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Oxidoreductase activity of chromatophores and purified cytochrome bc_1 complex from *Rhodobacter sphaeroides*: a possible role of cardiolipin

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Abstract Osmotic shock was used as a tool to obtain cardiolipin (CL) enriched chromatophores of *Rhodobacter sphaeroides*. After incubation of cells in iso- and hyper-osmotic buffers both chromatophores with a physiological lipid profile (Control) and with an almost doubled amount of CL (CL enriched) were isolated. Spectroscopic properties, reaction centre (RC) and reducible cytochrome (cyt) contents in Control and CL enriched chromatophores were the same. The oxidoreductase activity was found higher for CL enriched than for Control chromatophores, raising from 60±2 to 93±3 mol cyt *c* s^{-1} (mol total cyt *c*)$^{-1}$. Antymicin and myxothiazol were tested to prove that oxidoreductase activity thus measured was mainly attributable to the cyt bc_1 complex. The enzyme was then purified from BH6 strain yielding a partially delipidated and almost inactive cyt bc_1 complex, although the protein was found to maintain its structural integrity in terms of subunit composition. The ability of CL in restoring the activity of the partially delipidated cyt bc_1 complex was proved in micellar systems by addition of exogenous CL. Results here reported indicate that CL affects oxidoreductase activity in the bacterium *Rhodobacter sphaeroides* both in chromatophore and in purified cyt bc_1 complex.

Keywords *Rhodobacter sphaeroides* · Osmotic stress · Cardiolipin · Oxidoreductase activity · Cytochrome bc_1 complex

Abbreviations

CL	cardiolipin
cyt	cytochrome
His-tagged	histidine-tagged
LH1	light harvesting complexes I
LH2	light harvesting complexes II
PC	phosphatidylcholine
Q_i	ubiquinone reduction site
Q_o	ubiquinone oxidation site
RC	reaction center

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Introduction

Rhodobacter sphaeroides is a well-known purple photosynthetic bacterium generally considered a model organism in the photosynthesis studies. All photosynthetic apparatus elements, indeed, have been carefully studied in their structural and functional aspects. The bacterial photosynthetic cycle can be briefly illustrated as follows: light harvesting complexes, LH1 and LH2, absorb light and transfer the resulting excitation to the reaction centre (RC). Here a series

of electron transfer reactions lead to the formation of a stable ubisemiquinone near the cytoplasmic site and to the oxidation of a cyt c_2 protein in the periplasmic space; in a second light-induced turnover the ubisemiquinone is fully reduced to ubiquinol with the uptake of two protons from the cytoplasmic space and a second cyt c_2 is oxidized. The ubiquinol is released by the RC and moves through the lipid bilayer reaching the ubiquinol:cyt c_2 oxidoreductase (cyt bc_1 complex) which, in turn, catalyzes the re-oxidation of ubiquinol to ubiquinone and the reduction of the cyt c_2 releasing the protons into the periplasmic space (Crofts et al. 1999). In *Rhodobacter sphaeroides* actions performed by RC, cyt bc_1 complex and cyt c_2 generate a cyclic electron flow and a proton gradient through the cytoplasmic membrane which is necessary for ATP synthesis, for ion transport and other forms of work (Verméglio and Joliot 1999). The entire photosynthetic apparatus is housed in a series of invaginations in cell membrane called *intracytoplasmic membranes* (ICM) formed during the photosynthetic growth of the bacterium; these membranes can be isolated as a set of small vesicles named chromatophores (Drews and Golecki 1995).

Phospholipids play a major role in the photosynthetic process not only providing the solubilizing environment and separating the cytoplasmic and periplasmic sides, but also directly affecting the enzymatic activity of the membrane proteins involved (Jones 2007; Fyfe et al. 2005). Phospholipids in particular are essential for the activity of cyt bc_1 complex: previous studies carried out on bovine enzyme showed that the delipidated enzyme was completely inactive though it exhibited all the spectral properties of native cyt bc_1 complex. Cardiolipin (CL) has proved to be essential for restoring the full electron transfer activity, probably acting as allosteric stabilizer for the active conformation of the enzyme (Gomez and Robinson 1999). These results point out that the activating effect depends on the nature of the polar head group and is not merely due to a hydrophobic phase effect (Schägger et al. 1990). Similarly, lipid-depleted yeast cyt bc_1 complex resulted stable but catalytically inactive. Addition of CL reversed this effect: a specific role for CL in respiratory supercomplex formation was suggested (Wenz et al. 2009).

