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Stimulation of cytochrome *c* oxidase activity by detergents

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ABSTRACT

Cytochrome *c* oxidase (CytcO) is an integral membrane protein, which catalyzes four-electron reduction of oxygen linked to proton uptake and pumping. Amphipathic molecules bind in sites near the so-called K proton pathway of CytcO to reversibly modulate its activity. However, purification of CytcO for mechanistic studies typically involves the use of detergents, which may interfere with binding of these regulatory molecules. Here, we investigated the CytcO enzymatic activity as well as intramolecular electron transfer linked to proton transfer upon addition of different detergents to bovine heart mitoplasts. The CytcO activity increased upon addition of alkyl glucosides (DDM and DM) and the steroid analog GDN. The maximum stimulating effect was observed for DDM and DM, and the half-stimulating effect correlated with their CMC values. With GDN the stimulation effect was smaller and occurred at a concentration higher than CMC. A kinetic analysis suggests that the stimulation of activity is due to removal of a ligand bound near the K proton pathway, which indicates that in the native membrane this site is occupied to yield a lower than maximal possible CytcO activity. Possible functional consequences are discussed.

1. Introduction

Cytochrome *c* oxidase (CytcO) is an integral membrane-bound enzyme, which is a terminal component of the respiratory chains in eukaryotes and aerobic prokaryotes. The enzyme catalyzes oxidation of cytochrome (cyt.) *c* and reduction of O_2 to H_2O, which is linked to transmembrane charge separation and proton pumping to maintain a transmembrane electrochemical proton gradient. During catalysis, reduced cyt. *c* binds to CytcO at the membrane positive (*p*) side, near the primary electron acceptor, Cu_A. Electrons are then transferred from cyt. *c*, consecutively to Cu_A, heme *a* and the catalytic site, which is composed of heme a_3 and Cu_B. Each electron transfer from cyt. *c* to the catalytic site is linked to proton uptake from the membrane negative (*n*) side, which results in a charge separation across the membrane. In addition, each electron transfer from cyt. *c* is linked to transmembrane proton pumping, from the *n* to the *p* side (for review, see [1–3]).

Initial reduction of the oxidized catalytic site by two electrons is linked to the uptake of two protons from the *n* side to the catalytic site through the K pathway [4,5], named after a conserved Lys319 residue (*Bos taurus* CytcO amino-acid residue numbering) (Fig. 1). Upon two-electron reduction, heme a_3 binds dioxygen and further reduction of O_2 is linked to proton uptake from the *n* side through the D pathway, which starts near a conserved Asp91 that is connected by a number of water molecules to the conserved Glu242 residue (Fig. 1). A third, H pathway has also been suggested to be used for transfer of pumped proton in the mammalian CytcO, but the involvement of this pathway remains controversial and it has neither been identified in the bacterial [6] nor in the *S. cerevisiae* mitochondrial CytcOs [7,8].

The electron flux through the respiratory chain is presumably regulated by CytcO, which determines the oxidative phosphorylation rate [9–12]. Several regulatory mechanisms have been identified, for example, by expression of subunit isoforms [13], by phosphorylation of specific sites [14] or binding of thyroid hormones [15]. In addition, several conserved lipid binding sites have been identified in structures of CytcOs [16,17]. These lipid molecules are in many cases integral, structural components, but binding to hydrophobic sites may also regulate the enzyme's activity [18]. Detailed functional and structural studies of the *Rhodobacter sphaeroides* CytcO revealed that binding of detergents, steroids, and bile acids at a site near the K proton pathway display complex effects on proton transfer and thereby the activity of CytcO [19]. In a recent study, the interaction of mammalian CytcO with the steroids glycodiosgenin (GDN) and cholesteryl hemisuccinate (CHS) was investigated using cryogenic electron microscopy (cryo-EM), kinetic studies and molecular simulations [20]. Binding of these steroids results in reversibly lowering the CytcO activity and the kinetic studies showed that binding of both GDN and CHS results in slowing proton transfer

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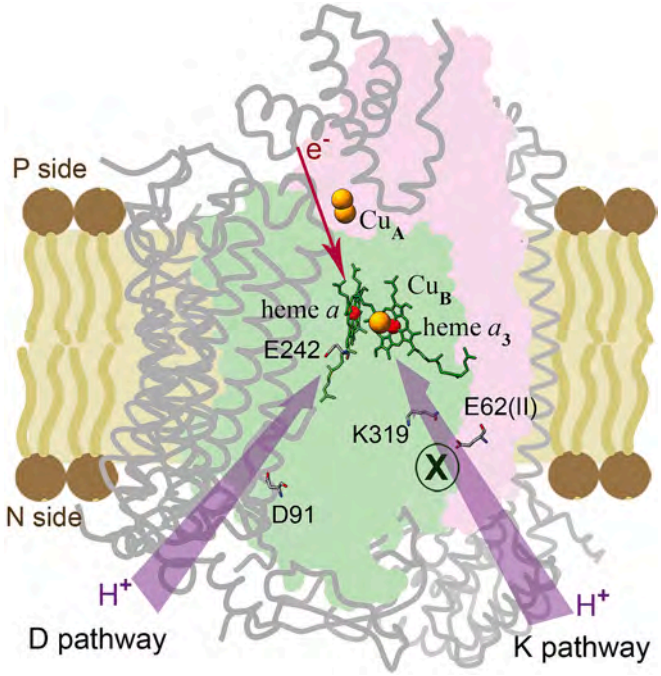

