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Properties of Arg481 mutants of the *aa*₃-type cytochrome *c* oxidase from *Rhodobacter sphaeroides* suggest that neither R481 nor the nearby D-propionate of heme *a*₃ is likely to be the proton loading site of the proton pump

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Abstract

Cytochrome *c* oxidase utilizes the energy from electron transfer and reduction of oxygen to water and pumps protons across the membrane, generating a proton motive force. A large body of biochemical work has shown that all the pumped protons enter the enzyme through the D-channel, which is apparent in X-ray structures as a chain of water molecules connecting D132 at the cytoplasmic surface of the enzyme, to E286, near the enzyme active site. The exit pathway utilized by pumped protons beyond this point and leading to the bacterial periplasm is not known. Also not known is the proton loading site (or sites) which undergoes changes in pK_a in response to the chemistry at the enzyme active site and drives the proton pump mechanism. In this paper we examine the role of R481, a highly conserved arginine that forms an ion pair with the D-propionate of heme *a*₃. The R481H, R481N, R481Q and R481L mutants were examined. The R481H mutant oxidase is about 18% active and pumps protons with about 40% of the stoichiometry of the wild type. The R481N, R481Q and R481L mutants each retain only about 5% of the steady state activity, and this is shown to be due to inhibition of steps in the reaction of O₂ with the reduced enzyme. Neither the R481N nor the R481Q mutant oxidases pump protons but, remarkably, the R481L mutant does pump protons with the same efficiency as the R481H mutant. Since the proton pump is clearly operating in the R481L mutant, these results rule out an essential role in the proton pump mechanism for R481 or its hydrogen bond partner, the D-propionate of heme *a*₃.

Keywords

Cytochrome *c* oxidase; *Rhodobacter sphaeroides*; proton pumping; kinetics

Cytochrome *c* oxidase is the terminal enzyme of the aerobic respiratory chains of most prokaryotes as well as all eukaryotic mitochondria. The enzyme couples the chemistry of reduction of O₂ (to 2 H₂O) to proton translocation across the membrane, generating a transmembrane electrochemical potential (1–10). The proton electrochemical gradient produced is then used to drive many energy-requiring processes, including the synthesis of

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ATP by the ATP synthase. A number of substantial questions about the mechanism of the proton pump remain to be answered, including identifying the exit pathway(s) of pumped protons and the site or sites which must bind and release protons during the catalytic cycle. Of particular interest in the current work is the potential protonation site formed by the ion pair between a highly conserved arginine, R481 (in the *Rhodobacter sphaeroides* oxidase) and the D-propionate of heme a_3 . This arginine/propionate ion pair has been suggested to play critical roles in the exit pathways for both pumped protons and water (11–15).

In the *R. sphaeroides* and related oxidases, all of the pumped protons are transferred from the bacterial cytoplasm through the D-channel (16–19) to a highly conserved glutamate (E286) which is near the active site (Figure 1). From E286, the pumped protons are transferred to the exit channel and then to the bacterial periplasm. The question of the pathway used by pumped protons beyond E286 remains unknown, although a number of studies have addressed this question (12,15,20–27). The proton pump requires at least one protonatable site which, during each electron transfer, binds a proton from the N-side of the membrane and then releases it to the P-side. Several candidates have been suggested as the “proton loading site” (19,28–35), including the A-propionate of heme a_3 , W280, Cu_B-ligands H333 and H334, and any of a cluster of interacting residues consisting of the D-propionate of heme a_3 , R481, R482 and W172. The candidates that are closest to E286 are the D-propionate of heme a_3 , along with R481 and W172, which each hydrogen bond to the D-propionate of heme a_3 . The location of these residues, just “above” E286 has focused attention on these groups, either as the proton loading site or, at least, as water binding components within the exit pathway of pumped protons.

A set of mutations of R481 has been examined in the cytochrome bo_3 quinol oxidase from *Escherichia coli* (12,36). It was concluded (12) that proton pumping requires stabilization of the D-propionate of heme o_3 (equivalent to heme a_3) in the anionic state. The mutations of the *E. coli* oxidase indicate that although a positively charged side chain in the R481 position is not absolutely required for proton pumping, stabilization by hydrogen bonding of the deprotonated carboxylate of the D-propionate of heme o_3 is critical to pumping (12). The R481Q mutation is 70% active and pumps protons, whereas R481L (about 40% active) did not pump protons. The main purpose of the current work is to examine the equivalent mutations of R481 in the oxidase from *R. sphaeroides* to determine whether the previous conclusions can be universally applied. The results show that neither the positive charge at position 481, nor the capacity for hydrogen bonding to the adjacent D-propionate of heme a_3 are absolutely required for proton pumping. Neither R481 nor the D-propionate of heme a_3 is a viable candidate as the proton loading site in the mechanism of the proton pump.

