Cardiolipin increases in chromatophores isolated from *Rhodobacter sphaeroides* after osmotic stress: structural and functional roles

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Abstract **Chromatophores isolated from cells of *Rhodobacter sphaeroides* exposed to hypertonic solutions were enriched in cardiolipin (CL). Because CL levels are raised by increasing the incubation time of *R. sphaeroides* in hypertonic solutions, it was possible to isolate chromatophores containing different CL amounts by starting from cells incubated in hypertonic solutions for different times. The functionality and stability of the photosynthetic proteins in chromatophore membranes having different CL levels were investigated. Reaction center (RC) stabilization with respect to thermal denaturation and photoxidative damage was observed by flash photolysis and fluorescence emission experiments in CL-enriched chromatophores. To gain detailed information about the structures of endogenous CLs, this lipid family was isolated and purified by preparative TLC, and characterized by high-resolution mass spectrometry. We conclude that osmotic shock can be used as a tool to modulate CL levels in isolated chromatophores and to change the composition of the RC lipid annulus, avoiding membrane artifacts introduced by the use of detergents.**—De Leo, V., L. Catucci, A. Ventrella, F. Milano, A. Agostiano, and A. Corcelli. **Cardiolipin increases in chromatophores isolated from *Rhodobacter sphaeroides* after osmotic stress: structural and functional roles.** ***J. Lipid Res.*** **2009.** 50: **256–264.**

Supplementary key words lipid-protein interactions • reaction center • thermal denaturation • photoxidative damage • flash photolysis

Rhodobacter sphaeroides is a Gram-negative bacterium of the Proteobacteria group, which is capable of growing in the presence of light and in anaerobic conditions by means of photosynthesis. The photosynthetic apparatus is located within the invaginated inner membrane that is formed during photosynthetic growth. This apparatus contains a number of defined transmembrane protein complexes: the light-harvesting complexes LHCI and LHCII, reaction center (RC), cytochrome bc_1 complex, and the ATP synthase (1).

The membrane system containing the photosynthetic apparatus can be isolated as a rather homogeneous population of small vesicles named chromatophores, originating from the so-called intracytoplasmatic membranes, i.e., invaginations of the cell membrane, formed during photosynthetic growth and particularly enriched in photosynthetic proteins. Chromatophores have been used to elucidate many biochemical and topological aspects of the bioenergetic systems present in photosynthetic bacteria, and recently, a molecular model of chromatophore vesicles from *R. sphaeroides* has been proposed (2).

Chromatophore vesicles are also an ideal model system for investigating the structures and properties of lipids bound to photosynthetic enzymes, the protein/lipid reciprocal interaction, and organization in the native membrane. The interest in this last aspect has recently grown, considering that membrane lipids are not only a structural support for membrane protein complexes but also key players in energy conversion processes and in enzyme activity and stabilization (3).

One of the most characteristic phospholipids present in chromatophores, as well as in other membranes involved in the generation of electrochemical potential for substrate transport and ATP synthesis, is the acidic phospholipid cardiolipin (CL), or bisphosphatidylglycerol. Several data in the literature have pointed out the importance of CL in establishing interactions with membrane protein complexes. As an example, the respiratory protein cytochrome *c* oxidase has been found to be inactivated by

This work was supported by PRIN 2006 (Complessi Porfirinici Autoorganizzati su Scala Nanoscopica: Proprietà e Applicazioni Tecnologiche) of the Ministero Italiano dell'Università e della Ricerca Scientifica.

Manuscript received 12 June 2008 and in revised form 18 August 2008.

Published, JLR Papers in Press, August 20, 2008.
DOI 10.1194/jlr.M800312-JLR200

Abbreviations: Bchl, bacteriochlorophyll; Bpheo, bacteriopheophytin; CL, cardiolipin or bisphosphatidylglycerol; CR, charge recombination; ESI-MS, electrospray ionization mass spectrometry; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; RC, reaction center; SQDG, sulfoquinovosyldiacylglycerol.