Fig. 1. Schematic view of bovine heart CytcO. Subunit I, harboring the redox centers heme *a* and the catalytic site composed of heme a_3 and Cu_B, is shown in light green. Subunit II harboring the electron entry point from Cu_A is shown in light-purple. All other subunits are shown as grey cartoons. Selected amino acid residues of the D (E242 and D91) and K (K319 and E62(II)) pathways are shown. The "X" marks the presence of a native regulator molecule, which binds and slows proton transfer through the K pathway. The figure is based on the PDB ID: 2dyr.pdb [46]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

through the K pathway upon reduction of the catalytic site. Cryo-EM revealed a novel steroid binding site near the K proton pathway while simulations showed that the conformational dynamics of key K pathway residues is modulated upon steroid binding to this site [20].

Mechanistic studies of CytcO are typically conducted with purified CytcO, extracted from native phospholipid membranes using detergents. These detergents primarily replace the native bilayers by forming a belt around a hydrophobic surface of the membrane protein [21]. However, detergent molecules may also replace integral lipid molecules or act to remove these lipid molecules thereby resulting in functional changes. For example, solubilization of rat liver mitoplasts results in a substantial increase of the CytcO activity [22], which presumably is a manifestation of changes in electron and proton transfer reactions in CytcO [23].

In this study, we investigated the effect of addition of a number of detergents to mitoplasts of bovine heart, *S. cerevisiae* and *R. sphaeroides* membranes. The activity of CytcO was measured as a function of added detergent concentration and we also investigated the effect of detergent on intramolecular electron and proton transfer. The data show that in the native environment, proton transfer through the K pathway is slower than that after treatment with e.g. DDM. In other words, the CytcO activity in the native setting is not maximized, presumably due to binding of a steroid moiety near the K proton pathway in the native membrane.

2. Results

2.1. CytcO activity upon addition of detergents to native membranes

Addition of increasing concentrations of DDM to bovine heart mitoplasts resulted in a progressive increase of CytcO activity to reach a maximum ~10-fold stimulation at 0.02 % DDM (Fig. 2, grey trace). For each addition, the increase in activity occurred instantaneously, i.e.

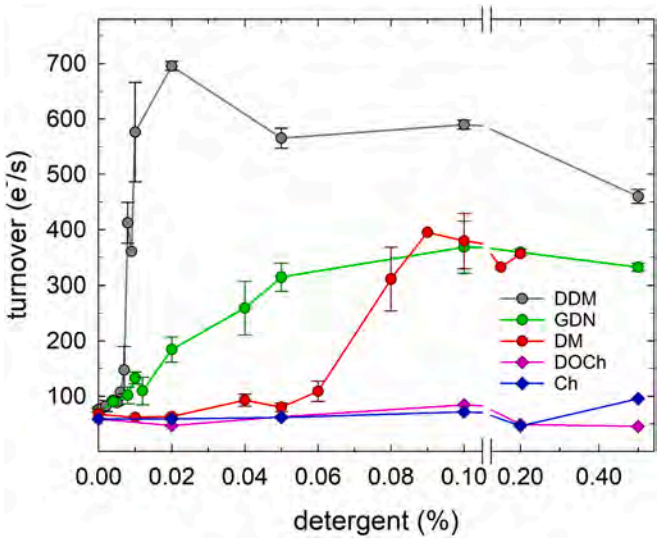

Fig. 2. Effect of addition of alkyl glucosides and GDN on CytcO activity in bovine mitoplasts. Grey, green and red circles are for titrations with DDM, GDN or DM, respectively. Pink and blue diamonds are for sodium deoxycholate and sodium cholate, respectively. The assay medium was composed of: 20 mM KH_2PO_4-KOH (pH 7.4), 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.25 M mannitol, 10 mM sodium ascorbate, 100 μM TMPD and 50 μM equine cyt. *c*. The protein concentration was 0.016 mg/ml. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

within ~10 s. Further increase of the DDM concentration up to 0.5 % resulted in a gradual decline of the CytcO activity. The initial increase in activity is unrelated to uncoupling of the membrane as it occurred also in the presence of the H^+ and K^+ ionophores FCCP (carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone) and valinomycin. The stimulating effect of DDM was also observed for yeast mitoplasts from *S. cerevisiae* where the addition of 0.05 % DDM enhanced the activity about 4-fold, from 220 ± 30 e^-/s to 960 ± 100 e^-/s (SD, 7 measurements).

DDM belongs to a group of non-ionic detergents, alkyl glucosides. Addition of two other alkyl glucosides to bovine heart mitoplasts, DM (*n*-decyl β-D-maltopyranoside) (Fig. 2, red trace) or OG (octyl β-D-glucopyranoside) (Table 1), also resulted in stimulation of the CytcO activity, by factors of ~7 or ~ 3, respectively.

