogene 20 (2001) 5195–5198.
[7] J.B. Jones, J.J. Song, P.M. Hempen, G. Parmigiani, R.H. Hruban, S.E. Kern, Detection of mitochondrial DNA mutations in pancreatic cancer offers a "mass"-ive

advantage over detection of nuclear DNA mutations, Cancer Res. 61 (2001) 1299–1304.
[8] J.A. Petros, A.K. Baumann, E. Ruiz-Pesini, M.B. Amin, C. Qi Sun, J. Hall, S. Lim, M.M. Issa, W.D. Flanders, S.H. Hosseini, F.F. Marshall, D.C. Wallace, mtDNA mutations increase tumorigenicity in prostate cancer, PNAS 102 (2005) 719–724.
[9] M.E. Gallardo, R. Moreno-Loshuertos, C. López, M. Casqueiro, J. Silva, F. Bonilla, S. Rodríguez de Córdoba, J.A. Enríquez, m.6267G>A: a recurrent mutation in the humna mitochondrial DNA that reduces cytochrome c oxidase activity and is associated with tumors, Human mutat. 27 (2006) 575–582.
[10] Y. Liu, G. Fiskum, D. Schubert, Generation of reactive oxygen species by the mitochondrial electron transport chain, J. Neurochem. 80 (2002) 780–787.
[11] D. Pye, D.S. Kyriakouli, G.A. Taylor, R. Johnson, M. Elstner, B. Meunier, Z.M.A. Chrzanowska-Lightowlers, R.W. Taylor, D.M. Turnbull, R.N. Lightowlers, Production of transmitochondrial cybrids containing naturally occurring pathogenic mtDNA variants, Nucleic Acids Res. 34 (2006).
[12] M. Svensson-Ek, J. Abramson, G. Larsson, S. Törnroth, P. Brzezinski, S. Iwata, The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome *c* oxidases from *Rhodobacter sphaeroides*, J. Mol. Biol. 321 (2002) 329–339.
[13] L. Qin, C. Hiser, A. Mulichak, R.M. Garavito, S. Ferguson-Miller, Identification of conserved lipid/detergent-binding sites in a high-resolution structure of the membrane protein cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 16117–16122.
[14] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å, Science 272 (1996) 1136–1144.
[15] J. Cao, J. Hosler, J. Shapleigh, A. Revzin, S. Ferguson-Miller, Cytochrome aa3 of Rhodobacter sphaeroides as a model for mitochondrial cytochrome c oxidase, The J. Biol. Chem. 267 (1992) 24273–24278.
[16] M. Bratton, D. Mills, C.K. Castleden, J. Hosler, B. Meunier, Disease-related mutations in cytochrome c oxidase studied in yeast and bacterial models, Eur. J. Biochem. 270 (2003) 1222–1230.
[17] B. Meunier, Site-directed mutations in the mitochondrially encoded subunits I and III of yeast cytochrome oxidase, Biochem. J. 354 (2001) 407–412.
[18] S. Lucioli, K. Hoffmeier, R. Carrozzo, A. Tessa, B. Ludwig, F.M. Santorelli, Introducing a novel human mtDNA mutation into the Paracoccus denitrificans COX I gene explains functional deficits in a patient, Neurogenetics 7 (2006) 51–57.
[19] B. Meunier, J.W. Taanman, Mutations of cytochrome c oxidase subunits 1 and 3 in Saccharomyces cerevisiae: assembly defect and compensation, Biochim. Biophys. Acta 1554 (2002) 101–107.
[20] M.W. Mather, H. Rottenberg, Intrinsic uncoupling of cytochrome c oxidase may cause the maternally inherited mitochondrial diseases MELAS and LHON, FEBS Lett. 433 (1998) 93–97.
[21] I. Namslauer, P. Brzezinski, A mitochondrial DNA mutation linked to colon cancer results in proton leaks in cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3402–3407.
[22] G. Brändén, R.B. Gennis, P. Brzezinski, Transmembrane proton translocation by cytochrome *c* oxidase, Biochim. Biophys. Acta 1757 (2006) 1052–1063.
[23] P. Brzezinski, P. Ädelroth, Design principles of proton-pumping haem-copper oxidases, Curr. Opin. Struct. Biol. 16 (2006) 465–472.
[24] P. Brzezinski, R.B. Gennis, Cytochrome c oxidase: exciting progress and remaining mysteries, J. Bioenerg. Biomembranes (2008) 1–11.
[25] M. Wikström, M.I. Verkhovsky, Mechanism and energetics of proton translocation by the respiratory heme-copper oxidases, Biochim. Biophys. Acta 1767 (2007) 1200–1214.
[26] J.P. Hosler, S. Ferguson-Miller, D.A. Mills, Energy transduction: proton transfer through the respiratory complexes, Ann. Rev. Biochem. 75 (2006) 165–187.
