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Supporting Information

# A Self-Assembled Respiratory Chain that Catalyzes NADH Oxidation by Ubiquinone-10 Cycling between Complex I and the Alternative Oxidase

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# Supporting Information

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## Experimental Methods

### Preparation of complex I and AOX

Complex I was prepared from *Bos taurus* (bovine) heart mitochondria using the method of Sharpley et al.[1] except that the phospholipids present in the Q-sepharose chromatography buffers (20 mM Tris-HCl pH 7.55, 10 % ethylene glycol, 0.2% *n*-dodecyl-β-D-maltoside (DDM, Anatrace) and 2 mM EDTA) were 25 μg $mL^{-1}$ of *B. taurus* heart cardiolipin (CL), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Avanti Polar Lipids Inc.), and size exclusion chromatography was carried out using a 24 mL Superose-6 Increase column (GE Healthcare).

The alternative oxidase from *Trypanosoma brucei brucei* (AOX) was over-expressed in *Escherichia coli* strain FN102/pTbAO[2,3]. The FN102 strain is auxotrophic for 5-aminolevulinic acid as it lacks the glutamyl-tRNA reductase (gene *hemA*) required for its production in the first step of *E. coli* heme biosynthesis;[4] the inability to produce heme prevents formation of the cytochrome *bd* quinol oxidase. FN102/pTbAO cells were plated onto LB supplemented with 100 mg $L^{-1}$ ampicillin (Sigma-Aldrich), 50 mg $L^{-1}$ kanamycin (Merck & Co. Inc.) and 100 mg $L^{-1}$ of 5-aminolevulinic acid (Sigma-Aldrich) and incubated for 18 hours at 37 ˚C. Large colonies were used to inoculate two 50 mL aliquots of base medium (10.4 g $L^{-1}$ $K_2HPO_4$, 3 g $L^{-1}$ $KH_2PO_4$, 0.74 g $L^{-1}$ Na-citrate.$2H_2O$, 2.5 g $L^{-1}$ $(NH_4)_2SO_4$, 10 g $L^{-1}$ tryptone, 5 g $L^{-1}$ yeast extract, 5 g $L^{-1}$ casamino acids and 0.2 % (w/v) glucose[3]) supplemented with carbenicillin (Melford Laboratories Ltd.), kanamycin, and 5-aminolevulinic acid as above. The cultures were grown aerobically at 37 ˚C for ~3 hours (until $A_{600}$ reached 0.6-0.8) and the cells collected by centrifugation. The medium was then exchanged for base medium supplemented with 100 mg $L^{-1}$ carbenicillin, 50 mg $L^{-1}$ $MgSO_4$, 25 mg $L^{-1}$ $FeSO_4$ and 25 mg $L^{-1}$ $FeCl_3$; following a single wash step all the cells were resuspended into a common 5 mL aliquot. The exchange removes the 5-aminolevulinic acid required for heme synthesis[4] and introduces the iron required for AOX over-expression. The cells were then used to innoculate eight 1 L cultures of the same supplemented medium; the cultures were incubated aerobically at 30 ˚C for ~6 hours (until $A_{600}$ reached ~0.6), 100 μM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce AOX expression, and the cultures were incubated aerobically at 30 ˚C overnight.

All steps from hereon were carried out at 4 ˚C. The cells, typically 20 g, were harvested by centrifugation and resuspended to 10 mL/g in buffer comprising 50 mM Tris-HCl (pH 7.5); a protease inhibitor cocktail (Roche, 4 tablets per 200 mL), ~500 U per 200 mL of benzonase and 1 mM $MgSO_4$ were added. Then, the cells were disrupted using a Constant Systems Ltd cell disruptor (one pass at 15 kpsi and two at 30 kpsi), and the cell debris removed by a slow-speed centrifugation (15 min., 8300 x g). The membrane fragments were collected using a high-speed centrifugation (80

