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Comparison of redox and ligand binding behaviour of yeast and bovine cytochrome c oxidases using FTIR spectroscopy

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1. Introduction

Mitochondrial cytochrome c oxidase (CcO) is a member of the A1 branch of the haem-copper oxidase (HCO) superfamily of respiratory oxidases that catalyse the four electron reduction of molecular oxygen to water [1–3], conserving the free energy in the transmembrane proton electrochemical gradient. In mammals, each monomer has at least 13 different subunits (~204 kDa) and may form dimers or integrate into supercomplexes with other respiratory complexes in the inner mitochondrial membrane [4]. Subunits I, II and III are encoded by mitochondrial DNA, whereas subunits IV and V are encoded by nuclear DNA [4]. Subunits I, II and III are encoded by mitochondrial DNA and form the catalytic core. The remaining ‘super-numerary’ subunits, several of which can occur in tissue-specific isoforms, are nuclear DNA-encoded; their functions are receiving increasing interest due to possible roles in assembly, stability, allosteric control [5] and supercomplex formation [6].

Particularly in mammalian mitochondrial CcO, the detailed atomic mechanism by which proton and electron transfers are coupled during catalysis remains controversial [7–9]. Electrons are provided one at a time by reduced cytochrome c in the intermembrane space to a dicopper centre (CuA) within subunit I. They are then transferred through subunit I via a histidine-coordinated A-type haem (haem a) to the O2 reducing binuclear centre (BNC), composed of another A-type haem (haem a3) and a copper atom (CuB). A large body of experimental data indicates that each electron transfer into the BNC is coupled to the uptake of two protons from the mitochondrial matrix: one pumped proton, ultimately translocated across the membrane, and one substrate proton, directed to the O2 reduction site [10]. These protons must traverse the interior of the largely hydrophobic enzyme along hydrophilic channels formed by arrays of protonatable/polar residues and associated water molecules [11–13]. Three candidate structures, the D, K and H channels have been identified within subunit I from the X-ray derived atomic structures of both mitochondrial (bovine) and bacterial A1-type HCOs [14,15]. In bacterial A1-type HCOs, a large body of kinetic and mutant data indicates that the D and the K channels provide the route for substrate protons at different stages of the oxygen reduction cycle, and that the D channel provides a part of the pathway for all pumped protons [10]. Briefly, in the most widely accepted mechanism [9,10], for each electron transfer from haem a into the BNC, a pumped proton is transferred from E242 (bovine numbering) at the top of the D channel via a gated route into a temporary proton trap in the region above the BNC, possibly close to the bound Mg2+ ion [16]. The opposite charges of the trap proton and the negative BNC...
electrostatically stabilise each other. However, reduction of the BNC creates a high pK protonatable site for a substrate proton. Protonation of this site via the K or D channel neutralises the BNC charge, which in turn induces release of the trap proton into the P phase via a route that is not yet well-defined. This same mechanism may be operative in mammalian CoxOs, though a different mechanism involving transfer of the pumped protons via the H channel, rather than through the D channel, has been proposed, based on structural and more limited functional studies [8].

The D channel extends from D91 at the N phase surface to E242, located roughly equidistant from both haem edges. E242 (with equivalents of E243 in S. cerevisiae, E278 in P. denitrificans and E286 in R. sphaeroides CoxOs and E286 in E. coli bo3 quinol oxidase) and the D channel are conserved throughout the A1-type HCOs [17]. Mutagenesis studies in bacterial [7,18] and yeast [19,20] systems have shown that they are essential for activity. The most recent proposal of H channel studies in bacterial [7,18] and yeast [19,20] includes a proton pathway from D407 in the N phase towards the edge of haem a that is gated by a redox- and ligand-sensitive movement of S382. When open, this pathway provides a protonic connection to the path leading to the proton trap site around Y440 and S441, finally arriving at D51 at the P phase subunit I/II interface [23].

Fourier transform infrared (FTIR) spectroscopy has revealed functional aspects of E242 and D51 [24], particularly from the characteristic bands of their protonated forms in the relatively uncluttered 1800–1700 cm$^{-1}$ range [25]. Here we compare new redox and CO photolysis difference FTIR data of yeast CoxO with equivalent published spectra of bovine and bacterial CoxOs.