[27] M. Wikström, M.I. Verkhovsky, Towards the mechanism of proton pumping by the haem-copper oxidases, Biochim. Biophys. Acta, Bioenerg. 1757 (2006) 1047–1051.
[28] S. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, H. Aoyama, T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, Proton pumping mechanism of bovine heart cytochrome c oxidase, Biochim. Biophys. Acta, Bioenerg. 1757 (2006) 1110–1116.
[29] M. Brunori, A. Giuffre, P. Sarti, Cytochrome c oxidase, ligands and electrons, J. Inorg. Biochem. 99 (2005) 324–336.
[30] A. Namslauer, P. Brzezinski, Structural elements involved in electron-coupled proton transfer in cytochrome c oxidase, FEBS Lett. 567 (2004) 103–110.
[31] D.M. Mitchell, R.B. Gennis, Rapid purification of wildtype and mutant cytochrome c oxidase from Rhodobacter sphaeroides by Ni2+-NTA affinity chromatography, FEBS Lett. 368 (1995) 148–150.
[32] N.T. Keen, S. Tamaki, D. Kobayashi, D. Trollinger, Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria, Gene 70 (1988) 191–197.
[33] Y. Zhen, J. Qian, K. Follmann, T. Hayward, T. Nilsson, M. Dahn, Y. Hilmi, A.G. Hamer, J.P. Hosler, S. Ferguson-Miller, Overexpression and purification of cytochrome c oxidase from Rhodobacter sphaeroides, Protein Expr. Purif. 13 (1998) 326–336.
[34] K. Faxén, P. Brzezinski, The inside pH determines rates of electron and proton transfer in vesicle-reconstituted cytochrome c oxidase, Biochim. Biophys. Acta, Bioenerg. 1767 (2007) 381–386.
[35] P. Ädelroth, M. Ek, P. Brzezinski, Factors determining electron-transfer rates in cytochrome *c* oxidase: investigation of the oxygen reaction in the *R. sphaeroide*s and bovine enzymes, Biochim. Biophys. Acta 1367 (1998) 107–117.
[36] A. Jasaitis, M.I. Verkhovsky, J.E. Morgan, M.L. Verkhovskaya, M. Wikström, Assignment and charge translocation stoichiometries of the major electrogenic phases in the reaction of cytochrome c oxidase with dioxygen, Biochemistry 38 (1999) 2697–2706.
[37] S. Ferguson-Miller, G.T. Babcock, Heme/copper terminal oxidases, Chem. Rev. 96 (1996) 2889–2907.
[38] M. Wikström, Cytochrome c oxidase: 25 years of the elusive proton pump, Biochim. Biophys. Acta 1655 (2004) 241–247.
[39] A. Namslauer, A. Aagaard, A. Katsonouri, P. Brzezinski, Intramolecular proton-transfer reactions in a membrane-bound proton pump: the effect of pH on the peroxy to ferryl transition in cytochrome c oxidase, Biochemsitry 42 (2003) 1488–1498.
[40] U. Pfitzner, K. Hoffmeier, A. Harrenga, A. Kannt, H. Michel, E. Bamberg, O.M.H. Richter, B. Ludwig, Tracing the D-pathway in reconstituted site-directed mutants of cytochrome c oxidase from Paracoccus denitrificans, Biochemistry 39 (2000) 6756–6762.
[41] J.R. Fetter, J. Qian, J. Shapleigh, J.W. Thomas, A. García-Horsman, E. Schmidt, J. Hosler, G.T. Babcock, R.B. Gennis, S. Ferguson-Miller, Possible proton relay pathways in cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 1604–1608.
[42] K. Faxén, G. Gilderson, P. Ädelroth, P. Brzezinski, A mechanistic principle for proton pumping by cytochrome c oxidase, Nature 437 (2005) 286–289.
[43] M.I. Verkhovsky, J.E. Morgan, M.L. Verkhovskaya, M. Wikström, Translocation of electrical charge during a single turnover of cytochrome-c oxidase, Biochim. Biophys. Acta 1318 (1997) 6–10.
[44] A.S. Pawate, J. Morgan, A. Namslauer, D. Mills, P. Brzezinski, S. Ferguson-Miller, R.B. Gennis, A mutation in subunit I of cytochrome oxidase from Rhodobacter sphaeroides results in an increase in steady-state activity but completely eliminates proton pumping, Biochemistry 41 (2002) 13417–13423.
[45] A. Namslauer, A.S. Pawate, R.B. Gennis, P. Brzezinski, Redox-coupled proton translocation in biological systems: proton shuttling in cytochrome c oxidase, PNAS 100 (2003) 15543–15547.
