Plasticity of Proton Pathway Structure and Water Coordination in Cytochrome c Oxidase

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Andreas Namslander, Håkan Lepp, Magnus Brändén, Audrius Jasaitis, Michael I. Verkhovsky, and Peter Brzezinski

From the Department of Biochemistry and Biophysics, The Arrhenius Laboratories for Natural Sciences, Stockholm University, SE-106 91 Stockholm, Sweden and the Helsinki Bioenergetics Group, Institute of Biotechnology, University of Helsinki, P.O. Box 56 (Viikinkaari 5), 00014 Helsinki, Finland

Cytochrome c oxidase (CytcO) is a redox-driven, membrane-bound proton pump. One of the proton transfer pathways of the enzyme, the D pathway, used for the transfer of both substrate and pumped protons, accommodates a network of hydrogen-bonded water molecules that span the distance between an aspartate (Asp132), near the protein surface, and glutamate Glu286, which is an internal proton donor to the catalytic site. To investigate how changes in the environment around Glu286 affect the mechanism of proton transfer through the pathway, we introduced a non-hydrogen-bonding (Ala) or an acidic residue (Asp) at position Ser197 (S197A or S197D), located ~7 Å from Glu286. Although Ser197 is hydrogen-bonded to a water molecule that is part of the D pathway "proton wire," replacement of the Ser by an Ala did not affect the proton transfer rate. In contrast, the S197D mutant CytcO displayed a turnover activity of ~35% of that of the wild-type CytcO, and the O2 reduction reaction was not linked to proton pumping. Instead, a fraction of the substrate protons was taken from the positive ("incorrect") side of the membrane. Furthermore, the pH dependence of the proton transfer rate was altered in the mutant CytcO. The results indicate that there is plasticity in the water coordination of the proton pathway, but alteration of the electrostatic potential within the pathway results in uncoupling of the proton translocation machinery.

Cytochrome c oxidase (CytcO) is a membrane-bound enzyme, which catalyzes the oxidation of cytochrome c and reduction of oxygen to water (for reviews, see Refs. 1–5). The protons used for oxygen reduction are taken up specifically at the catalytic site upon reaction of the fully reduced CytcO with O2, which is then reduced stepwise to H2O, accompanied by the oxygen binding (11–13). The second, D pathway, "starts" with an Asp, Asp132, located at the N-side surface, and leads to a Glu, Glu286, in subunit I (all residues that are discussed here are located in subunit I). The residue is a branching point from which protons may be transferred either to the catalytic site or toward the heme propionates, which have been proposed to accept pumped protons (14–17) and shuttle these protons to an acceptor located further away (18). Reaction of the two-electron reduced (mixed valence CytcO) with O2 results in formation of a state that is called PM in which the O–O bond is broken (19, 20). Formation of PM is not associated with proton uptake from solution (21–23). Upon reaction of the fully reduced CytcO (i.e., all four redox centers reduced) with dioxygen (see Fig. 2), binding of O2 to reduced heme a3 is followed by electron transfer from heme a to the catalytic site with a time constant of ~50 μs (24), forming an intermediate, P3, which presumably has the same chemical structure as P5, with the only difference being an additional electron at the catalytic site in P5 (25, 26). As with P5, formation of P3 is not associated with proton uptake from solution (22, 23). However, due to the extra electron in the catalytic site, a group with high proton affinity is present in the P3 state. This group is spontaneously protonated with a time constant of ~100 μs, giving rise to the next detectable intermediate, the
oxoferryl state (F). Formation of F is rate-limited by internal proton transfer from Glu\textsuperscript{286} (see below) that is followed by immediate reprotonation of Glu\textsuperscript{286} from solution via the D pathway (27–30). In addition, during the P\textsubscript{r} → F transition, the electron on Cu\textsubscript{a} equilibrates with heme a, which has been shown to be controlled by the proton uptake from the bulk solution (28, 29, 31). Also, during the next reaction (i.e. electron transfer from the heme a-Cu\textsubscript{a} equilibrium to the catalytic site forming the fully oxidized state (O)), proton uptake takes place through the D pathway via Glu\textsuperscript{286} (22, 30, 32). The residue is a highly conserved component of the D pathway, and replacement of Glu\textsuperscript{286} by its nonprotonatable analog, Gln, results in impaired proton transfer through the pathway (8, 13, 27). In both the P\textsubscript{M/R} → F and F → O transitions, the residue itself (or together with a cluster of water molecules) is the primary proton donor to the catalytic site, displaying an apparent pK\textsubscript{a} of 9.4 (30). During enzyme turnover, the P\textsubscript{M/R} → F and F → O transitions are linked to proton pumping across the membrane (33–37) (see also Ref. 38).

