Proton-transfer pathways in the mitochondrial *S. cerevisiae* cytochrome *c* oxidase

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In cytochrome *c* oxidase (CytCO) reduction of *O*₂ to water is linked to uptake of eight protons from the negative (n) side of the membrane: four are substrate protons used to form water and four are pumped across the membrane. In bacterial oxidases, the substrate protons are taken up through the K and the D proton pathways, while the pumped protons are transferred through the D pathway. On the basis of studies with CytCO isolated from bovine heart mitochondria, it was suggested that in mitochondrial CytCOs the pumped protons are transferred through a third proton pathway, the H pathway, rather than through the D pathway. Here, we studied these reactions in *S. cerevisiae* CytCO, which serves as a model of the mammalian counterpart. We analyzed the effect of mutations in the D (Asn99Asp and Ile67Asn) and H pathways (Ser382Ala and Ser458Ala) and investigated the kinetics of electron and proton transfer during the reaction of the reduced CytCO with *O*₂. No effects were observed with the H pathway variants while in the D pathway variants the functional effects were similar to those observed with the *R. sphaeroides* CytCO. The data indicate that the *S. cerevisiae* CytCO uses the D pathway for proton uptake and presumably also for proton pumping.

Cytochrome *c* oxidase (CytCO) is a membrane-bound enzyme that catalyzes reduction of *O*₂ to water and uses part of the free energy of this reaction for proton pumping from the negative (n) to the positive (p) side of the membrane. The electron donor for CytCO is cytochrome *c* (cyt. *c*), which binds near the electron-entry site, Cuₙ. From Cuₙ, electrons are transferred consecutively to heme *a₁* and then to the catalytic site, composed of heme *a₃* and Cuₚ, and Cuₚ₉. For each *O*₂ reduced to water, a total of eight protons are taken up from the n-side of the membrane. Four of these protons are used for reduction of *O*₂ to *H*₂*O* at the catalytic site (*O*₂ + 4*H*⁺ + 4*e⁻ — > 2*H*₂*O*); and the other four protons are taken up from the *n* side and released to the *p* side of the membrane (pumped).

In the A-type bacterial CytCOs, protons are taken up through two pathways named the K pathway, after a conserved Lys residue approximately in the middle of the pathway, and the D pathway, named after a conserved Asp residue at the orifice of the pathway (for review, see ref 3) (Fig. 1). The K pathway is used for uptake of ~2*H*⁺ upon reduction of the catalytic site while the D pathway is used for transfer of the remaining 6 protons, i.e. four that are pumped and the remaining two protons used for reduction of *O*₂ to *H*₂*O* at the catalytic site. In the B-type oxidases all protons are transferred through a single pathway, which approximately overlaps in space with the K pathway in the A-type oxidases 4,6,7. Hence, there are alternative pathways for transfer of the pumped protons from the n-side in different oxidases.

The mammalian mitochondrial CytCOs belong to the A-class, i.e. they harbor both the K and D proton pathways. However, structural studies have revealed structural changes in another region of the protein, highlighting a putative third functional pathway called the H pathway 8 (Fig. 1). The H pathway starts on the *n*-side, near a His residue (His413, bovine CytCO numbering) and spans the membrane domain close to heme *a* toward the *p*-side, via Asp51 and Ser205 (subunit II), and the Tyr440-Ser441 peptide bond, which has been proposed to control unidirectional proton transfer 9. It is characterized in its lower part by a water-containing cavity, which was shown to adopt open or closed configuration in different redox and ligand states depending on the interaction of Ser382 with the hydroxyfarnesyl-ethyl group of heme *a*. Pumping measurements were performed on mammalian H
pathway variants using a chimeric human/bovine mutagenesis system and the authors concluded that the H pathway, and not the D pathway, was the route taken by pumped protons in mammalian CytcOs. Equivalent studies in CytcOs from confirmed that the H pathway is not involved in proton conduction in bacterial CytcOs. Mammalian and bacterial CytcOs have thus been proposed to operate with different proton-pumping mechanisms. An alternative role has also been suggested for the H pathway as a dielectric well that could modulate the effects of buried charge changes on heme a.