Materials and Methods

Mutagenesis

The Quik-Change mutagenesis kit from Stratagene was used to introduce the mutations. PJS3-SH plasmid (37) was used as the template for the mutations and then pRK415-1 plasmid (38) was used as expression plasmid. The expression plasmid with the mutation was transferred into S-17-1 cells by electroporation. The plasmid was transferred into *R. sphaeroides* JS100 strain by conjugation. Restriction enzymes are from Invitrogen. Sequencing was performed by UIUC Biotech Center.

Protein Purification

Cells were grown in Sistrom's minimum media with 50 $\mu\text{g/mL}$ spectinomycin, 50 $\mu\text{g/mL}$ streptomycin, and 1 $\mu\text{g/mL}$ tetracycline at 30 °C until early stationary phase.

His-tagged wild type and mutant enzymes were purified by histidine affinity chromatography. Cell pellets were homogenized in 50 mM potassium phosphate buffer at pH 6.5 with final concentration of 1 mM EDTA, 8 mM MgSO₄, DNase, and protease inhibitor cocktail. In order to break the cell, the suspended cell mixture went through a microfluidizer 5 times at 20,000 psi. Cell debris was spun down at 8000 rpm for 30 min, and the supernatant was ultracentrifuged at 40,000 rpm for at least 5 h to pellet the cell membrane. The membrane was homogenized with 50 mM potassium phosphate buffer at pH 8.0, and solubilized with 2% (final concentration) dodecyl maltoside (DM), stirring for two hours at 4 °C. The solubilized membrane was then ultracentrifuged at 40,000 rpm for 1.5 h, and supernatant was added to the Ni-NTA resin and stirred for 2 h at 4 °C. The amount of Ni-NTA resin used was 1 mL resin per mg of cytochrome oxidase estimated to bind (approximately 1 mg per liter of growth medium). The resin with bound enzyme was loaded into the column. The column was washed until the flow-through is colorless, usually around 30 column volumes, with 50 mM potassium phosphate buffer at pH 8.0, 10 mM imidazole, and 0.1% DM. The oxidase was eluted with 50 mM potassium phosphate buffer at pH 8.0, 150 mM imidazole, and 0.1% DM. The enzyme was concentrated to about 20 mg/mL with a 50 kD-cutoff concentrator (Millipore) and the concentrated enzyme was desalted with a PD10 column (Amersham). The protein was aliquoted, fast frozen in liquid nitrogen and stored at -80 °C.

For the proton pumping assay, the enzyme was further purified by anion exchange chromatography. The concentrated protein (5 – 10 mg) eluted from the Ni-NTA column was diluted at least 10-fold to a final volume of no more than 4 mL with buffer A (10 mM potassium phosphate buffer, 1 mM EDTA, and 0.2% DM at pH 7.2) and loaded onto tandem DEAE-5PW column (Toso-Haas) attached to FPLC system (Amersham, model ÄKTA Basic). The column was washed with buffer A and then eluted using a gradient with buffer B (buffer A plus 1 M KCl). The enzyme elutes when the gradient is about 20% Buffer B. The eluted protein was then concentrated, aliquoted, fast frozen in liquid nitrogen and stored at -80 °C.

UV-vis Spectroscopy

A Shimadzu UV-vis-2101PC spectrophotometer was used to obtain the spectra of the enzyme (1.5 μM) in 50 mM potassium phosphate buffer at pH 8.0, 0.1% DM. The concentration of the oxidase was determined from the dithionite reduced-minus-air oxidized spectrum using the following relationships. Concentration of oxidase (mM) = $[A_{605}(\text{red-ox}) - A_{630}(\text{red-ox})]/24 \text{ mM}^{-1} \text{ cm}^{-1}$ or Concentration of oxidase (mM) = $[A_{606}(\text{red}) - A_{640}(\text{red})]/40 \text{ mM}^{-1} \text{ cm}^{-1}$.

Steady-state Activity Measurements

The steady-state activity of the enzyme was determined by the rate of oxygen consumption, monitored polarographically using a YSI model 53 oxygen meter equipped with a water-jacketed and stirred-glass measuring vessel. The reaction chamber was filled (1.8 mL) with 50 mM potassium phosphate buffer pH 6.5, 0.1% DM, 10 mM ascorbate, 0.5 mM TMPD, and 30 μM horse heart cytochrome *c*. The enzyme was added to initiate the reaction.

Reconstitution of Oxidase into Phospholipid Vesicles

Cytochrome *c* oxidase vesicles (COVs) were used to measure the proton pumping stoichiometry (protons pumped per electron). Asolectin (soybean)(80 mg/mL), 2% cholic acid, and 100 mM HEPES-KOH at pH 7.4 were mixed and sonicated using a model W-375 sonicator (Heat Systems-Ultrasonics, Inc.). The solution was sonicated under a stream of Argon gas for at least 5 cycles (until the sonicated mixture becomes clear), each cycle consisting of 1 min on followed by 1.5 min off. The oxidase was added to the sonicated lipid/cholate mixture to a final concentration of 0.9 μM. The detergent was slowly removed by the addition of aliquots of Bio-Beads (Bio-Rad). The Bio-Beads (66 mg/mL) were added every 30 min for 4 h at 4 °C. After this, the sample was diluted 1.5-fold with the 100 mM HEPES-KOH buffer, pH 7.4.

