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Communication between R481 and Cu_B in Cytochrome *bo*₃ Ubiquinol Oxidase from *Escherichia coli*

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Abstract

The R481 residue of cytochrome *bo*₃ ubiquinol oxidase from *E. coli* is highly conserved in the heme-copper oxidase superfamily. It has been postulated to serve as part of a proton loading site that regulates proton translocation across the protein matrix of the enzyme. Along these lines, proton pumping efficiency has been demonstrated to be abolished in many R481 mutants. However, R481Q in *bo*₃ from *E. coli* has been shown to be fully functional, implying that the positive charge of the arginine is not required for proton translocation [Puustinen, A. and Wikström, M. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 35–37]. In an effort to delineate the structural role of R481 in the *bo*₃ oxidase, we used resonance Raman spectroscopy to compare the non-functional R481L mutant and the functional R481Q mutant, to the wild type protein. Resonance Raman data of the oxidized and reduced forms of the R481L mutant indicate that the mutation introduces changes to the heme *o*₃ coordination state, reflecting a change in position and/or coordination of the Cu_B located on the distal side of heme *o*₃, although it is ~10 Å away from R481. In the reduced-CO adduct of R481L, the frequencies of the Fe-CO and C-O stretching modes indicate that, unlike the wild type protein, the Cu_B is no longer close to the heme-bound CO. In contrast, resonance Raman data obtained from the various oxidation and ligation states of the R481Q mutant are similar to those of the wild type protein, except that the mutation causes an enhancement of the relative intensity of the β conformer of the CO-adduct, indicating a shift in the equilibrium between the α and β conformers. The current findings, together with crystallographic structural data of heme-copper oxidases, indicate that R481 plays a keystone role in stabilizing the functional structure of the Cu_B site through a hydrogen-bonding network involving ordered water molecules. The implications of these data on the proton translocation mechanism are considered.

Heme-copper oxidases are the terminal enzymes of the electron transport chains in the inner mitochondrial membrane of eukaryotes, and in the cytoplasmic membrane of bacteria [1]. The enzymes catalyze the four-electron reduction of dioxygen to water at a catalytic site formed by a binuclear center, consisting of a high-spin heme (heme *a*₃ of cytochromes *aa*₃ and *ba*₃ or heme *o*₃ of cytochrome *bo*₃)¹ and a copper atom (Cu_B). In addition to the heme-copper binuclear center, the enzymes have a low-spin heme group (heme *a* of cytochromes

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Supporting Information available. Figures showing the power dependence of the resonance Raman spectra and a Table of the maxima in the optical spectra. This material is available free of charge via the internet at <http://pubs.asc.org>.

aa₃ or heme *b* of cytochromes *ba₃* and *bo₃*), which mediates electron transfer to the binuclear center [1].

In heme–copper oxidases, four protons are taken up from the negative side (n-side) of the mitochondrial/cytoplasmic membrane, which are consumed for the reduction of a dioxygen molecule to water. During the oxygen reaction, four additional protons are pumped across the membrane from the n-side to the positive side (p-side) of the mitochondrial intermembrane space or the periplasmic space [1]. Proton conducting pathways in oxidases were first identified in bacterial enzymes by site-directed mutagenesis studies more than two decades ago [2, 3]. Later X-ray crystallographic studies of the bacterial as well as mammalian oxidases have further clarified the structure of the proton conducting pathways. So far three pathways, H-, D-, or K-channels (named after one of the conserved amino acid residues in each postulated pathway) have been identified [4–6]. The functional importance of the three channels in proton translocation, as well as in providing chemical protons for the dioxygen reduction reaction to water, has been extensively investigated [2, 3, 7–15]; the data suggest that the D- and K-channels are essential for the function of the bacterial heme-copper oxidases [2, 3, 7–9, 11–13], while the H-channel is functionally unique to mammalian oxidases [10].

It is well accepted that the D- and K-channels (Figure 1a) provide proton-conducting pathways from the n-side surface of the protein to a proton loading site in the vicinity of the heme-copper center, ~13 Å away from the p-side surface. It is, however, unclear as to how the protons are expelled from the proton loading site to the p-side. The propionate side chain groups of the two hemes, pointing towards the p-side of the membrane (Figure 1a) have been postulated to be part of the proton loading site [16–22]. FTIR studies showed that the propionate groups of cytochrome *c* oxidase from *Paracoccus denitrificans* (PdCcO) undergo conformational changes in response to the redox state changes of the two hemes, consistent with the possible functions of the propionate groups as proton traps during the catalytic cycle of the enzyme [16, 17].

In the heme-copper oxidases, an arginine (R481 in the *bo₃* numbering) located within hydrogen-bonding distances to the D-propionates of the two hemes (Figure 1b), is highly conserved. The presence of such a positively charged amino acid is expected to stabilize the anionic form of the carboxyl groups, and thereby regulate the protonation state of the propionates, forming an essential element of the proton loading site. However, the proposed role of the conserved R481 residue is not fully sustained by site-directed mutagenesis studies. Puustinen and Wikstrom observed that the mutation of R481 to glutamine in cytochrome *bo₃* did not cause a significant loss of the pumping activity, although the mutation of R481 to nonpolar amino acids, such as methionine and leucine, or to neutral/polar asparagine, did abolish the proton pumping activity of the enzyme [21]. More recently, Lee *et al.* [23] found 40% pumping efficiency in the R481L mutant of the *Rhodobacter sphaeroides aa₃* oxidase (RsCcO), implying that the positive charge of R481 is not essential for the proton pumping function of the heme-copper oxidases, but resonance Raman (RR) data suggest that R481 is critical for the enzymatic function by stabilizing or maintaining the active site structures in a functional state [24]. To examine the role of R481 in cytochrome *bo₃*, in this work we have systematically investigate the structural properties of R481Q and R481L mutants in various oxidation and coordination states, by using RR and electronic absorption spectroscopy.

¹Abbreviations: cytochrome *bo₃*: cytochrome *bo₃* quinol oxidase from *E. coli*; CcO: cytochrome *c* oxidase; bCcO: cytochrome *c* oxidase from bovine; RsCcO: cytochrome *c* oxidase from *Rhodobacter sphaeroides*; PdCcO: cytochrome *c* oxidase from *Paracoccus denitrificans*; RR: resonance Raman, ICP-OES: inductively coupled plasma optical emission spectroscopy; FTIR: Fourier Transform Infrared Spectroscopy.