The bacterial A-type CytcOs are typically composed of the three core subunits I-III, in some cases having an additional fourth subunit that in e.g. consists of a single transmembrane helix. The mammalian CytcOs are bigger with an additional 10–11 accessory subunits. The S. cerevisiae mitochondrial CytcO is an ideal model of the mammalian counterpart. It is composed of the three core subunits as well as 9 accessory subunits. An atomic model of S. cerevisiae CytcO determined recently confirmed its similarity to mammalian CytcOs, including the core subunits responsible for catalysis and pumping.

In the present study, we prepared structural variants of the S. cerevisiae CytcO in which residues of the proposed D and H pathways were modified. The kinetics of internal electron transfer, were studied in these structural variants. Similar effects were observed with the S. cerevisiae CytcO as with the R. sphaeroides CytcO for the D pathway variants. We did not observe any functional effects of changes in the H pathway of the S. cerevisiae CytcO. Collectively, the data indicate that the mitochondrial S. cerevisiae CytcO displays similar functional characteristics to those seen with bacterial CytcOs.

Results
We investigated two D pathway structural variants (Asn99Asp and Ile67Asn) and two H pathway variants (Ser382Ala and Ser458Ala). These structural variants were chosen from a series of modified yeast strains that displayed apparent respiratory growth defects. In earlier studies, reduced minus oxidized difference spectra were recorded of samples of each mutant CytcO after affinity chromatography purification. These difference spectra of Asn99Asp and Ser382Ala were identical to that of the wild-type CytcO whereas those of Ile67Asn and Ser458Ala both displayed a 2–3 nm shift of their alpha-bands to 601 nm and 606 nm, respectively. A comparison of the numbering of these residues in CytcOs from S. cerevisiae, R. sphaeroides and bovine heart mitochondria is shown in Table 1.
Reaction of the reduced CytC with O₂. To investigate the kinetics of electron transfer in the wild-type and structural variants of the CytC, the enzyme was fully reduced (by four electrons) and incubated under an atmosphere of carbon monoxide, which binds at heme a₃, i.e. the ligand blocks access to O₂. The sample was then rapidly (~2 ms) mixed with an O₂-saturated solution in a stopped-flow apparatus. Because CO dissociation in the dark is slow (~30 s), the CytC stays essentially fully reduced over a time scale of milliseconds. About 200 ms after mixing, the ligand was dissociated by means of a short (~10 ns) laser flash, which initiates the reaction of the rescued CytC with O₂ simultaneously in the entire CytC population. Figure 2 shows absorbance changes associated with reaction of the reduced wild-type CytC at 445 nm at pH 7.5 and pH 10. The initial increase in absorbance at \( t = 0 \) is associated with CO dissociation. The decrease in absorbance with a time constant of ~40 µs is associated with electron transfer from heme a to the catalytic site forming the peroxy state (PR). This reaction is not associated with any proton uptake from solution and displays a very small pH dependence. In the next step a proton is taken up from solution to form the ferryl state, F with a time constant of ~100 µs at pH 7.5. This reaction is mainly seen at 580 nm where absorbance changes are small and it could not be resolved in this study. At 445 nm the reaction is observed as a small lag before the decrease in absorbance with a time constant of ~1 ms at pH 7.5. In this final step of the reaction the F state decays to form the oxidized CytC (state O), which is associated with proton uptake from solution and proton pumping.

