Integral membrane proteins: fundamental medical biochemistry studies towards novel antibiotic targets

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CHAPTER 4

Cardiolipin enhances the enzymatic activity of cytochrome *bd* and cytochrome bo_3 solubilized in dodecyl-maltoside

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ABSTRACT

Cardiolipin (CL) is a lipid that is found in the membranes of bacteria and the inner membranes of mitochondria. CL is a phospholipid that is required for optimal performance of many integral membrane proteins, in particular components of respiratory pathways. We here report that CL is needed for optimal function of detergent-solubilized cytochrome *bd,* a terminal oxidase from *Escherichia coli.* CL enhanced the oxygen consumption activity ~2.5 fold and decreased the apparent K_M value for ubiquinol-1 as substrate from 95 μM to 35 μM. Activation by CL was also observed for cytochrome *bd* from two Gram-positive species, *Geobacillus thermodenitrificans* and *Corynebacterium glutamicum,* and for cytochrome bo_3 from *E. coli*. These findings reveal a role of CL for maximal activity of cytochrome *bd* and cytochrome bo_3.

Cardiolipin (CL) is an anionic phospholipid that consists of two phosphatidyl groups connected by a glycerol moiety. CL is important for optimal function of various eukaryotic and prokaryotic membrane protein complexes[1-4]. CL can interact with bacterial respiratory complexes from phylogenetically diverse species, such as *Mycobacterium phlei*[5], *Rhodobacter sphaeroides*[6] and *Escherichia coli*[7]. Among *E. coli* respiratory chain complexes, CL was shown to activate purified, detergent-free cytochrome bo_3[8] and was the most efficient phospholipid for activation of detergent-solubilized NADH dehydrogenase[9] and of liposome-reconstituted nitrate reductase[10]. Defined binding sites for CL have been determined in crystal structures of *E. coli* formate dehydrogenase N[11], succinate dehydrogenase[12], and nitrate reductase[10].

The respiratory chain in *Escherichia coli* features a heme-copper-type terminal oxidase, cytochrome bo_3, which transfers electrons from quinol-type substrates onto molecular oxygen. Next to this energetically efficient terminal oxidase, *E. coli* utilizes cytochrome *bd* as an alternative branch of the respiratory chain. Cytochrome *bd* oxidizes quinols, like ubiquinol or menaquinol, coupled with reduction of molecular oxygen to water (Figure 1A)[13,14]. Cytochrome *bd* is particularly important under conditions of stress, such as O_2-limitation[15], in the presence of nitric oxide[16,17] hydrogen peroxide[18-20] and hydrogen sulfide[21,22]. Lack of cytochrome *bd* in uropathogenic *E. coli* strains led to attenuation in mouse infection models[23].

Purified active cytochrome *bd* has been prepared from several bacterial species, including *E. coli*[24,25] *Azotobacter vinelandii*[26], *Corynebacterium glutamicum*[27] and *Geobacillus thermodenitrificans*[28,29]. However, to our knowledge there are no data available concerning the effect of CL on cytochrome *bd* activity.

In this report, we investigated the influence of CL on detergent-purified cytochrome *bd*. We found that CL activated the enzymatic activity of cytochrome *bd* from *E. coli*, *G. thermodenitrificans* and *C. glutamicum*. We then extended our experimentation and also assessed the impact of CL on the activity of purified cytochrome bo_3 from *E. coli*.

RESULTS

CL enhances the activity and decreases the K_M value of purified cytochrome *bd*. Cytochrome *bd* was purified from *E. coli* strain MB43 using streptactin affinity chromatography and β-D-dodecyl-maltoside (DDM) as detergent, as described earlier[30,31]. In line with previous data[20,30,31], the purified enzyme showed a specific oxygen consumption activity of ~110 μmol O_2*mg^{-1}*min^{-1} in buffer containing 0.025 % DDM, using ubiquinol-1 as substrate. We examined the effect of CL and observed ~2-fold activation of the oxygen consumption activity (Figure 1B). The activity of cytochrome *bd* in both the absence and the presence of CL was strongly suppressed by the aurachin D (Figure 1C), an inhibitor of *E. coli* cytochrome *bd*[32].