MATERIALS AND METHODS

Sample preparation

The wild type and R481 mutants of *E. coli* *bo*₃ quinol oxidase were expressed and purified as described elsewhere [25]. The proteins were dissolved in 50 mM potassium phosphate (pH 8.2) with 0.1 % dodecyl maltoside. The fully-reduced ligand-free form was prepared by reducing the samples with sodium dithionite under an argon atmosphere, while the reduced-CO form was obtained by introducing CO to the fully-reduced samples. All chemicals were purchased from Sigma, and used without further purification.

Metal Analysis

Metal content was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Spectro Genesis spectrometer, as previously described [26]. Before analysis, in order to eliminate adventitious divalent metals, the wild-type and R481L cytochrome *bo*₃ samples were dialyzed overnight into 10 mM Tris-HCl, 20 mM KCl, 1 mM EDTA and 0.05 % dodecyl maltoside at pH 8. The method yields the concentrations of Fe, Cu, Mg, Mn, Ca, Zn Mo and Ni as well as sulfur and phosphorous. Assuming the protein is pure, the concentration of the protein can be determined by the sulfur content as the sum of methionine and cysteine residues. For cytochrome *bo*₃, this comes to 80 moles of S per mole of enzyme.

Heme Analysis

Hemes were extracted from cytochrome *bo*₃ samples and analyzed by HPLC as previously reported [27]. 500 μ L of fresh cold acidic acetone (acetone:HCl:H₂O; 9:1:1) was added to a sample containing 10 nmol of enzyme. The mixture was vortexed vigorously and centrifuged at 16000 $\times$ g for 2 min at 4 °C. The hemes in the supernatant were extracted with 600 μ L of diethylether, washed with 600 μ L H₂O and dried under a nitrogen stream. The dried heme extract was dissolved in 20 μ L of an ethanol:acetic acid:H₂O mixture (70:17:13) and loaded onto a Miscrosorb-MV 100–5 C18 reverse-phase HPLC column (Varian, Palo Alto, CA) fitted to a Waters 600 HPLC pump with a mobile phase of ethanol:acetic acid:H₂O mixture (70:17:13). The heme peaks were monitored at 402 nm by a Waters 2487 dual wavelength absorbance detector. At a flow-rate of 0.5 mL/min, heme B and heme O have retention times of about 4 and 8 minutes respectively.

Quinone Analysis

Ubiquinone from cytochrome *bo*₃ was extracted and analyzed by HPLC as reported before [27]. Ten nmol of cytochrome *bo*₃ and 10 nmol of ubiquinone-10 (Q-10) were mixed and diluted to 1 mL with water. The Q-10 was added as an internal standard. Three mL of a methanol:petroleum ether (3:2) solvent was added to the mixture which was then vortexed vigorously and centrifuged briefly. The upper organic layer containing the ubiquinones was transferred to a fresh tube and the remaining mixture was extracted with 1.5 mL of petroleum ether two more times. All the extracts were combined and dried under a nitrogen stream. The ubiquinone extracts were dissolved in 20 μ L ethanol and analyzed with the same HPLC equipment used in the heme analysis with a mobile phase of ethanol:methanol:acetonitrile (4:3:3). At a flow rate of 0.8 mL/min, the 278 nm peaks for ubiquinone-8 (Q-8) and Q-10 have retention times of about 9 and 19 minutes respectively. It was found the each of the proteins contained ~1 Q-8 molecule per protein molecule.

Raman and optical measurements

The resonance Raman spectra were carried out as previously described [28]. Briefly, the 413.1 nm excitation from a Kr ion laser (Spectra-Physics, Mountain View, CA) was focused

to a $\sim 30\ \mu\text{m}$ spot on the spinning quartz cell rotating at $\sim 1,000\ \text{rpm}$. The scattered light, collected at a right angle to the incident laser beam, was focused on the $100\ \mu\text{m}$ -wide entrance slit of a 1.25 m Spex spectrometer equipped with a 1200 grooves/mm grating (Bausch & Lomb, Analytical Systems Division, Rochester, NY), where it was dispersed and then detected by a liquid nitrogen-cooled CCD detector (Princeton Instruments, Trenton, NJ). A holographic notch filter (Kaiser Optical Systems, Ann Arbor, MI) was used to remove the laser line. The Raman shift was calibrated with indene. Unless otherwise stated, the laser power was kept $<4\ \text{mW}$ for all measurements to avoid photo-damage to the protein. Optical absorption spectra were measured on Shimadzu UV2100U spectrophotometer.

RESULTS

To explore the structural and functional roles of the R481 residue in cytochrome bo_3 quinol oxidase from *E. coli*, we have replaced the positive charged arginine with a neutral amino acid, leucine or glutamine. It has been shown that the proton pumping activity in R481L was abolished, while that in R481Q was retained [21], although the steady state activity is partially preserved in both mutants (*vide infra*). The present preparations of the mutant proteins confirmed the reported functional properties, the details of which will be reported elsewhere (Lin *et al.*, manuscript in preparation).

Analyses of the Metal and Heme Content of Cytochrome bo_3

To examine if the abolishment of the proton pumping activity in R481L is a result of the loss of heme O and/or Cu_B in the binuclear center, we performed metal and heme content analysis. As listed in Table 1, the wild type enzyme has the expected copper content (1 copper per enzyme molecule) but is slightly deficient (1.7 vs 2.0) in Fe, perhaps due to some loss of heme during the sample preparation. The R481L mutant, on the other hand, shows that there is a substantial loss of both Fe and Cu. This coincides with a deficiency of heme O compared to heme B. Altogether, the data indicate that the R481L mutation results in severe disruption of the heme o_3 - Cu_B active site, corresponding to the loss of the metals in the heme o_3 - Cu_B site in about 40% of the enzyme population. The observed steady state activity of the R481L mutant is hence attributed to the fraction of the protein population that retains the active-site metals.

Electronic Absorption Spectra

Figure 2 shows the absorption spectra of the R481L (b) and R481Q (c) mutants, as compared to the wild type (wt) bo_3 enzyme (a), in the *as-isolated* (oxidized) state (thin solid lines), reduced state (dotted lines) and reduced CO-bound state (dot-dashed lines). The absorption peak positions are summarized in Table S1 in the supporting information. Although the positions of the absorption maxima of the mutant proteins are similar to those of the wt in each of the three states, the relative band intensities are significantly different. For example, R481L has a more intense Soret maximum and weaker α band in the CO-bound state, as compared to the wt protein, while R481Q has a more intense Soret maximum in the reduced state. The data indicate that the mutations perturb the electronic structure of hemes b and/or o_3 , although some of these differences could result from the slight differences in the metal and heme contents. To differentiate whether the spectral changes originated from heme b or o_3 , we subtracted the reduced spectrum from the CO-bound spectrum. The contributions from heme b are expected to be the same in both spectra so they cancel out in the difference spectrum, resulting in an o_3^{2+} -CO minus o_3^{2+} spectrum. When we generated the CO difference spectra, the R481Q mutant showed a spectrum very close to that of the wt protein (bold lines in Figure 2, Panels (a) and (c), and Table S1). This suggests that the changes in the intensities in the absorption spectra of the reduced and CO-bound forms of the R481Q mutant do not originate from the o_3 heme but rather from the b^{2+} heme.