Figure 3A shows the pH dependence of the F → O reaction. The rate constant for the wild-type enzyme dropped from ~2·10³ s⁻¹ at low pH to ~20 s⁻¹ at pH 10.5. This pH dependence is qualitatively similar to that observed previously with the CytC from R. sphaeroides or from bovine heart mitochondria²³,²⁴. A single kinetic component was observed in the pH range 6–9 and at pH 10.5, but at the intermediate pH values the decay was biphasic with approximately equal amplitudes of the two components. For these points an average of the two rates is plotted in Fig. 3A. With the two H pathway variants, Ser382Ala and Ser458Ala, the rates at pH 7 and pH 10 were similar to those observed with the wild-type CytC (Table 2).

Table 1. Residues in the D and H pathways. Equivalent residues in the bovine heart and R. sphaeroides CytC are listed.

| S. cerevisiae | Bovine | R. sphaeroides |
|--------------|--------|---------------|
| D pathway    |        |               |
| D92          | D91    | D132          |
| N99          | N98    | N139          |
| I67          | I66    | M107          |
| E243         | E242   | E286          |
| H pathway    |        |               |
| R37          | R38    | R52           |
| S52          | S51    | G92           |
| F55          | F54    | W95           |
| Y371         | Y371   | Y414          |
| S382         | S382   | S425          |
| E407         | E407   | E450          |
| Q413         | Q413   | Q456          |
| M428         | M428   | Q471          |
| Y440         | Y440   | Y483          |
| P441         | P441   | P484          |
| N451         | N451   | N494          |
| A454         | A454   | A497          |
| S455         | S455   | S498          |
| S458         | S458   | A501          |
| S464         | S464   | S507          |
| A461         | A461   | S504          |
| A230i        | S205i  | A261i         |

Residue Asn99 is found near Asp92, which is located at the orifice of the D pathway (see Fig. 1). With the Asn99Asp mutant CytC, we observed F → O rate constants of 1200 ± 100 s⁻¹ at pH 7 and 780 ± 100 s⁻¹ at pH 10 (10 measurements on each of two samples), respectively. The rate constant was larger with the Asn99Asp than with the wild-type CytC at pH 10, consistent with earlier observations made with the R. sphaeroides CytC²⁵.

Residue Ile67 is located at a distance of ~3 Å from Glu243, a key D pathway residue, at the end of the pathway (see Fig. 1). With the Ile67Asn, the F → O rate was about a factor of 10 slower than that seen with the wild-type CytC, which is consistent with proton uptake through the D pathway in this reactions step. To test the effect of this structural alteration in a bacterial CytC, we introduced a mutation at the equivalent residue, Met107 in the R. sphaeroides CytC, which is also located ~3 Å from Glu286 (equivalent of Glu243 in S. cerevisiae). In both S. cerevisiae (Ile67Asn) and R. sphaeroides (Met107Cys) mutation at this site resulted in lower CytC activity of ~10%²⁶ and ~6%, respectively (see “Materials and Methods”). With the R. sphaeroides mutant CytC we obtained
The composition of the CytcO solution was: 150 mM KCl, 10 mM Bis-tris propane pH 7 and 0.035% DDM. The pumping and an altered pH dependence of the reaction rate was altered resulting in an increase in the pH dependence of the reaction rate constant by a factor of ~1.6 (Table 2). Hence, we observed a similar behaviour with the Asn99Asp CytcO variant (Fig. 3B), the effect of the mutation is a factor of ~1.325 at pH 7 to ~330 s⁻¹ at pH 10, i.e. by a factor of ~1.6 (Table 2). Hence, we observed a similar behaviour with the S. cerevisiae CytcO variant decreased by a factor of five from ~1000 s⁻¹ to ~200 s⁻¹ upon increasing the pH from 6 to 10, in Asp139Asn variant it decreased from 1200 s⁻¹ to 900 s⁻¹, i.e. by a factor of ~1.325

With the S. cerevisiae wild-type CytcO, the F → O rate constant decreased from ~2200 s⁻¹ at pH 7 to ~330 s⁻¹ at pH 10, i.e. by a factor of ~2.5, while with the Asn99Asp CytcO the rate decreased from 1200 s⁻¹ at pH 7 to ~780 s⁻¹ at pH 10, i.e. by a factor of ~1.6 (Table 2). Hence, we observed a similar behaviour with the S. cerevisiae enzyme as with the R. sphaeroides CytcO.