Additional Bio-Beads were added at room temperature: 133 mg/mL, every 30 min for 2 h and, finally, 266 mg/mL every 30 min for 1 h. The solution was then dialyzed overnight against 60 mM KCl. These treatments result in the incorporation of the enzyme into small unilamellar vesicles. The lipid/protein ratio is selected to result in an average of less than 1 oxidase molecule per vesicle.

Proton-pumping Assay

Two different methods were employed to determine the stoichiometry of proton pumping, and both techniques yielded compatible results.

In the stirred-cell method, proton pumping is directly measured with a pH electrode. The stirred-cell (1.5 mL) was filled at 25 °C with the enzyme solution, containing 60 mM KCl, 40 μ M horse heart cytochrome *c*, 10 μ M valinomycin, and 0.4 μ M oxidase. Most of the O₂ was removed from this solution by stirring under the stream of water-saturated argon gas. At this point, ascorbate was added to a final concentration of 300 μ M. After equilibration, in which the final remnants of O₂ were removed by the oxidase, the reaction was initiated by injection of 10 μ L of air-saturated water, equilibrated at 25 °C. The oxygen-saturated solution contains approximately 2.5 nmol O₂, which is promptly consumed. Protons released or consumed are recorded by the pH electrode. After each determination, the system was calibrated by adding 10 μ L of anaerobic 1 mM HCl solution (10 nmol H⁺).

Each of these experiments was repeated again in the presence of protonophore, CCCP (10 μ M) to equilibrate the protons on the inside and outside of COVs. In all cases, the data indicated 1 proton consumed per electron.

Proton-pumping was also measured using a pH-sensitive dye using a stopped-flow method (39) with an SX.17-MV model stopped-flow spectrophotometer from Applied Photophysics equipped with diode array detector. Proton pumping of the COVs was measured by monitoring the absorption changes of phenol red at 557 nm, the isosbestic point of reduced and oxidized cytochrome *c* (39). Stopped-flow measurements were done by mixing a solution containing 60 mM KCl, 5 μ M valinomycin, and 0.4 μ M COVs at pH 7.4 with a solution containing 60 mM KCl, 10 μ M reduced cytochrome *c*, and 40 μ M phenol red at pH 7.4. The experiment was repeated with 10 μ M CCCP added to the COV solution. The data analysis was done using SPLINE function of MATLAB (The Mathworks, Inc). Evaluation of the proton pumping is done by comparing the proton consumption determined in the presence of CCCP to the proton release, determined in the absence of CCCP, but with valinomycin present to discharge any membrane potential.

Stopped-flow Kinetics

The rate of reduction of the fully oxidized enzyme (reduction kinetics) was measured using the SX.17-MV model stopped-flow spectrophotometer from Applied Photophysics. The enzyme was freshly oxidized to avoid problems associated with the resting vs pulsed forms of the enzyme (40). A solution containing 50 mM Tricine at pH 8.0 and 0.1% DM with 5 μ M oxidase was placed in syringe barrel at the loading position of the stopped-flow system. Argon gas was applied to remove the oxygen in the syringe barrel. 10 mM ruthenium(III) hexamine and 30 mM dithionite were added to fully reduce the enzyme. In the second syringe, air-saturated 50 mM Tricine at pH 8.0 and 0.1% DM was loaded. Upon mixing, the reaction of the reduced enzyme with O₂ is rapid, but the excess reductant present then re-reduces the enzyme. This re-reduction is monitored spectroscopically.

Preparation of Fully Reduced CO-bound Cytochrome c Oxidase

The buffer was exchanged to 100 mM HEPES, 0.1% DM and 50 μ M EDTA at pH 7.5 using an Amicon Ultra (Millipore, Billerica, MA). The sample with a final enzyme concentration of 5–10 μ M was transferred to an anaerobic cuvette and the atmosphere was exchanged to N₂ on a vacuum line. The anaerobic sample was reduced with 1–2 mM ascorbate plus 0.5–1 μ M ruthenium(III) hexamine. The atmosphere was then exchanged to CO.

Optical Flow-flash Measurements

Fully reduced CO-bound oxidase, at a concentration of 5–10 μ M in a buffer composed of 100 mM HEPES, 0.1% DM, 50 μ M EDTA at pH 7.5 was mixed 1:5, in a modified stopped-flow apparatus (Applied Photophysics, Surrey, UK), with an O₂-saturated buffer of the same composition. About 200 ms after mixing the CO ligand was dissociated by an 8-ns laser flash at 532 nm (Quantel, Brilliant B) and the enzyme reaction with O₂ was monitored optically as absorbance differences at single wavelengths. Data were analyzed using the ProK software (Applied Photophysics).