On the other hand, the difference spectrum of the R481L mutant is significantly different from that of the wt protein, indicating that the heme o_3 site is altered in this mutant.

Resonance Raman (RR) Spectra of the Oxidized Forms

In prior RR studies of the bo_3 oxidase, the oxidized enzyme was found to be photoreduced when exposed to a laser beam for the Raman measurements [29, 30]. To avoid photoreduction that may complicate spectral assignments, we first examined the laser power-dependence of the RR spectra of the oxidized forms. The two wt spectra shown in Figure 3a were obtained with laser powers of 1 mW (solid line) and 3 mW (dotted line). The intensity of the 3 mW spectrum was normalized, so that the amplitude of the most intense RR band (ν_4 at 1375 cm^{-1}) was comparable to that of the 1 mW spectrum. As can be seen, at 1 mW, a small shoulder on the low frequency side of the ν_4 mode was present, indicating a small amount of photoreduction. This shoulder is slightly more pronounced at 3 mW. As highlighted in the 1 mW *minus* 3 mW difference spectrum [Figure 3b, difference spectrum (i)], a derivative-like feature in the 1375 cm^{-1} region was observed, confirming the additional photoreduction at the higher power. Similar power-dependence experiments were performed with the R481L and R481Q mutants. Interestingly, the mutant proteins did not show detectable photoreduction (difference spectra (ii) and (iii) in Figure 3b), giving additional evidence that the electronic structures of the heme(s) are somewhat altered in the mutants. Based on the power-dependence experiments, we compared the RR spectra of the wt protein and mutants obtained with the minimum laser power (1 mW).

The high frequency RR spectra of oxidases are in general dominated by the spin and coordination state marker lines of the two hemes [31, 32]. In the wt spectrum (Figure 3a, Table 2), the bands at 1585 , 1506 and 1641 cm^{-1} are assigned to the ν_2 , ν_3 and ν_{10} modes, respectively, of the 6-coordinate low spin (6C-LS) heme b ; while those at 1571 and 1481 cm^{-1} are assigned to the ν_2 and ν_3 modes of the 6-coordinate high spin (6C-HS) heme o_3 . In the R481L mutant, new bands appeared near 1493 and 1633 cm^{-1} at the expense of the ν_3 (1481 cm^{-1}) and ν_{10} (1641 cm^{-1}) band intensity of heme o_3 in the wt protein (see the R481L spectrum and the R481L-wt difference spectrum shown at the bottom). Such results are consistent with changes at heme o_3 upon the mutation.

The frequencies of the new ν_3 and ν_{10} bands are indicative of the presence of a 5-coordinate high spin (5C-HS) heme o_3 [31, 32]. On the other hand, the RR intensities of the ν_3 (1506 cm^{-1}) and ν_{10} (1641 cm^{-1}) modes of heme b were unchanged, although the ν_2 RR band at 1585 cm^{-1} is weaker in the mutant and may have broadened and/or shifted to lower frequency. In addition, the intensity of ν_{37} at 1598 cm^{-1} from heme b has increased in intensity. Both ν_2 and ν_{37} are C_β - C_β stretching modes of the porphyrin core [31, 32]. It is noted that the ν_2 mode may also broaden and/or shift to lower frequency upon the mutation. Taken together the RR data show that the distal water ligand coordinated to the heme o_3 in the wt protein [33, 34] is absent in R481L as indicated by its 5C-HS configuration; in addition, the data indicate that heme b , like that in the wt protein, has a 6C-LS configuration, although the electronic structure of the mutant is slightly different from that of the wt protein. In contrast to R481L, changes were not detected in the heme o_3 marker bands of R481Q, although a small change in ν_2 of heme b , similar to that found in R481L, was observed. These observations are consistent with the results of the electronic absorption measurements, which indicated that there were changes in heme b of R481Q but not in heme o_3 .

In addition to the changes in the spin and coordination marker bands, small differences in the ν_4 mode at 1375 cm^{-1} in the mutants are evident in the difference spectrum. However, owing to a small amount of photoreduction in the wt protein (even at a power of 1 mW), a

possible small shift in the mode from either of the hemes in both mutants would be obscured.

RR Spectra of the Reduced Forms

Figure 4 shows the RR spectra of the reduced forms of the R481L and R418Q mutants, as compared to the wt protein. The wt spectrum exhibits bands from the 5C-HS heme α_3 at 1473 (ν_3), 1575 (ν_2) and 1611 (ν_{10}) cm^{-1} , and those from the 6C-LS heme b at 1491 (ν_3) and 1587 (ν_2) cm^{-1} . The RR band at 1621 cm^{-1} is attributable to the vibrational modes of the vinyl side chains of both hemes [35], although ν_{10} of heme b might also contribute to the RR intensity at this frequency.

In the spectrum of R481L, the spectral intensity in the 1490–5 cm^{-1} region slightly increased at the expense of the ν_3 mode at 1473 cm^{-1} from heme α_3 ; in addition, a new band appeared at 1583 cm^{-1} with a concomitant weakening of the ν_2 mode at 1575 cm^{-1} from heme α_3 (see the R481L-wt difference spectrum). Furthermore, the ν_{10} band of heme α_3 at 1611 cm^{-1} weakened, although it is unclear if a new ν_{10} band emerged, because the nearby 1621 cm^{-1} band also shifted slightly, which masked the possible spectral changes in the ν_{10} mode. These observations imply that the heme α_3 in R481L is mainly in a native-like 5C-HS form, with a minor population of a 6C-LS form. The spectral changes found in R481L were not observed in R481Q, although there were small spectral changes in the 1620 cm^{-1} region.

It is noted that the ν_4 band increased in frequency in both mutants. As in the oxidized enzyme, ν_4 from both heme b and α_3 exhibit similar frequencies; thus, the origin of the shift can not be determined unequivocally from these spectra alone. However, as the difference is significantly larger in the difference spectrum of the R481L mutant than that of the R481Q mutant, it is likely that the shift originates from the α_3 heme. Additional insights into the origin of the differences in the ν_4 region were obtained from a comparison with the CO spectra (*vide infra*).

