Assignment of the CO-sensitive carboxyl group in mitochondrial forms of cytochrome c oxidase using yeast mutants ☆

Amandine Maréchal [a], Brigitte Meunier [b], Peter R. Rich [a],*

[a] Glynn Laboratory of Bioenergetics, Institute of Structural and Molecular Biology, University College London, Gower Street, London, WC1E 6BT, UK
[b] Centre de Génétique Moléculaire du CNRS, UPR 3404, avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France

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ABSTRACT

Point mutations of E243D and I67N were introduced into subunit I of a 6histidine-tagged (6H-WT) form of yeast *Saccharomyces cerevisiae* mitochondrial cytochrome c oxidase. The two mutants (6H-E243D$_I$ and 6H-I67N$_I$) were purified and showed $\approx$50 and 10% of the 6H-WT turnover number. Light-induced CO photolysis FTIR difference spectra of the 6H-WT showed a peak/trough at 1749/1740 cm^{-1}, as seen in bovine CcO, which downshifted by 7 cm^{-1} in D$_2$O. The bands shifted to 1736/1762 cm^{-1} in 6H-E243D$_I$, establishing that the carboxyl group affected by CO binding in mitochondrial CcOs is E243. In 6H-I67N$_I$, the trough at 1740 cm^{-1} was shifted to 1743 cm^{-1} and its accompanying peak intensity was greatly reduced. This confirms that the I67N mutation interferes with conformational alterations around E243. This article is part of a Special Issue entitled: 17th European Bioenergetics Conference (EBEC 2012).

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1. Introduction

Mitochondrial cytochrome c oxidase (CcO) is part of a large superfamily of haem-copper terminal respiratory oxidases that reduce molecular oxygen to water (for a recent review see [1]). Structures of bovine heart mitochondrial CcO and various bacterial homologues have been solved. Bovine CcO is a homodimer with each monomer composed of 13 subunits with a combined molecular weight of 204 kDa. Subunits I and II host the four redox active metal centres of Cu$_A$, haem a, haem a_3 and Cu$_B$ that receive electrons from reduced cytochrome c. Haem a_3 and Cu$_B$ form the binuclear centre (BNC) where reduction of molecular oxygen to water occurs. Subunit III may provide a channel for the substrate O$_2$ to diffuse to the BNC. The remaining ten 'supernumerary' subunits are not found in bacterial CcOs and might have additional functions such as assembly/stabilisation, protection and, possibly, allosteric regulation [2]. The mechanism of oxygen reduction is fairly well understood but the molecular details by which the released free energy are coupled to proton translocation remain in dispute. Each catalytic cycle consumes eight protons from the negative side of the membrane. Four are substrate protons required for reduction of O$_2$ to water; the additional four are translocated across the membrane, thereby increasing the energy stored in the protonmotive force for ATP synthesis. The protons must travel along hydrophilic channels formed from protonatable amino acids and associated waters. Three possible channels have been identified in X-ray structures of various CcOs [3–5]. The K and the D channels are thought to provide paths to the BNC for the four substrate protons at different steps in the oxygen reduction cycle. The D channel leads to a key glutamic acid (E242 in bovine, E243 in yeast) close to both haems a and a_3. It has been proposed that, at least in bacterial CcOs, it is also the pathway for transfer of all of the pumped protons to an exit route above the haems [6,7]. This dual function would require a strict gating mechanism to prevent short-circuiting and possible mechanisms involving structured water chains [8], conformational changes of E242 [9] and other factors [10] have been proposed. Bacterial mutations in the D channel that result in uncoupling [7,11,12] provide support for such gated connectivity of E242 to both pumping exit route and BNC, though molecular details have yet to be established by more direct methods. The H channel was initially identified in bovine CcO [4] where it spans the entire subunit I, passing close to haem a but spatially separated from the BNC. Structural changes have been observed in the H channel of bovine CcO in different redox and ligation states that may reflect its function as the gated proton translocation channel [13,14]. However, although mutations in the H channel of a bovine/human hybrid CcO caused loss of proton translocation without inhibition of electron transfer [15], similar mutations in bacteria did not exhibit such uncoupling [12,16]. These differences in behaviour have yet to

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be consolidated and have raised the possibility that bovine and bacterial C*c*Os may even have different coupling pathways.