min., 160,000 x g), resuspended to 12 mg $mL^{-1}$ (typically 20 mL) in the same buffer, and snap frozen in liquid nitrogen for storage if required. The membrane proteins were solubilized by addition of an equal volume of solution containing 40% glycerol, 400 mM $MgSO_4$ and 2.8% octyl-glucoside, stirred for 1 hour, and centrifuged (30 min., 165,000 x g). The supernatant was filtered, then mixed with TALON cobalt metal affinity resin (Clontech, pre-equilibrated in 50 mM Tris-HCl (pH 7.3), 20% glycerol, 100 mM $MgSO_4$, 1.4% octyl-glucoside) in a ratio of 5:1 supernatant:sedimented resin and agitated gently for 45 min.[2] Then, the mixture was centrifuged (5 min., 1000 x g), the supernatant discarded, and the resin washed twice with five volumes of chromatography buffer (20 mM Tris-HCl (pH 7.3), 20% glycerol, 50 mM $MgSO_4$, and 0.042 % DDM) supplemented with 20 mM imidazole. Following the final centrifugation the resin was resuspended using two volumes of the same buffer, poured into an XK50 column casing (GE Healthcare) and allowed to settle. The column was assembled, transferred to an Äkta Prime Plus FPLC (GE Healthcare) and washed at 2.5 mL $min^{-1}$ in the same buffer until $A_{280}$ reached the baseline. The column was washed again with chromatography buffer supplemented with 135 mM imidazole and 80 mM NaCl, before AOX was eluted using chromatography buffer supplemented with 250 mM imidazole and 160 mM NaCl. AOX-containing fractions were pooled, concentrated ten-fold to ~1.3 mg $mL^{-1}$ using a 30 KDa molecular weight cut-off centrifugal filter (Amicon), and dialysed against 2 L of chromatography buffer supplemented with 160 mM NaCl for 5 hours, to remove the imidazole. The final product was aliquoted and snap frozen in liquid nitrogen for storage. Typically, ~8 mg of AOX were obtained from 8 L of cell culture, and the purity was estimated to be 95% by SDS-PAGE analyses.

**Preparation of complex I:AOX:$Q_{10}$ proteoliposomes**

Proteoliposomes were prepared using a protocol adapted from that used previously to reconsitute complex I[5]. First, stock solutions of 25 mg $mL^{-1}$ *B. taurus* PC, PE and CL in chloroform were mixed in a glass homogeniser at a mass ratio of 8:1:1, along with the required amount of ubiquinone-10 (Sigma-Aldrich), also in chloroform. A typical preparation used a total of 10 mg phospholipids, and the following protocol is based on this amount. The chloroform was removed by evaporation under $N_2$ and by applying a vacuum, then the phospholipid/$Q_{10}$ mixture was resuspended in ~675 µL of proteoliposome buffer (10 mM Tris-$SO_4$ (pH 7.5) and 50 mM KCl), and extruded nine times through a Whatman 0.1 µm pore tracked edge membrane. The mixture was partially solubilised by addition of 160 µL of octyl-glucoside (OG, supplier) from an aqueous 10% stock solution, sonicated in a Grant XB3 Ultrasonic bath sonicator for 10 min., and placed on ice for 10 min. All subsequent steps were at 4 °C. 0.2 mg of AOX (~150 µL of ~1.3 mg $mL^{-1}$) and 0.2 mg of complex I (~15 µL of ~13 mg $mL^{-1}$) were added to the solubilised lipids and incubated for 10

min., then 100 μL of sedimented SM2 Biobeads (Bio-Rad Laboratories Ltd.) were added and the mixture agitated gently for 4 hours, with a further 100 μL of Biobeads added after each hour. The mixture was passed through an empty micro bio-spin column (Bio-Rad Laboratories Ltd.) to remove the Biobeads, then centrifuged (45 min., 57,500 x g) to collect the proteoliposomes. Following resuspension in 100 μL of proteoliposome buffer the yield was typically 200 μL at ~1.5 mg protein $mL^{-1}$. Activity assays were carried out on freshly prepared material, as the activity was observed to decrease by ~25% upon each freeze-thaw cycle.