We then investigated whether activation of cytochrome *bd* by CL is only observed at saturating substrate concentrations, or if the K_M value is affected as well. In the absence of CL, cytochrome *bd* showed a K_M value of 95 μM for ubiquinol-1 as substrate, in line with previously published results[24,25,33]. In the presence of 10 μM CL, the K_M value decreased to 35 μM (Figure 1D). These results suggest that CL is important for optimal activity of DDM-solubilized *E. coli* cytochrome *bd*.

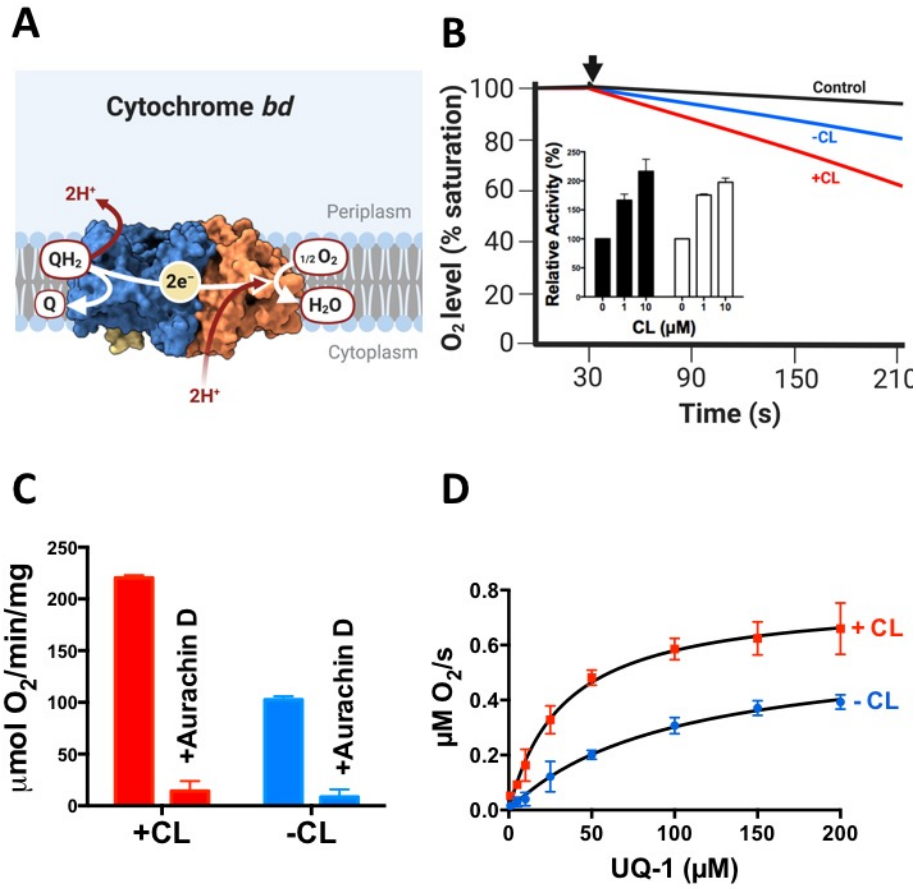

Figure 1: Activation of *E. coli* cytochrome *bd* by CL.

A: Enzymatic function of cytochrome *bd*. **B**: The effect of CL on oxygen consumption activity by cytochrome *bd* purified from *E. coli* was determined using a Clark-type electrode. The reaction was initiated by addition of ubiquinone-1 + DTT (arrow), the negative control contained ubiquinone-1 and DTT, but no cytochrome *bd*. Inset: Dependency of activation on the pre-incubation time at the indicated CL concentrations. The enzyme was incubated with CL prior to starting the reaction for either 3 min (black bars) or 60 min (white bars). **C**: Impact of the inhibitor aurachin D (400 nM) on oxygen consumption by cytochrome *bd* in the presence or absence of CL. **D**: Effect of CL on the K_M value of *E. coli* cytochrome *bd*.. Curve fit was done with a simple Michaelis-Menten analysis; R^2 values in the absence and presence of CL were 0.978 and 0.969, respectively. Average values were calculated from at least two independent experiments at 37 °C; error bars represent standard deviations.