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, 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 6-histidine-tag on the nuclear DNA-encoded subunit Cox13 of yeast Saccharomyces cerevisiae CoxO and the subsequent construction of mutant strains with additional single point mutation of E243D, I67N or S52D in the mitochondrial DNA-encoded subunit Cox1 were described in [26]. Growth of the yeast cells in galactose medium, and the protocols for the preparation of mitochondria and the purification of the resulting 6H-WT, 6H-E243D, 6H-I67N and 6H-S52D CoxOs with n-dodecyl-$\beta$-D-maltoside by Ni$^{2+}$-affinity and DEAE Sepharose CL-6B ion exchange chromatography were as in [19,26]. WT P. denitrificans CoxO was provided by Märtten Wikström (University of Helsinki, Finland) and was prepared as in [27].

2.2. 'ATR-ready' protein sample preparation and electrochemically induced ATR-FTIR difference spectroscopy

Depletion of detergent for the preparation of ‘ATR-ready’ protein samples was performed from 250 pmol of purified yeast 6H-WT or mutated CoxO as described in [28]. The resulting ‘ATR-ready’ CoxO was immediately placed on a 3-reflection silicon attenuated total reflectance (ATR) prism (DuraSamIR II, SensIR/Smith Detection). After drying with a gentle stream of N$_2$ gas, the protein film was rehydrated with a buffer of 100 mM K-phosphate, 100 mM KCl at pH 6.0. The same procedure was followed for analyses of H/D exchange effects except that all solutions were prepared with D$_2$O and at pH 6.0. pH values were adjusted with a standard glass pH electrode assuming pD = pH (meter reading) + 0.4 [29].

An electrochemical cell built in-house that allows simultaneous recording of UV/visible and IR absorption spectra [30] was assembled on top of the protein film. The working electrode was a platinum grid held approximately 0.2 mm above the protein film. A platinum sheet counter electrode and Ag/AgCl/KCl reference electrode in 100 mM K-phosphate, 100 mM KCl at pH 6.0 were connected to the sample chamber by a Vycor frit [31]. The sample chamber was filled with the same solution containing in addition 50 μM anthraquinone-2-sulfonate (E$^{\text{red}}_{\text{m,7}} = -225$ mV) and 1 mM ferricyanide (E$^{\text{red}}_{\text{m,7}} = 430$ mV) as redox mediators. All redox potentials are quoted versus the standard hydrogen electrode.

Reduced minus oxidised mid-IR difference spectra were recorded at 4 cm$^{-1}$ resolution at room temperature in ATR mode on a Bruker IFS 66/S spectrometer equipped with a liquid nitrogen-cooled MCT-A detector. All frequencies cited have an accuracy of ± 1 cm$^{-1}$. For each IR spectrum recorded, 500 interferograms were averaged before Fourier transformation. Redox transitions were induced by applying potentials of approximately 350 mV/+500 mV (reduction/oxidation) with a potentiostat (Princeton Applied Research) following the protocol described in [31] for a typical redox cycle. Equilibration times for stabilisation of all UV/visible and IR absorbance changes were typically 12 min in both directions. Redox cycles were repeated to improve signal to noise depending on the IR signal size and stability of the protein film. Spectra presented are averages of data from at least two distinct samples and the number of redox cycles used to produce each IR spectrum is given in figure legends.

2.3. Light induced CO photolysis FTIR difference spectroscopy

The preparation of fully reduced CO bound samples of yeast and P. denitrificans CoxOs and recording of light minus dark mid-IR difference spectra in transmission mode was as described in [19]. The data were acquired at 4 cm$^{-1}$ resolution on a Bruker Vertex 80v spectrometer equipped with a liquid nitrogen-cooled MCT-C detector with the optics compartment kept under vacuum (< 2 hPa). A narrow band filter (Northumbria Optical Coatings Ltd.) was used to isolate the high-frequency region (3800–3600 cm$^{-1}$) and increase signal/noise [32].

2.4. Spectra correction and data treatment

When necessary, IR spectra were corrected for the contribution of water vapour, redox mediators, pH change and total protein or lipid changes due to a slight swelling of the protein film on recording in ATR mode. This was done with OPUS 6.5 software (Bruker) by iterative subtraction of model spectra recorded under the conditions of the experiments. All figures were subsequently produced using OriginPro 2015 (OriginLab Corporation).

