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Mutations which decouple the proton pump of the cytochrome *c* oxidase from *Rhodobacter sphaeroides* perturb the environment of glutamate 286

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Abstract **Mutants that decouple the proton pump of cytochrome *c* oxidase from *Rhodobacter sphaeroides* are postulated to do so by increasing the pK_a of glutamate 286, which is 20 Å away. The possibility that a conformational change near E286 is induced by the decoupling mutations (N139D and N207D) was investigated by FTIR difference spectroscopy. In both decoupled mutants, the reduced-*minus*-oxidized FTIR difference spectra show a shift of 2 cm^{-1} to lower frequency of the band resulting from the absorbance of E286 in the oxidized enzyme. The decoupling mutants may influence E286 by altering the chain of water molecules which runs from the site of the mutations to E286.**

Keywords: Oxidase; FTIR; Water; Glutamate; Conformation; Proton pump

1. Introduction

Cytochrome oxidase is the terminal enzyme in the mitochondrial respiratory chain, and homologues of this heme–copper oxidase are present in most aerobic prokaryotes [1–3]. These enzymes catalyze the four-electron reduction of O_2 to 2 H_2O and use the free energy made available from this reaction to electrogenically pump protons across the membrane and, thus, generate a transmembrane potential and proton motive force [4–10]. The mechanism by which the chemistry at the active site of the enzyme is coupled to the proton pump has been of great interest. Recently, a set of remarkable mutants have been isolated and characterized in the heme–copper oxidases in which proton pumping is completely eliminated while there is little or no effect on the steady-state oxidase activity [11–15]. Minimally, this demonstrates that the proton pump operates by an indirect mechanism, and is not an integral part of the catalytic mechanism. The mutations that result in the "decoupling" phenotype are located near the entrance of the D channel, which is one of the two proton-conducting input channels that have been characterized in the oxidases [8,16–18].

The D channel starts at an aspartate (D132 in the *Rhodobacter sphaeroides* oxidase) near the negative side (N-side) enzyme surface and runs about 26 Å to a glutamate (E286) which is near the enzyme active site (see Fig. 1) [19–22]. There are about a dozen crystallographically resolved water molecules that form a pathway between D132 and E286 that facilitates rapid proton transfer from the bulk aqueous phase to the enzyme interior. The decoupling mutations are within a cluster of three asparagines that are near D132 [11,12,15]. The best characterized decoupling mutant is N139D in the *R. sphaeroides* oxidase, which introduces a new ionizable residue within the proton input channel, about 20 Å from E286, located at the opposite end of the channel (Fig. 1). A second mutation, N207D, has recently been characterized and shown to have very similar properties as N139D (Han et al., submitted).

Single-turnover rapid kinetics studies have been used to characterize both N139D [11,13] and N207D (Han et al., submitted). In these "flow-flash" experiments, the reaction of the fully reduced enzyme with O_2 is monitored [23,24]. Upon initiating the reaction, an oxygenated product is formed at the active site, called intermediate P_R[25], in which the O–O bond has been split. Following this step, a proton is transferred from E286 to the active site, resulting in the formation of a second spectroscopically distinct species, intermediate F. The rate of the $P_R \rightarrow F$ transition is, thus, a measure of a specific internal proton transfer reaction from E286 to the active site [26,27]. The rate of the $P_R \rightarrow F$ transition depends on the extent of protonation of E286 and is thus one way to measure the apparent pK_a of E286 [27]. The values obtained with both N139D [9,11] and N207D (manuscript in preparation) show changes that imply a perturbation of E286 due to these mutations.

The wild type oxidase has an apparent pK_a of about 9.4, whereas the apparent pK_a of E286 is greater than 11 for the N139D mutant [13] and even higher for the N207D mutant (manuscript in preparation). These observations form the basis

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Abbreviations: ATR, attenuated total reflectance; FTIR, Fourier transform infrared; R4, refers to the fully reduced oxidase; O, refers to the fully oxidized oxidase