Results from a number of experimental studies have shown that proton pumping in Cyt\textsubscript{c}O can be uncoupled from oxygen reduction by modification of amino acid residues within the D pathway (39–42). More systematic studies of selected structural variants (e.g. N139D and N207D mutant Cyt\textsubscript{c}Os) showed that the uncoupling is correlated with an alteration in the apparent pK\textsubscript{a} of Glu\textsuperscript{286}. This pK\textsubscript{a} change was suggested to be due to direct electrostatic interactions (40) or an altered hydrogen bonding pattern around Glu\textsuperscript{286} due to structural changes in the water molecules around the site (41). Results from electrostatic calculations suggested that the latter explanation is more likely (43). The involvement of water molecules as “protonatable sites” is also implied from theoretical calculations, which indicate that a water cluster surrounded by Ser\textsuperscript{200}, Ser\textsuperscript{201}, and Ser\textsuperscript{197} may act as a transient proton donor to Glu\textsuperscript{286} (44). Furthermore, results from detailed theoretical studies suggest that the uncoupling of oxygen reduction from proton pumping in the N139D mutant Cyt\textsubscript{c}O is due to an alteration in the proton energy landscape within the D pathway (44, 45). Because such alterations may result in changes in the proton equilibrium involving Glu\textsuperscript{286}, they may be reflected in an altered apparent pK\textsubscript{a} of the residue.

To investigate the effect of changes in the water coordination pattern and electrostatic environment of the D pathway, we have studied the effect on function of two mutant forms of Cyt\textsubscript{c}O, one in which a hydrogen bond to a water molecule near Glu\textsuperscript{286} was removed (Ser\textsuperscript{197} → Ala, about 7 Å from Glu\textsuperscript{286}; see Fig. 1) without altering the electrostatics of the pathway and one in which the electrostatics and/or structure around Glu\textsuperscript{286} was altered by introducing an acidic residue, Asp, at the position of Ser\textsuperscript{197}.#MATERIALS AND METHODS

Mutagenesis, Growth of Bacteria, and Cyt\textsubscript{c}O Purification—Site-directed mutagenesis of \textit{R. sphaeroides} Cyt\textsubscript{c}O was done as described in detail in Ref. 46 (see also Ref. 47). Bacteria were grown in Sistrom’s medium, aerobically in a fermentor. Ni\textsuperscript{2+} -nitritotriacetic acid affinity chromatography was used to purify the histidine-tagged enzyme (48).#Measurements of the Catalytic Activity—The catalytic activity was determined from the initial oxidation rate of reduced cytochrome \textit{c} (2 mM) upon mixing with Cyt\textsubscript{c}O (10 nM) in the presence of oxygen. Absorbance changes associated with cytochrome \textit{c} oxidation were recorded at 550 nm. The measurements were done in a 50 mM phosphate buffer with 0.1% dodecyl-β-d-maltoside at pH 6.5.

Potentiometric Titration of Heme \textit{a}—Chemical redox titrations were performed as in Ref. 49. Frozen stock solutions of Cyt\textsubscript{c}O were diluted to a concentration of ∼5 μM in 0.1 M phosphate buffer and 0.1% n-dodecyl-β-d-maltoside at pH 7. The sample was transferred to an anaerobic cuvette to which the standard and reference electrodes were inserted to monitor the reduction potential of the solution. The sample was flushed with N\textsubscript{2}(g) for 30 min, and KCN at a concentration of 5 mM was added to keep heme \textit{a} oxidized during the titration. The redox mediators diaminodurene, quinhydrone, ferrocene, and phenazine methosulfate were added at concentrations of 10–20 μM. Heme \textit{a} was first gradually oxidized by additions of potassium ferricyanide and then reduced in steps by additions of potassium ferrocyanide. Optical absorption spectra were recorded at each E\textsubscript{o1} value, and the degree of heme \textit{a} reduction was determined from the absorbance peak at 605 nm (∆A\textsubscript{605 nm} – ∆A\textsubscript{530 nm}).

Proton Pumping Measurements—Phospholipid vesicles containing Cyt\textsubscript{c}O were prepared as described in Ref. 34. The argon-flushed proteoliposome sample contained 5 μM valinomycin, 12.5 μM (8 nmol) cytochrome \textit{c}, and 0.5 mM ascorbate. The release of pumped protons upon small additions of air-saturated water (<2 nmol O\textsubscript{2}) was measured using a pH electrode. The pH response at the O\textsubscript{2} pulse was calibrated by the addition of a known amount of H\textsubscript{2}SO\textsubscript{4} to the same sample. The reoxidation rate of cytochrome \textit{c} by ascorbate was measured simultaneously by following absorbance changes at 550 nm to make sure that the measured changes in pH take place during Cyt\textsubscript{c}O turnover (i.e. when cytochrome \textit{c} becomes oxidized) and not upon the slower reoxidation of cytochrome \textit{c} by ascorbate.

Preparation of Fully Reduced Cyt\textsubscript{c}O—Frozen stock solutions of Cyt\textsubscript{c}O were diluted to a concentration of 10–15 μM in 100 mM Heps and 0.1% n-dodecyl-β-d-maltoside at pH 7.4, and the redox mediator phenazine methosulfate was added at a concentration of 1 μM. In experiments in which pH changes of the bulk solution were investigated, buffer was removed with a PD-10 column (Amersham Biosciences) equilibrated with 0.1 M KCl and 0.1% n-dodecyl-β-d-maltoside at pH 7.8 (see Ref. 24). The solution was transferred to a modified anaerobic cuvette, which was repetitively evacuated on a vacuum line and flushed with N\textsubscript{2}. Ascorbate at a concentration of 2 mM was added to the anaerobic Cyt\textsubscript{c}O solution, after which the N\textsubscript{2} was replaced by CO. The reduction and CO binding was confirmed by inspection of optical absorbance spectra in the visible region that were recorded during the process.