In the case of *Rhodobacter sphaeroides*, biochemical data suggest that CL is not homogeneously distributed in the plasma membrane: most of CL present in the total lipid extract of the microorganism grown in anaerobic conditions, arises from chromatophore membrane domains (De Leo et al. 2009; Russel and Harwood 1979). Interestingly CL enriched chromatophores can be isolated from *Rhodobacter sphaeroides* cells exposed to osmotic shock (De Leo et al. 2009). These CL enriched vesicles can be used to investigate possible modifications of enzymatic activity induced by subtle changes in lipid composition of the plasma membrane, avoiding the use of detergents or addition of exogenous lipids.

In this work we have studied the oxidoreductase activity both in chromatophores having a physiological lipid profile and in those enriched in CL. Specific inhibitors were tested to prove that oxidoreductase activity measured in vesicles was mainly attributable to cyt bc_1 complex. This enzyme was then purified from BH6 strain in two ways, using the original protocol (Guergova-Kuras et al. 1999) and a modified one yielding a partially delipidated cyt bc_1 complex. These enzyme preparations were characterized in terms of subunit composition and activity. The effect of CL in restoring the activity of partially delipidated cyt bc_1 complex was tested in micellar systems by addition of exogenous CL.

Experimental procedure

Materials

All chemicals were purchased of the highest purity available and were used without further purification. The reagent grade salts for the bacterial liquid medium and for Tris–HCl (pH 8.0) buffer solutions, organic solvents for lipid extraction, horse heart cyt *c*, decylubiquinol, antimycin, myxothiazol, kanamycin and tetracycline were purchased from Sigma. Standard CL (from bovine heart) and PC (from soybean) were purchased from Avanti Polar Lipids. TLC plates (Silica gel 60A) were obtained from Merck. Ni-NTA agarose resin was purchased from Quiagen. All aqueous solutions were prepared by using water obtained by Milli-Q Gradient A-10 system (Millipore).

Bacterial strains, cultivation and chromatophores preparation

Rhodobacter sphaeroides wild-type (WT) 2.4.1 was photosynthetically grown in saturating light conditions and in liquid medium 27 of the German collection of microorganisms and cell cultures (http://www.dsmz.de/) which contains Zn^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and MoO_4^{2-} as trace elements, at room temperature. The cells were collected in the early stationary state by centrifugation and suspended in Tris–HCl 20 mM, pH 8.00 (TRIS buffer).

Rhodobacter sphaeroides BH6 mutant strain was photosynthetically grown in Sistrom medium supplemented with 10 μg/ml kanamycin and 2 μg/ml tetracycline in order to maintain the mutant plasmid. The temperature was kept at 29 °C. The strain was kindly provided by prof. A. R. Crofts, Department of Biochemistry, University of Illinois at Urbana-Champaign, USA.

Chromatophores were prepared as previously described (Bowyer et al. 1979). The cells were washed with TRIS buffer and then broken by French press (150 MPa), and after removal of the unbroken cells by centrifugation (30 min, 5 °C,

13000 g), chromatophores were sedimented by ultracentrifugation (105 min, 4 °C, 270000 g) and suspended in Tris–HCl 20 mM pH 8.00, EDTA 1 mM, NaCl 100 mM to a final OD_{850}=50.

Osmotic shock for altering the CL level in WT Rhodobacter sphaeroides membranes

An aliquot of cells (approximately 1.4 g wet weight), representing the Control, was suspended in 40 ml of TRIS buffer (isosmotic) and incubated for 5 h at room temperature; an equivalent aliquot of cells from the same batch, was suspended in the TRIS buffer supplemented with 0.7 M NaCl, and incubated in the same conditions in order to obtain the shocked sample. After the incubation, chromatophores were obtained from both Control and shocked cells and suspended in Tris–HCl 20 mM pH 8.00, EDTA 1 mM, NaCl 100 mM. Lipids were extracted from each sample of chromatophores and CL relative amount (expressed as percentage w/w of CL in the lipid extract) of the two different preparations was estimated by densitometric analysis of thin layer chromatography plates based on a calibration curve as reported in a previous work (De Leo et al. 2009).