3. Results and discussion

3.1. Comparison of redox-induced FTIR spectra

Fig. 1A presents an electrochemically-induced reduced minus oxidised ATR-FTIR difference spectrum of yeast 6H-WT CoxO recorded at pH 6.0 in the mid-IR ‘fingerprint’ region (middle trace). This spectrum arises from band changes of IR-active groups that are sensitive to redox changes of the enzyme in its unligated state. Typical redox spectra of bovine mitochondrial and P. denitrificans A1-type enzymes are also displayed for comparison (Fig. 1A, top and bottom traces, respectively; similar data have been published in [32,33]). All spectra have been scaled on their amide II feature at 1562(−)/1545(+) cm$^{-1}$. Major features of the spectra are similar and tentative assignments have been made for many bands (for a recent review see [31] and references therein).

However, one difference resides in the extent of their amide I band
changes in the 1650 cm$^{-1}$ region. In bovine and $P.\text{denitrificans}$ CcOs, a prominent peak at 1662–1660 cm$^{-1}$ dominates the redox spectrum and has been linked to the redox changes of $\text{Cu}_a$/haem a [31,34]. It has an intensity 2–3 times greater than their amide II changes. In yeast CcO, the amide I peak at 1658 cm$^{-1}$ relative to the amide II change is much smaller. Visible absorption spectra recorded simultaneously with IR spectra (Fig. 1B) indicated that the Soret and $\alpha$-band features had fully formed and were at a ratio consistent with reduction of both haems [35]; hence, the smaller amide I band does not appear to arise from incomplete redox cycling. Isotope labelling of $P.\text{denitrificans}$ CcO has shown that amide bonds of histidine and tyrosine are major contributors to this band [33]. Comparisons of the high resolution X-ray structures for bovine [21,23] and $R.\text{sphaeroides}$ [36,37] CcOs in their fully oxidised and reduced states have revealed three regions of redox-induced structural flexibility (Fig. 2). In $R.\text{sphaeroides}$ significant changes occur in the region of the BNC associated with movements of haem $a_3$, helix VIII, water molecules at the top of the D- and the K-channels and an H-bond between Y244 of the HPEVY pentapeptide and the haem $a_3$ hydroxyfarnesyl [36]. Equivalent redox-induced structural changes have not been observed in all bovine CcO structures, despite

Fig. 1. Electrochemically induced reduced minus oxidised ATR-FTIR difference spectra of bovine, yeast 6H-WT and $P.\text{denitrificans}$ A1-type oxidases. A. The spectra of bovine (top) and yeast 6H-WT CcOs (middle trace) are the average of 120 redox cycles recorded at pH 8.5 and 6.0, respectively. The spectrum of $P.\text{denitrificans}$ CcO (bottom trace) is the average of 25 redox cycles at pH 8.5. All were scaled on their amide II band change at 1562(−)/1545(+) cm$^{-1}$. B. Typical reduced minus oxidised visible absorption difference spectra of bovine (dashed) and yeast 6H-WT (solid line) recorded simultaneously with IR recordings. C. Expansion of the 1800–1725 cm$^{-1}$ region and effects of H/D exchange (labelled D$_2$O, average of 190 redox cycles at pD 6.0) and S52D mutation on the yeast 6H-WT redox spectrum.

Fig. 2. Structural comparison of bovine cytochrome c oxidase in oxidised and reduced states. Figure was composed using coordinates from PDB ID: 5B1A (oxidised) and PDB ID: 5B1B (reduced) structures [21] after alignment of their haem $a$ moieties. Selected stretches of polypeptide backbones, amino acids and the haem $a$ farnesyl hydroxyl group are shown in pink (oxidised subunit I, PDB ID: 5B1A) and cyan (reduced subunit I, PDB ID: 5B1B).
their similar amino acid composition, and some interesting suggestions have been made as to why those changes might not have been consistently evident (see discussion in [37]). In contrast, in both R. sphaeroides and bovine CcOs changes are observed around S382, including reorientation of its side chain, though in bovine structures this also includes reorientation of part of the haem α hydroxyfarnesyl chain (Fig. 2). Amino acids in those two regions are conserved in yeast CcO and so might be expected to contribute similar amide I changes in all redox spectra, though this will require structural confirmation. A third region of redox-induced structural flexibility has been observed around residues D50/D51 in bovine CcO and the change of D51 in particular has been proposed to have significant functional importance [23]. D51 and its adjacent amino acids are not conserved at all in bacterial CcOs and no redox-induced structural changes in the equivalent region have been observed. The region in both cases is close to the subunit I/II interface, which is formed also in part from a short sequence that includes Y440 and two arginines (R438 and R439) that interact with haem propionates. The C=O amide of Y440 interacts with the D-ring propionate of haem α via a bridging water and its head group projects towards Cuα in subunit II. Y440 is conserved in P. denitrificans and R. sphaeroides CcOs, but is an isoleucine in yeast CcO. This replacement could decrease the polarity, and hence IR intensity, of its amide I band. Studies of further amino acid replacements may help to resolve this issue.