Preparation of Mixed Valence Cyt\textsubscript{c}O—Cyt\textsubscript{c}O at a concentration of 10 μM in 100 mM Tris-HCl (pH 8.5), 0.1% dodecyl-β-d-maltoside was transferred to a modified anaerobic cuvette. The gas phase in the cuvette was exchanged to N\textsubscript{2}, and the cuvette was shaken to equilibrate the solution with the gas phase after each gas exchange cycle. After removal of all oxygen, N\textsubscript{2} was exchanged for CO at ∼1 mM, which results in formation of a
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state in which heme a and Cu₆ are oxidized, and the binuclear center (heme a₃/Cu₆) is reduced, with CO bound to the reduced heme a₃ (mixed valence state). The CO itself acts as a reductant (reducing CytO with two electrons, forming CO₂, (50)). Hence, a small fraction of CO is consumed (same amount as that of CytO; i.e. negligible compared with the total amount of CO), and another CO molecule binds to heme a₃, stabilizing the electron at that site.

Flash Photolysis and Flow-Flash Measurements—The kinetics of internal electron transfer between hemes a and a₃ was studied after flash photolysis of the CO ligand from the mixed valence CytO in the absence of oxygen as described in Ref. 51. The flow-flash method was used to measure absorbance changes associated with the reaction of the fully reduced and mixed valence CytO with oxygen. The CO-bound CytO was mixed rapidly with an oxygen-saturated buffer solution (ratio 1:5), after which the CO ligand was photodissociated with a 5-ns laser flash (Quantel Brilliant; frequency-doubled Nd-YAG, 532 nm). The reaction was monitored by measuring absorbance changes at single wavelengths with a time resolution of ~0.5 μs (bandwidth of the amplifier). Complete absorbance spectra (360–700 nm) were also collected with a time resolution of 2.5 ms (time between two consecutive spectra) using a diode-array detector (Applied Photophysics Ltd., Surrey, UK). The measurements were done in a modified custom built combined stopped flow/flash photolysis apparatus (LK.60 FF-60; Applied Photophysics Ltd., Surrey, UK) as described in detail in Ref. 52. In measurements of the proton uptake kinetics upon oxidation of the fully reduced CytO, the oxygen-saturated buffer was replaced by 100 mM KCl, pH 7.8, 0.1% dodecyl-β-D-maltoside, supplemented with the pH-sensitive dye phenol red at a concentration of 48 μM. The amount of reacting CytO was calculated from the CO dissociation absorbance change at 445 nm upon illumination, using an absorption coefficient of 67 mM cm⁻¹ cm⁻¹. The relation between the observed absorbance changes and changes in the proton concentration was determined by several consecutive additions of well defined amounts of HCl to the enzyme-dye solution, as described (24).

Optical Absorption Difference Spectra of the “Peroxy” and “Oxoferryl” Intermediates—The absorption spectra of the P₇₅ and F intermediates were obtained using a stopped flow apparatus equipped with a diode array detector (see above) as described in Ref. 53. Briefly, the wild-type R. sphaeroides CytO at a concentration of 10 μM in 100 mM Hepes at pH 7.4 and 0.1% n-dodecyl-β-D-maltoside was reduced with a slight excess of dithionite and transferred anaerobically to one of the syringes of the stopped flow apparatus. The other syringe contained H₂O₂ at a concentration of 10 mM in the same buffer as the CytO solution. Upon mixing the two solutions (ratio 1:1), the CytO is rapidly oxidized (τ ≈ 1 ms), and the excess dithionite is consumed within a few ms by the oxygen present in the H₂O₂ solution. The pulsed, oxidized CytO then reacts with H₂O₂ to initially form P₇₅ on the millisecond time scale, followed by formation of F on the second time scale. The spectral contributions, relative to that of the fully oxidized pulsed CytO, were calculated from a global fit of the data to the sequential model O → P₇₅ → F.

Measurements of Electric Membrane Potential Generation during Reaction between Reduced CytO and Oxygen—The time-resolved electrometric measurements were based on a method originally developed for photosynthetic applications (54) and applied later to CytO (55). In the present system, Ag/AgCl electrodes recorded the voltage between the two compartments of a cell separated by a lipid-impregnated Teflon mesh. The proteoliposome solution was added to one of the two compartments of the cell, and the proteoliposomes were fused to the membrane by the addition of 12 mM CaCl₂ followed by a 2-h incubation at pH 7 (100 mM MOPS). Then the liquid in both compartments was exchanged to remove excess proteoliposomes that did not adsorb to the membrane. Finally, the Ag/AgCl electrodes were inserted, and the air in the chamber was exchanged, first to N₂(g) and then to CO(g). A laser flash (Quantel Brilliant; frequency-doubled YAG; pulse energy 50 mJ) was used to start the reaction. The measuring system consisted of a home-made operational preamplifier, the output of which was recorded using an IBM-PC-based digitizer, a 12-bit CompuScope 512 (Gage Applied Sciences, Montreal, Canada), and running data acquisition software written by Nikolai Belevich. A CTM-05 counter-timer board (Metabyte) was used to control the timing.