Electrochemistry

The FTIR difference spectra were obtained using the techniques previously described (41). A 3-bounce attenuated total reflectance (ATR) attachment with a 3 mm diamond prism (SensIR now Smiths Detection) was used with a BioRad (now Varian Inc.) FTS-575C FTIR spectrophotometer equipped with a liquid-nitrogen cooled MCT detector. A thin film containing the enzyme was adhered to the surface of the diamond prism. The initial step is to remove the detergent from the purified enzyme and pellet the enzyme. 10 μ L of 150 μ M enzyme solution was diluted 300-fold with water. The solution was concentrated using an Amicon 50 K membrane concentrator to a final volume of 500 μ L. This dilution and concentration was repeated. The final suspension of enzyme was pelleted using a bench-top centrifuge. The pellet was re-suspended in 10 μ L of water and could be stored at –80 °C.

To prepare the protein film, 6 μ L of this sample was pipetted onto the ATR diamond prism and air-dried for a few minutes. This caused the protein to stick firmly to the crystal surface. The presence of residual phospholipids in the preparation appears to help stabilize the enzyme and assist in the adherence to the surface. The protein film was rehydrated by the first humidifying the air around the film until a stable FTIR spectrum is recorded. Then a 1 mL solution of the titration buffer (30 mM HEPES, 100 mM KCl, 5 mM MgCl₂, pH 7.5, in H₂O) is put on the film in order to re-wet the sample. The protein concentration is estimated to be approximately 300 μ M. The sample was sealed with an acrylic lid, designed to allow the space above the film to be perfused with buffer of any composition. In this way, the redox status of the enzyme was altered, as previously described (41), to obtain the fully reduced and fully oxidized states. Upon changing the buffer composition, the state of the enzyme in the film was monitored by visible spectroscopy using a home-built apparatus with an Ocean Optics USB2000 spectrometer. The absorption spectrum in the visible was obtained by reflectance off the surface of the sample on the diamond ATR crystal. Thus, one can record the visible spectrum simultaneously with the infrared spectrum as the applied potential is changed. In general, the sample was equilibrated with a buffer by flowing the solution over the sample for about 2 h. A peristaltic pump (Cole-Parmer, Masterflex C/L) is used for the flow of the buffer. All experiments were performed at 22 °C with a flow speed of 0.33 mL/min.

In order to achieve the electrochemical titration, a potentiostat (CV-27, BAS) is connected to the flow-electrochemical cell that is mounted on the ATR unit. The flow-electrochemical cell is designed as previously described (41), with gold particles (1–2 mm) as the working electrode and platinum plated titanium electrode as the counter electrode. These two electrodes are separated by two ion-exchange membranes and a compartment that is filled with 400 mM

phosphate buffer and continuously bubbled with argon gas in order to prevent any oxygen diffusion into the titration buffer. Three mediators are used to equilibrate the potential of the protein film with the titration buffer; potassium ferrocyanide (+420 mV vs NHE), ruthenium (III) hexamine (+50 mV vs NHE) and menadione (−12 mV vs NHE). A Ag/AgCl reference electrode is located on the ATR unit close to protein film.

Titration were repeated with at least two different sample films. Each high potential step was followed by equilibration at −292 mV (vs NHE) to record a background and also reduce any oxygen that might have leaked into the sample compartment. Equilibration times for each point were about 30 min.

Since there are 4 redox-active centers in the enzyme, a quantitative fit to the data was not attempted. The results were analyzed qualitatively in order to observe whether the mutations influenced the electrochemical properties of the hemes.

Results

The following mutants were made: R481H, R481N, R481Q and R481L. Each mutant oxidase was purified to homogeneity and characterized.

UV-vis Spectra

The wild type *aa₃*-type oxidase from *R. sphaeroides* has a characteristic absorption spectrum with a Soret band at 424 nm and an α band at 600 nm in the oxidized state. Upon reduction by adding dithionite, the Soret band and α band shift to 445 nm and 605 nm, respectively. The Soret band at 445 nm is contributed by both low-spin heme *a* and high-spin heme *a₃*. In the α band, heme *a* mainly contributes up to 80% of the peak (42).

In spectra of the dithionite-reduced enzymes, both the Soret band and α band are shifted for all of the mutants. In R481H, the absorption peaks are shifted to 442 nm and 603 nm, respectively and, for the rest of mutants, the two peaks are shifted slightly more, to 441 nm and 602 nm for the Soret band and α band, respectively (Figure 2). In other words, the data indicate that the environments of heme *a* and heme *a₃* are perturbed by all of the R481 mutations.

Steady-state Activity

All the mutants exhibit lower steady state activity than the wild type oxidase (Table 1). R481H, which can maintain a salt bridge and hydrogen bond to the carboxyl of the D-propionate has 18% of the oxidase activity. R481N, R481Q and R481L each have about 5% of the wild type oxidase activity. These values compare to the activity of the equivalent mutations in the *E. coli bo₃*-type quinol oxidase (12): R481N (~60%), R481Q (~50%) and R481L (~40%).