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removing this phospholipid (4). In addition, it is widely indicated in the standard procedures for isolation and purification of mitochondrial carrier proteins, that addition of exogenous CL is required to preserve the stability and functions of isolated complexes (5). Moreover, CL is also known to interact with the RC of photosynthetic bacteria, influencing the protein structure and function, and affecting RC thermal stability, as has recently been reported for *R. sphaeroides* by Jones et al. (6) and Fyfe et al. (7).

Most studies on lipid-protein-specific interactions have been performed in reconstituted systems or liposomes, which offer the possibility of easily changing the lipid composition. However, these studies often use detergents, which could alter the protein supramolecular organization, and exogenous lipids that are different from the endogenous ones for the acyl chain lengths and saturation degrees.

Although it is known that various organisms may change their lipid membrane composition in response to fluctuations in environmental parameters such as temperature and salinity, there are very few literature reports describing these changes at the level of isolated membranes. Recently, it has been reported that the levels of CL increase upon exposure of prokaryotes (both bacteria and archaea) to osmotic stress (8–14). In particular, the CL content in the lipid extracts of *R. sphaeroides* cells increases with the rise in environmental osmolarity and with the time of exposure to hyperosmotic media (3).

Here we show that it is possible to isolate CL-enriched chromatophore membranes from *R. sphaeroides* exposed to high-salt-containing media, and we describe the effects of increased levels of CL on the RC functionality and stability in native membranes, avoiding artifacts introduced by the use of detergents.

EXPERIMENTAL PROCEDURES

Materials

All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich, St. Louis, MO). TLC plates (Silica gel 60A) obtained from Merck KGaA (Germany) were washed twice with chloroform-methanol (1:1, v/v) and activated at 120°C before use. Lipid standards were purchased from Sigma-Aldrich.

Bacterial strains, cultivation, and chromatophore preparation

R. sphaeroides wild-type 2.4.1 was grown photosynthetically in saturating light conditions 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 resuspended in 20 mM Tris-HCl, pH 8.00 (control buffer).

Chromatophores were prepared from control cells and cells exposed to osmotic shock (see below) as previously described (15). The cells were broken by French press (150 MPa), and after removal of the unbroken cells by centrifugation (30 min, 5°C, 13,000 *g*), the chromatophores were sedimented by ultracentrifugation (105 min, 4°C, 270,000 *g*) and resuspended in 20 mM Tris-HCl, pH 8.00, 1 mM EDTA, and 100 mM NaCl.

Induction of the increase of CL level in bacterial membranes

Equivalent aliquots of cells (approximately 1.4 g wet weight) were suspended in 40 ml of control buffer containing NaCl in variable amounts. Then the cell suspensions were incubated in an open flask under gentle stirring for variable times at room temperature (23–25°C). In particular, to study the time course of the CL increase in the bacterial membranes, equivalent aliquots of cells (grown in the same conditions) were resuspended in control buffer containing 0.7 M NaCl and incubated for 0, 5, and 14 h, respectively; after the incubation in hyperosmotic medium, aliquots of the above samples were used to isolate chromatophores. The chromatophore samples were labeled as "control," "high salt 5 h," and "high salt overnight." Lipids were extracted from each sample (cells and chromatophores) by the standard procedure (see below).