RESULTS

We have investigated the effect of modifying the local hydrogen-bonding pattern and/or electrostatic environment around glutamate 286 on the proton transfer in CytO from R. sphaeroides. The modification was done by inserting an acidic amino acid residue (Asp, S197D) or an Ala (S197A) at the position of S197, located about 7 Å from Glu286 (see Fig. 1, distance measured between the oxygen of the Ser¹⁹⁷ side chain and the closest oxygen atom of the carboxyl group of Glu²⁸⁶).

Basic Functional Characterization of the Mutant Cytochrome c Oxidases—The CytO steady-state turnover activity was measured as the initial oxidation rate of reduced cytochrome c upon mixing with oxidized, detergent-solubilized CytO. The proton pumping efficiency (i.e., number of pumped protons per electron) was measured using the O₂ pulse method (see “Materials and Methods”). In the S197A mutant CytO at pH 6.5, both the steady-state activity and the proton pumping efficiency were the same as with the wild-type CytO. In contrast, the S197D mutant CytO displayed an activity of ~35% of that of the wild-type CytO. No proton pumping was detected with the S197D mutant CytO (i.e., the oxygen-reduction reaction was uncoupled from proton pumping). Instead, a small proton consumption (0.2 H⁺/e⁻) was observed from the outside of the vesicles.

To test whether the mutations had any effect on the internal electron transfer rate between hemes a and a₃, we investigated the kinetics of electron equilibration between these sites after flash photolysis of carbon monoxide from the two-electron reduced (mixed valence) CytO (50). Both the rate and extent of this electron transfer were the same in the S197D mutant as in the wild-type CytO (data not shown). Also, the midpoint potential of heme a, at equilibrium conditions with CN⁻ bound to heme a₃, was the same in the S197D as in the wild-type CytO (Eₘₒ = 370 mV, not shown). Furthermore, both the S197A and
S197D mutant CytcOs in the mixed valence state reacted with O_2 to form the P_M state (23) with the same rates and extents as the wild-type CytcO (data not shown).

Reaction of the Fully Reduced CytcO with Oxygen—The reaction of the fully reduced CytcO with O_2 was studied using the flow flash technique, which makes it possible to follow internal electron transfer (changes in redox state of the co-factors) and the formation and decay of the oxygen intermediates at the catalytic site. Four different kinetic phases are resolved with the wild-type CytcO (24); Fig. 2 shows a reaction scheme, and Fig. 3 shows the kinetic traces.

The rapid absorbance change at t = 0 at all wavelengths shown in Fig. 3 is associated with CO dissociation. The first resolved kinetic phase has a time constant of ~8 μs (at 1 mM O_2), and it is associated with binding of O_2 to reduced heme a_3, forming the so-called A intermediate (see Fig. 2). The rate of oxygen binding was the same in the S197D mutant CytcO as in the wild-type CytcO. This phase is followed by electron transfer from heme a to the catalytic site, associated with formation of intermediate PR and breaking of the O–O bond, with a time constant of ~50 μs in the wild type CytcO. The transition is seen as an absorbance decrease at 445 nm (Fig. 3A), 605 nm (Fig. 3B) and at 580 nm (Fig. 3D). None of these first two reaction steps are associated with proton uptake from solution (23).

Next, in the wild-type CytcO, the oxoferryl (F) intermediate is formed with a time constant of ~100 μs (at pH < 8). Formation of the F intermediate can be monitored directly at 580 nm (increase in absorbance in the time range 0.1–0.5 ms (τ ≈ 100 μs); Fig. 3D) or indirectly at 830 nm, where a fractional oxidation of CuA, concomitant with F formation, is seen as an absorbance increase (Fig. 3C). Proton uptake upon formation of F is seen as an increase in absorbance at 560 nm of the pH dye phenol red (Fig. 3E).

The last transition has a time constant of ~1.2 ms, and it involves transfer of the fourth electron from the CuA-heme a equilibrium to the catalytic site. The oxidation of CuA (the fraction that remained reduced after F formation) is seen as an absorbance increase at 830 nm (Fig. 3C), whereas the decay of F, oxidation of heme a, and formation of the fully oxidized state is seen as a decrease in absorbance at 445 nm (Fig. 3A), 605 nm (Fig. 3B), and 580 nm (Fig. 3D). The transition is also associated with proton uptake from solution (see Fig. 3E). With the S197A mutant CytcO, all rates and extents of absorbance changes during reaction of the fully reduced enzyme and O_2 were the same as in the wild-type CytcO.

