Probing the putative H-channel of Cytochrome c oxidase from Paracoccus denitrificans using site-directed mutagenesis

S M Tavender, J Bennett

Central Laser Facility, CCLRC Rutherford Appleton Laboratory, Chilton, Didcot, Oxon., OX11 0QX, UK

J Salje

University of Oxford, Oxford, UK

Main contact email address: *s.tavender@rl.ac.uk*

Introduction

Cytochrome c oxidase (COX) is a heme-copper oxidase and is the terminal electron acceptor of the respiratory chain in which it catalyses the reduction of oxygen to water using electrons donated by cytochrome c. The free energy released by this exergonic reaction is harnessed by the enzyme and used to translocate protons across the membrane, contributing to the generation of an electrochemical gradient which is used by ATP-synthase to generate ATP[1,2,3]. The mechanism by which heme-copper oxidases couple oxygen reduction to proton translocation has still not been determined.

High-resolution X-ray structures of mammalian and several bacterial oxidases show substantial structural similarity with a conserved core of three subunits[4]. An image of the structure of the unit is shown in Figure 1.

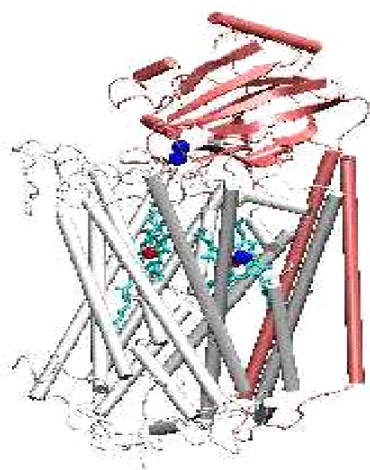
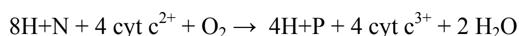


Figure 1. High-resolution structure of cytochrome c oxidase from Paracoccus denitrificans. Subunits I (white) and II (pink), heme prosthetic groups (cyan), Fe centres (red); Cu atoms (green).

The overall reaction catalysed by cytochrome c oxidase is:



$$8H^{+}N + 4\ \text{cyt}\ c^{2+} + O_2 \rightarrow 4H^{+}P + 4\ \text{cyt}\ c^{3+} + 2\ H_2O$$

where N corresponds to the negative side (cytoplasm in bacteria; matrix in mitochondria) and P to the positive side (periplasm in bacteria; intermembrane space in mitochondria). The net charge translocation across the membrane is 8 q+ per 4 e-, since four protons are pumped across the membrane, and the four electrons donated come in from the positive side thus contributing to charge separation.

Proton-translocation pathways must provide a continuous hydrogen-bonded pathway, usually comprising protonable amino-acid side-chains and bound water molecules, in addition to some mechanism to ensure unidirectional transfer[5]. Two such pathways, the K- and D-pathways, have been identified in both bacterial and mammalian oxidases (Figure 2). Whereas the K-pathway feeds protons directly to the binuclear centre, the D-pathway can transfer protons both across the membrane and to the binuclear centre. It is thought that a glutamate residue located in the D-pathway acts as a proton valve through a conformational change during the catalytic cycle. A putative third and distinct proton-translocation channel (H-proton translocation) has been proposed which bypasses the binuclear centre completely and which lies instead near to the binuclear centre and in addition to usual protonable side-chains, this pathway employs both the heme, a propionate group and a peptide bond[6]. It is thought that changes in the redox state of heme a provide the driving force for proton-transfer[7]. However, mutagenesis experiments in bacteria have repeatedly undermined any role for this pathway[8]. The debate has been reopened with a recent report of a series of decoupled H-channel mutants of bovine-heart cytochrome c oxidase using a novel expression system[9].

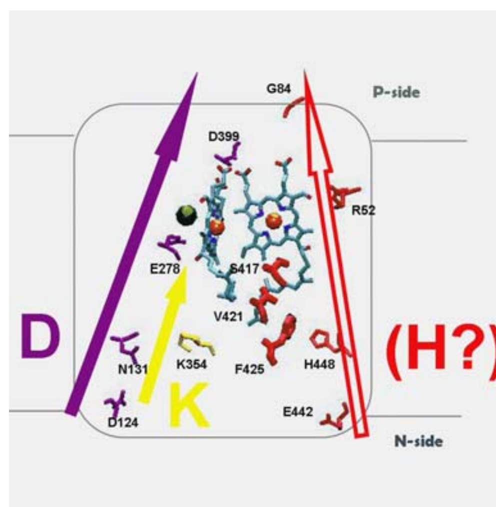


Figure 2. Sub unit I of P. denitrificans cytochrome c oxidase showing the D-, K- and putative H-proton translocation pathways. Heme a is shown on the right heme a3 on the left; CuB is shown in green and Fe atoms in orange. Residues targeted are shown in bold.