Lipid extraction

Polar lipids were extracted from cells and chromatophores, according to the Bligh and Dyer standard method (16). Briefly, 6 ml of methanol-chloroform (2:1, v/v) was added to 1.6 ml of suspension of cells or chromatophores fraction in aqueous buffer. The mixture was shaken for 15 min, then centrifuged (at 1,800 *g* for 15 min, at room temperature) and the supernatants were transferred by means of a Pasteur pipette to another tube. The residual precipitated material was then resuspended in 7.6 ml of methanol-chloroform-water (2:1:0.8, v/v), and the mixture was again shaken for 15 min and centrifuged (at 1,800 *g* for 15 min, at room temperature). The combined extracts were diluted with 8 ml of chloroform-0.2 M KCl (1:1, v/v) in order to have a final mixture of methanol-chloroform-water (1:1:0.9, v/v). Then the phase separation was obtained by centrifugation (at 8,500 *g* for 10 min, at 10°C); the lower chloroform phase was withdrawn, and about 2 ml of chloroform was added to the methanol-water phase to optimize the lipid recovery. The combined chloroform phases were dried in a rotatory evaporator, and the lipids obtained were weighed and redissolved in chloroform-methanol (1:1, v/v) at a final concentration of 10 μg/μl and stored at −20°C.

TLC

Total lipid extracts were analyzed by TLC on silica gel (20 × 10 cm, layer thickness 0.2 mm). The plate was developed with the solvent chloroform-methanol-acetic acid-water (85:15:10:3.5, v/v), and detected by spraying with 5% sulfuric acid in water, followed by charring at 120°C for 30 min.

The quantitative analyses of CL content were performed by video densitometry, using the software Image J (http://rsb.info.nih.gov/ij).

Isolation and purification of CL. Preparative TLC of total lipid extract was carried out on silica gel 60 plates in the solvent chloroform-methanol-acetic acid-water (85:15:10:3.5, v/v). After scraping the silica in a band of CL from the plate, the lipid was extracted from the silica five times with chloroform-methanol (1:1, v/v). After centrifugation, the supernatants were combined and dried under a stream of N_2.

Mass spectrometry

Mass spectrometry (MS) determinations were carried out in flow injection analysis with dried lipid samples that were dissolved in chloroform-methanol (1:1, v/v). Samples (2 μl) injected via a 10 μl loop were transferred into the MS electrospray interface with a flow rate of 0.1 ml/min of chloroform-methanol (1:1, v/v) delivered by a Perkin-Elmer 200 chromatographic system.

Electrospray ionization mass spectra were obtained with a QSTAR hybrid Qq-TOF mass spectrometer (Applied Biosystems/MSD Sciex, Canada) equipped with a turbo ion spray interface. Interface conditions for the detection of negative ions were as follows: nebulizer gas (air) = 1.2 l/min, curtain gas (nitrogen) = 1 l/min, needle voltage = −4,500 V, declustering potential = −50 V, focusing potential = −300 V. Mass mass spectrometry (MS/MS) measurements were carried out by fragmenting the target ions at proper collision energy (usually −35 eV).

Optical measurements

Photochemical activity was determined by flash-induced absorption change at 600 nm that reflects the $P^+Q^- \rightarrow PQ$ charge-recombination (CR) kinetics, where P stands for primary donor and Q for quinone. The measurements were performed with a home-built kinetic spectrometer previously described (17), and the excitation pulse was provided by a Xenon flash lamp (Hamamatsu, model L4634-01) with a duration of ≈1 μs, well below the time constant of the fastest recorded process.

RC thermal stability. Aliquots (400 μl) of chromatophore samples (Abs_{865} = 50) were simultaneously incubated and subjected to different increasing temperatures for 30 min. For this purpose, a thermostatic system with temperature control at the first decimal digit was employed. After incubation, the different samples were cooled to room temperature and then diluted in control buffer, adjusting their absorption at Abs_{600} = 0.6; subsequently, the CR kinetic constant was evaluated by means of flash photolysis.

Fluorescence spectra were recorded using a Varian Eclipse spectrofluorimeter. Chromatophore samples were incubated at 60°C for variable periods of time. Appearances of fluorescence at 760 nm, in response to excitation at 531 nm, were recorded. Emission values were recorded without cooling the samples; in fact, keeping the 60°C temperature constant avoided possible denaturation reversibility.

RC photodegradation. Chromatophores were diluted in control buffer, adjusting their absorption at Abs_{600} = 0.6, and were illuminated with a QTH (quartz-tungsten-halogen) 240 W lamp in the presence of air at 20°C. Every 30 min, the RC functionality was evaluated by sampling the CR constant values.