FTIR spectroscopy has been a useful tool for probing the roles of carboxyl groups in C*c*O (reviewed in [17,18]). Bacterial and bovine forms of C*c*O have redox-sensitive bands that can be assigned to the protonated form of E242 that undergoes a decrease in extinction coefficient and/or a frequency decrease when haem a/Cu$_A$ become reduced. In bovine C*c*O, this is accompanied by the loss of an additional band at 1736 cm^{-1} that has been proposed to arise from deprotonation of conformationally-flexible residue D51 [18–20]. Less clear has been the assignment of a prominent carboxylic group band in bovine C*c*O that shifts from 1741 to 1749 cm^{-1} when CO is photolysed from the fully reduced CO-bound (FR-CO) form [18,21,22]. In the homologous quinol oxidase, cytochrome bo_3, CO photolysis also results in the upshift of a carboxyl band (in this case from 1724 to 1731 cm^{-1} in D$_2$O media) and mutations have established that it arises from E286 [23]. However, there is no clear bandshift in equivalent CO photolysis spectra of bacterial forms of C*c*O [24–26]. Furthermore, the signal in bovine C*c*O appeared to be heterogeneous in terms of accessibility to H/D exchange [18,21] and the 1749 cm^{-1} positive induced by CO photolysis did not correspond with the frequency of E242 if it were assumed to have downshifted (to 1742 cm^{-1}) in the fully reduced (FR) protein, as occurs in bacterial C*c*Os [27–30]. As a result, questions have remained as to whether the CO-sensitive carboxyl signal of bovine C*c*O might arise primarily from group(s) other than E242. Resolution of the origin of the CO-sensitive carboxyl band of mitochondrial forms of C*c*O is directly relevant to understanding the coupling mechanism since CO photolysis/rebinding mimics the oxygen binding step of the catalytic cycle. The core structure of yeast (*Saccharomyces cerevisiae*) mitochondrial C*c*O closely resembles that of bovine C*c*O [31]. In this study, we have used two mutant forms, E243D (equivalent to bovine E242) and I67N (equivalent to I66 in bovine C*c*O, a residue that is spatially adjacent to E242) in order to assign definitively the CO-sensitive carboxyl signal of mitochondrial forms of the enzyme.

2. Materials and methods

Yeast extract was purchased from Ohly GmbH, Germany, detergents were from Melford Laboratories, UK, Ni^{2+}-affinity resin (His-bind®) from Novagen, CO gas from BOC Ltd, UK and D$_2$O (D, 99.9%) from Cambridge Isotope Laboratories, Inc. All other reagents were purchased from Sigma Aldrich.

2.1. Mutant constructs and enzyme preparation

The addition of a 6histidine-tag on the nuclear encoded subunit Cox13 of yeast *S. cerevisiae* C*c*O and introduction of point mutations of E243D and I67N in the mt-DNA encoded subunit I were performed as described in [32]. The resulting yeast strains containing the 6histidine-tagged wild type (6H-WT) C*c*O and forms with subunit I mutations were grown under the same conditions of 16H in YPGal medium (yeast extract 1%, peptone 2%, galactose 2%) at 28 °C. This yielded to 16, 10 and 5 g of wet cells per litre of culture for the 6H-WT, 6H-E243D$_I$ and 6H-I67N$_I$ strains, respectively. Nevertheless, all contained the same relative amounts of optically-detectable C*c*O (2 nmol C*c*O per g wet weight). Mitochondrial membranes were prepared from 80 g of wet cells and solubilised with n-dodecyl-β-D-maltoside (DDM). C*c*O was purified by Ni^{2+}-affinity and DEAE Sepharose CL-6B ion exchange chromatographies [32].