Oxidoreductase activity

Enzymatic activity was assayed as previously described (Guergova-Kuras et al. 1999) with horse heart cyt *c* as electron acceptor and decylubiquinol as electron donor. The reaction was triggered by adding 100 μM of decylubiquinol into the cuvette containing 2.5 ml Tris–HCl 20 mM pH 8.00, NaCl 100 mM, DM 0.01 %, 25 μM horse heart cyt *c* and either 20 μl of chromatophores at OD_{850}=50 or 30 nM purified cyt bc_1 complex. The reduction of horse heart cyt *c* was monitored at 550 nm. All the determinations of enzymatic activity were conducted in the dark to avoid interference from the RC. When needed, antymicin and myxothiazol were added from ethanol stock solutions to the samples five minutes before triggering the reaction with decylubiquinol, in order to inhibit the cyt bc_1 complex activity. For testing the lipid effect on the purified enzyme, the activity assays were conducted directly in solution, delivering lipids by an ethanol lipid solution in the buffer containing the cyt bc_1 complex purified using lipid-free buffers. Reported data represent mean values ± standard deviations obtained from four replicates of two independent experiments.

Cyt *b*, c_1 and RC quantification

Chromatophores were diluted in Tris–HCl 20 mM pH 8.00, EDTA 1 mM, NaCl 100 mM to a final OD_{850}=6. The concentrations of cyt *b* and cyt c_1 in chromatophores were measured from reduced minus oxidized difference spectra as previously described (Vanneste 1966). The concentration of RC was assayed by flash-induced absorbance change at 600 nm using $\Delta\varepsilon$=20 mM^{-1} cm^{-1} (De Leo et al. 2009).

His-tagged cyt bc_1 complex purification

Purification of His-tagged cyt bc_1 complex was performed as described by Guergova-Kuras (Guergova-Kuras et al. 1999). Two types of purification were performed: the first in presence of PC 15 μg/ml (in all buffers used for the procedure), the second using lipid-free buffers. The integrity of the purified complex was verified by Tricine-SDS Polyacrylamide Gel Electrophoresis (Schägger and von Jagow 1987).

Results

Lipid profile of bacteria can be modulated by genetic mutations or through metabolic approaches (Zhang et al. 2011a). We have previously shown that by varying the NaCl concentration of the extracellular medium and the incubation time, the CL content of chromatophores can be even doubled (De Leo et al. 2009). Table 1 shows that the CL content increases from about 2 % (w/w of total lipid extract) for chromatophores isolated from Control cells, to 5 % for chromatophores isolated from osmotically stressed cells. No further changes in lipid composition were observed (data not shown) after osmotic shock. The UV–vis spectroscopic analysis of the isolated vesicles also shows identical patterns for the two samples and no shift in the absorption peaks was observed. In detail Fig. 1a shows the main absorption bands centered at 800 and 850 nm that correspond to the Q_Y transition of LH2 complexes while bacterio-chlorophylls *a* of the LH1 complexes bring about the shoulder at 875 nm. In the blue region the Q_X transition of the LH1 complexes originates the peak at 590 nm whereas carotenoid bands range from about 440 to 505 nm (Hunter 1995). The RC content was found the same in Control and CL enriched

Table 1 Characteristics of chromatophores used for oxidoreductase activity assay

Chromatophores	Cardiolipin (% *w/w*)	[RC] (*μM*)	[Total cyt *c*] (*μM*)	Oxidoreductase activity (mol cyt *c* s^{-1} (mol total cyt *c*)$^{-1}$)
Control	2.1±0.1	1.7±0.1	0.84±0.05	60±2
CL enriched	5.2±0.1	1.7±0.1	0.80±0.05	93±3

Reported data are referred to chromatophores at OD_{850}=50 and represent mean values ± standard deviations obtained from four replicates of two independent experiments