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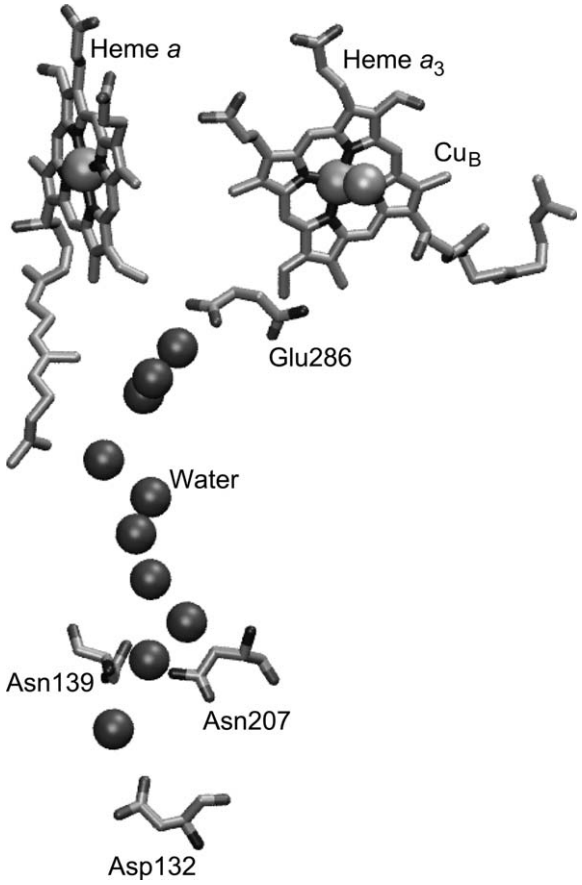

Fig. 1. A view of the proton-conducting D channel of the cytochrome *c* oxidase from *R. sphaeroides*. The D channel connects D132 near the protein surface and E286, near the enzyme active site. Also shown are heme a and heme a_3, as well as the sites of the decoupling mutations, N139 and N207. Spheres represent the water molecules that are observed in the crystal structure [19]. The figure was generated by using VMD software [51].

of one model of how these mutants result in decoupling the proton pump [9,11]. Essentially, it is postulated that all the pumped protons are taken up through the D channel to E286, and then transferred from E286 to a proton acceptor in the pump exit pathway. If the pK_a of the proton acceptor is near 11, then the increase in the pK_a of E286 to 11 or higher can explain why proton transfer through the pumping pathway is effectively stopped in these mutants. At the same time, proton transfer from E286 to the reaction intermediates at the active site continues because the proton affinity of the groups within the active site is very high, and a modest increase of the pK_a of E286 will not significantly influence the rate of proton transfer to the chemical site.

The question being addressed in the current work is how the mutations in residues that are about 20 Å away from E286 can cause the apparent pK_a to shift. One obvious possibility is that there is a direct electrostatic interaction, presuming the newly introduced Asp139 is ionized. At the least, the large distance raises doubts about whether a direct coulombic interaction between Asp139 and Glu286 is responsible for the effect. Continuum electrostatic calculations predict very little influence on the pK_a of E286 from the N139D mutation [28]. Another possibility that has been suggested is that the N139D mutation stabilizes a proton within the D channel and, therefore, slows the proton transfer rate to the proton acceptor within the exit pathway, destroying the critical timing necessary for the pump to function [29]. A third possibility is a conformational change induced by the decoupling mutations that alters the immediate environment around E286, leading to the increased proton affinity of this residue. Such a conformational change could be propagated either through the protein or, more likely, through the unusual chain of water molecules that connects the site of the decoupling mutations to E286 (see Fig. 1). This question has been addressed by using Fourier transform infrared (FTIR) difference spectroscopy.

Several studies with different heme–copper oxidases have shown that reduction of the enzyme causes the C=O stretching band of E286, to move to lower frequencies (1746–1735 cm^{-1} for the *R. sphaeroides* oxidase) [30–36]. This shift is consistent with a loss of a hydrogen bond to E286 upon reduction of the metal centers. It appears that it is the reduction of low spin heme a that is responsible for this conformational change [32,33,35]. The heme a Fe is located about 12 Å from the E286 carboxyl group. In the current work, the four-electron-reduced-*minus*-oxidized FTIR difference spectra are compared for the wild type, N139D and N207D mutant oxidases from *R. sphaeroides*. The presumption is that if there is a conformational change that influences the apparent pK_a of E286, then this conformational interaction may also be apparent in either the fully reduced or fully oxidized state of the enzyme.

The results show that the N139D and N207D mutations each cause a small (2 wavenumber) shift to lower frequency in the infrared absorbance of E286 in the fully oxidized form of the enzyme, detected in the reduced-*minus*-oxidized (R_4–O) difference spectrum. The simplest interpretation is that the decoupling mutations result in an alteration of the interactions of E286 with its immediate environment in the fully oxidized state of the enzyme, perhaps due to a small repositioning of water molecules. In principle, therefore, the effect of the uncoupling mutations on the apparent pK_a of E286 could be due to a direct structural change propagated through the water chain connecting the residues.

2. Materials and methods

2.1. Cell growth and enzyme purification

R. sphaeroides cell growth and purification of both wild type and mutant cytochrome *c* oxidase enzyme was achieved as described in Mitchell et al. [37]. The enzyme was kept in 50 mM potassium phosphate buffer with 0.1% dodecyl maltoside at pH 8.0.