To investigate if the stimulating effect was unique for alkyl glucosides, we studied the effect of other detergents used for solubilization of CytcO, such as steroid derivatives GDN (Fig. 2, green trace) and digitonin (supplementary Fig. S1, red trace), and a PEG-derivative Triton X100 (supplementary Fig. S1, blue trace), all being non-ionic detergents. The activity enhancement was observed for all these detergents. However, with Triton X100 the fold of stimulation was much lower than for the other detergents. Here, an initial stimulation was followed by a

Table 1
Enhancement of CytcO activity in bovine heart mitoplasts by detergents. The protein concentration was 0.016 mg/ml (for reaction medium, see Materials and Methods).

Detergent concentration at half-stimulation	Maximum activity (e^-/s) (the fold of enhancement in parenthesis)	CMC (mM)
no detergent	60	
0.01 % DDM (0.2 mM)	600 (10)	0.18
0.07 % DM (1.4 mM)	400 (6.7)	2.2
0.025 % GDN (0.21 mM)	400 (6.7)	0.018
0.07 % Digitonin (0.57 mM)	300 (5.0)	0.2
0.75 % OG (25.7 mM)	160 (2.7)	19–25
0.015 % Triton X100 (0.2 mM)	120 (2.0)	0.25

CMC values are from [21], except for GDN [32] and for Digitonin [63].

decrease in activity at higher detergent concentrations to reach a level below that seen without addition of detergent, qualitatively similar to earlier observations [24].

As seen in Table 1, 50 % activity enhancement was observed at concentrations close to the corresponding CMC values, independently of the degree of stimulation. An exception was GDN, for which the increase in activity was observed at concentrations one order of magnitude higher than the CMC value. For a structurally similar detergent digitonin, the stimulating concentration was 2–3-times higher than its CMC.

Addition of anionic detergents, such as salts of bile acids, sodium cholate or sodium deoxyholate, did not result in any changes in CytcO activity (Fig. 2, blue and pink traces, respectively, and Table 1), which is different from the small lowering in activity observed previously [24].

2.2. Activity at different cytochrome c concentrations

The increased CytcO activity upon solubilization of the mitochondrial membrane was explained in terms of changes in the affinity for cyt. *c* due to changes in the lipid environment [22]. To investigate if the observed modulation of activity upon addition of detergent was caused by changes in the binding constant of cyt. *c* to CytcO, we also measured the turnover activity as a function of detergent concentration at different cyt. *c* concentrations: 1 μM, 50 μM and 150 μM cyt. *c*. As seen in supplementary Fig. S2A, the stimulation pattern for DDM was unaltered, which indicates that the binding constant for cyt. *c* was independent of detergent concentration. For GDN, the stimulating effect was slightly shifted to higher or lower GDN concentrations for 150 μM cyt. *c* or 1 μM cyt. *c*, respectively (supplementary Fig. S2B).

2.3. Solubilization of CytcO

We examined whether or not CytcO was solubilized at DDM or GDN concentrations at which the stimulation in activity was observed. The experiments were designed to reproduce conditions in the oxygraph chamber during titration with detergents. Mitoplasts were incubated with the detergents 0.02 % DDM or 0.05 % GDN (the concentrations, which are sufficient to reach maximal stimulation of the CytcO activity (c.f. Fig. 2) and the samples were centrifuged to separate the solubilized material from membranes (see Materials and Methods). The solubilized CytcO concentration in supernatants was determined from the reduced minus oxidized difference absorption spectra. The fraction of solubilized CytcO was 45 ± 6 % (SD of 4 experiments) for 0.02 % DDM, but only 7 ± 3 % (SD of 4 experiments) for 0.05 % GDN.

The fractional solubilization as a result of addition of detergent to mitoplasts is also evident from changes in light scattering of the mitoplast suspension. For example, upon addition of DDM or DM, light scattering decreased by 40 % or 30 %, respectively, for bovine heart mitoplasts (supplementary Fig. S3AB, respectively). Increase of the membrane concentration in the samples resulted in a shift of the detergent effect toward higher concentrations because the detergent: membrane lipid ratio controls solubilization. The effect of GDN was small (supplementary Fig. S3C), consistent with a low degree of solubilization.

2.4. Effect of detergents on R. sphaeroides CytcO

The influence of DDM or GDN on the CytcO activity was also investigated with bacterial *R. sphaeroides* membranes. As with the mammalian mitochondria membranes, we observed stimulation of activity with increasing detergent concentrations, but the stimulating effect was smaller; the activity increased by factors of 2 or 1.5 for DDM and GDN, respectively (supplementary Fig. S4). Interestingly, with GDN the stimulation in activity occurred at substantially lower concentrations than for mitoplasts and the half maximum value was observed closer to the CMC value.

2.5. Detergent effect on CytcO incorporated into liposomes

To test whether or not the effects of detergent addition was related to the presence of a surrounding membrane, we investigated the effect of added detergent to azolectin proteoliposomes with reconstituted detergent-purified bovine CytcO. After the reconstitution was completed, the concentration of remaining detergent in the sample was estimated to be <1 ppm, based on the degree of dilution during each step of dialysis.

In these experiments the CytcO O_2-reduction activity was ~40 electrons/s (Fig. 3, no additions). The lower activity than that for detergent-solubilized CytcO is caused by the buildup of an electrochemical proton gradient during turnover; upon dissipation of the electrochemical proton gradient by addition of FCCP and valinomycin the O_2-reduction activity increased to ~250 s^{-1} (Fig. 3, FCCP+valinomycin). Addition of DDM to the uncoupled liposomes did not result in any changes in activity (Fig. 3, black bars), while addition of GDN resulted in inhibition of the activity (Fig. 3, grey bars), consistent with results from earlier studies [20].