2.2. Steady state O$_2$ consumption rate

C*c*O was diluted to 10–20 nM in 67 mM KPi at pH 6.2 containing 40 μM TMPD, 50 μM horse heart cytochrome c and 0.05% DDM in the chamber of a Clark-type oxygen electrode at 24 °C. The reaction was initiated by addition of 20 mM sodium ascorbate. Turnover numbers (expressed in electrons per sec, e.s^{-1}) were calculated from the steady state rates of oxygen consumption.

2.3. FTIR spectroscopy

Mid-IR spectra were recorded in transmission mode at 4 cm^{-1} resolution with a Bruker VERTEX 80v FTIR spectrometer fitted with a liquid nitrogen-cooled MCT-C detector, giving an accuracy of cited frequencies of ±1 cm^{-1}. During measurements, the optic compartment was kept under vacuum and the sample chamber, isolated from the optics by two KBr windows, was purged with N$_2$ gas. 2–3 nmol of purified protein was concentrated to 5 μl by ultrafiltration and reduced by addition of 1 μl of CO-saturated 0.2 M dithionite in 1 M KPi at pH 8.5. After equilibration for 2 min, the sample was placed in the centre of a CaF$_2$ window and exposed to a stream of CO for 1 min. A second window was placed on top and the sample was sealed with vacuum grease. 'Light *minus* dark' difference spectra were recorded at 277 K as described in [21]. For samples in D$_2$O, the protein was pre-incubated at 4 °C for 16H in D$_2$O containing 10 mM KPi pD 8.5, 5 mM Na$^+$-ascorbate, 10 μM ruthenium hexamine and 0.1% octylglucoside. The same protocol was then followed except that all buffers were prepared with D$_2$O.

3. Results and discussion

The turnover number of the purified 6H-E243D$_I$ mutant has already been reported to be 52% of that of the 6H-WT level [32]. Dithionite-reduced *minus* oxidised UV/visible spectra of purified 6H-I67N$_I$ (not shown) showed the same downshift of the α-band from 603 to 601 nm that was reported in an untagged form of the same mutant obtained by random mutagenesis [33]. In addition, its turnover number had decreased to 9% of that of the WT (16 e.s^{-1}), consistent with [33].

3.1. FTIR difference spectra induced by CO photolysis from fully reduced yeast C*c*O

FR *minus* FR-CO FTIR difference spectra of the 6H-WT C*c*O are shown in Fig. 1. Negative and positive bands correspond to the FR-CO and FR forms, respectively. As already described for other homologous C*c*Os, the 1964 cm^{-1} band (1963 cm^{-1} in bovine C*c*O) arises from haem-bound CO. Its narrowness (FWHM ≈ 6 cm^{-1}) suggests a very restricted environment of CO within the BNC, and its high frequency of 1964 cm^{-1} is indicative of weak H-bonding. The positive band at 2063 cm^{-1} arises from CO transiently bound to Cu$_B$ [34]. 'β' forms of haem-bound CO are also evident; simulation with Gaussian components indicated two principal 'β' forms at 1975 (11%) and 1960 (19%) cm^{-1}, both with FWHM ≈ 13 cm^{-1}. The origin of these forms, which are also present in other C*c*Os [35], is still not clear. As expected, the CO bands are unaffected by H/D exchange (Fig. 1, black/red traces) or by the E243D or I67N mutations in subunit I (Fig. S1).