The current study shows that structural alterations close to the D pathway, at the site of residue Ile67 in S. cerevisiae or Met107 in R. sphaeroides CytcO, result in a dramatically slower F → O reaction rate (by factors of ~100 and ~50, respectively). This residue is located at hydrogen-bonding distance from the Glu243 (or Glu286 in the R. sphaeroides CytcO). Results from earlier studies have shown that replacement of Glu286 by other residues results in a dramatic decrease or impaired proton transfer through the D pathway. Hence, this segment of the D pathway is particularly sensitive to structural alterations. Because with the R. sphaeroides CytcO we observed a ~50-fold slower F → O reaction rate with the Met107Cys variant (Fig. 3), the effect of the mutation is

![Figure 2](https://doi.org/10.1038/s41598-019-56648-9)
Figure 3. pH dependence of reaction rate constants. (A) The rates were determined by fitting an exponential function to traces obtained at 445 nm, after initiation of the reaction of reduced CytcO with O₂. Data with the wild-type and I67N CytcOs. Standard errors ((SD)/√n; SD, standard deviation, n, number of measurements) were typically ~10% of the measured value. Experimental conditions were the same as those in Fig. 2. The different buffers used at different pH values are listed in the Fig. 2 legend. (B,C) Data with the R. sphaeroides CytcO. Absorbance changes were measured at 580 nm with the Met107Cys variant and the P → F (B) and F → O (C) rate constants were determined from a fit of a sum of exponential functions (see e.g.37). Standard errors were typically ~5% of the measured value (SD of ~20 traces measured with 2 samples). The wild-type data are from38. The experiments were carried out in the same way as that in Fig. 2, except that the composition of the CytcO solution was 10 mM Bis-tris propane pH 7.5, 0.05% DDM and 0.1 mM EDTA. The O₂-containing buffer solution was composed of ~1.2 mM O₂, 100 mM buffer (Bis-tris propane pH 6.5, 7.5, 8 and 9 or CAPS for pH 10.5), 0.05% DDM and 0.1 mM EDTA. The solid line is a guide for the eye.
Table 2.  F → O rate constants. Standard errors are shown. Number of measurements in parentheses. For the S382A mutant CytcO the error obtained at pH 10 is estimated based on the range of rate constants that could be used to fit an average trace composed of data from eight measurements. In this case, the signal-to-noise ratio did not allow fits of data obtained with the individual samples.

| Sample       | rate constant ($s^{-1}$) pH 7 | rate constant ($s^{-1}$) pH 10 |
|--------------|-------------------------------|---------------------------------|
| wild type    | 2200 ± 140 (6)                | 330 ± 30 (12)                  |
| N99D         | 1200 ± 70 (18)                | 780 ± 40 (18)                  |
| S382A        | 1200 ± 60 (8)                 | 250 ± 150 (8)                  |
| S458A        | 2500 ± 60 (5)                 | 350 ± 30 (5)                   |

Methods
Preparation of mutants.  The construction of the modified yeast strains is described in21. To prepare the Met107Cys CytcO variant in R. sphaeroides a Quik-Change II site-directed mutagenesis kit (Agilent technologies) was used. The resulting amino-acid replacement was verified by sequencing. The PJS3-SH plasmid was used as a template plasmid for making mutations while pRK415-1 plasmid was used for expression35.