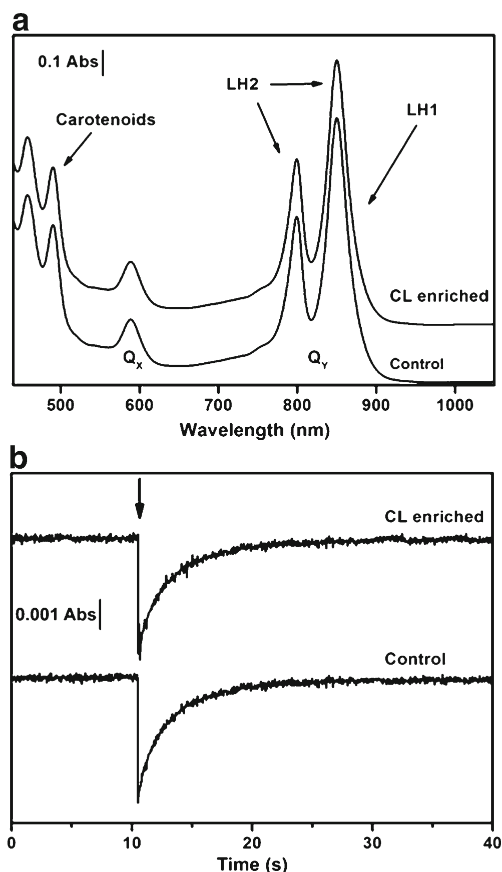

Fig. 1 **a** Absorption spectra of chromatophores obtained from *Rhodobacter sphaeroides* cells after 5 h of incubation in isosmotic (TRIS) and hyperosmotic (NaCl) buffer solutions. **b** Absorption changes of RC in Control and CL enriched chromatophores at 600 nm induced by flash illumination. The arrow indicates the triggering of charge separation by light

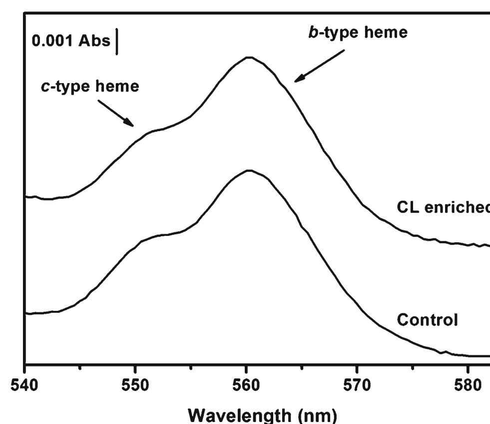

Fig. 2 Reduced minus oxidized difference spectra of Control and CL enriched chromatophores

vesicles, as shown by the same flash-induced absorbance change at 600 nm, reported in Fig. 1b.

Dithionite-reduced minus oxidized difference spectra recorded between 540 and 590 nm of the vesicles are displayed in Fig. 2. The 562 nm and 553 nm peaks correspond to *b*-type and *c*-type heme mainly due to the cyt bc_1 complex although a cbb_3 oxidase and a membrane-bound cyt *c* could contribute to the 560 and to the 550 nm region respectively (Zhang et al. 2011b). No appreciable differences in the amplitude and in the absorption wavelengths of the peaks have emerged. Overall these findings indicate that the two samples contain similar amount of reducible cytochromes (see also Table 1) and that they retain native structure in suspended membranes even after treatment with hyperosmotic solution, as indicated by unaltered spectral patterns.

Table 1 shows that oxidoreductase activity raised from 60±2 for the Control to 93±3 mol cyt *c* s^{-1} (mol total cyt *c*)$^{-1}$ for CL enriched chromatophores, taking into account a non-enzymatic reaction rate (measured in absence of chromatophores) of about 2.5 mol cyt *c* s^{-1} (mol total cyt *c*)$^{-1}$. In order to test if this oxidoreductase activity can be attributed to cyt bc_1 complex exclusively or also to other enzymes of the chromatophores, we measured kinetics in presence of two specific inhibitors of cyt bc_1 complex: antimycin, which irreversibly binds to the Q_i site, and myxothiazol which locks the Q_o site (Gray and Daldal 1995). The oxidoreductase activity dropped to about 6 mol cyt *c* s^{-1} (mol total cyt *c*)$^{-1}$ for both Control and CL enriched samples. This is a modest fraction of the reported data, accounting for at most the 10 % of overall reaction rate. Therefore the oxidoreductase activity measured in vesicles can be attributed reasonably to cyt bc_1 complex. Consequently it can be deduced that in CL enriched chromatophores the cyt bc_1 oxidoreductase activity becomes stimulated.