3.2. Assignment of the CO-sensitive protonated carboxyl group

The 1800–1000 cm^{-1} 'fingerprint' region contains band changes of amide I/II backbone, amino acids and haems that are also very similar to those of bovine C*c*O [18,21] (Fig. S1). Of particular interest in relation to the CO-sensitive carboxyl group [18,21,25,36] is the 1800–1700 cm^{-1} region where protonated carboxylic amino acids absorb. Since an equivalent signal has not been reported to date in bacterial C*c*Os [24,25], its identity has not been confirmed by mutagenesis. Fig. 1 shows that a similar peak/trough at 1749/1740 cm^{-1} is present in the yeast 6H-WT CO photolysis spectrum (trace *a* in inset, black), hence providing a second example of its
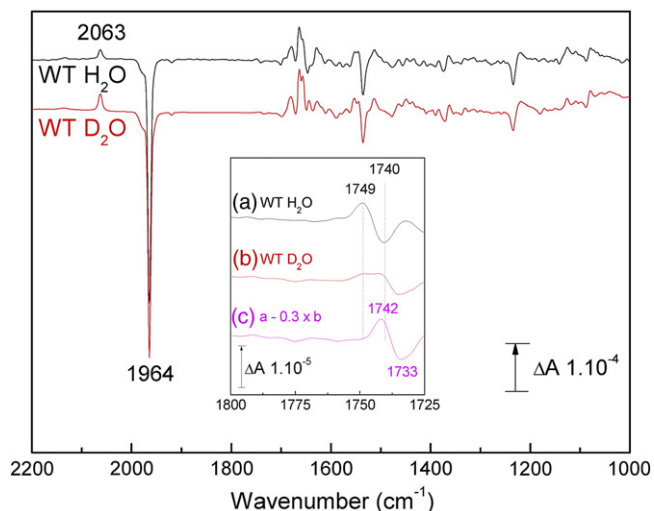
Fig. 1. CO photolysis FTIR difference spectra of the 6H-WT yeast C*c*O. Light *minus* dark FTIR difference spectra of the CO compound of fully reduced 6H-WT yeast C*c*O are shown at pH (black trace) and pD (red trace) 8.5. All spectra were scaled to the intensity of their CO band at 1964 cm^{-1}. Each trace is the average of 2000 transitions. Inset: expansion of the 1800–1725 cm^{-1} region: (a), 6H-WT in H$_2$O; (b), 6H-WT in 70% D$_2$O — see text for details; (c), calculated spectrum for 100% D$_2$O medium.

presence in a mitochondrial form of C*c*O. The signal was downshifted in D$_2$O media (trace *b* in inset, red), as expected for a COOH group with an exchangeable proton, though it became asymmetric in shape, as had also been observed in bovine C*c*O [18,21]. However, the absolute spectrum of the 6H-WT photolysis sample in D$_2$O showed that free H$_2$O had increased to 30% during sample mounting for IR measurements (calculated from the areas of free H$_2$O and D$_2$O in supplementary Fig. S2). By subtracting a 30% contribution of the H$_2$O
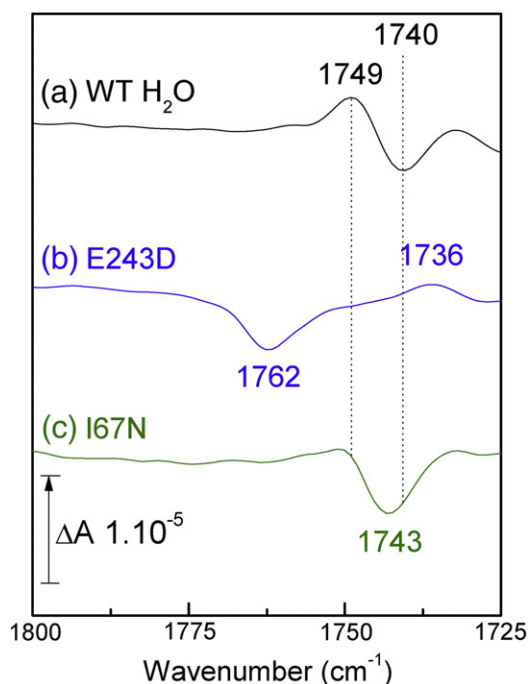