RR Spectra of the CO Forms

Figure 5a shows the high frequency RR spectra of the CO derivatives of the R481L and R481Q mutants, as compared to the wt protein. The laser power used for the RR measurements was kept low (1 mW) to avoid photo-dissociation of CO (the RR spectra shown here, as well as the low frequency spectra that cover the Fe-CO stretching bands, did not show photo-dissociation dependent changes within 100 μW –1 mW laser power under our experimental conditions; see Figures S1 and S2 in the supporting information). In the CO-derivative, the ν_4 mode from heme b and heme α_3 are expected to be well separated, with the former in the 1360 cm^{-1} region (as found in the reduced spectrum) and the latter in the 1370–75 cm^{-1} region as found in the CO-adducts of other heme proteins [28]. Surprisingly, large differences were found in the relative intensities of these two modes in the wt protein and the two mutants. In the wt protein, the ν_4 mode is broad and centered at 1363 cm^{-1} , indicating that the spectrum is dominated by the heme b . A similar spectrum was observed for the R481Q mutant, although a prominent shoulder appeared at higher frequency. In contrast, in the R481L mutant, the ν_4 line was located at 1372 cm^{-1} with a shoulder at 1363 cm^{-1} , indicating that the spectrum is dominated by the CO-bound heme α_3 . It is important to note that there is no evidence in the data for photooxidation that would give an oxidized heme b , yielding a spectrum with a strong ν_4 band in the 1372 cm^{-1} region as that observed in the R481L mutant; in addition, there is no evidence in the data for photodissociation of the CO from heme α_3 , which would give a strong line in the 1360 cm^{-1} region as that observed in the wt protein and the R481Q mutant. Thus, we attribute the observed differences in the ν_4 region of the spectra to a difference in the electronic transitions, resulting in differences in the relative enhancements of the heme b and heme α_3 .

However, we are cognizant that this difference in the R481L mutant could be affected by the metal and heme loss.

To sort between the contributions from the two hemes, difference spectra of the CO-bound minus the ligand-free reduced form were calculated as was done for the optical absorption spectra (Figure 2). Prior to subtraction, we normalized the intensity of two spectra with respect to the heme *b* mode at 1556 cm^{-1} , which was seen in the spectra of both the CO-bound and the ligand-free reduced forms, with the same center frequency and width. In the difference spectra, the spectral contributions from heme *b* are canceled out and a pure CO-bound minus reduced heme α_3 difference spectrum of is expected. Figure 5b shows the CO difference spectra of the wt protein and the R481L and R481Q mutants thus obtained. As, in the difference spectra, the ν_4 modes of the CO-adduct and reduced form of the heme α_3 show up as a positive and negative peak, respectively, we assign the ν_4 mode of the reduced heme α_3 of the wt protein, the R481Q mutant and R481L mutant at 1354 , 1355 and 1359 cm^{-1} , respectively, and the corresponding frequencies of the CO-bound heme α_3 at 1369 , 1370 and 1373 cm^{-1} . The data show significant changes in the heme α_3 of R481L, but not R481Q, although those in the reduced heme of R481L may be a consequence of its conversion to a mixture of the 5C-HS and 6C-LS forms (see above). Based on the difference spectra, frequencies of the other marker bands of heme α_3 , ν_{10} at 1636 cm^{-1} and ν_2 at 1592 cm^{-1} , were also estimated for the wt protein, and were found to be unchanged in the mutants. The frequencies of the marker bands are summarized in Table 2, together with the assigned coordination and spin states of the hemes.

CO has been shown to be a useful probe for the investigation of the distal environment of heme proteins [28, 36]. To determine the Fe-CO stretching mode ($\nu_{\text{Fe-CO}}$), C-O stretching mode ($\nu_{\text{C-O}}$) and Fe-C-O bending ($\delta_{\text{Fe-C-O}}$) mode, we performed $^{12}\text{C}^{16}\text{O}$ - $^{13}\text{C}^{18}\text{O}$ isotope substitution experiments. As shown in Figure 6a, the isotope sensitive bands of the wt protein at 521 and 573 cm^{-1} are assigned as the $\nu_{\text{Fe-CO}}$ and the $\delta_{\text{Fe-C-O}}$ modes, respectively, based on the isotope shifts shown in Figure 6b [29, 30, 37]. Similar spectra were obtained from the R481Q mutant. In contrast, the R481L protein exhibits a new $\nu_{\text{Fe-CO}}$ mode at 495 cm^{-1} together with a weaker $\nu_{\text{Fe-CO}}$ mode at 521 cm^{-1} . The detection of two $\nu_{\text{Fe-CO}}$ modes in R481L indicates the presence of two distinct conformers of the Fe-C-O moiety. Close examination of the wt and R481Q spectra revealed that both proteins contain a minor conformer with $\nu_{\text{Fe-CO}}$ at $\sim 495\text{ cm}^{-1}$, as evident by the shoulder of the 521 cm^{-1} band in the raw data (Figure 6a) and a weak trough at 478 cm^{-1} in the isotope difference spectra (Figure 6b). The data also show that the contribution of the minor conformer in R481Q is slightly larger than that in the wt protein. The $\delta_{\text{Fe-C-O}}$ mode at 573 cm^{-1} , on the other hand, appeared to be unaffected by either mutation, although its intensity in the R481L mutant was significantly weaker than that in the wt.

In addition to the $\nu_{\text{Fe-CO}}$ and $\delta_{\text{Fe-C-O}}$ bands, the wt protein and mutants exhibit a weak isotope dependent band at 363 cm^{-1} (Figure 6b), as that reported for several other CO-bound heme proteins [38, 39]. Through a series of studies with deuterated heme, Rajani and Kincaid concluded that isotope sensitive bands in this region originate from vibrational coupling between $\nu_{\text{Fe-CO}}$ and heme deformation modes in this region [39]. Although R481L exhibits two $\nu_{\text{Fe-CO}}$ modes, no additional isotope sensitive lines other than the 363 cm^{-1} band are observed, implying that the 363 cm^{-1} band arose through a vibrational coupling between the major $\nu_{\text{Fe-CO}}$ component at 521 cm^{-1} and a low-frequency mode of the porphyrin. Consistent with this hypothesis, the intensity of the 363 cm^{-1} band is much weaker in R481L than in the wt and the R481Q mutant.