[46] H. Lepp, L. Salomonsson, J.P. Zhu, R.B. Gennis, P. Brzezinski, Impaired proton pumping in cytochrome c oxidase upon structural alteration of the D pathway, Biochim. Biophys. Acta 1777 (2008) 897–903.
[47] A. Namslauer, H. Lepp, M. Brändén, A. Jasaitis, M.I. Verkhovsky, P. Brzezinski, Plasticity of proton pathway structure and water coordination in cytochrome c oxidase, J. Biol. Chem. 282 (2007) 15148–15158.
[48] D. Han, A. Namslauer, A. Pawate, J.E. Morgan, S. Nagy, A.S. Vakkasoglu, P. Brzezinski, R.B. Gennis, Replacing Asn207 by aspartate at the neck of the D channel in the aa3-type cytochrome c oxidase from Rhodobacter sphaeroides results in decoupling the proton pump, Biochemistry 45 (2006) 14064–14074.
[49] D.A. Mills, S. Ferguson-Miller, Influence of structure, pH and membrane potential on proton movement in cytochrome oxidase, Biochim. Biophys. Acta 1555 (2002) 96–100.
[50] C. Ribacka, M.I. Verkhovsky, I. Belevich, D.A. Bloch, A. Puustinen, M. Wikström, An elementary reaction step of the proton pump is revealed by mutation of tryptophan-164 to phenylalanine in cytochrome c oxidase from Paracoccus denitrificans, Biochemistry 44 (2005) 16502–16512.
[51] E. Olkhova, V. Helms, H. Michel, Titration behavior of residues at the entrance of the D-pathway of cytochrome c oxidase from Paracoccus denitrificans investigated by continuum electrostatic calculations, Biophys. J. 89 (2005) 2324–2331.
[52] M.H. Olsson, A. Warshel, Monte Carlo simulations of proton pumps: on the working principles of the biological valve that controls proton pumping in cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 6500–6505.
[53] J. Xu, G.A. Voth, Free energy profiles for H+ conduction in the D-pathway of cytochrome c oxidase: a study of the wild type and N98D mutant enzymes, Biochim. Biophys. Acta 1757 (2006) 852–859.
[54] R.M. Henry, C.H. Yu, T. Rodinger, R. Pomès, Functional hydration and conformational gating of proton uptake in cytochrome c oxidase, J. Mol. Biol. 387 (2009) 1165–1185.
[55] V.R.I. Kaila, M.I. Verkhovsky, G. Hummer, M. Wikström, Mechanism and energetics by which glutamic acid 242 prevents leaks in cytochrome c oxidase, Biochim. Biophysi. Acta, Bioenerg. 1787 (2009) 1205–1214.
[56] R. Sugitani, E.S. Medvedev, A.A. Stuchebrukhov, Theoretical and computational analysis of the membrane potential generated by cytochrome c oxidase upon single electron injection into the enzyme, Biochim. Biophys. Acta, Bioenerg. 1777 (2008) 1129–1139.
[57] D.M. Popovic, A.A. Stuchebrukhov, Proton pumping mechanism and catalytic cycle of cytochrome c oxidase: Coulomb pump model with kinetic gating, FEBS Lett. 566 (2004) 126–130.
[58] D.M. Popovic, A.A. Stuchebrukhov, Electrostatic study of the proton pumping mechanism in bovine heart cytochrome c oxidase, J. Am. Chem. Soc. 126 (2004) 1858–1871.
[59] J. Xu, G.A. Voth, Computer simulation of explicit proton translocation in cytochrome c oxidase: the D-pathway, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 6795–6800.
[60] J. Xu, M.A. Sharpe, L. Qin, S. Ferguson-Miller, G.A. Voth, Storage of an excess proton in the hydrogen-bonded network of the D-pathway of cytochrome *c* oxidase: identification of a protonated water cluster, J. Am. Chem. Soc. 129 (2007) 2910–2913.
[61] A. Aagaard, G. Gilderson, D.A. Mills, S. Ferguson-Miller, P. Brzezinski, Redesign of the proton-pumping machinery of cytochrome c oxidase: proton pumping does not require Glu(I-286), Biochemistry 39 (2000) 15847–15850.
[62] K.L. Duerr, J. Koepke, P. Hellwig, H. Mueller, H. Angerer, G. Peng, E. Olkhova, O.M.H. Richter, B. Ludwig, H. Michel, A D-pathway mutation decouples the Paracoccus denitrificans cytochrome c oxidase by altering the side-chain orientation of a distant conserved glutamate, J. Mol. Biol. 384 (2008) 865–877.
[63] A.S. Vakkasoglu, J.E. Morgan, D. Han, A.S. Pawate, R.B. Gennis, Mutations which decouple the proton pump of the cytochrome c oxidase from Rhodobacter sphaeroides perturb the environment of glutamate 286, FEBS Lett. 580 (2006) 4613–4617.