2.6. Detergent effect on intramolecular electron transfer in CytcO

Results from earlier studies showed that proton transfer through the K pathway in detergent-solubilized CytcO is inhibited upon addition of steroids [19,20,25]. The kinetics of proton transfer via this pathway is investigated by measuring absorbance changes associated with intramolecular electron transfer, linked to proton transfer, upon flash photolysis of the heme a_3-CO bond in mixed-valence CytcO in the absence of O_2. In this state the catalytic site (heme a_3 and Cu_B) is reduced while heme *a* and Cu_A are oxidized. Laser flash-induced dissociation of CO results in lowering the apparent midpoint potential of heme a_3, which results in electron equilibration between hemes a_3 and *a* [26,27].

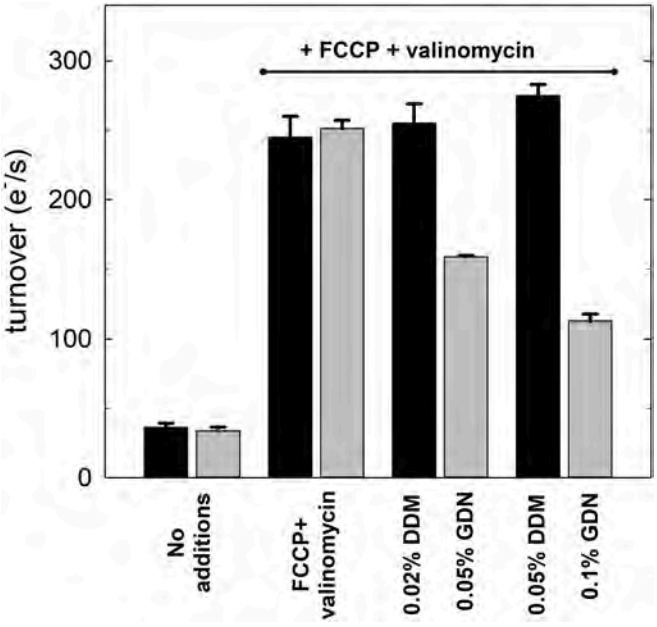

Fig. 3. Effect of detergents on CytcO activity in liposomes. The incubation medium was composed of: 20 mM KH_2PO_4-KOH (pH 7.4); 10 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 10 mM sodium ascorbate; 100 μM TMPD and 50 μM equine cytochrome *c*. The concentrations of lipids was 0.2 mg/ml; CytcO, 11 nM. Uncouplers (when present): 1 μM FCCP and 0.1 μM valinomycin. Except for the 1st two bars, all measurements were done in the presence of uncouplers with sequential additions of detergents, where separate samples were titrated with DDM (black bars) or with GDN (grey bars). Additions are shown in the graph. In all measurements, an 80 % CytcO orientation with the cyt. *c*-binding site to the outside is taken into account.

Results from earlier studies with detergent-solubilized CytcO showed that the electron transfer occurs in two main kinetic phases with rate constants of ~3*10^5 s^{-1}(τ ≅ 3 μs) and ~ 10^3 s^{-1} at pH 8 (τ ≅ 1 ms) [28–30]. The first component corresponds to electron transfer from heme a_3 to heme *a* while the slower component, referred to as the "ms phase", corresponds to further electron transfer to heme *a*, linked to rate limiting proton release to the bulk solution through the K-pathway [5]. The latter intramolecular electron transfer is followed in time spectrophotometrically at 598 nm, which is an isosbestic point for spectral changes associated with CO-dissociation/recombination reaction. In this wavelength region the molar extinction of reduced heme *a* is about 4-fold higher than that of the heme a_3. Therefore, an increase in absorbance is indicative of electron transfer from heme a_3 to heme *a*. Here, we investigated the proton-coupled electron transfer (ms phase) (*i*) in native membranes (SMPs) from bovine heart mitochondria (Fig. 4, black trace), (*ii*) after treatment of SMPs with SMA (styrene-maleic acid copolymer) and separation of a light membrane fraction, which results in a lower degree of light scattering (Fig. 4, red trace), (*iii*) in SMP after addition of 0.5 % DDM (at the same detergent: protein ratio as used in polarographic studies) (Fig. 4, grey trace), (*iv*) in proteoliposomes with reconstituted CytcO (Fig. 4, green trace). Solubilized CytcO from bovine heart in the presence of 0.05 % DDM was used as a reference (Fig. 4, blue trace). Submitochondrial particles (SMPs) were used instead of mitoplasts because the former yield less light scattering. Treatment with SMA allowed to "extract" CytcO in the presence of the native membrane in CytcO-SMA nanodiscs [31].