CL activates purified cytochrome *bd* from Gram-positive bacteria. Next, we evaluated if activation by CL is restricted to cytochrome *bd* from *E. coli,* a Gram-negative bacterium, or if it represents a more general property of this enzyme across species. Previously, purification of cytochrome *bd* from the two Gram-positive strains *Geobacillus thermodenitrificans* (formerly called *Bacillus stearothermophilus*)[28,29] and *Corynebacterium glutamicum*[27] was described.

We examined the oxygen consumption activity of purified cytochrome *bd* from both strains with the same protocol as for *E. coli* cytochrome *bd*, except for using menaquinol-1 instead of ubiquinol-1 as substrate, as these Gram-positive bacteria use menaquinone as main constituent of the quinone pool. Cytochrome *bd* from *G. thermodenitrificans* showed lower oxygen consumption activity (~18 µmol O_2*mg^{-1}*min^{-1} in the initial phase) as compared to the *E. coli* enzyme, consistent with previous data[34]. After the initial phase of the reaction, time-dependent inactivation was observed (Figure 2A). CL significantly increased the activity of cytochrome *bd* from this strain (Figure 2A). As observed above for the *E. coli* enzyme, the activity of *G. thermodenitrificans* cytochrome *bd* was sensitive to inhibition by aurachin D in the presence and absence of CL (Figure 2B).

Consistent with previous results[27], the oxygen consumption activity of cytochrome *bd* from *C. glutamicum* (~50 µmol O_2*mg^{1}*min^{-1}) was lower than that of the *E. coli* enzyme, but higher than that of *G. thermodenitrificans* cytochrome *bd*. Importantly, the activity was significantly enhanced by CL (Figure 2C). We confirmed that the observed oxygen consumption activity in

the presence and absence of CL was sensitive to inhibition by aurachin D (Figure 2D).

These results suggest that CL can enhance the activity of purified cytochrome *bd* from phylogenetically diverse bacteria, which indicates that the requirement for CL may be a general feature of cytochrome *bd* across species.