The $\nu_{\text{C-O}}$ modes of the wt protein and the R481L and R481Q mutants were examined in the 1800 – 2050 cm^{-1} window. As shown in the $^{12}\text{C}^{16}\text{O}$ - $^{13}\text{C}^{18}\text{O}$ isotope difference (Figure 7),

the $\nu_{\text{C-O}}$ mode of the wt protein was identified at 1960 cm^{-1} , in agreement with the reported IR data [29]. It shifted to 1869 cm^{-1} with $^{13}\text{C}^{18}\text{O}$. The isotope shift of 91 cm^{-1} agrees well with the theoretical value predicted by assuming a C-O harmonic oscillator. The major $\nu_{\text{C-O}}$ modes at 1960 and 1869 cm^{-1} with $^{12}\text{C}^{16}\text{O}$ and $^{13}\text{C}^{18}\text{O}$ are accompanied by shoulders at 1947 and 1859 cm^{-1} , respectively. The shoulders are more prominent in R481Q, although the main peak positions at 1960 and 1869 cm^{-1} are unchanged. The R481L mutant has a significantly broader $\nu_{\text{C-O}}$ mode with center position shifted to higher frequency by $\sim 3\text{--}4\text{ cm}^{-1}$ as compared to the major $\nu_{\text{C-O}}$ modes of the wt protein and R481Q mutant. In addition, a weak but sharp contribution is present at 1960 cm^{-1} , the same frequency as the major $\nu_{\text{C-O}}$ modes of the wt protein and R481Q mutant. The significance of these data is discussed in the following section.

DISCUSSION

Substitution of the Arginine at position 481 with a leucine residue dramatically alters the structure and stability of the binuclear center, as indicated by the loss of the heme $\text{o}_3\text{-Cu}_\text{B}$ active site metals in about 40% of the population. To a first approximation, therefore, a maximum of 60% of the enzyme population must be responsible for the observed steady state activity (Table 3). The turnover number of the R481L mutant is $\sim 35\%$ to 40% of the wild type, normalized by the total heme content. Hence, the turnover per active enzyme molecule is likely to be somewhat higher than this value. Also, some of the differences in the spectroscopic properties of the mutant as compared to the wt may have resulted from the reduced fraction of the heme $\text{o}_3\text{-Cu}_\text{B}$ content in the R481L mutant.

The Effects of Mutations on Heme b

Our recent study of cytochrome *c* oxidase from *Rhodobacter sphaeroides* has shown that mutations of residue R481 results in significant perturbations to both the low spin heme (heme *a*) and the heme $\text{a}_3\text{-Cu}_\text{B}$ active site [24]. In the current work, we have shown that the R481L mutant of cytochrome *bo*₃ does not substantially perturb the low-spin heme *b*, although some modifications do occur. In the oxidized forms of R481L and R481Q of *bo*₃, the ν_3 mode of heme *b* at 1506 cm^{-1} was not perturbed, indicating that the heme remained 6C-LS. In contrast, the ν_2 and ν_{37} modes of heme *b*, which involve the $\text{C}_\beta\text{-C}_\beta$ stretching mode of porphyrin core [31, 32], were slightly altered upon the mutations. In the wt protein, R481 forms an H-bond with the propionate group attached to the D ring of heme *b* (see Figure 1b). Recent experimental and theoretical studies showed significant electronic conjugation between the propionate orbitals and porphyrin π -orbitals [40, 41]. Accordingly, we attribute the changes in the ν_2 and ν_{37} modes of heme *b* to the change in structural and the electronic properties of the C_β to which the propionate group is covalently linked. We hypothesize that the mutations alter the structure of the propionate which, in turn, change the planarity of the heme macrocycle and thus its vibrational spectrum. In addition, the proximal ligand of heme o_3 is H419, which is 2 residues in sequence away from H421, an axial ligand of heme *b*; the change in heme o_3 (*vide infra*) perhaps could affect the structural properties of heme *b* via the H419/H421 moiety. In summary, our data demonstrate that the mutations in R481 slightly affect the structural and electronic properties of the heme *b*, although the origin of the spectral changes in heme *b* in the oxidized proteins remains to be further investigated.

The Effects of Mutations on the Structure of Heme o_3

In contrast to the modest effects of the mutations on heme *b*, substituting the R481 with a leucine residue dramatically alters the structure of the binuclear center. Our RR data, show that the distal environment of heme o_3 is perturbed significantly in this mutant, but it is not perturbed in R481Q. In particular, in the oxidized form of R481L, the 6th water ligand

coordinated to the heme o_3 found in the wt protein is absent; in addition, in the reduced form, the vacant 6th ligand binding site observed in the wild type protein is partially occupied by a nonnative ligand. The latter finding suggests the coordination of an endogenous amino acid residue to the ferrous heme o_3 in R481L, as it is unlikely that a water molecule could be a 6th ligand of the ferrous heme. The distal pocket of heme o_3 is occupied by the Cu_B atom, which is coordinated by three histidine ligands (Figure 8). We hypothesize that the structure of the Cu_B site is perturbed in the R481L mutant, which destabilizes the coordination of the water ligand of heme o_3 in the oxidized state, and allows one of the histidine ligands of Cu_B (possibly H334, see Figure 8) to coordinate to the heme o_3 in the reduced state, as no other endogenous ligands are accessible to the heme iron.

These large structural perturbations are somewhat surprising, because the 481 position is quite remote from the Cu_B site (Figures 1 and 8). However, the reported crystallographic structures of hemecopper oxidases reveal the possible linkages between R481 and Cu_B. Although a set of full atomic coordinates including those of the ordered water molecules has not been reported for cytochrome *bo*₃, the positions of the ordered water molecules are available in crystal structures of cytochrome *c* oxidase from *Rhodobacter sphaeroides* [42], from *Paracoccus denitrificans* [43] and from bovine [44]. Remarkably, in RsCcO [42], PdCcO [43] and bovine CcO (bCcO) [44], three water molecules were found to occupy virtually identical positions in the three distinct oxidases, on the basis of the crystallographic data. The three water molecules establish an H-bonding network linking the R481 to the two heme propionate groups and one of the histidine ligands of Cu_B as shown in Figure 8. Based on the identity of the positions of these water molecules, we posit that related structured water molecules are present in the *bo*₃ structure as well, and they serve to stabilize the structure of the Cu_B center and its regioorientation with respect to the heme macrocycle. In the R481L mutant, the leucine can not maintain the H-bonding interactions; the structural integrity of the binuclear center is hence modified, resulting in the changes in the coordination state of the heme o_3 .