As observed earlier, for detergent solubilized Cytc*O*, the "ms phase" displayed a time constant of ~1.2 ms at pH 8.2–8.3 (Fig. 4, blue trace,

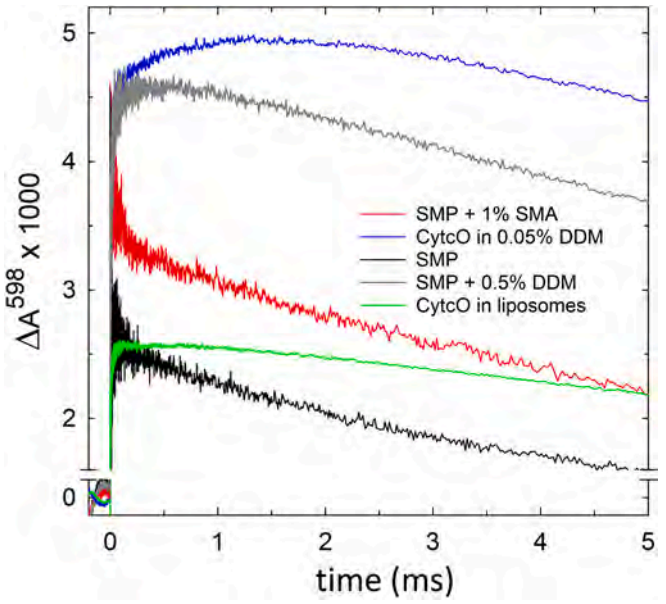

Fig. 4. Internal electron transfer in CytcO in a membrane environment and in detergent. Black: SMP; Red: SMP treated with 1 % SMA, the supernatant containing SMA-CytcO nanodiscs; Grey: SMP in the presence of 0.5 % DDM; Blue: purified CytcO in 0.05 % DDM; green: CytcO incorporated into proteoliposomes. The protein concentration for SMPs was 3 mg/ml. The CytcO concentration was ~2 μM for SMPs; 1.5 μM for the SMA-CytcO nanodiscs and 4 μM for solubilized CytcO. The reaction medium for SMPs and SMA-CytcO nanodiscs was: 0.25 M sucrose; 50 mM KH_2PO_4-KOH (pH 8.2); 0.1 mM EDTA. The reaction medium for proteoliposomes and purified CytcO was: 50 mM KH_2PO_4-KOH (pH 8.2); 20 mM KCl; 0.1 mM EDTA; it was supplemented with 0.05 % DDM in case of purified CytcO. The traces were normalized to 1 μM reactive CytcO, as calculated from the amplitude of a laser-induced absorbance change at time = 0 at 445 nm using a molar extinction coefficient of 67 mM^{-1} cm^{-1} [64]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increase in absorbance in the time range 0 - ~1.5 ms). The following decrease in absorbance is associated with CO recombination while the electron is transferred back to heme a_3. The ms-phase was also observed upon reconstitution of the detergent-solubilized CytcO into proteoliposomes (Fig. 4, green trace), although here the amplitude was smaller and therefore seen as flat region in the time range 0 - ~2 ms. In the native membrane environment, in SMPs (Fig. 4, black trace) or SMA-nanodiscs (Fig. 4, red trace), the ms-phase was not observed as reflected in a decrease in absorbance (CO recombination) over the entire time range. Addition of DDM to the SMPs resulted in appearance of the ms-phase component (Fig. 4, grey trace), seen as an increase in absorbance over a time scale of 0 - ~1 ms.

3. Discussion

The detergents used in this study are classified as ionic, such as bile acids cholate and deoxycholate, or non-ionic such as alkyl glucosides (*n*-dodecyl β-D-maltoside, DDM; *n*-decyl-β-D-maltopyranoside, DM and octyl β-D-glucopyranoside, OG), steroid derivatives (digitonin, glycodiosgenin, GDN) or PEG derivatives (Triton X100). The non-ionic detergent DDM is commonly used for purification of CytcO. The steroid analogue GDN is a synthetic mimic of digitonin [32,33], which has the ability to maintain integrity of respiratory supercomplexes (see e.g. [34–37].

Addition of alkyl glucosides (DDM, DM), at concentrations close to their CMC values, to bovine heart mitoplasts caused enhancement of the Cytc*O* oxidase activity (Fig. 2 and Table 1). This effect of detergent addition was also observed for Cytc*O*-SMA nanodiscs, i.e. CytcO initially purified without the use of detergent [31]. Addition of the steroid analogues, GDN or digitonin, also caused an increase of Cytc*O* activity in native membranes although for GDN the stimulation was obtained at concentrations 10-fold higher (0.025 %) than its CMC value (0.002 %) (see Fig. 2 and Table 1). Results from an earlier study showed that GDN binds to a sterol binding site in mammalian CytcO after purification using a detergent that presumably removes the native sterol moiety, and binding of GDN results in inhibition of the enzyme [20]. Here, the distinct effect of GDN addition to mitoplasts, in which the inhibitory sterol binding site is occupied, could be explained by competition of GDN and the native sterol moiety for the same site. Upon addition of GDN, the native sterol would be displaced, but at the same time GDN would bind. If the inhibitory effect of GDN [20] is weaker than that of the native sterol, it could result in an increase in activity at higher GDN concentrations irrelevant to its CMC. The two overlaid effects of GDN could also explain the more complex effect of cyt. *c* concentration on turnover activity observed with GDN than with DDM (supplementary Fig. S2).