With the S197D mutant CytcO, it was difficult to resolve formation of state F. The P_R state was formed with a time constant of ~65 μs, and after this kinetic phase, a lag was seen at 580 nm (Fig. 3D), followed by a slow (compared with the wild-type CytcO) biphasic decay to the absorbance level of the fully oxidized state with time constants of 30 and 100 ms. Because the absorbance level at 580 nm in the time range around 100–300 μs was higher than the final absorbance level of the E286Q mutant CytcO, which was shown earlier to stop at the P intermediate (27), it is likely that the F intermediate is formed on a ~100-μs time scale in the S197D mutant CytcO. However, it is difficult to resolve kinetically its formation, because it is accelerated and displays a rate that is similar to that associated with formation of P_R. As evidenced from the absorbance changes at 830 nm shown in Fig. 3C, CuA was not significantly oxidized on
the 100-μs time scale (τ = 100 μs for the oxidation of Cu₄ during F formation in the wild-type CytCO). Instead, two major kinetic phases attributed to Cu₂ oxidation were observed with time constants of 30 and 100 ms (i.e. the same time constants as those associated with formation of the fully oxidized state, as seen at other wavelengths).

**Kinetic Difference Spectrum of the F → O Transition**—To determine the spectra of the intermediates formed in the S197D mutant CytCO, a photo-diode array detector (with a time resolution of 2.5 ms) was used to record full kinetic difference spectra of the 30- and 100-ms phases. The dashed line in Fig. 4B shows the kinetic difference spectrum of the first minus the last component spectra from the global fit, in other words the sum of the spectral changes associated with both the 30- and the 100-ms kinetic phases, which are associated with formation of the fully oxidized CytCO. The spectrum was compared with the P → O and F → O difference spectra obtained in the reaction between wild type CytCO and H₂O₂, shown in Fig. 4A (see “Materials and Methods”). After subtraction of a small amount of reduced heme a (~10% of the total CytCO concentration), the spectrum shows the characteristics of mainly an “oxoferryl” minus oxidized (F → O) difference spectrum with absorbance peaks at 580 and 436 nm (compare solid lines in Fig. 4, A and B). However, the kinetic difference spectrum from the flow-flash experiment and that of F → O obtained with H₂O₂ were not identical, and a better fit of the S197D kinetic difference spectrum was obtained when using a combination of 80% of F → O and 20% P → O (compare solid lines in Fig. 4, B and C).

For comparison, we also determined a kinetic difference spectrum of the F → O transition by measuring absorbance changes upon reaction of the fully reduced E286A/I112E double mutant CytCO with oxygen (Fig. 4C, dashed line). In this mutant, CytCO formation of both the F and O intermediates is slowed dramatically (56), and the full kinetic F → O spectrum can easily be recorded at 4°C using a diode array detector. This kinetic difference spectrum was similar to that obtained with the S197D mutant CytCO (compare the dashed line in Fig. 4C with the solid line in Fig. 4B).

**Proton Uptake during O₂ Reduction**—Proton uptake from the bulk solution during oxygen reduction was investigated by monitoring absorbance changes of the pH-sensitive dye phenol red at 560 nm in a buffer-free CytCO solution. Three kinetic phases were observed in the S197D mutant CytCO with time constants of ~100 μs, ~30 ms, and ~100 ms, respectively (Fig. 3E). The first phase displayed a time constant typical for proton uptake associated with F formation. It corresponds to the uptake of 0.4 ± 0.1 H⁺/CytCO, which is less than observed with the wild-type CytCO (~0.7 H⁺/CytCO). The following proton uptake reactions in the S197D mutant CytCO displayed time constants of 30 and 100 ms, associated with the uptake of ~0.5 and ~1 H⁺/CytCO, respectively. The time constants of the two slower proton uptake reactions are the same as those associated with formation of the fully oxidized state. The total number of protons taken up during oxidation of the fully reduced CytCO was the same in the S197D mutant as in wild-type CytCO (~1.9 H⁺/CytCO).
FIGURE 3. Absorbance changes at 445 nm (A), 605 nm (B), 830 nm (C), and 580 nm (D) after flash-induced dissociation (at t = 0) of CO from the fully reduced wild-type (WT) and S197D mutant CytC0 in the presence of O2.

The pH Dependence of the O Formation Rate—The kinetics of the 30-ms and 100 ms phases associated with F → O transition during the reaction of the fully reduced CytC0 with O2 was...
investigated in the pH range 5–10 (see Fig. 5). The wild type CytO displayed a pH-dependent rate of the F to O transition in the range between pH 6 and 11 that was best fitted to a titration with two pKₐ values, 6.3 and 8.9 (57, 58). With the S197D mutant CytO, the rate was pH-independent in the range above pH 7. Below pH 7, the rate increased with decreasing pH and the pKₐ value was estimated to be ~6.