**Analytical methods**

Total $Q_{10}$ contents were quantified spectroscopically under anaerobic conditions, by the change in absorbance observed upon addition of 1.5 or 2 mM $KBH_4$ at 275 nm ($\varepsilon_{ox\text{-}red}$ = 12.9 $mM^{-1}$ $cm^{-1}$);[6] when necessary, 1% SDS was added to minimize light scattering. Total phospholipid contents were determined using the Ames method.[7] 100 μL of each sample (at several different dilutions up to 0.4 mg phospholipid $mL^{-1}$) or standard (0 to 500 μM $KH_2PO_4$) was transferred into a boiling tube along with 50 μL of methanol and 30 μL of 390 mM $Mg(NO_3)_2$ in ethanol. The tubes were heated over a roaring blue flame in a fumehood (with care being taken not to boil or bump the samples) until no further brown fumes were formed and the residue in the tubes had turned white. The tubes were left to cool for 5 min., then 0.3 mL of 500 mM HCl was added to each tube, and the tubes were lightly stoppered (using glass marbles) and incubated at 99 ˚C for 15 min. in the fumehood in a heat block. Once the tubes were cool, 0.7 mL of an aqueous solution of 114 mM ascorbic acid, 2.72 mM $(NH_4)_6Mo_7O_{24}$ and 400 mM $H_2SO_4$ was added to each one, and the tubes were lightly stoppered and incubated at 37 ˚C for 1 hour. 200 μL aliquots were transferred into the wells of a quartz 96-well plate, the absorbance (from reduced phosphomolybdate) measured at 820 nm, and the phospholipid contents of the samples determined by reference to known standards, accounting for the fact that each PC or PE contributes one orthophosphate, whereas each CL contributes two. Protein concentrations in $Q_{10}$PLs were determined using the Pierce bicinchoninic acid (BCA) assay. Complex I was quantified using the rate of NADH:$APAD^+$ oxidoreduction (measured at 400-450 nm ($\varepsilon$ = 3.16 $mM^{-1}$ $cm^{-1}$) with 100 μM NADH and 500 μM $APAD^+$)[8] with $Q_{10}$ reduction by complex I inhibited with 1 μM piericidin A and 15 μg $mL^{-1}$ alamethicin (a pore forming antibiotic[9]) to activate all the complexes I. The AOX concentration was taken to be the difference in the total protein and complex I concentrations. Density gradient centrifugation used a glycerol gradient of 2.5–25 % (1.004–1.060 g $mL^{-1}$) in proteoliposome buffer; samples were applied to the top of the gradient and centrifuged for 80 minutes at 310,000 x g.

**Catalytic activity measurements**

All catalytic activity assays were carried out at 32 ˚C in solutions containing 10 mM Tris-$SO_4$ (pH 7.5) and 50 mM KCl. Complex I and AOX were inhibited, when required, by 1 μM piericidin A or 1 μM ascofuranone,[3,10] respectively. Rates of NADH:$O_2$ oxidoreduction were measured spectroscopically at 340-380 nm ($\varepsilon_{NADH}$ = 4.81 $mM^{-1}$ $cm^{-1}$) using a Spectra max 348 plus 96-well plate reader (Molecular Devices), initiated with 200 μM NADH. NADH:DQ oxidoreduction was measured similarly using 200 μM NADH and 100 μM DQ (added from an DMSO stock solution), with AOX inhibited by 1 μM ascofuranone. Fluorescence measurements were carried out in an RF-5301PC spectrofluorometer (Shimadzu). The fluorescent dye 9-amino-6-chloro-2-methoxyacridine (ACMA, excitation at 419 nm and emission at 484 nm[11]) was used to monitor the formation of ΔpH in the presence of 75 mM $KNO_3$ with nitrate acting as a permeant ion to diminish Δψ and allow ΔpH to take the full value of Δp.