A second clear difference is in the 1800–1725 cm⁻¹ spectral region, expanded in Fig. 1C, where only protonated carboxyl groups and lipids of the samples absorb. The yeast enzyme shows a single trough at 1748 cm⁻¹, as do bacterial A1-type CcOs at 1746 or 1745 cm⁻¹ [38,39], whereas two troughs have consistently been reported in bovine CcO, independent of pH, at 1749 and 1736 cm⁻¹ (Fig. 1C, top two traces). These bands have also been associated with reduction of Cuα/haem α [24,31,34]. The 1736 cm⁻¹ feature has been assigned to D51 [24], consistent with the absence of the band and this residue in both bacterial and yeast CcOs ([40] and Fig. 1). Based on structural data, it was interpreted as arising from deprotonation of D51 in reduced CcO and was proposed to be a key function in the alternative H-channel proposal [8,23]. Direct evidence for protonated D51 in oxidised CcO is yet to be produced. A sufficiently high pK is difficult to reconcile with its position at the top of a helix with nearby waters and polar residues, or with the known Eₜₜₒₒ/pH dependencies of haem α and Cuα [41–43].

3D model of yeast CcO built by sequence homology onto the bovine X-ray structure shows a good alignment of features in that region, including the short helix that extends from the haem α histidine ligand (bovine H61, yeast H62) to the yeast equivalent of D51 (SS2) [44]. Based on this, we have introduced a D residue in yeast CcO at the SS2 locus. The mutant (6H-SS2D) cells had a WT growth phenotype [26] and the O₂-reduction rate measured on mitochondrial membrane preparation indicated WT CcO activity at 1200 e⁻·s⁻¹ (measured as in [19]). As shown in Fig. 1C, lower trace, the mutation did not result in an additional redox-induced carboxylic acid IR signal equivalent to that in bovine CcO. Hence, if the bovine CcO band is indeed due to protonated D51, the data suggest that additional structural differences between bovine and yeast CcOs govern the band change in this region. For example, the H-bonding partners of bovine D51 (subunit I S441 and subunit II S205) that are proposed to create the protonating environment of D51 are replaced by proline and alanine, respectively, in yeast. However, at this stage assignment of the 1736 cm⁻¹ signal in the bovine spectrum to another buried carboxyl group [40] or to a lipid molecule cannot be definitively ruled out.

3.2. Effect of mutations in yeast

The trough at 1749–1748 cm⁻¹ in bovine and yeast CcOs (Fig. 1C, top two traces) is consistent with the same band of protonated E242 (E243 in yeast) observed in their CO photolysis spectra [19,24,45]. On H/D exchange in both yeast (Fig. 1C, lower trace) and bovine CcO redox spectra [24,46] the trough has a 7 cm⁻¹ downshift to 1741 cm⁻¹, consistent with its carboxylic acid origin. The 1748 cm⁻¹ trough reported here for the yeast enzyme seems devoid of an associated peak, and hence is most likely to arise from a decrease in polarity of the E243 carboxyl on reduction of the enzyme. The same assignment and interpretation are likely for the equivalent 1749 cm⁻¹ band of bovine CcO.

Yeast CcO mutants E243D and I67N have already been reported to decrease O₂-reduction activity with turnover numbers in mitochondria at 43% and 1% of that of 6H-WT [19,26]. Mutant 6H-E243D led to a single net positive at 1765 cm⁻¹ in redox spectra, downshifted by 8 cm⁻¹ to 1757 cm⁻¹ in D₂O (Fig. 3B, second and third traces from top), providing definitive assignment to E243. Equivalent E/D replacements have been made previously in bacterial CcOs. These resulted in the disappearance of the trough observed at 1746 cm⁻¹ in WT.
P. denitrificans CcO [47] and of the 1745(−)/1735(+) cm⁻¹ pair in WT E. coli bo₃ [48] and R. sphaeroides aa₃ oxidases [49]. In the latter case, a new signal from the replacement aspartic acid was observed at 1738(−)/1729(+) cm⁻¹. All three studies provided definitive assignment of the redox-induced IR signals to their E243 equivalents.