Furthermore we have examined the effect of exogenous CL on the isolated and purified cyt bc_1 complex in a micellar system where the only variables are represented by the enzyme and a lipid counterpart. To this end, polyhistidine-tagged cyt bc_1 complex was purified from BH6 mutant strain of *Rhodobacter sphaeroides*, using the one-step purification procedure described by Guergova-Kuras et al. (Guergova-Kuras et al. 1999). The protein was purified both according to the original protocol, i. e. in the presence of 15 μg/ml PC (Prep. I), and without the addition of exogenous lipids (Prep. II); the electrophoretic analysis confirmed that the purified enzyme preserved all its four subunits in both preparations (Fig. 3a). As expected the activity of the cyt bc_1 complex isolated in absence of exogenous lipids was much lower than that of the enzyme supplemented with PC during the purification (Fig. 3b). Activity assays showed that the oxidoreductase activity for Prep. I was about twice than that measured for the Prep. II (see Table 2). This finding supports the idea that lipids play an important role in modulating the enzymatic activity. Furthermore when PC was added in large excess to the Prep. II (PC/cyt bc_1 complex molar ratio=500) after the isolation, a full restoration of its functionality was found, reaching values comparable to those of Prep. I (Table 2).

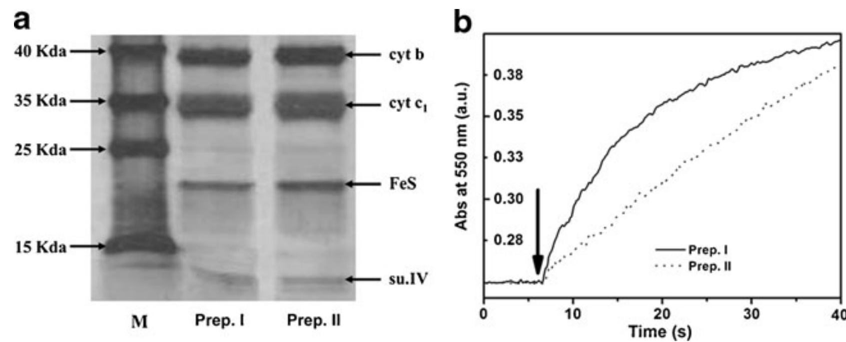

Fig. 3 **a** Tricine-SDS Polyacrylamide Gel Electrophoresis (Schagger-von Jagow gel) of His-tagged cyt bc_1 complex. Lane 1 (M), molecular weight marker; lane 2 (Prep. I), complex purified in presence of PC 15 µg/ml; lane 3 (Prep. II), complex purified in absence of lipids. **b** Initial slope of the cyt c^{+3} reduction signal by the two preparation of cyt bc_1 complex. The arrow indicates the triggering of reaction by decylubiquinol addition

In addition Table 2 shows that the oxidoreductase activity of the Prep. II at CL/cyt bc_1 complex molar ratio of 500 reached values of 38±3 s^{-1}, more than five times the value found for the purified enzyme (7±2 s^{-1}) and about twice with respect to the activity measured in the presence of PC (18±2) at the same molar ratio.

Therefore we were able to demonstrate the existence of a direct relationship between the amount of CL and the increase of specific activity of the cyt bc_1 complex in vitro, supporting the results we obtained in the isolated native membranes of chromatophores. These data represent the first biochemical evidence that CL has a stimulating effect on bacterial cyt bc_1 complex activity of *Rhodobacter sphaeroides*.

Discussion

In a cellular membrane different types of lipids can be distinguished depending on the residence time at the protein-lipid interface and ultimately on the degree of interaction that these lipids have with the integral membrane protein (Lee 2003). Lipids that diffuse rapidly in the bilayer plane and which are not in contact with a protein are defined *bulk lipids*; lipid molecules that form an annular shell around the protein and that have a significant reduction of exchange rate with the bulk lipids are referred to as *boundary* or as *annular lipids*; tightly bound lipids at the protein-protein interface in multi-subunit proteins or between transmembrane α-helices are referred to as *non-annular lipids* (Lee 2004; Contreras et al. 2011). There are several experimental evidences showing the importance of phospholipids for the proper functioning of the mitochondrial bc_1 complex. It was demonstrated that progressive steps in protein delipidation lead to a gradual decrease of enzyme activity (Lange et al. 2001; Schägger et al. 1990; Yu and Yu 1980). An extensive delipidation performed using high amount of detergent followed by ion-exchange chromatography purification, may result even in the destabilization of the protein quaternary structure (Gomez and Robinson 1999). In this work we obtained a partially lipid-depleted bacterial enzyme with a lower activity with respect to the control preparation, but with an intact multimeric structure. The outcome is in agreement with what found for the mitochondrial cyt bc_1 complex (Schägger et al. 1990) and probably derives from the destruction of the annular shell of lipid molecules around the dimeric array. About 100 lipid molecules/enzyme correspond to a single bilayer annulus for bovine enzyme (Schägger et al. 1990). In our experiments of relipidation, 500 lipid molecules/cyt bc_1 complex were used in order to ensure the complete reconstitution of the lipid environment at the protein interface. Our data clearly show that while exogenous PC proved to effectively restore the oxidoreductase activity of cyt bc_1 complex, CL addition not only restored but also stimulated the enzymatic activity, thus highlighting the specificity of this lipid in modulating the cyt bc_1 complex function. On the other hand an increase in oxidoreductase activity of about 1.5 times, induced in chromatophores by smaller variations in the CL content (from about 2 to 5 % of total lipids), represents a further and stronger confirmation of the role played by CL in regulating specifically the activity of the bacterial cyt bc_1 complex.