Electrochemical Titrations

Electrochemical titrations of the wild type oxidase were obtained using the enzyme deposited as thin film on a diamond ATR, perfused with buffer at pH 7.5 (Figure 3). The titrations were obtained by monitoring the absorbance at 602 nm and varying the electrochemical solution potential using a potentiostat. The data could be roughly viewed as exhibiting two redox steps with a high potential step at +452 mV (NHE) and a low potential step at 136 mV (NHE). FTIR difference spectra, recorded simultaneously (not shown), displayed the same two steps and confirmed that each of the two steps contains contributions from both heme *a* and heme *a₃*.

The same two-step titration is observed with the R481L mutant, but with the midpoints shifted to 419 mV (NHE) and 78 mV (NHE). The shape of the redox titration curve for the wave at

78 mV is steeper than the equivalent redox process with the wild type oxidase, suggesting cooperativity. However, this is an artifact due to incomplete equilibration with the mutant. The data should not be considered quantitatively, therefore, but the trend is clear. The leucine substitution for arginine results in destabilizing the reduced form of the hemes, reducing the midpoint potentials by about 35 mV and 60 mV for the high and low potential steps, respectively. The shifts are in the expected direction based on removing a positive charge from the vicinity of the hemes.

Respiratory Control and Proton Pumping

To measure proton pumping of the isolated enzymes, both wild type and mutant enzymes were reconstituted in the vesicles. The extent to which the vesicles are able to maintain a proton motive force is indicated by the respiratory control ratio (RCR). This is the ratio of the steady state oxidase activity of the vesicle-reconstituted enzyme in the presence of uncoupler (uncontrolled activity) to the activity in the absence of uncoupler (controlled activity). The controlled oxidase activity is limited if a proton motive force is generated across the vesicle membrane, and this activity is increased upon the addition of CCCP (protonophore) and valinomycin (ionophore), which collapse the proton electrochemical gradient. Each of the mutant enzymes exhibits an RCR substantially greater than 1 (Table 1), indicating that each enzyme is generating a proton-motive force.

The proton pumping assay was performed using the stir-cell method with a pH-sensitive electrode and stopped-flow method using the enzyme-reconstituted vesicles. Data are shown only for the stopped-flow method (Figure 4), but the results of both methods are essentially the same.

Using a stopped-flow method, the pH changes in solution are monitored with a pH-sensitive dye. After mixing the solutions containing the enzyme and excess oxygen, the reaction that ensues is limited by the amount of reduced cytochrome *c* that is present. The data from the stopped-flow definitively show that the wild type pumps protons (Figure 4A) and that R481L (Figure 4B) also pumps protons but at a lower stoichiometry. There is no indication of proton pumping by R481Q (Figure 4C) or by R481N (not shown). R481H pumps protons with about the same stoichiometry as does R481L (not shown). In all cases, in the presence of the protonophore CCCP, net alkalinization was observed due to proton consumption from the reduction of oxygen to water. Note that for the wild type enzyme, the rate of alkalinization is considerably faster than the rate of proton pumping (Figure 4A). This reflects the fact that in the presence of the uncoupler, the enzyme specific activity increases 10-fold (see Table 1).

Stopped-flow Reduction Kinetics

To determine the extent to which the mutations in R481 alter the rate of reduction of the fully oxidized enzyme, a stopped-flow spectrophotometer was used. The fully oxidized enzyme was mixed with ruthenium(III) hexamine. The reduction rate of the hemes (not resolved into heme *a* and heme *a*₃) was monitored using the Soret band at 445 nm. The wild type and R481H mutants are reduced by ruthenium(III) hexamine at about equal rates ($\sim 160 \text{ s}^{-1}$), and the greatest influence on the rate of reduction, observed for the R481Q mutant is slower by only a factor of 2 ($\sim 80 \text{ s}^{-1}$) (Table 2).

Flow-flash Measurement of the Oxidation of the Fully Reduced Enzyme

To determine how the R481 mutants slow down the rate of oxidation of the fully reduced enzyme, the flow-flash assay was utilized to compare the wild type enzyme with the R481H, R481Q and R481L mutants. In this assay, the reaction is initiated by photolysis of the CO-adduct of the fully reduced enzyme in the presence of O₂. Since the reaction is not rate-limited by the process of mixing solutions, fast processes can be time-resolved. Earlier experiments

with the wild type enzyme showed that there are 4 sequential steps (43) (Table 3). 1) $R \rightarrow A$, formation of the initial complex of the reduced enzyme with O_2 ; 2) $A \rightarrow P_R$, reaction splitting the O-O bond to form the oxoferryl form of heme a_3 with the concomitant oxidation of heme a , but without proton transfer into the active site; 3) $P_R \rightarrow F$, proton transfer from E286 to the active site, converting ^-OH to H_2O associated with $Cu_B(II)$; 4) $F \rightarrow O$, coupled electron transfer from heme a and proton transfer from E286 to the active site. The two latter reactions are linked to proton pumping (44,45). The reaction of fully reduced R481H with O_2 is nearly the same as the wild type oxidase, except that the $F \rightarrow O$ step is slowed by about 4-fold (Figure 5, Table 3). This correlates with the 18% steady state turnover of the R481H mutant.