In the present study, a double-mutation was introduced with the aim of blocking the putative passage of protons through the supposed H-channel of cytochrome c oxidase from Paracoccus denitrificans by site-directed mutagenesis of residues proposed to line this channel, and to test the purified protein for catalytic activity by measuring its ability to pump protons.

Materials and Methods

Site-directed mutagenesis of subunit (SU) I (Figure 2) was carried out and expressed with wild-type SUs II-IV in a SUI knockout strain of P. denitrificans. Complete four-subunit COX was purified and analysed using UV-Visible absorption and resonance Raman spectroscopy, and tested for catalytic activity. Purified COX was tested for proton pumping activity using stopped-flow methods. Two separate classes of mutants were constructed. First, the double-mutant was introduced into the bacterial enzyme, (see Figure 2). Residues V421 and F425

lie in the vicinity of the proposed channel and were targeted and replaced by residues designed to block the passage of protons without perturbing global protein structure. A second serine→alanine mutation, also located in the proposed H-channel region, was repeated following criticism that the introduced residue was not large enough to block proton movement[10]. In this case, the serine was changed to a valine residue, which has both increased diameter and loss of hydrogen-bonding capability.

Resonance Raman spectra were recorded using back scattering geometry and a spinning quartz sample cell containing approx. 200 µL sample (10 µM COX ; 20 mM KPi, 50 mM NaCl, 0.5 mM EDTA buffer). Scattered light was focused into a Spex Triplemate 1877 spectrometer and data collected by a CCD camera (Andor iDus CCD, model DU420A-BU2). The excitation wavelength was at 457.9 nm (Coherent Innova 300C FreD argon-ion laser), with 20 mW at the sample focused to 500 µm spot. Spectra were recorded for 15 minutes and were back-ground subtracted with buffer-only spectra.

Results and Discussion

Catalytic activity of purified V421L, F425W and V421L/F425W mutants was measured to be between 82 % and 128 % of wild-type. This, together with spectroscopic evidence, suggests that neither the oxygen reducing active site, nor the electron transfer pathway from the cytochrome c docking site, have been affected by these mutations. Protein characterisation and Raman spectroscopy showed normal subunit association, heme incorporation and heme spectral properties in these mutants. The residues lie in the postulated proton-translocating H-pathway, and these results strongly suggest that this channel does not exist in bacteria. The results are in disagreement with similar experiments carried out in bovine COX, in which the analogous double-mutant was constructed and reported to have an ideally decoupled phenotype[10]. This presents the rather surprising possibility that, despite a high degree of similarity in structure and both biochemical and biophysical properties, the mammalian and bacterial COXs may employ distinct proton-translocation pathways[11].

The S417V mutant lost almost all its catalytic activity and showed minor changes in UV-Visible and resonance Raman spectra. The same residue was previously changed to alanine in Rhodobacter sphaeroides with no loss of catalysis[12], hence it is unlikely that a loss of hydrogen-bonding by the serine 417 side-chain hydroxyl group is the primary cause of the observed S417V inactivity. Other studies on the putative H-channel have yielded comparable inactive mutants, such as H448L in Paracoccus denitrificans[13] and R54A/Q in Rhodobacter sphaeroides[12]. The H448L mutant showed distorted heme incorporation, although it was not clear why this should occur. The same experiment was later repeated in Rhodobacter using alanine[13], and found to have wild-type activity. R54 is located near the formyl group of heme a and extensive spectral analysis led to the conclusion that R54 participates in hydrogen-bonding to the formyl group which is necessary for catalytic activity. Additionally, structural perturbations caused by the mutations led to conversion of the a3 heme to the low-spin configuration, as shown by resonance Raman experiments. In contrast to the S417V results presented here, mutants suffered major structural perturbations around the heme a site.