Similarly to what hypothesized for the yeast bc_1 complex, it is possible to argue that some molecules of CL sitting in

Table 2 Enzymatic activity of purified cytochrome bc_1 complex

Sample	Lipids/cyt bc_1 (*molar ratio*)	Oxidoreductase activity (mol cyt *c* s^{-1}(mol cyt c_1)$^{-1}$)
Prep. I	/	16±3
Prep. II	/	7±2
Prep. II + PC	500	18±2
Prep. II + CL	500	38±3

Prep. I: purified in presence of 15 µg/ml of PC; Prep. II: purified without lipids. Reported data represent mean values±standard deviations obtained from four replicates of two independent experiment

specific sites on the protein surface are more tightly bound respect to common annular molecules but less tightly bound than the non annular lipids (Lange et al. 2001). Some authors suggest that individual lipids of a shell may vary their residence time depending on the local architecture of the membrane protein and their affinity for protein surface (Contreras et al. 2011; Anderson & Jacobson 2002). This would explain both the great influence that CL molecules exert on the bacterial enzyme activity (by occupying special positions on the protein surface) and the attitude to be easily removed and replaced (since less tightly bound). These CL could be placed in proximity of the proton uptake pathways acting as a buffer capable of concentrating protons as suggested for the yeast enzyme (Lange et al. 2001; Haines 1983). The hypothesis is strengthened by the great structural analogy existing between crystal structures of the cyt bc_1 complex of *Rhodobacter sphaeroides* and its mitochondrial counterparts and by the evidence that even the lipids resolved in the structures show some degree of similarity (Esser et al. 2008). In analogy with the mitochondrial complex (Lange et al. 2001), we can suppose that, besides CL molecules possibly present in the homodimer cleft, also CLs inside lipid annulus surrounding cyt bc_1 complex could influence enzyme activity and stability, as boundary lipids. Therefore inside a *not necessarily homogenous shell* (Contreras et al. 2011), a short range lipid domain enriched in CL around cyt bc_1 complex would favour its enzymatic activity helping the redistribution of protons along the membrane surface and therefore contributing to create a proton gradient across the membrane. Moreover CL could play an active role in quinol substrate binding, as recently shown for nitrate reductase A of *E. coli*, another bacterial respiratory complex (Arias-Cartin et al. 2011).

Conclusions

The osmotic stress is an effective environmental tool to modulate the level and distribution of CL in bacterial membranes (De Leo et al. 2009; Romantsov et al. 2008). In this work CL enriched chromatophores isolated from the photosynthetic bacterium *Rhodobacter sphaeroides* exposed to hyperosmotic shock have been used as an experimental model to investigate the CL influence on the oxidoreductase activity. We found that the enzymatic activity, mainly attributable to bc_1 complex, was stimulated in CL enriched chromatophores in comparison with Control membranes and that the cyt bc_1 complex exhibited a CL concentration dependence similar to that previously described for the corresponding enzyme in yeast mitochondria (Lange et al. 2001). The stimulating effect of CL was also demonstrated for the purified cyt bc_1 complex by addition of exogenous lipids. The modulation of CL content in *Rhodobacter sphaeroides* membrane under osmotic stress might represent a dynamic homeostatic factor which is able to optimize not only the functioning and the stability of the photosynthetic apparatus protein complexes (De Leo et al. 2009) but also the bioenergetic performance of the cells.

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