Mutant 6H-I67N, led to disappearance of the E243 band in redox difference spectra (Fig. 3B, lower trace), consistent with the lack of a bandshift of E243 in its CO photolysis spectra [19]. In addition, this mutation further decreased band changes in the amide I region. As discussed above (Section 3.1), the HPEVY pentapeptide is conformationally flexible in R. sphaeroides CcO [36,50]. If this is also the case in yeast CcO, a contribution of these residues to amide I changes in WT redox spectra, which are lost due to constraints imposed in the 6H-I67N mutant, can be suggested.

3.3. Water molecule rearrangements

Water molecules are key in the transfer of both substrate and translocated protons within the channels. Their role can be structural, for instance in orienting protonatable groups, or dynamic by forming transient H-bonded networks to conduct protons in a Grotthuss mechanism. Changes have been reported in the 3700–3600 cm⁻¹ range on oxidoreduction of P. denitrificans [51] and bovine [32] CcOs. More extensive changes were also reported in that region on CO photolysis from the fully reduced state of these CcOs [32] and were attributed to weakly H-bonded water molecule rearrangements. Fig. 4A shows equivalent data for yeast 6H-WT CcO (middle trace). Above 3600 cm⁻¹, the difference spectrum was simulated with 8 Gaussian functions with full widths at half maximum fixed at 6 cm⁻¹, similar to what was required to simulate spectra from bovine and P. denitrificans CcOs [32].

In bovine and yeast CcOs, signals of these weakly H-bonded waters and of protonated E242 appear in the same CO photolysis spectra (Fig. 4B, top and middle traces), suggesting a possible mechanistic connection. To date a signal from E242 equivalents has only been reported in CO photolysis spectra from the mixed valence CO forms of bacterial A1-type CcOs, and never from their fully reduced CO state [52,53]. However, Fig. 4B (lower trace) presents a CO photolysis spectrum of P. denitrificans at high signal/noise, revealing a trough at 1734 cm⁻¹ with no clear positive. Hence, it is likely to arise from an extinction coefficient decrease of E278 (equivalent to bovine E242) due to a polarity decrease on CO photolysis, consistent with the wave-number of the band attributed to E278 in the fully reduced enzyme at 1736–1734 cm⁻¹ in redox-induced IR spectra [38,47]. Hence, in all CcOs tested to date, perturbations of water molecules and E242 can be observed.

4. Conclusions

Yeast is a genetically-amenable system which can be used to investigate mitochondrial CcOs. By comparison with bacterial and mammalian CcOs, important common functional features can be identified. In this report, we have discussed assignment and interpretation of published redox and CO photolysis IR spectra of bovine, yeast and P. denitrificans CcOs together with new IR redox data for yeast CcO (Fig. 1A and C, 6H-WT, -E243DI, -I67NI and -S52DI) and new IR CO photolysis data for P. denitrificans (Fig. 4B) and yeast (Fig. 4A) WT CcOs. In both types of spectra, all CcOs exhibit changes of their E242 equivalent, highlighting its central mechanistic importance. Bovine CcO displays an additional redox sensitive band at 1736 cm⁻¹ which was suggested to arise from protonated D51. This signal is absent from yeast (Fig. 1A) and bacterial CcOs which indeed lack D51 equivalent and are structurally different in this region. Reintroduction of D51 in yeast CcO (Fig. 1C) failed to induce the signal. Redox spectra of yeast CcO also show much smaller changes in the amide I region, which may also relate to differences in the region around D51 and the subunit I/II interface. The yeast mutation I67N appears to constrain E242 ([19] and Fig. 3) and also further decreases amide I region changes, which may be related to constraint of the HPE243VY pentapeptide in which E243 resides. In all three CcOs, CO photolysis results additionally in perturbation of signals attributable to weakly H-bonded water molecules.

Fig. 4. CO photolysis induced FTIR spectra. A. Water molecule rearrangements in the 3775–3600 cm⁻¹ region. Yeast 6H-WT data (middle trace) are the average of 4000 light minus dark difference spectra. Data from bovine and P. denitrificans (top and lower traces) are from [32]. B. Associated protonated E242 signals in the 1775–1720 cm⁻¹ region. The spectrum presented for P. denitrificans is the average of 2000 light minus dark difference spectra. All spectra are scaled on the extent of their carbon monoxide photolysis band at 1963 cm⁻¹.
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