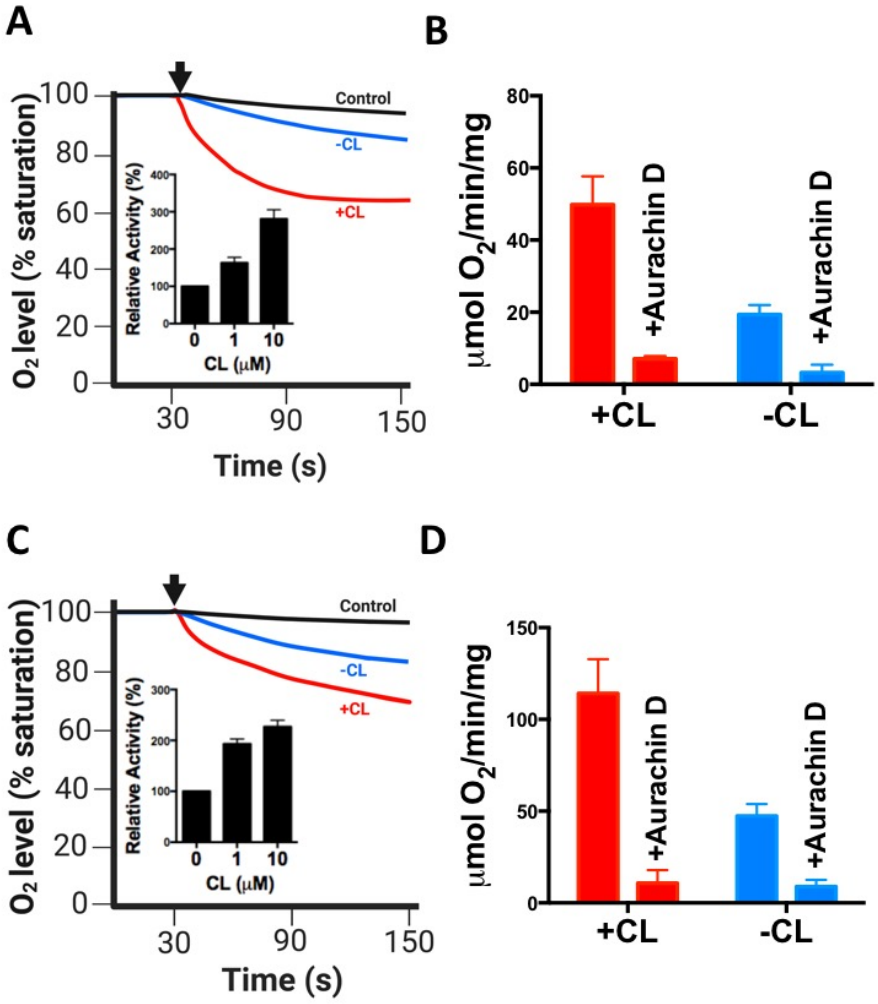

Figure 2: Activation of cytochrome *bd* from Gram-positive bacteria by CL. The oxygen consumption activity of cytochrome *bd* purified from *G. thermodenitrificans* (**A, B**) and *C. glutamicum* (**C, D**) was determined using a

Clark-type electrode at 37 °C. The reaction was initiated by addition of menaquinone-1 + DTT (arrow), the negative control contained menaquinone-1 and DTT, but no cytochrome *bd*. Insets (**A, C**): Dependency of activation on the CL concentration. **B, D**: Impact of the inhibitor aurachin D (400 nM) on the oxygen consumption activity. Average values were calculated from two independent experiments; error bars represent standard deviations.

Cardiolipin activates enzymatic activity of cytochrome *bo*$_3$ from *E. coli*.

We then extended our efforts to the second terminal oxidase found in *E. coli*, cytochrome *bo*$_3$. Cytochrome *bo*$_3$ was purified from *E. coli* strain GO105/pJRhisA using DDM as detergent, as described earlier[35, 36]. Like cytochrome *bd*, cytochrome *bo*$_3$ can accept ubiquinol-1 as electron donor and reduces molecular oxygen (Figure 3A). In the absence of CL, cytochrome *bo*$_3$ displayed a specific oxygen consumption activity of 47 μmol O_2*mg^{-1}*min^{-1}, comparable to previously reported values[36,37]. Addition of CL caused a pronounced increase in activity (Figure 3B). Oxygen consumption by cytochrome *bo*$_3$ in both the absence and in the presence of CL was highly susceptible to the inhibitor potassium cyanide (KCN) (Figure 3C). The K_M value in presence of CL (38 μM) was found considerably lower as compared to the K_M measured in the absence of CL (56 μM) (Figure 3D). Taken together, our results show that CL is important for optimal enzymatic function of both terminal oxidases in *E. coli*.