The α and β conformers of CO-bound form

The binuclear center of CcO family of proteins has been shown to exhibit two conformations in the presence of CO in the resonance Raman spectra [45, 46]. The discovery of 2 conformers of the CO-complex of CcO was first made by Alben and coworkers in low temperature photolysis studies of bovine mitochondria, rat heart myocytes and opossum heart tissue slices with FTIR [47–50]. Similar conformers of the CO-adduct of bacterial CcO were identified in RsCcO with FTIR by Gennis and coworkers [51–53]. One conformer, termed the α form, has a higher ν_{C-O} frequency (1964 cm⁻¹ for bCcO and 1966 cm⁻¹ for RsCcO) as compared to the other conformer, termed the β form (1952 cm⁻¹ for bCcO and 1955 cm⁻¹ for RsCcO) [45, 46]. The ν_{Fe-CO} frequencies in these conformers were also determined for RsCcO at 519 (α form) and 493 (β form) cm⁻¹, although for bCcO only the α conformer with a ν_{Fe-CO} at 520 cm⁻¹ was observed [46].

The ν_{Fe-CO} and ν_{C-O} frequencies of heme proteins are typically related by the well-established inverse correlation curve. For example, most of the data from histidine-ligated heme proteins, such as hemoglobins and myoglobins, fall on a linear line (diagonal crosses in Figure 9). The offset of a correlation line is determined by the electronic properties of the proximal heme ligand. Consequently, the data associated with heme proteins with histidine as a proximal ligand stay on a line distinct from the P450 line with thiolate as a proximal ligand (not shown), and from the line associated with five coordinate CO-bound heme complexes. The positioning of a data point in a given correlation line, on the other hand, depends on the electrostatic environment of the heme-bound CO; when the CO is exposed to a positive polar environment, the data point is located in the upper-left corner correlation; as the positive electrostatic environment of the CO is reduced, the data points shift along the

correlation line to the lower right corner. Although the CcO family of enzymes possesses a heme group with histidine as the proximal ligand, the data points associated with the α conformers of bCcO, RsCcO, and cytochrome *bo*₃ (with $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ at 1960 and 521 cm^{-1}) do not lie on the inverse correlation line defined by the heme proteins with histidine as the proximal ligand (Figure 9) [54]. The origin of this observation has been under intense debate. Nonetheless, it is generally agreed that the displacement of the CcO data from the histidine correlation line is a consequence of a strong interaction between Cu_B and the heme-bound CO. Consistent with this conclusion, the reported crystal structure of the CO-bound CcOs show that the CO is highly bent due to its close proximity to Cu_B [6]. In contrast to the behavior of the α -conformer, the β conformer lies close to the histidine correlation line (as demonstrated by the data associated with the β conformer of RsCcO shown in Figure 9), indicating a weaker interaction between CO and Cu_B.

Interestingly, like RsCcO, the R481Q mutant of cytochrome *bo*₃ possesses both α and β conformers. The data point for the major component, the α conformer with $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ at 1960 and 521 cm^{-1} , like the wt protein, lies far above the histidine inverse correlation line in the plot (Figure 9, closed square), whereas the minor component has a data point ($\nu_{\text{C-O}}$, 1947 cm^{-1} ; $\nu_{\text{Fe-CO}}$, 495 cm^{-1}) near the histidine correlation line (Figure 9, open square). Our RR data (Figure 6) indicate that, in the R481Q mutant, the equilibrium between the α and β conformers shifts slightly towards the former as compared to the wt protein, suggesting that the hydrogen bonding network linking R481 to the Cu_B site (Figure 8) regulates the α - β equilibrium.

On the other hand, the major component of R481L (with $\nu_{\text{C-O}}$ and $\nu_{\text{Fe-CO}}$ at 1965 and 495 cm^{-1}) lies on the histidine line, indicating that the strong influence of Cu_B exerted on CO is absent in this mutant. The position of the data point for the R481L mutant differs from that of the β -forms of the wt and the R481Q mutant. The frequency of the $\nu_{\text{C-O}}$ mode is more akin to that of the Y288F mutant of *bo*₃ in which it was concluded that the Cu_B was lost from the protein. This conformer was labeled the δ -form of the protein by Das et al [55]. This indicates that in R481L the hydrogen bonding network connecting R481 to the Cu_B site is disrupted such that there is no longer any interaction between Cu_B and the bound CO. It is also noteworthy that the β conformer of R481Q, as well as the major component of R481L mutant, were found to be more resistant to the photo-dissociation than their α conformers (Figure S2 in the supporting information), although the origin of such difference in the photo-sensitivity is unclear.

Implications on the proton translocation mechanism

In the catalytic cycle of heme-copper oxidases, the protons taken up from the n-side of the mitochondrial/cytoplasmic membrane are either consumed by the dioxygen to form water molecules (chemical protons) at the binuclear center, or pumped across the protein matrix to the p-side (pumped protons). The impact of mutations in the highly conserved R481 residues on the activity and proton pumping efficiency in various oxidases, including RsCcO, PdCcO and *bo*₃ from *E. coli*, have been extensively studied. It was found that nearly all of the mutants retained some degree of activity, but only some mutants are competent to pump protons (see Table 3 and references therein).

The R481Q mutants are particularly interesting. In PdCcO, the mutation abolishes the activity and proton pumping capability; consistent with this, the RR data showed that the binuclear center was significantly modified [56]. In RsCcO, the mutation leads to significant reduction of the activity (to 4% the native level) and total abolishment of the pumping efficiency [23]; again the RR data are consistent with a modified binuclear center [24]. In the *bo*₃ oxidase from *E. coli*, the mutation causes ~50% reduction in activity, while the proton pumping capability is retained (M. T. Lin et al., to be published) [21]; as revealed in

the present work, the mutation only resulted in small changes in the binuclear center. These data indicate that, depending on the protein, the R481Q mutation exerts differing effects on the stability of the binuclear center. Along these same lines, our present data show that the R481L mutant of *bo*₃ oxidase retains ~35–45% activity, but it does not pump protons, and its binuclear center is significantly perturbed. However, in the R481H and R481L mutants of RsCcO, proton pumping was observed but the RR data showed that the catalytic site was significantly modified [24]. In contrast, in the R481K mutant of RsCcO, both the activity and proton pumping capability are maintained, and the electronic absorption spectrum appears to be the same as the wt, indicating an intact binuclear center (although its RR data have not been obtained) [22, 57]. In summary, it is clear that mutations at this position may alter the structure of the heme sites and the catalytic activity, but whereas catalytic activity is necessary for proton pumping it is not sufficient in proteins with mutations at this position.