The reaction of fully reduced R481Q with O_2 is much more perturbed (Figure 5, Table 3). The initial combination of O_2 with heme a_3 proceeds at the same rate as does the wild type, indicating that the structure of the active site and pathway for O_2 are not altered. The rate of the $A \rightarrow P_R$ step is slowed by about 6-fold. The steps following, $P_R \rightarrow F$ and $F \rightarrow O$, are not clearly resolved in part because of the spectroscopic changes due to the mutation. However, it is estimated that the rate of the $P_R \rightarrow F$ rate is about 100-fold slower than the wild type. The reaction to form the O state may not be complete on the 1 second time scale. These data indicated substantial perturbation, perhaps due to slow proton transfer to the active site that is required for both the $P_R \rightarrow F$ and $F \rightarrow O$ steps of the reaction. This mutant does not pump protons.

The most interesting mutant, R481L, displays even greater indications of perturbation (Figure 5, Table 3). The rate of binding of O_2 is about 3-fold slower, likely a perturbation to the structure of the active site or of the normal pathway used by O_2 to reach heme a_3 . The rate of the $A \rightarrow P_R$ step is substantially slower than the wild type (25-fold), indicating a slower electron transfer rate from heme a to the active site. As with the R481Q mutant, the steps following the formation of the P_R state are not clearly resolved, though it is evident that there is a very large decrease in the rates of these steps. Most remarkably, despite the large perturbations evident from the R481L mutation, the mutant oxidase still pumps protons.

Discussion

The motivation for the current work was to test the importance of the ion pair of R481 and the D-propionate of heme a_3 in the mechanism of the proton pump of the heme-copper oxidases. Previous work (12) on the cytochrome bo_3 quinol oxidase from *E. coli* indicated that proton pumping requires stabilization of the anionic form of the carboxylate of the D-propionate of heme a_3 . Most notably, with the *E. coli* oxidase, R481Q (60% active after isolation), which can hydrogen bond to the D-propionate can pump protons, whereas R481L (40% active after purification), which cannot hydrogen bond to the D-propionate, does not pump protons. The most important result from the current work is that the R481L mutant oxidase from the aa_3 -type oxidase from *R. sphaeroides* can pump protons. This result virtually rules out R481 as well as the associated D-propionate of heme a_3 as being the proton loading site in the proton pump mechanism. Assuming a common mechanism, this conclusion should apply generally to all the proton pumping heme-copper oxidases.

Previously, the R481K mutant has been characterized in both the *R. sphaeroides* oxidase (15, 46–48) as well as in the *P. denitrificans* oxidase (49). Under most circumstances, these enzymes behave as do the wild type oxidases. The R481K mutation in the *R. sphaeroides* oxidase resulted in lowering the midpoint potentials of heme a and heme a_3 by 40 mV and 15 mV, respectively. It was also deduced that the R481K mutation alters the pK_a of the heme a_3 D-propionate. This propionate is hydrogen bonded in the wild type oxidase by R481 and also by W172, and the protonation state of this cluster appears to modulate the rate of internal electron transfer during catalytic turnover. The D-propionate cluster (including R481 and W172) has also been suggested to be the proton acceptor for pumped protons (15). Computational studies

have also indicated that either the D-propionate of heme a_3 or R481 or W172 could function as the proton loading site proposed in the mechanism of the proton pump (46).

Computational approaches have also been used to examine a plausible mechanism of proton transfer from E286 to the D-propionate of heme a_3 (14,21,25,46). There is a hydrophobic cavity that can accommodate water molecules between the presumed proton donor (E286) and the proton acceptor. These water molecules would provide a pathway for rapid proton transfer. Furthermore, the orientation of the water molecules between E286 and the D-propionate of heme a_3 has been computationally shown to depend on the charge distribution on the hemes. The water orientation within this cavity could act as a kinetic valve or gate, allowing pumped protons to exit but not to leak backward (14). Conformational changes of W172 and/or the collapse and formation of the water chain between E286 and the D-propionate of heme a_3 have also been suggested as mechanisms to gate the proton pump (46). The dynamics of R481/D-propionate ion pair has been examined using molecular dynamics methods and the movement of these residues is proposed to be a key factor in the water-mediated transfer of pumped protons as well as the transport of water out of the enzyme (11). In addition, such dynamics could also regulate the pK_a values of the D-propionates of both heme a and heme a_3 (47).