Electrogenic Reactions of S197D Mutant CytO—Fig. 6 shows the kinetics of electric potential generation upon reaction of the fully reduced S197D mutant CytO with oxygen, whereas the inset shows the fast phase on a millisecond time scale. For comparison, the kinetics measured with the wild-type CytO, normalized by the amplitude of the fast phase, is shown (dashed line). The reaction starts at time 0, where CO is dissociated by means of a laser flash. There was no generation of electric potential during the first 28 μs for the wild-type and 14 μs for the mutant CytO (R → P₅ transition). Development of the electric potential after the primary lag was slightly faster for the mutant (τ = 75 μs, amplitude 9.6 mV) than for the wild-type CytO (τ = 100 μs), which is consistent with an accelerated formation of the F intermediate (see above). Because the mutant CytO does not pump protons, the amount of charge translocated across the membrane is determined only by the transfer of electrons and protons involved in the reduction of O₂ to H₂O. The rapid phase was followed by a slower biphasic process, corresponding to charge translocations associated with formation of the oxidized state, with time constants of 30 and 120 ms and amplitudes of 5.5 and 10.3 mV, respectively. Thus, the amplitude ratio of the rapid phase and the sum of the slow phases is 1:1.6 (fast to slow). In addition, a transition with a time constant of ~6 ms that is not associated with a change in voltage is seen between the fast and slow phases.

DISCUSSION

We have investigated the effect of altering the local environment within the D pathway, near Glu²⁸⁶, on proton pumping and the kinetics of specific reaction steps of the R. sphaeroides CytO. The modification was introduced by mutating Ser¹⁹⁷, located ~7 Å from Glu²⁸⁶, to Asp (S197D mutant CytO). Furthermore, we investigated the S197A mutant CytO, which displayed 100% activity, the same kinetic phases during reaction of the fully reduced enzyme with O₂ as the wild-type CytO, and full proton-pumping activity. Similar results were obtained previously from studies of the S201A mutant CytO (59). The results with the Ser → Ala mutants indicate that Ser¹⁹⁷ or Ser²⁰¹ themselves are not necessary for the function of the D pathway, although these Ser residues are hydrogen-bonded to water molecules that are part of a proton-conducting water chain (see a recent special issue on proton transfer in biological systems (60)). Results from molecular dynamics simulations using the program HyperChem (Fig. 7) show that although the hydrogen bond between Ser¹⁹⁷ and a nearby water molecule (H₂O, number 44) is removed, the water structure does not change significantly, which suggests that there is flexibility in the design of the residues lining the water chain.

The S197D mutant CytO, on the other hand, showed a number of striking differences compared with the wild-type CytO: (i) the steady state turnover rate of oxygen reduction was decreased by a factor of 3; (ii) the proton pumping was impaired, and instead a fractional proton uptake from the “wrong” side (P-side) was observed during catalytic turnover; (iii) the formation rate of the F intermediate in the reaction between the fully reduced CytO and oxygen was slightly increased; however, the amount of proton uptake associated with this reaction was decreased; (iv) the formation rate of the O intermediate was slowed.

It is seen that the rates of transitions associated with proton uptake through the D pathway were affected, whereas the rate of internal electron transfer within the enzyme, the rate of O–O
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The P → F and F → O Transitions—The P_R to F transition has previously been investigated extensively in both wild-type and different mutant CytOs from R. sphaeroides and other species. As seen in Fig. 2, in P_R there is an uncompensated negative charge in the catalytic site, because an electron is transferred from heme a to the catalytic site upon P_R formation. The electron transfer is then followed by proton uptake from the N-side of the membrane to the catalytic site (τ ≈ 100 μs) forming the F state. The results from earlier studies have shown that during the P_R → F transition, the proton is initially transferred from Glu286 to the catalytic site, followed by rapid reprotonation of the glutamate from the bulk solution (22, 28). Thus, the F intermediate can be formed even if proton transfer through the D pathway is impaired “below” Glu286 (28, 29), in which case Glu286 presumably stays unprotonated after the proton donation to P_R at the catalytic site. Hence, Glu286 functions as an internal proton donor to the catalytic site, and its apparent pK_a was determined to 9.4 during the P_R → F transition (30).

The next transition, F → O, includes electron transfer from Cu_A/heme a to the catalytic site and concomitant uptake of a proton through the D pathway. Its observed rate was modeled to be a function of the fraction of F intermediate reduced with another electron times the intrinsic rate of the proton transfer reaction (31, 58). The pH dependence of the rate is more complex than that of the P_R → F transition, displaying two different pK_a values (6.4 and 8.9; see Fig. 5) (57, 58).

It has been shown that insertion of aspartates close to the entrance of the D pathway (ND139 and ND207) results in impaired proton pumping (39–42) and increased apparent pK_a values of the P_R → F and F → O transitions (40, 42), without slowing the rate of oxygen reduction. Hence, these mutations seem to affect the water structure or energy landscape (44, 45) of the D pathway in a way that increases the proton affinity of Glu286, thus uncoupling oxygen reduction from proton pumping.

With the S197D mutant CytO, the aspartate is inserted closer to Glu286, and if there is an electrostatic interaction between the groups, the pK_a of one of them is expected to increase, whereas that of the other one should decrease (61). As shown in Fig. 7, it is also likely that the mutation would alter the water structure around Glu286.