Figure 3

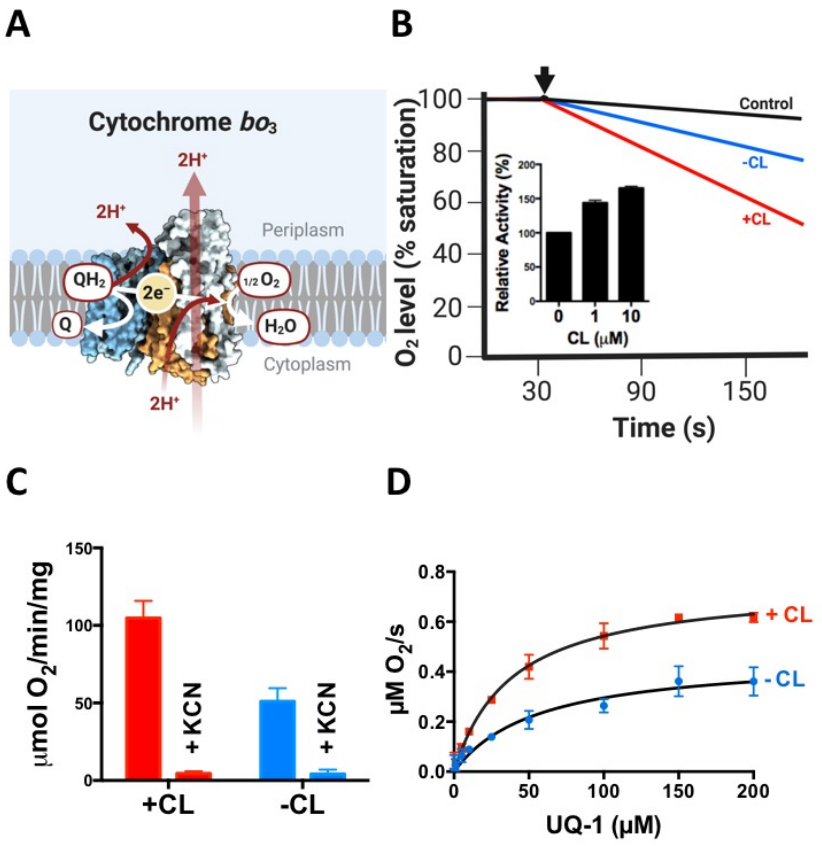

Figure 3: Activation of *E. coli* cytochrome bo_3 by CL.

A: Enzymatic function of cytochrome bo_3. **B**: The effect of CL on oxygen consumption activity by cytochrome bo_3 purified from *E. coli* was determined using a Clark-type electrode. The reaction was initiated by addition of ubiquinone-1 + DTT (arrow), the negative control contained ubiquinone-1 and DTT, but no cytochrome bo_3. Inset: dependency of activation on the CL concentration. **C**: Impact of KCN (2 mM) on oxygen consumption by cytochrome bo_3 in the presence or absence of CL. **D**: Effect of CL on the K_M value of purified *E. coli* cytochrome bo_3. Curve fit was done with a simple Michaelis-Menten analysis, R^2 values in the absence and presence of CL were 0, 974 and 0.983, respectively. All experiments were carried out at 37 °C. Average values were calculated from at least two independent experiments; error bars represent standard deviations.

DISCUSSION

It has been established that CL can enhance the activity of various bacterial membrane proteins, including complexes of both aerobic and of anaerobic respiration[1,2,8,10,11]. Previously, activation of purified cytochrome *bo*$_3$ by CL and activation of purified cytochrome *bd* by asolectin was reported[8,25]. However, these experiments were carried out in detergent-free state. In the absence of detergent, membrane protein aggregation likely causes a significant decrease in activity, which subsequently is relieved by addition of lipid. In this study, we found that CL enhanced the activity of both terminal oxidases of the *E. coli* respiratory chain in the detergent-solubilized state. In line with our results, recently high enzymatic activity of *E. coli* cytochrome *bd* solubilized in nano-discs has been reported[38], reflecting the importance of the lipid environment for enzyme performance.

CL can be located at the outer surface of a detergent-solubilized membrane protein, enabling proper vertical positioning of the protein, or it may bind to clefts or cavities on the protein surface[1,3]. CL may play a structural role, e.g. by binding at the interface between individual subunits, as previously reported for formate dehydrogenase N[11]. Alternatively, CL may enhance the interaction with the quinol substrate and/or facilitate the electron transfer reaction, as reported for nitrate reductase, where CL binds to a niche near the quinol-binding site[10]. Three-dimensional structures are available for cytochrome *bd* from *Geobacillus thermodenitrificans*[29] and from *E. coli*[38,39], however, the presently achieved resolution might not allow for identification of all bound lipid molecules. The decreased K_M value of *E. coli* cytochrome *bd* for ubiquinol-1 measured here indicates that CL influences the substrate binding process.

In our study we investigated cytochrome *bd* and cytochrome bo_3 in the detergent-solubilized state and our results therefore do not clarify if CL is needed for optimal performance of these enzymes in the native membrane. CL as high-curvature lipid is predominantly localized at the poles in rod-shaped bacteria and may thereby influence the cellular localization of membrane protein complexes[40], as suggested for the SecYEG translocon[41]. Previously, for cytochrome *bd*, a distribution in mobile patches in the *E. coli* cytoplasmic membrane has been reported [42]. It needs to be investigated if CL can influence function, localization or dynamics of cytochrome *bd* or cytochrome bo_3 in the native plasma membrane.

MATERIALS & METHODS

Chemicals. Aurachin D was synthesized as described earlier in Li *et al*. 2013[43] and was kindly provided by Dr. Jennifer Herrmann (Helmholtz Centre for Infection Research and Pharmaceutical Biotechnology, Saarbrücken). All other chemicals were bought from Sigma, unless indicated otherwise.