Fig. 2. Effects of point mutations in yeast C*c*O subunit I on the 1800–1725 cm^{-1} region of CO photolysis FTIR difference spectra. The 1800–1725 cm^{-1} region is shown of the light *minus* dark FTIR difference spectra of photolysis of the CO compounds of fully reduced forms of 6H-WT yeast C*c*O (trace a, black) and of the 6H-E243D$_I$ (trace b, blue) and 6H-I67N$_I$ (trace c, green) mutants at pH 8.5. All spectra were scaled to the intensities of their CO band at 1964 cm^{-1} (Fig. S1). Each trace is the average of 2000 transitions.

spectrum from the D$_2$O spectrum, and rescaling of the 1964 cm^{-1} CO bands, the signal became equivalent in shape and size to the H$_2$O spectrum, homogenously downshifted by 7 cm^{-1} (inset Fig. 1, trace *c*, pink). Hence, it may be concluded that the bandshift arises from a single carboxyl group and not from heterogeneous species as suggested from prior bovine C*c*O studies [18,21].

The overall photolysis spectra of both 6H-E243D$_I$ and 6H-I67N$_I$ mutants were very similar to that of the 6H-WT (Fig. S1), except in the carboxyl group region (Fig. 2). In the case of 6H-E243D$_I$ (Fig. 2, trace *b*, blue), the trough at 1740 cm^{-1} was upshifted +22 cm^{-1} to 1762 cm^{-1}, and the peak at 1749 cm^{-1} downshifted −13 cm^{-1} to 1736 cm^{-1} and its extinction coefficient decreased. Hence, the ligand-sensitive group can be assigned to protonated E243 of subunit I. The simplest explanation for the observed shifts is weaker and stronger H-bonding interaction of the protonated carboxyl group in its FR-CO and FR environments, respectively, due to the modification of the side chain length.

The ubiquinol oxidase, cytochrome *bo*$_3$ of *E. coli*, is the only bacterial homologue reported to exhibit a similar CO-sensitive carboxyl group. Its positive and negative bands at 1731/1724 cm^{-1} were also shifted by different extents in an E286D mutant (equivalent to yeast E243D) such that the peak/trough became inverted and shifted to 1756/1761 cm^{-1}. This, together with the loss of the bandshift altogether in an E286C mutant, also allowed assignment to its equivalent E286 residue [23].

The CO-sensitive IR signal of E243 was also affected by the I67N mutation (Fig. 2, trace *c*, green). The negative band of the CO-bound form was shifted to 1743 cm^{-1} and the peak was greatly reduced in intensity. This confirms the original suggestion [37] that the I67N mutation affects the environment of E243. This is consistent with structural data since I67 is spatially close to E243. Fig. 3 illustrates one possible rotamer of an asparagine at position 67 that is in close proximity to E243. The perturbation of E243 appears to be particularly severe in the reduced state, resulting in a decreased extinction coefficient or possibly a pK$_a$ decrease of E243. It is this effect of I67N on E243, rather than its relatively weak effects on the redox midpoint potential of haem *a* [33,37], that is most likely to cause the severe inhibition of turnover number, presumably through
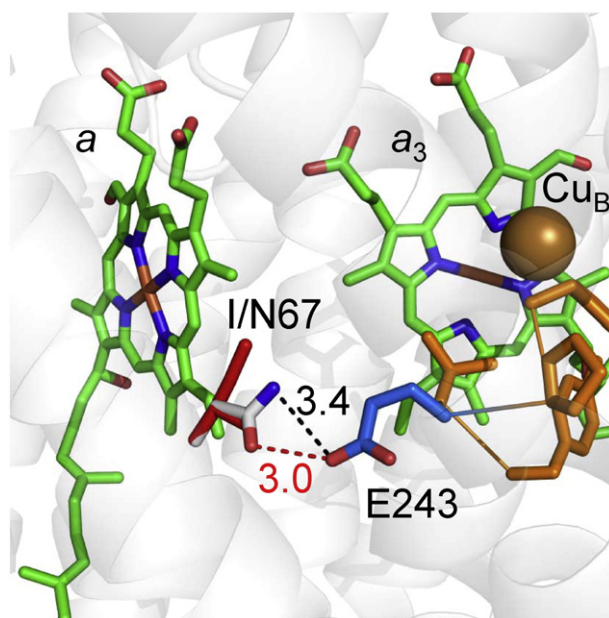

Fig. 3. Illustration of proximity of residue E243 and the I67N mutation. The I67N mutation was inserted into the yeast C*c*O homology model (atomic coordinates available in [31]) using PyMOL (DeLano Scientific; http://www.pymol.org) and rotated until in close proximity with E243.

interference with the E243 protonation reactions that are required in concert with reduction of the BNC [1].