The increase in activity upon addition of DDM or DM occurred under conditions where partial solubilization of the membranes was observed. However, with GDN no visible solubilization was observed under conditions when the activity increased, which suggests that solubilization itself does not result in an enhancement of the activity. This conclusion is also supported by the observation that the activity of Cytc*O* in uncoupled liposomes was similar to that in detergent solution.

Qualitatively, the Cytc*O* activity enhancement was also observed for *S. cerevisiae* mitoplasts and in membrane particles from the bacterium *R. sphaeroides*. Notably, cholesterol is not present in the *R. sphaeroides* membranes. However, based on *in vitro* binding and computational studies, Buhrow et al. [25] concluded that the regulatory conserved bile acid site is polyspecific, i.e. different ligands such as ATP, GDP, steroids, retinoic acid, T3 thyroid hormone and porphyrins could bind to this site. *S. cerevisiae* harbors the sterol moiety ergosterol, which is enriched in the inner mitochondrial membrane [38,39].

Results from earlier studies revealed detergent-binding sites in CytcO [17,25,40,41]. For example, a bile acid/sterol binding site was identified at the entrance to the K pathway in the purified CytcO from *R. sphaeroides*. A recent combined kinetic, structural and molecular

simulations study showed that sterols, such as GDN or CHS inhibit Cyt*c*O reversibly by binding to a site near the K pathway of the mammalian Cyt*c*O [20]. Because Cyt*c*O turnover is rate limited by reduction of the catalytic site and this reduction rate is proportional to the proton-uptake rate through the K pathway [42] (see also below), any change in proton uptake kinetics would act to modulate the turnover rate. This effect is also evident from studies in which residues of the K pathway were modified by site-directed mutagenesis [43]. Furthermore, molecular simulations suggested that upon binding of the sterol, the conformational dynamics of key residues of the K pathway is modulated, which acts to decrease proton-transfer kinetics through this pathway [20]. Thus, it is likely that proton uptake through the K pathway would be sensitive to binding of any ligand that would either interact directly with K pathway residues or result in steric changes of these residues. [42].

In the present study, in native membranes of SMPs or for Cyt*c*O isolated in SMA nanodiscs, the ms-phase was impaired, indicating that the slower activity of Cyt*c*O in the native membrane is explained by binding of a ligand near the K pathway. Accordingly, addition of DDM to native membranes resulted in acceleration of proton transfer through the K pathway, observed as an appearance of the ms-phase (see Fig. 4, compare grey and black traces) and an increase in activity (Fig. 2). The ms-phase was observed with detergent-solubilized Cyt*c*O that was reconstituted into proteoliposomes, which suggests that appearance of the ms-phase in SMPs upon addition of detergent is related to exposure of the Cyt*c*O to detergent and not to removal of the lipid membrane. We also note that earlier studies with bacterial Cyt*c*O showed an acceleration of the ms-phase component upon reconstitution into membrane environment [44] due to a proton equilibrium of Cyt*c*O of the K pathway surface protonatable groups with phosphate groups of the lipid "head groups" [45]. This acceleration was not observed for the bovine enzyme reconstituted into proteoliposomes in the current study, presumably due to the presence of additional subunits that protonically "isolate" the K pathway entrance from the membrane surface. Alternatively, the proton uptake in mammalian Cyt*c*O may be guided by cardiolipin [18] that co-purifies with the enzyme and is thus present in both the detergent-purified and membrane-bound Cyt*c*O [46].

A comparison of results from the earlier study [20] with those from the current study, suggests that in the native membrane the sterol-binding site is occupied, presumably by cholesterol, which is a component of the mitochondrial membrane (see X mark in Fig. 1). Removal of this moiety upon addition of the non-ionic detergents DDM or DM would result in increasing the Cyt*c*O activity, which coincides with solubilization. This observation is also consistent with the data with liposome-reconstituted Cyt*c*O as the enzyme is exposed to detergent during purification and therefore the sterol-binding site would be unoccupied already before membrane reconstitution.

The observation that addition of all tested detergents qualitatively resulted in an enhancement in Cyt*c*O activity suggests that this effect is not specific (except for GDN, see above). One possibility is that the regulatory binding site in Cyt*c*O is polyspecific as suggested for the *R. sphaeroides* Cyt*c*O [25]. Another, more likely, possibility is that the native sterol moiety is soluble in all tested detergents and partitions into solution resulting in an increase in the activity.

The data from the present study indicates that in the native membrane, the sterol-binding site in Cyt*c*O is occupied, which results in lower activity than that measured with an unoccupied site. The question then arises, why the Cyt*c*O activity is lower than the maximally possible. We note that in native membranes the fraction reduced cyt. *c* at steady state in a mitochondrion is ~10 % [47,48], which indicates sufficient Cyt*c*O activity to keep ~90 % of the cyt. *c* pool in the oxidized state. Internal electron transfer from Cu_A to the catalytic site is fast (ns-μs range) [29,49] and predetermined by conserved distances between the redox sites and reorganization energy [50]. Verkhovsky et al. [42] noted that when reduction begins during turnover, the midpoint potentials of heme a_3 and Cu_B are low such that only a small fraction of the redox sites is reduced. Reduction of the catalytic site is then stabilized by uptake of protons through the K pathway. In other words, the reduction rate of Cyt*c*O and, hence, the turnover rate, is determined by the fraction of reduced catalytic site and the proton-transfer rate through the K pathway. Therefore, modulation of the latter, e.g. by binding of a ligand to a regulatory site near the K pathway, directly controls the turnover rate of Cyt*c*O. As pointed out by Verkhovsky et al. [42], this control is key to assuring that the redox state of the respiratory chain as a whole is maintained at a fixed and stable level [42].