Purification of cytochrome *bd*. Cytochrome *bd* from *E. coli* was purified based on Hoeser *et al*. 2014[30], with modifications as described by Goojani et al 2020[31]. Briefly, *E. coli* MB43 carrying the pET17cydABX-Strep-tag plasmid was grown in Luria-Bertani (LB) medium with 100 µg/ml Ampicillin at 37 °C overnight with shaking at 200 rpm. The bacteria were diluted to OD_{600} ~ 0.01 in 800 ml LB medium with 100 µg/ml Ampicillin and incubated until reaching OD_{60} ~ 0.4. Then IPTG (0.45 mM final conc.) was added and the bacteria were incubated again at 37 °C, 200 rpm until reaching OD_{600} ~ 2.0. Cells were sedimented by centrifugation at 6000 g for 20 minutes (JA-10 rotor). The pellets were washed by phosphate buffer saline, pH 7.4, and spun down at 6000

g for 20 minutes. Each 15 grams of wet cells were re-suspended with 75 ml of MOPS solution (50 mM 3-N-morpholino-propanesulfonic acid, 100 mM NaCl and protease inhibitor (cOmplete^{TM}, Roche). The cells were disrupted by passing three times though a Stansted cell homogenizer at 1.8 kb. Unbroken cells were centrifuged at 9500 g (Ja-3050-ti rotor) for 20 minutes. Subsequently, the supernatant was pelleted by ultracentrifugation 250,000 g (70-ti rotor) for 75 minutes at 4°C. The pellet was re-suspended in MOPS solution and the protein concentration was measured using the BCA Protein Assay kit (Pierce) as described by the manufacturer. The concentration was adjusted to 10 mg/ml and incubated in MOPS solution containing 1% DDM (final conc.) at 4 °C for an hour with gentle shaking. Un-solubilized material was sedimented by ultracentrifugation at 250,000 g at 4 °C for 15 min (70-ti rotor). The collected supernatant was applied on streptactin column at 4 °C (cold room) and the flowthrough was collected. The column was washed with washing buffer (50 mM sodium phosphate, 300 mM NaCl, protease inhibitor (cOmplete), containing 0.01% DDM, pH 8.0) to remove unspecific protein binding and the flow-through was collected again. The elution buffer (50 mM sodium phosphate, 300 mM NaCl, protease inhibitor (C0mplete EDTA free), 0.01% DDM, and 2.5 mM desthiobiotin pH 8.0) was added to the column at 4 °C to elute the protein.

Purification of cytochrome *bd* from *Geobacillus thermodenitrificans* and from *Corynebacterium glutamicum*

Cytochrome *bd* from *G. thermodenitrificans* was extracted and purified from membrane fractions of *G. thermodenitrificans* K1041/pSTE-*cbdAB* recombinant cells with two consecutive column chromatography of DEAE-Toyopearl and hydroxyapatite in the presence of 0.5%(w/v) MEGA9+10, as

described previously in Arutyunyan *et al.* 2012[44]. Cytochrome *bd* from *C. glutamicum* was extracted and purified from membrane fractions of *C. glutamicum ΔctaD*/pPC4-*cydABDC* recombinant cells[45] with two consecutive chromatography of hydroxyapatite and then DEAE-Toyopearl in the presence of 0.05%(w/v) DDM.

Purification of *E. coli* cytochrome bo_3.

Cytochrome bo_3 was extracted and purified from *E. coli* cytoplasmic membranes based on Rumbley *et al*. 1997[35], with modifications as described in Hards et al. 2018[36]. *E. coli* cytoplasmic membranes were prepared from strain GO105/pJRhisA in which cbo_3 is overexpressed. *E. coli* was aerobically grown to mid-log phase at 37 °C in LB medium supplemented with 500 µM $CuSO_4$and 100 µg ml^{-1} carbenicillin. Cells were harvested by centrifugation at 10,000 × *g* for 10 min and the pellets were washed and repelleted twice with buffer A (20 mM (3-N-morpholino-propanesulfonic acid (MOPS), 30 mM Na_2SO_4, pH 7.4). Cells were then resuspended in buffer A containing a mini protease inhibitor tablet (cOmplete) per 50 mL, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml pancreatic DNase, and lysed by two passages through a French Press at 20,000 psi. Any debris and unbroken cells were removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was then ultracentrifuged (200,000 × *g*, 45 min, 4°C) and the membrane pellet resuspended in buffer B (20 mM MOPS, 30 mM Na_2SO_4, 25% w/w sucrose, pH 7.4). The suspension was applied to the top of a 30% w/w to 55% w/w sucrose gradient and ultracentrifugation (130,000 × *g*, 16 h, 4°C) with no deceleration or breaking to separate inner membrane from outer membrane. The inner membrane fraction was removed from the sucrose gradient and washed 3 times with buffer A by ultracentrifugation (200,000 × *g*, 45 min,