The mechanism by which the energy generated in the oxygen reduction process is coupled to proton translocation remains one of the major unsolved issues in the cytochrome oxidase field. Despite intense research, the identities of the proton pumping pathways remain controversial. Nonetheless, so far three proton pumping channels, D, K (Figure 1a) and H, have been suggested to pump the proton from the n-side of the mitochondrial/cytoplasmic membrane to a region near the binuclear center. However, albeit the fact that the D-channel has been clearly demonstrated in some bacterial oxidases, it is absent in the *ba*₃ oxidase from *Thermus thermophilus* [58]; likewise, the H-channel has been implicated in mammalian CcO [14], but it is not functional in bacteria CcO [10]. The proton loading sites, as well as the proton exit pathways are also not well-defined. In any case, it will be quite remarkable if the differing oxidases with nearly identical catalytic sites use totally different mechanisms to couple the redox chemistry to the proton translocation.

It is well-established that the D-channel in RsCcO, PdCcO and *bo*₃ from *E. coli* delivers both chemical and pumped protons [1]. The E286 residue at the end of the D-channel has been proposed to be a branching point, from which the protons are brought either to the binuclear center for the formation of water or toward a postulated proton exit channel [1]. The D-propionate group of the heme *o*₃ (*a*₃) is believed to be one of the proton loading site, accepting protons from E286 [16–22]; although it is unclear how protons are transferred over such a long distance (see P1 pathway in Figure 1b), as there is no clear hydrogen bonding connection found between them in the crystal structures. Nonetheless, computer simulation studies have suggested that E286 and the D-propionate are connected via a number of as yet unresolved water molecules [59–63]. In addition, an alternative route via the Cu_B site, indicated as the P2 pathway in Figure 1b, has been suggested [64–66].

The evidence presented in this study demonstrates that there is communication between R481 and the Cu_B site, possibly via a hydrogen bonding network involving the two propionates groups of heme *o*₃, three water molecules and a Cu_B ligand, H334 (see Figure 8), supporting the P2 pathway. Presumably, the water molecules could facilitate proton movement along the P2 pathway [67]. Consistent with this scenario, DFT and electrostatic calculations have implicated H334 as a critical proton pumping element [68]. Additional water molecules identified recently in the crystal structure of rsCcO could facilitate proton movement along this pathway [67].

In order to ensure that the protons translocate against a gradient and to prevent slippage of the protons back to the n-side of the membrane, conformational changes are required. Recently, a large change in the position of heme *a*₃ was detected in the comparison between the oxidized and reduced forms of the *aa*₃ oxidase from *Rhodobacter sphaeroides* [67]. This demonstrated a conformational flexibility of the heme that could be part of a regulatory mechanism for control of proton translocation. Moreover, the data reported here show that

the heme structure of the CO-bound adduct exists as an equilibrium between two conformers (α and β) of the protein and this equilibrium, which was shown previously to exist in mitochondria and heart tissues [47, 49, 50] and which was shown to be pH dependent in *rsCcO* [45, 52], is sensitively regulated by the 481 residue. While the presence of the two conformers has not been determined in the absence of CO, if the distinct conformers interchange during catalytic turnover, it suggests that they may play a role in controlling the delivery of protons through the H-bonding network by regulating the linkage between the Cu_β ligands and the heme propionates with the associated H-bonded arginines. Further studies are needed to clarify the postulated proton affinity changes of the Cu_β cluster upon its α - β conversion and the role of other nearby residues. In summary, these results reported here support the structural role played by R481 in maintaining the functional H-bonding network that stabilizes the catalytic site of *CcO* and provide evidence for a potential pathway and control mechanism for the passage of the pumped protons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

The X-ray crystallographic structure of cytochrome b_0 . The proton conducting D and K-channels and the relative locations of the R481 and E286 residues with respect to hemes b , heme o_3 and Cu_B are illustrated in (a). The two putative proton exit pathways, which shuttle protons from E286 to the p-side of the membrane are shown in (b); the P1 pathway represents a direct proton exit pathway from E286 to the H-bonded R481-heme propionate region, whereas the P2 pathway represents an alternative pathway via the Cu_B ligands. The hemes, Cu_B and all the amino acids shown here reside in the subunit I, except for the E89, which is from the subunit II. The structure was rendered from PDB: 1FFT with PyMOL (DeLano Scientific, LLC).

FIGURE 2.

Electronic absorption spectra of the wt (a), and the R481L (b) and R481Q (c) mutants of cytochrome *bo*₃ in the oxidized (thin solid line), reduced ligand-free (dotted line) and reduced CO-bound (dot-dashed line) states. The bold solid line at the bottom of each panel shows the CO-bound minus reduced ligand-free difference spectrum.

FIGURE 3.

Resonance Raman spectra of the oxidized forms of the wt and the R481L and R481Q mutants of cytochrome b_{03} (a) and their associated laser power difference (1 mW minus 3 mW) spectra (b). The R481L-wt and R481Q-wt difference spectra are shown at the bottom in (a). The frequencies of the new modes in the mutants are highlighted in bold. The wt spectra shown in (a) are those obtained with the laser power of 1 mW (solid line) or 3 mW (dotted line). The power difference spectra for wt, R481L and R481Q are shown as the top (i), middle (ii) and bottom (iii) traces, respectively, in (b). The excitation wavelength for the Raman measurements was 413.1 nm and the laser power was 1 mW unless indicated otherwise.

FIGURE 4.

Resonance Raman spectra of the reduced forms of the wt and the R481L and R481Q mutants of cytochrome *b*₀₃. The R481L-wt and R481Q-wt difference spectra are shown at the bottom. The 1445–1530 cm⁻¹ region of the difference spectra were expanded vertically by a factor of 5 as compared to the other region of the spectra to magnify the spectral changes. The frequencies of the new modes in the mutants are highlighted in bold. The excitation wavelength for the Raman measurements was 413.1 nm.

FIGURE 5.

Resonance Raman spectra of the CO-bound forms of the wt and the R481L and R481Q mutants of cytochrome *b*₀₃ (a) and their associated CO-bound minus reduced ligand-free difference spectra (b). To obtain the difference spectra shown in (b), the reduced spectrum of each protein taken from Figure 4 was subtracted from the corresponding spectrum shown in (a), and the two spectra were normalized with respect to the heme *b* mode at 1556 cm⁻¹ such that it was canceled out in the resulting difference spectra. The frequencies of the new modes in the mutants are highlighted in bold. The excitation wavelength for the Raman measurements was 413.1 nm.

FIGURE 6.