The equivalent of both the R481K (49) and W172F (13) mutants (R473K and W164F) have been characterized in the oxidase from *P. denitrificans*. The R481K mutant perturbs the FTIR reduced-minus-oxidized difference spectrum, and this was used to help assign the FTIR bands of the different heme propionates. It was concluded that the D-propionate of heme a , but not that of heme a_3 , is protonated upon reduction of the enzyme and, by extension, proposed to be protonated/deprotonated during the catalytic cycle (49).

The W164F mutant of the oxidase from *P. denitrificans* (equivalent to W172 in *R. sphaeroides*) retains 40% of steady state oxidase activity and has reduced proton pumping (0.5 proton/electron) (13). In single turnover (flow flash) experiments, the W164F mutation appears to result in a delay in reprotonating E286 (E278 in *P. denitrificans*) after the glutamate has donated its proton to the active site forming the P_R state, thus slowing down the $P_R \rightarrow F$ transition. The FTIR spectra of the W164F mutant indicate perturbation of the heme propionates. In addition, the midpoint potential of heme a_3 is decreased by about 50 mV by the W164F mutation.

The current work confirms that R481 is important for the optimal function of cytochrome c oxidase. Even the replacement of this residue by a lysine lowers the midpoint potential of both hemes, although both oxidase activity and proton pumping remain unaltered (47). The R481H mutant results in lower oxidase activity (18%) and the stoichiometry of the proton pump is about half that of the wild type. This is somewhat similar to the phenotype reported for the W164F oxidase of *P. denitrificans*. Proton pumping is not abolished but, rather, the stoichiometry is reduced.

The less conservative mutations of the *R. sphaeroides* enzyme, examined in the current work, R481N, R481Q and R481L, all reduce the oxidase turnover to about 5% of the wild type. The reduced turnover rate is accounted for by inhibition of steps in the reaction of the reduced enzyme with O_2 . For the R481H mutant, the mutation appears to selectively lower the rate of the last step in the reaction sequence, the $F \rightarrow O$ transition. Since the previous step, $P_R \rightarrow F$ is due to proton transfer from E286 to the active site, it is concluded that this proton transfer is not altered by the R481H mutation. Possibly, the slower $F \rightarrow O$ rate is due to a change in the electrochemical properties of the hemes, though this is pure speculation. The R481Q mutant clearly inhibits the rate of electron transfer from heme a to the binuclear center, measured by the $A \rightarrow P_R$ transition. More drastic, however, is the inhibition of steps following the formation of the P_R state ($P_R \rightarrow F$ and $F \rightarrow O$), suggesting the proton transfer from E286 is also be

influenced by the mutation. This is also observed for the W164F mutant of the oxidase from *P. denitrificans*, where the $P_R \rightarrow F$ step is also inhibited (49).

The most perturbed structural variant examined in the current work was the R481L mutant oxidase. Oxidase activity is similar to R481Q (about 5%), but the flow-flash single turnover study indicates that even the initial formation of the O_2 complex is delayed compared to the wild type. This suggests some conformational alteration limiting access to the active site by O_2 diffusing from the external medium. Once the initial complex with the fully reduced enzyme and O_2 is formed, each electron and proton transfer step leading to the fully oxidized enzyme is strongly inhibited.

Electrochemical titrations of the wild type and R481L mutant oxidases were performed (Figure 3). Results for the wild type were qualitatively similar to results reported for the oxidase from *P. denitrificans*, showing two waves corresponding to the oxidation of the interacting hemes (41). The midpoints of the two waves were shifted lower for the R481L mutant by 33 mV and 58 mV, respectively. These values do not represent the midpoint potentials of heme a and heme a_3 , since both hemes are represented in each wave, and a specific model would be required to obtain further detail. Furthermore, the shape of the titration curves of the mutant indicate that complete equilibration was not achieved over the entire redox range. For the purposes of this work, the observation that the R481L mutant results in lower midpoint potentials of the hemes is sufficient, and is qualitatively similar to what has been reported for the R481K mutant of the *R. sphaeroides* oxidase (47) and the W164F mutant of the *P. denitrificans* oxidase (13). Each of these residues hydrogen bonds to the D-propionate of heme a_3 .

Remarkably, despite the evidence for alterations of each step in the oxidation of the *R. sphaeroides* oxidase by the R481L mutation (Figure 5, Table 3), the enzyme still functions as a proton pump, albeit with about half the stoichiometry of the wild type. The slow rate of binding by O_2 ($R \rightarrow A$), slow electron transfer ($A \rightarrow P_R$), slow proton transfer ($P_R \rightarrow F$) and slow coupled electron/proton transfer ($F \rightarrow O$) exhibited by this mutant do not completely disable the proton pump. It is, therefore, difficult to imagine that the dynamics of the R481/D-propionate ion pair plays a critical part of the mechanism of proton pumping. Similarly, it is also very unlikely that any member of the cluster containing R481, R482, the D-propionate of heme a_3 , and W172 is the proton loading site, essential for the proton pump to function. Replacing R481 by a leucine should have a major influence on the pK_a values of all of these residues.

