



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

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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 ≈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 D₂O. 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 O₂ 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 O₂ 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

Abbreviations: BNC, binuclear centre; CcO, cytochrome *c* oxidase; DDM, n-dodecyl-β-D-maltoside; 6H-WT, yeast CcO containing a six-histidine tag on nuclear-encoded Cox13 subunit; 6H-E243D_I or 6H-I67N_I, the 6H-WT strain with an additional mutation of E243D or I67N in mtDNA-encoded subunit I; FTIR, Fourier transform infrared; FR, fully reduced and FR-CO, fully reduced CO-bound forms of CcO

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be consolidated and have raised the possibility that bovine and bacterial CcOs may even have different coupling pathways.

FTIR spectroscopy has been a useful tool for probing the roles of carboxyl groups in CcO (reviewed in [17,18]). Bacterial and bovine forms of CcO 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 CcO, 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 CcO 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_2O 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 CcO [24–26]. Furthermore, the signal in bovine CcO 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 CcOs [27–30]. As a result, questions have remained as to whether the CO-sensitive carboxyl signal of bovine CcO might arise primarily from group(s) other than E242. Resolution of the origin of the CO-sensitive carboxyl band of mitochondrial forms of CcO 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 CcO closely resembles that of bovine CcO [31]. In this study, we have used two mutant forms, E243D (equivalent to bovine E242) and I67N (equivalent to I66 in bovine CcO, 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_2O (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* CcO 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) CcO 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 CcO (2 nmol CcO per g wet weight). Mitochondrial membranes were prepared from 80 g of wet cells and solubilised with n-dodecyl- β -D-maltoside (DDM). CcO was purified by Ni^{2+} -affinity and DEAE Sepharose CL-6B ion exchange chromatographies [32].

2.2. Steady state O_2 consumption rate

CcO was diluted to 10–20 nM in 67 mM KPi at pH 6.2 containing $40\text{ }\mu\text{M}$ TMPD, $50\text{ }\mu\text{M}$ 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 $\pm 1\text{ cm}^{-1}$. 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 $5\text{ }\mu\text{l}$ by ultrafiltration and reduced by addition of $1\text{ }\mu\text{l}$ 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_2O , the protein was pre-incubated at 4°C for 16H in D_2O containing 10 mM KPi pH 8.5, 5 mM Na^+ -ascorbate, $10\text{ }\mu\text{M}$ ruthenium hexamine and 0.1% octylglucoside. The same protocol was then followed except that all buffers were prepared with D_2O .

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 CcO

FR minus FR-CO FTIR difference spectra of the 6H-WT CcO are shown in Fig. 1. Negative and positive bands correspond to the FR-CO and FR forms, respectively. As already described for other homologous CcOs, the 1964 cm^{-1} band (1963 cm^{-1} in bovine CcO) arises from haem-bound CO. Its narrowness ($FWHM \approx 6\text{ cm}^{-1}$) 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 $FWHM \approx 13\text{ cm}^{-1}$. The origin of these forms, which are also present in other CcOs [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 CcO [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 CcOs [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

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