4°C). Inner membranes were then resuspended in buffer A and either used immediately for purification or stored in aliquots at −80°C until use. To extract cytochrome bo_3, inner membrane fractions were diluted to 5 mg/mL protein content with solubilization buffer (20 mM Tris HCl, pH 8.0, 5 mM $MgSO_4$, 10% glycerol, 300 mM NaCl, 1% DDM, 10 mM imidazole) and incubated at 30°C for 30 min with gentle inversion every 5 min. The unsolubilized material was removed by ultracentrifugation (200,000 × *g*, 45 min, 4°C), and the supernatant was applied to a Nickel-Sepharose High Performance (GE Healthcare) column that was previously washed with water and equilibrated with IMAC buffer (50 mM Tris/HCl, pH 8.0, 5 mM $MgSO_4$, 10% glycerol, 0.01% DDM, 300 mM NaCl) containing 10 mM imidazole. To remove contaminating proteins, the resin was washed with IMAC buffer containing 30 mM imidazole and 150 mM NaCl, and cytochrome bo_3 was eluted with IMAC buffer containing with 200 mM imidazole, 150 mM NaCl, and 20% glycerol. The red cytochrome bo_3 containing fractions were pooled and concentrated to 6.57 mg mL^{-1} using an Amicon Ultra centrifugal filter devices with100,000 Da molecular weight cutoff.

Oxygen consumption activity assay

Oxygen consumption by purified cytochrome *bd* and cytochrome bo_3 was measured using a Clark-type electrode as previously described in Lu *et al*. 2018[46], with modifications as in Goojani *et al*. 2020[31]. Briefly, the electrode was fully aerated (212 µM O_2 at 37 °C) and calibrated with sodium hydrosulfite. The purified enzymes (final conc: 2 nM for cytochrome *bd* from *E. coli*, 10 nM for cytochrome *bd* from *G. thermodenitricifans*, 2.8 nM for cytochrome *bd* from *C. glutamicum*, 5 nM for cytochrome bo_3) were pre-incubated for three minutes with CL (and with inhibitors, if applicable) in a

pre-warmed (37°C) buffer containing 50 mM 3-N-morpholino-propanesulfonic acid (MOPS), 100 mM NaCl and 0.025% DDM, pH 7.5. Ubiquinone-1 (Sigma) and menaquinone-1 (Santa Cruz Biotechnology) were dissolved in absolute ethanol (20 mM stock) and the reducing agent dithiothreitol (1 M stock) in 50 mM HEPES (4- (2-hydroxyethyl) piperazine-1-ethanesulfonic acid), pH 7.75. Quinone stock and DTT stock were mixed in 1:1 volume ratio and incubated for 3 min (ubiquinone-1/DTT) or 6 min (menaquinone-1/DTT) at 37 °C. The oxygen consumption reaction was initiated by adding the quinone/DTT mixture (final concentration 200 μM quinone and 10 mM DTT) to the assay mixture, respiration was measured for 3 minutes. The enzymatic activity was calculated for the period from 30 s - 60 s after starting the reaction.

Data availability

All data generated or analyzed during this study are included in this published article.

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AUTHOR CONTRIBUTIONS

A.H.A. and A.G.H. performed experiments; A.H.A., H.L., A.G.H. and H.G.G. designed experiments and/or analyzed data; D.G.G.M. J.S. and D.B. supervised and coordinated experiments; A.H.A. D.G.G.M and D.B. wrote the manuscript with contributions from all co-authors, D.B. and D.G.G.M. supervised the overall research.

COMPETING FINANCIAL INTEREST

The authors declare no competing financial interests.