It is still quite likely, and seemingly unavoidable, that pumped protons must be transferred from E286 through the region of the protein occupied by R481 and the D-propionate of heme a_3 . In the wild type, this might well involve waters hydrogen bonded to any or all of the residues in this cluster. Such pathways must also exist in the R481L mutant. The driving force for the pumped proton, however, must be provided by formation of strong proton binding site outside of the cluster represented by R481, R482, W172 and the D-propionate of heme a_3 . The best candidates for the proton loading site that remain are the A-propionate of heme a_3 and one of the histidine ligands to Cu_B .

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Abbreviations

Ni-NTA, nickel nitrilotriacetic acid

DM, *n*-dodecyl β -*D*-maltoside
 HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
 EDTA, ethylenediaminetetraacetic acid
 TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine
 RCR, respiratory control ratio
 COVs, cytochrome oxidase vesicles
 CCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
 ATR, attenuated total reflectance
 FTIR, fourier transform infrared
 NHE, normal hydrogen electrode

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Figure 1.

Structure of *R. sphaeroides* cytochrome *c* oxidase, defining the residues discussed. Three redox active metal centers: heme *a* and the heme *a*₃-Cu_B binuclear center are in subunit I; Cu_A is in subunit II. The water molecules defining the D-channel used for the input of all the pumped protons from D132 to E286 is also shown. The figure was produced by using VMD from crystal structure 1M56 (12,15,20–27).

Figure 2. UV-visible spectra of the dithionite-reduced oxidases: wild type, R481H, R481N, R481L, and R481Q. Both the Soret band at 445 nm and α band at 605 nm are shifted in all of the mutants.

Figure 3.

Electrochemical titrations of the *R. sphaeroides* wild type and R481L mutant oxidases. The solution potential (vs NHE) is plotted versus the absorbance at 602 nm. The lines are simply a polynomial fit and not based on any model. The estimated midpoints of the two waves are indicated. The midpoints are shifted to lower values in the R481L mutant. The steep curve for the titration of the R481L mutant is due to incomplete equilibration, so the value of the midpoint (419 mV) should be considered to be an estimate.

Figure 4.

Proton pumping determined by monitoring the absorbance change of phenol red at 557 nm using a stopped-flow spectrophotometer. An absorbance increase indicates alkalinization, which is observed in the presence of the protonophore CCCP. In the presence of valinomycin, without CCCP, the change of the phenol red absorbance is downward, indicating acidification due to proton pumping for both (A) the wild type oxidase and (B) the R481L mutant. In contrast, the acidification is not observed with (C) the R481Q mutant oxidase. If it is assumed that the alkalinization is due to the consumption of 1 proton per electron, the amplitude of the absorbance change in the presence of CCCP can be used to estimate the stoichiometry of proton release, assuming that the buffering capacity before and after addition of the uncoupler remains

constant. For the wild type enzyme (A), the rate of enzyme turnover is about 10-fold larger for the decoupled enzyme, in the presence of CCCP, than in the absence of the uncoupler. For this reason, the rate of alkalinization is substantially faster than the rate of proton pumping, which is measured with the coupled enzyme.

Figure 5. Flow-flash reaction of fully reduced wild type, R481H, R481Q and R481L mutant oxidases. Panel A: reaction is monitored at 445 nm. Panel B: reaction is monitored at 595 nm. Experimental conditions: 5–10 μM fully reduced and CO-bound oxidase were mixed 1:5 with an oxygen saturated buffer containing 100 mM HEPES, 0.1% DM, 50 μM EDTA at pH 7.5. Traces are scaled to 1 μM of reacting enzyme.

Table 1

Comparison of activities of wild-type and mutant oxidases from *R. sphaeroides* before and after reconstitution.

oxidase	activity, $e^{-} s^{-1} aa_3^{-1}$			
	In detergent	In vesicles		RCR ^c
		Controlled ^a	Uncontrolled ^b	
WT	1100	32	312	10
R481H	201	17	150	9
R481L	54	25	96	4
R481N	63	25	71	3
R481Q	43	14	57	4

^a measured in the absence of ionophores.

^b measured in the presence of the protonophore CCCP.

^c the ratio of the (uncontrolled/controlled) enzyme turnover numbers.

Table 2

The rate constants for reduction of the wild type and mutant oxidases by ruthenium(III) hexamine.

oxidase	k_{red} (s^{-1})
WT	156
R481H	160
R481L	98
R481N	136
R481Q	82

Table 3

Time constants ($\tau = 1/k$) for the four steps time-resolved for the reaction of the fully reduced oxidase with O₂.

Oxidase Sample	R→A τ (μ s)	A→P τ (μ s)	P→F τ (ms)	F→O τ (ms)
WT ^a	8	40	0.1	1.1
R481H	6	40	0.1	3.7
R481L	26	1000	10 ^b	10 ^b
R481Q	9	250	10	10

^a data for the wild type are from (43).

^b Not resolved. Assumed to correspond to the F→O transition.