Resonance Raman spectroscopy can detect slight changes in the porphyrin ring environment of heme proteins as seen by changes in the resonance of in-plane vibrational modes in the 1300 – 1700 cm^{-1} frequency region. Figure 3 shows the resonance Raman spectra of purified WT, V421L/F425W and S417V COXs in the dithionite-reduced state. In general, there appear to be very few differences between WT and mutant

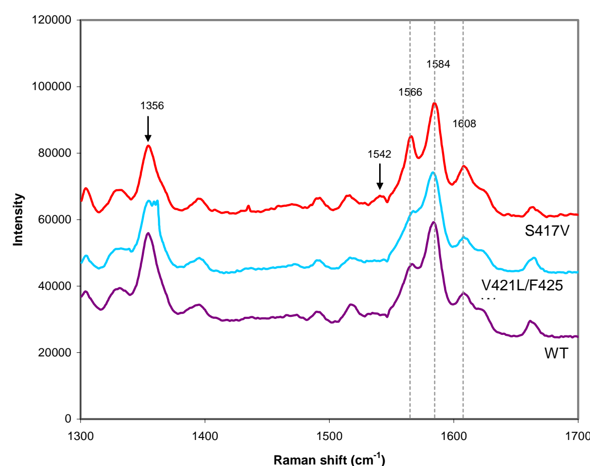


Figure 3. Resonance Raman spectra of purified wild-type and mutant dithionite-reduced COX.

spectra. The peak at 1356 cm^{-1} in all enzymes confirms that both hemes are in the ferrous oxidation state. The three peaks at 1566 cm^{-1}, 1584 cm^{-1} and 1608 cm^{-1} are the classic probes of heme conformation, corresponding to the formyl stretch of heme a, the vinyl stretch of heme a and the formyl stretch of heme a3 respectively[12]. These bands are very similar in the WT and V421L/F425W spectra suggesting a homogeneous porphyrin environment. S417V shows a minor shift in the 1584 cm^{-1} band, with a new band appearing at 1542 cm^{-1}. These observations may indicate a small structural perturbation may have occurred near the heme a site in this mutant.

Conclusions

Mutation of valine 421 to leucine, and phenylalanine 425 to tryptophan, both singly and in tandem, yielded enzymes with no significant attenuation of catalytic activity or proton pumping ability when compared with wild-type. The S417V mutant shows minor shifts in its Raman spectra. These observations indicate a small structural perturbation may have occurred near the heme a site in this mutant. These mutants may, therefore present an interesting insight into the catalytic action of cytochroms c oxidase.

Further work

Assignment of the new peak requires further Raman spectroscopy of COX in the oxidised state, at different excitation wavelengths and of the CO-bound form to determine the persistence of the peak, and isotope labelling to identify the contributing atom types.

References

1. D M Mitchell,
   Nature 191 144-148 (1961)
2. G T Babcock and M Wikström,
   Nature 356 301-309 (1992)
3. R B Gennis and S.Ferguson-Miller,
   Curr. Biol. 6 36-38, (1996)
4. S Iwata, C Ostermeier, B Ludwig, H Michel,
   Nature 376 660-669 (1995)
5. J F Nagle, H J Morowitz,
   Proc. Natl. Acad. Sci. USA 75 298-302 (1978)
6. S Yoshikawa,
   FEBS Lett, 555 8 – 12. (2003)

7. S Yoshikawa, K Shinzawa-Itoh, R Nakashima, R Yaono, E Yamashita, N Inoue, M Yao, M Jie Fei, C P Libeu, T Mizushima, H Yamaguchi, T Tomizaki, T Tsukihara
Science 280 1723-1729 (1998)

8. U Pfitzner, A Odenwald, T Ostermann, L Weingard, B Ludwig, O-M H Richter
J Biomembr. Bioenerg. 30 89-97 (1998)

9. K Shimokata, Y Katayama, H Murayama, K Muramoto, M Suematsu, T Tsukihara, S Yoshikawa, H Shimada
Biochem. Biophys. Acta. 1658 157 (2004)

10. K Shimokata, Y Katayama, H Murayama, K Muramoto, M Suematsu, T Tsukihara, S Yoshikawa, H Shimada
Biochem. Biophys. Acta. 1658 157 (2004)

11. R B Gennis
Science 280 1712-13. (1998)

12. H Lee, T K Das, D L Rousseau, D Mills, S Ferguson-Miller, R B Gennis
Biochemistry 39 2989–96 (2000)

13. H Lee, T K Das, D L Rousseau, D Mills, S Ferguson-Miller, R B Gennis
Biochemistry 39 2989–96 (2000)