Preparation of the S. cerevisiae CytcO.  Yeast cells were grown aerobically in YPGal medium at 28°C and harvested in late log phase as described in21. Mitochondrial membranes were prepared and the S. cerevisiae CytcO was purified as described by Meunier et al.21 with some modifications as outlined here. During solubilization the membranes were diluted to 2 mg/ml in buffer (50 mM KPi, 100 mM KCl and 1.5% n-dodecyl β-D-maltoside (DDM (w/v)) and solubilized for 1 hour. To remove cyt. c the supernatant from the centrifugation step after solubilization was run over a column loaded with a cation-exchanger (Bio-Rex 70, Bio-Rad) equilibrated with 50 mM KPi, 100 mM KCl and 0.035% DDM. After addition of 5 mM imidazole to the flow-through the ion-exchanger it was loaded on a column filled with 25 ml Ni-NTA resin (NI Sepharose 6 Fast Flow from GE healthcare), pre-equilibrated with (20 mM KPi, 150 mM KCl and 0.035% DDM). The column was washed with four column volumes of wash buffer (20 mM KPi, 150 mM KCl, 10 mM imidazole and 0.035% DDM). This was followed in time by a two-step elution of four column volumes at each imidazole concentration (20 mM KPi, 150 mM KCl, 40 or 100 mM imidazole and 0.035% DDM). The eluted fractions were pooled and concentrated in a 100 kDa cut-off filter (Merck Millipore). Buffer exchange (elution buffer without imidazole) was performed to lower the imidazole content to sub-μM concentrations.

Preparation of the R. sphaeroides CytcO.  Expression of the R. sphaeroides CytcO was achieved by growing bacteria aerobically (Sistrom medium) in the dark at 30°C. After harvesting the cells (at OD550 ≈ 1.5) they were re-suspended (50 mM Tris-buffer, pH 8.0) at 4°C in the presence of DNase I (0.05 mg/ml final concentration) and passed twice through a continuous-flow cell disruptor (Constant Systems LTD) operating at 170 MPa. The inner membrane fraction was collected by ultracentrifugation (138 000 g for 90 minutes at 4°C) and 1.5% DDM was added to solubilize the membrane fraction. The histidine-tagged CytcO was purified using Ni²⁺-NTA affinity chromatography, essentially as described in35,36.

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Activity. A Clark-type O₂ electrode (Hansatech instruments) was used to measure the oxygen reduction rate of the Met107Cys CytcO. A buffer composed of 50 mM KPi (pH 6.7) and 0.1% DDM was added to the reaction chamber and supplemented with 6 mM ascorbate, 670 mM N,N,N,N-tetramethyl-p-phenylenediamine (TMPD) and 32 µM cych. C A CytcO solution (in 100 mM Hepes, pH 7.5 and 0.05% DDM) was added (final concentration of 7 nM) to the reaction chamber and the oxygen-consumption rate during CytcO turnover was monitored (a background oxygen consumption before addition of the CytcO was subtracted). The steady state activity of the Met107Cyt CytcO variant was ~6% of that of the wild-type CytcO, which was ~570 e⁻/s/CytcO.

Flow-flash measurements. The sample was prepared by exchanging the CytcO buffer to 10 mM (wild-type, Ile67Asn and Met107Cys variants) or 3 mM (Asn99Asp, Ser382Ala and Ser458Ala)) Bis-tris propane pH 7, 150 mM KCl and 0.035% DDM. In addition, the buffer was supplemented with 150 mM KCl and 0.035% DDM. For measurements with R. sphaeroides CytcO: 100 mM Bis-tris propane buffer for pH 6.5, 7.5 and 9; CAPS for pH 10 and 0.1 mM EDTA. The CO ligand was dissociated at 0.2 s after mixing, which initiated the reaction. The reaction was monitored by following the time absorbance at specific wavelengths (see figure legends). An amplifier (C11184, Hamamatsu) was used to amplify the signal before recording using a digital oscilloscope.

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**Author contributions**

M.L.B., J.V., A.M.H. and L.N.O. performed experiments. P.B., A.M. and M.L.B. wrote the manuscript. P.B., J.V. and M.L.B. prepared figures. A.M., B.M. and P.B. planned research. All authors reviewed the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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