4. Materials and methods

4.1. Reagents

Detergents were purchased from the following companies: DDM (n-dodecyl β-D-maltoside), Glycon; DM (n-Decyl β-maltoside), Anatrace; GDN (GDN101), Anatrace; digitonin, Calbiochem; OG (n-Octyl-β-D-Glucopyranoside), Glycon; sodium cholate and sodium deoxycholate, Sigma; Triton X100, Sigma. A styrene-maleic acid copolymer solution was prepared from the anhydride powder SMA EF30 (styrene to maleic anhydride ratio of 3:1), Cray Valley, by alkali hydrolysis in water as previously described [31].

4.2. Bovine mitochondria and Cyt*c*O purification

Mitochondria (heavy fraction, see below) were isolated from bovine heart as described in [51], with some modifications. Briefly, grounded tissue (400 g) was mixed with 1.2 l of 0.25 M sucrose; 0.01 M Tris-HCl (pH 7.8), supplemented with 5 ml of 2 M Tris base and immediately treated in a blender for 45 s; the pH was measured and adjusted to 7.8, if necessary. Cell debris was separated by centrifugation at 1600 *g* for 10 min at 4 °C. The supernatant was filtered through two layers of cheese cloth and the mitochondrial fraction was collected by centrifugation at 17000 *g* for 30 min at 4 °C. The supernatant and lipid residues were removed, the pellet was suspended in the same buffer and collected by centrifugation as described above. This washing procedure was repeated two additional times. Finally, two fractions of the mitochondrial pellet were collected separately: a light-colored fraction (referred to as the light fraction) and a dense dark pellet fraction (referred as heavy fraction). Each fraction was flash-frozen in liquid nitrogen and kept at −80 °C. The heavy fraction was used for mitoplast preparation (see below).

The light fraction was used for purification of Cyt*c*O by the protocol [52] with some modifications. The original protocol, where solubilization of the membranes was achieved with Triton X100, was followed until the step involving chromatography on Sepharose CL-6B. This chromatography step was omitted and the pH of the pooled Cyt*c*O fraction was adjusted to 8.5 with a Tris base solution. Triton X100 was removed using Bio-Beads (Bio-Rad, SM-2). Before use, Bio-Beads were washed with ethanol and water, and the excess of water was removed by filtering through a filter paper (Munktell Filtrak Grade 3) using a Buchner funnel. Bio-Beads were added to the fraction containing Cyt*c*O (0.3 g wet weight per ml) and incubated while stirring at 5 °C for 3 h or overnight. After a change of the solution colour owing to precipitation of Cyt*c*O, it was filtered through a glass filter so that Bio-Beads were retained and the colored filtrate was collected. Bio-Beads on the filter were additionally rinsed with a small amount of 0.2 M NaCl; 20 mM Tris-HCl (pH 8.5). The precipitated Cyt*c*O was collected from the combined filtrate by centrifugation at 30000 *g* for 20 min at 4 °C. The dark-green pellet was gently dissolved in the minimal amount of the same buffer supplemented with 2 % DDM. A small amount of the denatured protein was removed by centrifugation at 500 *g* for 20 min at 4 °C. The supernatant (purified Cyt*c*O solution) was collected, aliquoted, flash-frozen in liquid nitrogen and stored at −80 °C. Protein concentration was estimated by Lowry assay in the presence of SDS as modified for membrane proteins [53].

4.3. Preparation of mitoplasts from bovine heart mitochondria

Mitoplasts were prepared from bovine heart mitochondria (heavy fraction) by incubation in a hypotonic solution. Mitochondria (2 ml; protein concentration 20–30 mg/ml) were pelleted by centrifugation (11,000 *g*; 15 min; 4 °C). The pellet was re-suspended in 10 ml of 60 mM sorbitol; 25 mM KH_2PO_4-KOH (pH 7.4) and incubated on ice for 10 min. Then the suspension was centrifuged as described above. The hypotonic treatment was repeated one more time with the same buffer and two times with a buffer supplemented with 250 mM KCl. Finally, the mitoplasts were suspended in a small volume of this buffer and flash-frozen in liquid nitrogen to be stored at -80 °C.

4.4. S. cerevisiae mitochondria and preparation of mitoplasts

Cultivation of *S. cerevisiae* strain W303a and purification of yeast mitochondria were performed according to [54]. Mitoplast were prepared by hypotonic treatment as described above for bovine heart mitochondria.

4.5. R. sphaeroides membrane purification

Membrane samples of *R. sphaeroides*, purified as described in [55], were kindly provided by Dr. J. Vilhjálmsdóttir, Department of Biochemistry and Biophysics, Stockholm University.

4.6. Preparation of inverted submitochondrial particles

Inverted submitochondrial particles (SMPs), prepared from bovine heart mitochondria (heavy fraction) as described in [56] were kindly provided by Dr. M. Björk, Department of Biochemistry and Biophysics, Stockholm University.