Hence, in conclusion, these data establish that the CO-sensitive carboxyl group change that is observed clearly only in mitochondrial forms of CcO, and whose origin to date had not been unequivocally resolved, does indeed arise from the conserved residue E243. In addition, they demonstrate that E243 can be strongly influenced by mutation of the nearby residue I67 (I66 in bovine CcO).

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Acknowledgements

References

[1] P.R. Rich, A. Maréchal, The mitochondrial respiratory chain, Essays Biochem. 47 (2010) 1–23.

[2] D. Pierron, D.E. Wildman, M. Hüttemann, G.C. Markondapatnaikuni, S. Aras, L.I. Grossman, Cytochrome c oxidase: evolution of control via nuclear subunit addition, Biochim. Biophys. Acta Bioenerg. 1817 (2012) 590–597.

[3] S. Iwata, C. Ostermeier, B. Ludwig, H. Michel, Structure at 2.8 Å resolution of cytochrome c oxidase from Paracoccus denitrificans, Nature 376 (1995) 660–669.

[4] T. Tsukihara, H. Aoyama, E. Yamashita, T. Tomizaki, H. Yamaguchi, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, S. Yoshikawa, The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å, Science 272 (1996) 1136–1144.

[5] M. Svensson-Ek, J. Abramson, G. Larsson, S. Törnroth, P. Brzezinski, S. Iwata, The X-ray crystal structures of wild-type and EQ(I-286) mutant cytochrome c oxidases from Rhodobacter sphaeroides, J. Mol. Biol. 321 (2002) 329–339.

[6] M.K.F. Wikström, M.I. Verkhovsky, Mechanism and energetics of proton translocation by the respiratory heme–copper oxidases, Biochim. Biophys. Acta Bioenerg. 1767 (2008) 1200–1214.

[7] P. Brzezinski, R.B. Gennis, Cytochrome c oxidase: exciting progress and remaining mysteries, J. Bioenerg. Biomembr. 40 (2008) 521–531.

[8] M. Wikström, M.I. Verkhovsky, G. Hummer, Water-gated mechanism of proton translocation by cytochrome c oxidase, Biochim. Biophys. Acta 1604 (2003) 61–65.

[9] V.R.I. Kaila, M.I. Verkhovsky, G. Hummer, M. Wikström, Mechanism and energetics by which glutamic acid 242 prevents leaks in cytochrome c oxidase, Biochim. Biophys. Acta Bioenerg. 1787 (2009) 1205–1210.

[10] M.R.A. Blomberg, P.E.M. Siegbahn, The mechanism for proton pumping in cytochrome c oxidase from an electrostatic and quantum mechanical perspective, Biochim. Biophys. Acta Bioenerg. 1817 (2012) 495–505.

[11] P. Brzezinski, A.-E. Johansson, Variable proton-pumping stoichiometry in structural variants of cytochrome c oxidase, Biochim. Biophys. Acta Bioenerg. 1797 (2010) 710–713.

[12] U. Pfitzner, A. Odenwald, T. Ostermann, L. Weingard, B. Ludwig, O.-M.H. Richter, Cytochrome c oxidase (heme aa_3) from Paracoccus denitrificans: analysis of mutations in putative proton channels of subunit I, J. Bioenerg. Biomembr. 30 (1998) 89–97.