Low frequency resonance Raman spectra of the reduced CO-bound forms of the wt and the R481L and R481Q mutants of cytochrome *b*₀₃ (a), and the associated ¹²C¹⁶O-¹³C¹⁸O isotope difference spectra (b). The RR spectra of the ¹²C¹⁶O and ¹³C¹⁸O-adducts shown in (a) are presented in the solid and dotted lines, respectively. The frequencies of the new modes in the mutants are highlighted in bold. The excitation wavelength for the Raman measurements was 413.1 nm.

FIGURE 7. $^{12}\text{C}^{16}\text{O}$ - $^{13}\text{C}^{18}\text{O}$ isotope difference spectra of the CO-adducts of the wt and the R481L and R481Q mutants of cytochrome *b*₀₃. The R481Q spectrum is overlaid with the wt spectrum (dotted line) to highlight the band intensity changes.

FIGURE 8.

The H-bonding network linking R481 to the Cu_B site in *R. sphaeroides* CcO. A, B and C indicate the three water molecules mediating the H-bonding network involving R481, the D propionate group of the heme *o*₃ and H334 (one of the three Cu_B ligand). The H-bonding interactions (< 3.1 Å) are indicated by the dashed lines. The structure was rendered from PDB: 2GSM with PyMOL (DeLano Scientific, LLC).

FIGURE 9.

The $\nu_{\text{Fe-CO}}$ versus $\nu_{\text{C-O}}$ inverse correlation plot of heme proteins. Data from hemoglobins, myoglobins and their distal mutant proteins are indicated by diagonal crosses. The solid line is the linear correlation obtained by a least-squares fit of the data. The data of heme-copper oxidases are indicated by closed triangle (bovine CcO), closed circle (α conformer of *R. sphaeroides* CcO), open circle (β conformer of *R. sphaeroides* CcO), closed square (the major component of wt and R481Q mutant of cytochrome bo_3), open square (minor component of R481Q mutant of cytochrome bo_3) and closed diamond (the major component of the R481L mutant of cytochrome bo_3). The data of hemoglobins and myoglobins are taken from ref. [36] and references therein; bovine CcO and rsCcO, are taken from ref. [46]; wt and mutants of cytochrome bo_3 are from this work.

Table 1

Metal and heme contents of wt and R481L mutant of cytochrome *b_o3* proteins.

	Cu ^a	Fe ^a	Heme B : Heme O
wt	0.96	1.7	1 : 0.88
R481L	0.53	1.3	1 : 0.54

^aThe Cu and Fe amount reported was per cytochrome *b_o3* complex as estimated from the amount of sulfur measured for the same samples by ICP-OES.

Table 2
Frequencies of the RR bands and Coordination/Spin States of the hemes *b* and *o*₃ of Cytochrome *bo*₃ Proteins.

	ν_2	ν_3	ν_4	ν_{10}	ν_{Fe-Co}	$\nu_{C=O}$	Coordination-Spin states*
Oxidized Forms							
wt							
<i>b</i> + <i>o</i> ₃			1375				
<i>b</i>	1585	1506	-	1641			6C-LS
<i>o</i> ₃	1571	1481	-	--			6C-HS
R481L							
<i>b</i> + <i>o</i> ₃			1375				
<i>b</i>	-	1506	-	1641			6C-LS
<i>o</i> ₃	1579	1493	-	1633			5C-HS
R481Q							
<i>b</i> + <i>o</i> ₃			1375				
<i>b</i>	-	1506	-	1641			6C-LS
<i>o</i> ₃	1571	1481	-	--			6C-HS
Reduced Forms							
wt							
<i>b</i> + <i>o</i> ₃			1360				
<i>b</i>	1587	1491	-	--			6C-LS
<i>o</i> ₃	1575	1473	1354 ^a	1611			5C-HS
R481L							
<i>b</i> + <i>o</i> ₃			1361				
<i>b</i>							
<i>o</i> ₃	1583 ^b	1471/1495 ^c	1379 ^{a,d}	--			5C-HS/6C-LS
R481Q							
<i>b</i> + <i>o</i> ₃			1360				
<i>b</i>	--	1491	-	--			6C-LS
<i>o</i> ₃	1575	1473	1355 ^a	--			5C-HS

CO-bound ⁶⁾ Forms	ν_2	ν_3	ν_4	ν_{10}	ν_{Fe-Co}	$\nu_{C=O}$	Coordination-Spin states [#]
wt - α_3	1592	1499	1369	1636	521	1960	6C-LS
R481L - α_3	1590	1501	1373	1634	521/495	1964 ^f	6C-LS
R481Q - α_3	1590	1499	1370	1636	521/495 ^g	1960/1947	6C-LS

“_” stands for “not determined”, due to overlap of RR signals from hemes *b* and α_3 α ;

“_” stands for not clearly observed;

“a” are approximate frequencies obtained from the trough positions in the CO-reduced difference spectra;

“b” is the RR band of the 6CLS component;

“c” is an approximate value of the 6CLS component determined on the basis of the difference spectrum with respect to the wt spectrum;

“d” is attributed to both the 5CHS and 6CLS components;

“e” are frequencies of porphyrin core modes obtained from the peak positions of the CO-reduced difference spectra;

“f” is the frequency of the major component.

“g” is the frequency of the shoulder.

* 5C and 6C stand for 5-coordinate 6-coordinate, respectively; HS and LS stand for high spin and low spin, respectively.

Table 3

Comparison of the properties of the R481 (R473 in PdCcO) mutants of various oxidases. The activity is expressed as the percent of the wild-type (wt) and the structure is based on detection of changes in the resonance Raman spectra as defined in the text.

Protein	Activity (%wt)	Pumping	Structure
<i>E. coli bo₃</i>			
R481M	Yes (2–8) ^a	No ^a	nd
R481L	Yes (35–45) ^a	No ^a	Modified ^b
R481N	Yes (51–65) ^a	No ^a	nd
R481Q	Yes (54–80) ^a	Yes ^a	Intact ^b
<i>RsCcO aa₃</i>			
R481H	Yes (18) ^c	Yes ^c	Modified ^d
R481K	Yes (100) ^e	Yes ^e	nd
R481L	Yes (5) ^c	Yes ^c	Modified ^d
R481N	Yes (6) ^c	No ^c	nd
R481Q	Yes (4) ^c	No ^c	Modified ^d
<i>PdCcO aa₃</i>			
R473Q	No ^f	No ^f	Modified ^f

^a: Ref. [21];

^b: This work;

^c: [23];

^d: Ref. [24];

^e: Refs. [22, 57];

^f: [56];

nd: not determined