4.7. SMA treatment of SMPs

Submitochondrial particles at 2 mg protein/ml were incubated with 1 % SMA in 0.25 M sucrose; 50 mM KH_2PO_4-KOH (pH 8.3); 0.1 mM EDTA at room temperature for 90 min. The SMA-solubilized fraction was separated by centrifugation at 102000 *g* using a TLA-55 rotor at 4 °C for 45 min.

4.8. Partial solubilization of CytcO from bovine heart mitoplasts

Mitoplasts at 0.017–0.02 mg/ml protein concentration were incubated at room temperature in 20 mM KH_2PO_4-KOH (pH 7.4); 10 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 0.3 M mannitol for 5 min in the presence of 0.02 % DDM or 0.05 % GDN. Then, the samples were centrifugated at 138000 g for 70 min at 4 °C. Supernatants were collected and concentrated with Amicon Ultra centrifugal filter units; cutoff 100 kDa (Merk Millipore).

4.9. Reconstitution of CytcO into proteoliposomes

Proteoliposome were obtained by sonication (see basics in [57–59]. Azolectin (L-α-phosphatidylcholine from soybean, Type IIS; Sigma) at 20 mg/ml was homogenized in 50 mM KH_2PO_4-KOH (pH 8.0), 20 mM KCl, supplemented with 2 % sodium cholate, purged with nitrogen and sonicated on ice for 2–3 min at 40 % amplitude with pulses of 15 s with 15 s pauses (Vibracell Ultrasonic liquid processor VCX130, Sonics). Non-dispersed lipid and titanium particles were removed by centrifugation (10,000 *g*, 10 min, 4^0C). An aliquot of CytcO was added to a final concentration of 0.5–0.6 μM and the sample was incubated at 4^0C for 40 min. Detergents were removed by dialysis against the same buffer (two changes). Proteoliposomes were kept on ice.

4.10. CytcO activity assay

Activity of CytcO was estimated from the oxygen consumption rate monitored with the Clark-electrode using an oxygraph (Hansatech) at a temperature of 25 °C. Activity of mitoplasts from bovine mitochondria was assayed in 20 mM KH_2PO_4-KOH (pH 7.4); 10 mM KCl; 5 mM $MgCl_2$; 0.1 mM EDTA; 0.25 M mannitol with 10 mM sodium ascorbate; 100 μM TMPD (*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride, Sigma) and 50 μM horse heart cyt. *c* (Sigma) as electron donor system. The activity of Cyt*c*O incorporated into liposomes was assayed in the same medium, but without mannitol. The O_2-reducution activity of *R. sphaeroides* membranes was assayed in 20 mM KH_2PO_4-KOH (pH 7.5); 10 mM KCl; 0.1 mM EDTA with the same electron donor system (ascorbate + TMPD + horse heart cyt. *c*). Where applicable, the concentrations of FCCP (Sigma) and valinomycin (Sigma) were 1 μM and 0.1 μM, respectively. The concentration of CytcO was determined from reduced-minus-oxidized difference optical absorption spectra using the molar absorption coefficient for hemes $a + a_3$, $\varepsilon_{604-630} = 24\ mM^{-1}\ cm^{-1}$ [60]. The sample was reduced using a small aliquot of sodium dithionite. Spectra were monitored using Cary100 or Cary 4000 spectrophotometer (Agilent Technologies).

4.11. Intramolecular electron transfer in two-electron reduced CytcO

Samples were placed in Thunberg cuvettes (light path 10 mm), air was exchanged for nitrogen and subsequently to carbon monoxide on a vacuum line. Incubation with carbon monoxide resulted in a partial reduction of redox centers of Cyt*c*O [61,62] and binding of CO to the reduced heme a_3. Reduction of the CytcO and ligand binding were followed spectrophotometrically. The two-electron reduced state with CO bound to heme a_3 has an absorption maximum at ~590 nm (supplementary Fig. S5). In cases where further reduction also of heme *a* was observed, this heme was carefully re-oxidized using a redox buffer composed of 100 μM of each of potassium ferri- and ferrocyanide obtaining a pure two-electron reduced state, as confirmed spectrophotometrically.

Internal electron transfer was initiated by flash-photolysis of the CO complex (Nd:YAG laser; Quantel; 10 ns, 532 nm, 200 mJ). The kinetic traces were recorded in the wavelength range 596–598 nm, adjusted to the isosbestic point for CO-recombination spectral changes, with a digital oscilloscope using a setup from Applied Photophysics Ltd. with a time resolution of ~100 ns.

4.12. Light scattering assay

Light scattering was monitored using fluorometer Eclipse (Agilent Technologies) at excitation/emission wavelength of 470 nm. The protein concentration was in the range 0.06 to 0.17 mg/ml (see Figure legends). The assay medium was the same as for the activity measurements. Where applicable, light-scattering caused by GDN was considered. Signals are presented as ratios of signal amplitudes (intensity of scattered light collected at 90° to the source beam) after detergent addition divided by that before detergent addition.

4.13. Software

Software used for data processing and preparation of figures are SigmaPlot, ChimeraX, Adobe Photoshop and Adobe Illustrator.

CRediT authorship contribution statement

Irina Smirnova: Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Fei Wu:** Investigation, Formal analysis, Data curation. **Peter Brzezinski:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbabio.2024.149509.

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