The results indicate that after the initial two kinetic phases (the latter with a time constant of ~65 μs), the major fraction of the enzyme population was in the F state, which decayed slowly in a biphasic manner to the oxidized state. This is evident from an inspection of the kinetic difference spectrum of the two slowest phases (30 and 100 ms), which shows the characteristics of an F → O difference spectrum after subtraction of a 10% heme a^2+-heme a^3+ contribution (see Fig. 4 and discussion below). This conclusion is further supported by the ~100-μs proton uptake (see Fig. 3E) and the charge translocation with a time constant of ~75 μs (Fig. 6). The relatively small fraction heme a oxidation is consistent with the small extent of electron transfer from Cu_A to heme a during the P_R → F transition (see Fig. 3C). It has been shown earlier that the electron transfer from Cu_A to heme a is controlled by the reprotonation of Glu286 (i.e. if Glu286 is not reprotonated during the P → F transition, the electron transfer does not occur) (31). The lack of electron transfer and the slow F → O transition indicate that Glu286 was not reprotonated on the time scale of F formation (i.e. the proton transfer from the bulk solution to Glu286 was slower than ~100 μs). However, we did observe a net proton uptake from the bulk solution (τ ≈ 100 μs), but the extent was ~50% of that observed with the wild-type CytO. Also, the smaller observed proton uptake indicates that Glu286 did not become immediately reprotonated. Instead, the fractional

![Figure 7: Effects of the S197A (A) and S197D (B) mutations on the orientation of adjacent water molecules and residues.](Image)

The geometry was optimized using HyperChem 7.1 (Hypercube, Inc.) software. All residues and water molecules within a sphere with a radius of about 16 Å around the C_a atom of S197 (CytO from R. sphaeroides; Protein Data Bank identification 1M56) were selected. Molecular mechanics with the Amber99 force field and the Polak-Ribiere algorithm was used to minimize the energy. No constraints were applied to the selected region. In both A and B, the mutant CytOs are shown in dark blue. The figure was prepared using the Visual molecular dynamics software (67).
Proton Pumping in Cytochrome c Oxidase

proton uptake is explained in terms of protonation of another group as a result of deprotonation of Glu\textsuperscript{286}, due to electrostatic interactions between the two groups. One possible such group is Asp\textsuperscript{197}, and another one is the aspartate at the entrance of the D pathway (Asp\textsuperscript{132}). According to this scenario, the extent of proton uptake is simply determined by the electrostatic interaction energy between Glu\textsuperscript{286} and the interacting group.

How is the accelerated F formation, the slow reprotonation of Glu\textsuperscript{286}, and the slow formation of O explained? It has previously been suggested that the side chain of Glu\textsuperscript{286} is flexible, and there are observations indicating that it changes conformation upon proton transfer to the catalytic site (9, 62, 63). The S197D mutation may induce a change in the environment of Glu\textsuperscript{286} that locks its side chain in a specific conformation. In this conformation, the initial proton donation to the catalytic site (e.g. upon F formation) occurs rapidly, but the reprotonation becomes very slow due to an impaired protonic contact with the bulk solution. The flexibility of the Glu\textsuperscript{286} side chain and the rapid reprotonation of Glu\textsuperscript{286} from the bulk solution on the N-side is presumably important for a functioning proton pumping machinery, and thus, this would provide an explanation for the impaired proton pumping and the proton uptake from the wrong side of the membrane.

The pH dependence of the F → O transition rate also shows that the rate in the S197D mutant Cyt\textsubscript{c}O is probably not determined by the proton transfer rate from Glu\textsuperscript{286} to the catalytic site. For the wild type Cyt\textsubscript{c}O, the rate of the F → O transition has been modeled to be a function of the rate of proton transfer to the catalytic site times the fraction of the F intermediate reduced with another electron (31). The pH dependence of the rate is best described by a titration of two groups with different pK\textsubscript{a} values, 6.3 and 8.9 (see Fig. 5), where the higher pK\textsubscript{a} is presumably associated with the titration of Glu\textsuperscript{286} (i.e. determined by the fraction of protonated Glu\textsuperscript{286}, see Refs. 30, 57, 58), whereas the lower is presumably associated with the titration of the Arg\textsuperscript{481}-heme a\textsubscript{3} D-ring propionate cluster, which modulates the rate of internal electron transfer to the catalytic site (58). The S197D mutant Cyt\textsubscript{c}O does not show any pH dependence at pH > 7, because the observed rate of the F → O transition is in this case not limited by the proton transfer from Glu\textsuperscript{286} to the catalytic site but rather by the reprotonation rate of Glu\textsuperscript{286}. In other words, the equilibrium between Glu\textsuperscript{286} and the bulk solution is slower than the rate of O formation.