[13] T. Tsukihara, K. Shimokata, Y. Katayama, H. Shimada, K. Muramoto, H. Aoyama, M. Mochizuki, K. Shinozawa-Itoh, E. Yamashita, M. Yao, Y. Ishimura, S. Yoshikawa, The low-spin heme of cytochrome c oxidase as the driving element of the proton-pumping process, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 15304–15309.

[14] K. Muramoto, K. Ohta, K. Shinzawa-Itoh, K. Kanda, M. Taniguchi, H. Nabekura, E. Yamashita, T. Tsukihara, S. Yoshikawa, Bovine cytochrome c oxidase structures enable O$_2$ reduction with minimization of reactive oxygens and provide a proton-pumping gate, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 7740–7745.

[15] K. Shimokata, Y. Katayama, H. Murayama, M. Suematsu, T. Tsukihara, K. Muramoto, H. Aoyama, S. Yoshikawa, H. Shimada, The proton pumping pathway of bovine heart cytochrome c oxidase, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 4200–4205.

[16] H.-M. Lee, T.K. Das, D.L. Rousseau, D. Mills, S. Ferguson-Miller, R.B. Gennis, Mutations in the putative H-channel in the cytochrome c oxidase from Rhodobacter sphaeroides show that this channel is not important for proton conduction but reveal modulation of the properties of heme a, Biochemistry 39 (2000) 2989–2996.

[17] P.R. Rich, A. Maréchal, Carboxyl group functions in the heme-copper oxidases: information from mid-IR vibrational spectroscopy, Biochim. Biophys. Acta Bioenerg. 1777 (2008) 912–918.

[18] D. Okuno, T. Iwase, K. Shinzawa-Itoh, S. Yoshikawa, T. Kitagawa, FTIR detection of protonation/deprotonation of key carboxyl side chains caused by redox change of the Cu$_A$-heme a moiety and ligand dissociation from the heme a_3-Cu$_B$ center of bovine heart cytochrome c oxidase, J. Am. Chem. Soc. 125 (2003) 7209–7218.

[19] P. Hellwig, T. Soulimane, G. Buse, W. Mäntele, Similarities and dissimilarities in the structure–function relation between the cytochrome c oxidase from bovine heart and from Paracoccus denitrificans as revealed by FT-IR difference spectroscopy, FEBS Lett. 458 (1999) 83–86.

[20] S. Yoshikawa, K. Shinzawa-Itoh, R. Nakashima, R. Yaono, E. Yamashita, N. Inoue, M. Yao, M.J. Fei, C.P. Libeu, T. Mizushima, H. Yamaguchi, T. Tomizaki, T. Tsukihara, Redox-coupled crystal structural changes in bovine heart cytochrome c oxidase, Science 280 (1998) 1723–1729.

[21] P.R. Rich, J. Breton, FTIR studies of the CO and cyanide compounds of fully reduced bovine cytochrome c oxidase, Biochemistry 40 (2001) 6441–6449.

[22] J.A. Bailey, F.L. Tomson, S.L. Mecklenburg, G.M. MacDonald, A. Katsonouri, A. Puustinen, R.B. Gennis, W.H. Woodruff, R.B. Dyer, Time-resolved step-scan Fourier transform infrared spectroscopy of the CO adducts of bovine cytochrome c oxidase and cytochrome $bo3$ from Escherichia coli, Biochemistry 41 (2002) 2675–2683.

[23] A. Puustinen, J.A. Bailey, R.B. Dyer, S.L. Mecklenburg, M. Wikström, W.H. Woodruff, Fourier transform infrared evidence for connectivity between Cu$_B$ and glutamic acid 286 in cytochrome bo_3 from E. coli, Biochemistry 36 (1997) 13195–13200.

[24] B. Rost, J. Behr, P. Hellwig, O.M.H. Richter, B. Ludwig, H. Michel, W. Mäntele, Time-resolved FT-IR studies on the CO adduct of Paracoccus denitrificans cytochrome c oxidase: comparison of the fully reduced and the mixed valence form, Biochemistry 38 (1999) 7565–7571.