**Table S1: Retention of each component in a set of $Q_{10}$PL preparations**

| $Q_{10}$ at start (nmol) | $Q_{10}$ | | Complex I | | AOX | | Phospholipid | |
|---|---|---|---|---|---|---|---|---|
| | nmol | % | mg | % | mg | % | mg | % |
| 0 | 1.17 ±0.14 | - | 0.12 ±0.003 | 60 | 0.21 ±0.007 | 104 | 4.81 ±0.189 | 48 |
| 10 | 7.88 ±0.19 | 79 | 0.13 ±0.002 | 63 | 0.22 ±0.022 | 109 | 5.31 ±0.019 | 53 |
| 20 | 16.13 ±0.02 | 81 | 0.14 ±0.003 | 68 | 0.19 ±0.039 | 96 | 6.4 ± 0.006 | 64 |
| 30 | 19.65 ±0.15 | 66 | 0.1 ±0.012 | 50 | 0.2 ±0.013 | 99 | 4.83 ±0.002 | 48 |
| 40 | 32.94 ±1.03 | 82 | 0.14 ±0.003 | 69 | 0.21 ±0.011 | 105 | 5.85 ±0.167 | 59 |
| 60 | 38.82 ±0.47 | 65 | 0.11 ±0.001 | 56 | 0.21 ±0.026 | 106 | 4.78 ±0.053 | 48 |
| 80 | 40.52 ±1.57 | 51 | 0.06 ±0.001 | 58 | 0.2 ±0.018 | 101 | 5.59 ±0.007 | 56 |
| 80 | 57.8 ±4.07 | 72 | 0.1 ±0.001 | 50 | 0.2 ±0.02 | 100 | 3.97 ±0.105 | 40 |
| 160 | 110.17 ±4.99 | 69 | 0.14 ±0.004 | 68 | 0.21 ±0.029 | 107 | 5.63 ±0.152 | 56 |

The amounts of each component added at the start of the preparation were 0.2 mg of complex I, 0.2 mg of AOX, 10 mg of phospholipids and variable amounts of $Q_{10}$ (see column 1). The amounts of each component present in the final preparations are given alongside the percentage retention. A preparation which began with 0.1 mg of complex I (half the usual amount) is shaded in grey.

**Table S2: Measurements of the $Q_{10}$ content of mitochondria and mitochondrial membrane preparations.**

| Study | System | Method of $Q_{10}$ quantitation | nmol $Q_{10}$ (mg protein)$^{-1}$ | nmol $Q_{10}$ (mg phospholipid)$^{-1}$ |
|---|---|---|---|---|
| Crane et al. [12] | SMPs (*B. taurus* heart) | 275 nm absorbance | 2.7 | 5.8[1] |
| Norling et al.[13] | SMPs (*B. taurus* heart) | 275 nm absorbance | 6 – 8 | 12.8 – 17.1[1] |
| Narabayashi et al.[14] | SMPs (*B. taurus* heart) | 275 nm absorbance | 3.4 – 3.98 | 7.2 – 8.5[1] |
| Suzuki and Ozawa [15] | Mitochondria (*B. taurus* heart) | 275 nm absorbance | 3.0 | n.d. |
| Estornell et al.[16] | Mitochondria (*B. taurus* heart) | HPLC | 2.4 ± 1 | n.d. |
| This work | Mitochondrial membranes (*B. taurus* heart)[2] | 275 nm absorbance | 5.67 ± 0.22 (n = 3) | 12.09 ± 0.47 (n = 3) |

1. Calculated using 0.469 ± 0.023 phospholipid (mg protein)$^{-1}$.

2. Due to the complexity of the membrane composition the $Q_{10}$ was extracted using 2:5 (v:v) ethanol:*n*-hexane[17] before spectroscopic quantification.

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