2.2. Sample preparation for ATR-FTIR experiments

The FTIR difference spectra were obtained using the techniques previously described [30,31]. 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 μl of 150 μM 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 μl. 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 μl of water and could be stored at −80 °C.

To prepare the protein film, 6 μl 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 first humidifying the air around the film until a stable FTIR spectrum is recorded. Then a 1 ml solution of the perfusion buffer (30 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$, pH 8.5, in H_2O) is put on the film in order to re-wet the sample. The protein concentration is estimated to be approximately 300 μM. 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, 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, similar to that described [38]. 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 buffer composition is changed. In general, the sample was equilibrated with a buffer by flowing the solution over the sample for about 1 h. A peristaltic pump (Cole-Parmer, Masterflex C/L) and a valve controller (Hamilton) are used for the flow and exchange of buffers. All experiments were performed at 22 °C with a flow speed of 0.33 ml/min.

2.3. The fully reduced-minus-oxidized difference spectrum (R_4–O)

The air-oxidized enzyme was obtained by flowing the perfusion buffer (30 mM HEPES, 20 mM KCl, 5 mM $MgCl_2$, pH 8.5) over the sample. To reduce the enzyme, an aliquot of a freshly prepared solution of dithionite was added to this buffer (3 mM final concentration). Each FTIR spectrum consisted of 1024 interferograms which were averaged. This "single-beam" (detector response) spectrum (1024 averaged interferograms) was recorded in one state and, after changing buffers, recorded in the second state. Triangle apodization was used for the Fourier transformation. The oxidized and reduced single-beam spectra were ratioed to obtain the R_4–O absorbance difference spectrum ($-\log_{10}$(oxidized/reduced)). This oxidation reduction cycle was repeated 40–60 times for each sample and the spectra from 4 to 6 samples were averaged. All experiments performed with 4 cm^{-1} spectral resolution.

3. Results

For each sample, the reduced-*minus*-oxidized difference spectrum at pH 8.5 in the visible part of the spectrum was obtained using reflectance spectroscopy from the same samples used in the FTIR experiments. Fig. 2 shows that the expected R_4–O spectra are obtained with a peak at 605 nm for the wild type and for both mutant oxidases. The FTIR reduced-*minus*-oxidized (R_4–O) perfusion difference spectrum of the wild type *R. sphaeroides* oxidase (Fig. 3) is virtually identical to those reported previously [30,31]. Fig. 3 (inset) shows the region between 1700 cm^{-1} and 1800 cm^{-1}. The peak at 1735 cm^{-1} and trough at 1745 cm^{-1} have been previously assigned as being due to a perturbation of the C=O stretch of the protonated carboxyl group of E286. Also shown in Fig. 3 are the equivalent spectra of the N139D and N207D mutants of the

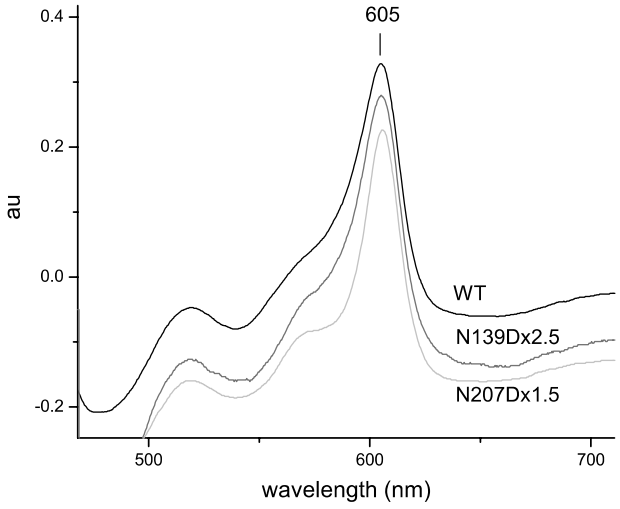

Fig. 2. Fully reduced-*minus*-oxidized (R_4–O) visible difference spectra of the wild type cytochrome oxidase from *R. sphaeroides* and of the N207D, N139D mutants. The samples were present as thin films briefly air-dried on the diamond ATR crystal and submerged under the perfusion buffers that alternatively converted the enzyme from the air-oxidized state to the dithionite-reduced form. These spectra were obtained by reflectance spectroscopy as described in the text, at the same time as the FTIR difference spectra.