In the S197D Cyt\textsubscript{c}O, the fully oxidized state was formed with time constants of 30 and 100 ms at pH 7.5, which gives an average time constant of ∼50 ms (fitting with one exponential function). During oxidation of the fully reduced Cyt\textsubscript{c}O, four electrons are transferred to O\textsubscript{2}, which means that a 50-ms time constant corresponds to an activity of ∼80 s\textsuperscript{-1}. This value is in good agreement with the overall activity (∼100 s\textsuperscript{-1}) of the mutant Cyt\textsubscript{c}O at pH 7.5 in HEPES buffer.

The Spectrum of the F Intermediate—As pointed out under “Results,” the kinetic difference spectrum of the 30- and 100-ms phases was not identical to that of the F → O spectrum obtained from the reaction with H\textsubscript{2}O\textsubscript{2} (compare the solid lines in Fig. 4, A and B), and a better fit was obtained by including a 20% contribution of the P → O spectrum (see solid line in Fig. 4C). Alternatively, the 30- and 100-ms phases could be well fitted with a kinetic F → O difference spectrum obtained from measurements with the E286A/I112E mutant Cyt\textsubscript{c}O in which formation of both the F and O states is slowed significantly (see dashed line in Fig. 4C). Probably, the spectrum of the F intermediate obtained when Cyt\textsubscript{c}O reacts with H\textsubscript{2}O\textsubscript{2} is slightly different from the spectrum of the F intermediate in the dioxygen reaction. Another possible explanation is that the F intermediate is not formed in 100% of the enzyme population in the S197D mutant Cyt\textsubscript{c}O. If, for example, the pK\textsubscript{a} of E286 is increased to a value close to that of the acceptor group at the catalytic site, the proton equilibrates between the two groups, and the F intermediate will only be formed in a fraction of the Cyt\textsubscript{c}O population, determined by the ΔpK\textsubscript{a} between the groups. In a study of the kinetic spectra during the reaction of the fully reduced bovine Cyt\textsubscript{c}O with oxygen, a branched reaction scheme was proposed, resulting in a mixture of P and F intermediates (see Ref. 64).

The Transfer of Charges across the Membrane—The electrometric measurements detect the transfer of charges (i.e. electrons and protons) across the membrane plane. In the electrometric measurements with the wild-type Cyt\textsubscript{c}O, the voltage changes associated with both the P\textsubscript{a} → F and F → O transitions reflect electron transfer from Cu\textsubscript{A} to heme a, the translocation of pumped protons, and the transfer of substrate protons to the catalytic site, and the ratio of the amplitudes is close to 1 (34). The S197D mutant Cyt\textsubscript{c}O does not pump protons, and, as evidenced from the optical measurements, there is at most 10% electron transfer from Cu\textsubscript{A} to heme a during the P\textsubscript{a} → F transition (see Fig. 3). Therefore, only proton transfer from the bulk solution to Asp\textsuperscript{197} gives a significant contribution to the rapid phase (see Fig. 6). Consequently, the slow phase includes electron transfer from Cu\textsubscript{A} to the binuclear site as well as proton uptake to complete the oxygen reduction chemistry. The ratio of the amplitudes of the electrogenic events associated with the P\textsubscript{a} → F and F → O transitions was 1:1.6. According to the optical absorption data, ∼0.4 protons were taken up during the P\textsubscript{a} → F transition, and, at most, 0.1 electrons were transferred from Cu\textsubscript{A} to heme a. During the F → O transition, 1.5 protons were taken up from the bulk solution, and 0.9 electrons were transferred from Cu\textsubscript{A} to the catalytic site. Assuming heme a and the catalytic site to be located at a depth from the N-side at ∼70% of the membrane dielectric, the expected ratio is 1:4.3. A possible explanation for this discrepancy between the measured amplitudes and the estimated ones might be that, due to the impaired proton transfer through the D pathway, the substrate proton is taken from the P-side of the membrane during the F to O transition, resulting in smaller electrogenic amplitude. This explanation is supported by the proton pumping measurement (see “Results”), where a small amount of protons (0.2 H\textsuperscript{+}/e\textsuperscript{-}) were taken from the outside of the vesicles. The S197D mutant Cyt\textsubscript{c}O does not have a functional proton pumping machinery, presumably because of the structural and electrostatic changes around Glu\textsuperscript{286}. Therefore, the proton pathway to the P-side of the membrane might be open, resulting in partial proton uptake from the wrong side. Proton uptake from the P-side of the membrane has previously been suggested to
occur when the D pathway is blocked in the D132N mutant CytcO (65).

Conclusions—The results from studies of the S197A mutant CytcO show that the boundary of the D pathway could be altered, still maintaining rapid proton conduction through the water chain. The S197D mutation presumably affects the local charge environment of Glu286, making the intrinsic proton transfer from Glu286 to the catalytic site faster, whereas the proton transfer from the bulk solution to reprotonate Glu286 is delayed. As a result, the proton affinity of Glu286 is altered such that the residue can transfer protons efficiently to the catalytic site, whereas proton transfer to the "pump site" is impaired. In conclusion, proton transfer through the pathway appears to be fairly robust and resistant to changes in the hydrogen bonding pattern (see Ref. 66). Alteration of the relative rates of proton transfer reactions can be accomplished by alteration of the electrostatic environment.

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