[25] D. Heitbrink, H. Sigurdson, C. Bolwien, P. Brzezinski, J. Heberle, Transient binding of CO to Cu$_B$ in cytochrome c oxidase is dynamically linked to structural changes around a carboxyl group: a time-resolved step-scan Fourier transform infrared investigation, Biophys. J. 82 (2002) 1–10.

[26] C. Koutsoupakis, T. Soulimane, C. Varotsis, Ligand binding in a docking site of cytochrome c oxidase: a time-resolved step-scan Fourier transform infrared study, J. Am. Chem. Soc. 125 (2003) 14728–14732.

[27] P. Hellwig, J. Behr, C. Ostermeier, O.-M.H. Richter, U. Pfitzner, A. Odenwald, B. Ludwig, H. Michel, W. Mäntele, Involvement of glutamic acid 278 in the redox reaction of the cytochrome c oxidase from Paracoccus denitrificans investigated by FTIR spectroscopy, Biochemistry 37 (1998) 7390–7399.

[28] M. Lübben, K. Gerwert, Redox FTIR difference spectroscopy using caged electrons reveals contributions of carboxyl groups to the catalytic mechanism of haem–copper oxidases, FEBS Lett. 397 (1996) 303–307.

[29] R.M. Nyquist, D. Heitbrink, C. Bolwien, T.A. Wells, R. Gennis, J. Heberle, Perfusion-induced redox differences in cytochrome c oxidase: ATR/FT-IR spectroscopy, FEBS Lett. 505 (2001) 63–67.

[30] M. Iwaki, A. Puustinen, M. Wikström, P.R. Rich, ATR-FTIR spectroscopy of the P$_M$ and F intermediates of bovine and Paracoccus denitrificans cytochrome c oxidase, Biochemistry 42 (2003) 8809–8817.

[31] A. Maréchal, B. Meunier, D. Lee, C. Orengo, P.R. Rich, Yeast cytochrome c oxidase: a model system to study mitochondrial forms of the haem–copper oxidase superfamily, Biochim. Biophys. Acta Bioenerg. 1817 (2012) 620–628.

[32] B. Meunier, A. Maréchal, P.R. Rich, Construction of histidine-tagged yeast mitochondrial cytochrome c oxidase for facile purification of mutant forms, Biochem. J. (2012) http://dx.doi.org/10.1042/BJ20120116.

[33] C. Ortwein, T.A. Link, B. Meunier, A.-M. Colson, P.R. Rich, U. Brandt, Structural and functional analysis of deficient mutants in subunit I of cytochrome c oxidase from Saccharomyces cerevisiae, Biochim. Biophys. Acta 1321 (1997) 79–92.

[34] O. Einarsdóttir, R.B. Dyer, D.D. Lemon, P.M. Killough, S.M. Hubig, S.J. Atherton, J.J. López-Garriga, G. Palmer, W.H. Woodruff, Photodissociation and recombination of carbonmonoxy cytochrome oxidase: dynamics from picoseconds to kiloseconds, Biochemistry 32 (1993) 12013–12024.

[35] S. Park, L.P. Pan, S.I. Chan, J.O. Alben, Photoperturbation of the heme a_3-Cu$_B$ binuclear center of cytochrome c oxidase CO complex observed by Fourier transform infrared spectroscopy, Biophys. J. 71 (1996) 1036–1047.

[36] B.H. McMahon, M. Fabian, F. Tomson, T.P. Causgrove, J.A. Bailey, F.N. Rein, R.B. Dyer, G. Palmer, R.B. Gennis, W.H. Woodruff, FTIR studies of internal proton transfer reactions linked to inter-heme electron transfer in bovine cytochrome c oxidase, Biochim. Biophys. Acta 1655 (2004) 321–331.

[37] B. Meunier, C. Ortwein, U. Brandt, P.R. Rich, Effect of mutation of residue I67 in yeast cytochrome c oxidase on redox-linked protonation processes, Biochem. J. 330 (1998) 1197–1200.