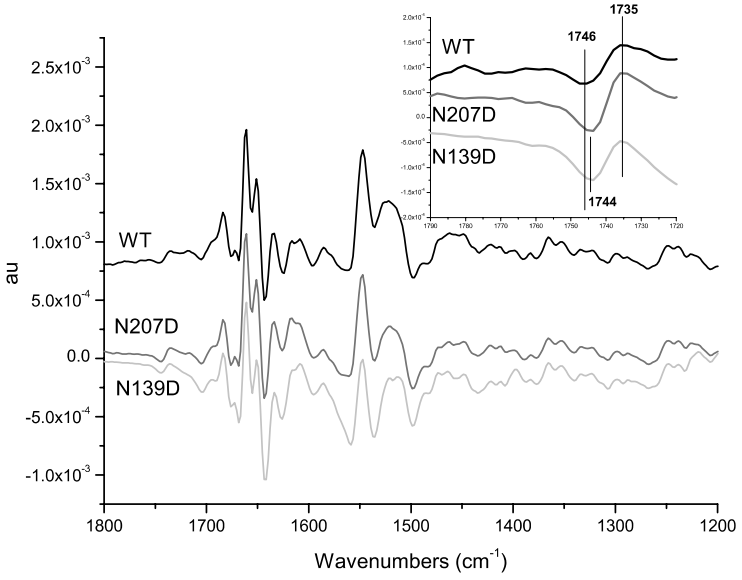

Fig. 3. Fully reduced-*minus*-oxidized (R_4–O) ATR-FTIR difference spectra of the wild type cytochrome oxidase from *R. sphaeroides* and of the N207D, N139D mutants. Inset shows the protonated carboxylic acid (COOH) region of the spectra. These difference spectra were obtained using perfusion buffers that alternatively converted the enzyme to the air-oxidized state and the dithionite-reduced form. Each spectrum is an average of 40–60 spectra, each constructed by co-addition of 1024 interferograms from 4 to 6 different samples.

oxidase. The spectra of the mutant oxidases are virtually identical with that of the wild type, with the notable exception of a small shift of the trough due to the C=O stretch of E286 in the oxidized form. The 2-wavenumber shift to lower frequency is observed with both mutants and is reproducible in multiple enzyme preparations of these mutants.

4. Discussion

The purpose of this work was to determine whether the decoupling mutations, N139D and N207D, caused a change in the local environment around E286, located about 20 Å away. FTIR difference spectroscopy is a very sensitive indicator of changes in protein structure [39–44] and has been particularly useful in showing changes in the immediate vicinity of E286 [30,31,33–36,45–49].

The results show that the introduction of either the N207D or N139D mutation, results in a small but reproducible shift of the E286 component of the R_4–O difference spectrum. The negative band due to the protonated carboxyl of E286 in the oxidized enzyme shifts from 1746 cm^{-1} in the wild type to 1744 cm^{-1} in both of the mutants.

The conclusion is that there must be some conformational coupling between the sites of the decoupling mutations (N207D and N139D) and the immediate vicinity of the E286 carboxyl. This could amount to a small reorientation of a water molecule, for example, influencing the hydrogen bonding to the COOH group, and thus altering the C=O stretching vibration. This is a likely explanation, considering the string of water molecules that connects E286 to the site of the decoupling mutations (Fig. 1). It is tempting to conclude that such changes may be directly responsible for the shift of the apparent pK_a of E286 measured by the pH-dependence of the proton transfer from E286 to the enzyme active site in the $P_R \rightarrow F$ transition [11,13]. However, the small shift of 2 cm^{-1} corresponds to only about 0.25 mV, which is much smaller than the free energy corresponding to a pK_a shift of 1.5 (about 90 mV). The spectroscopic changes cannot be equated with changes in bond strength of comparable magnitude to the energetics of the shift in proton affinity. However, the current data do establish the fact that there is a conformational connection between E286 and the site of the mutations. The spectroscopic changes that are observed are limited to the oxidized state of the enzyme, whereas the shift in the apparent pK_a of E286 is measured during turnover from a transient state of the oxidase. If the decoupling effect of the mutations is due to the shift in pK_a of E286, and if this shift in pK_a is due to a direct perturbation, then the magnitude of the perturbation must be more substantial in the transient states of the enzyme during actual turnover.

It is noted that the correlation of the shift in the apparent pK_a of E286 with the decoupling phenomenon observed with both the N139D and N207D mutations does not necessarily imply a causal relationship. The decoupling of the proton pump may be due to changes in the free energy barriers or wells for a proton moving through the D channel [29,50] Nevertheless, the current work does provide a rationale for the simplest explanation for the change in the apparent pK_a of E286, which is a direct structural change, likely propagated by the arrangement of water molecules being perturbed by the decoupling mutations.

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