RC quantitation. The RC concentrations in chromatophores were determined by the light-minus-dark absorption difference at 600 nm, as reported in the literature (15).

Total protein determination. The total proteins in chromatophores were determined colorimetrically through the bicinchoninic acid assay as previously described (18).

Bacteriochlorophyll a quantification. Pigments were extracted from chromatophores by acetone. Spectra in the range 500–900 nm were collected by a Varian Cary 5000 spectrophotometer. Bacteriochlorophyll (Bchl) *a* brings a near-infrared absorption band at 775 nm that has ε_{mM} = 20.1 (19).

Statistical analysis

GraphPad InStat software (Sigma) was used to process the data by ANOVA to indicate statistically extremely significant differences between means (one-way ANOVA with posthoc Tukey test, $P < 0.0001$). All reported data represent mean values ± standard deviations.

RESULTS

High osmolarity increases CL levels in *R. sphaeroides* wild-type strain

Previous results demonstrated that in carotenoidless *R. sphaeroides* R-26 strains, the exposure of cells to hyperos-

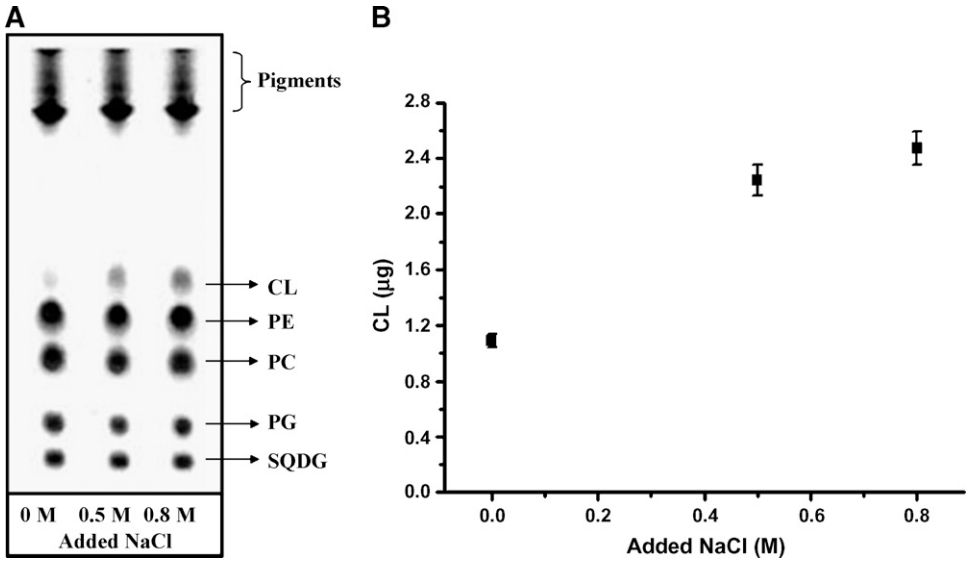

Fig. 1. Effect of high external osmolarity on the lipid composition of *R. sphaeroides* cells. A: TLC separation of polar lipids. Lipid extracts were obtained from equivalent aliquots of *R. sphaeroides* cells kept for 2 h at room temperature in control buffer and in control buffer containing increasing amounts of NaCl. Thirty micrograms of each total lipid extract was loaded onto the plate. B: Cardiolipin (CL) amounts as functions of NaCl concentration, quantified by using CL standard on the same plate for the calibration curve (not shown). Reported data represent mean values ± standard deviations obtained from three replicates. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

motic media affects the lipid composition of the plasma membrane. In particular, hypertonic solutions of NaCl caused a considerable increase in CL cell membrane content. Preliminary data in the same work indicated that the wild-type 2.4.1 strain shows a similar response to hyperosmotic stress (3).

In this study we have thoroughly examined the effects of hyperosmotic stress on the membrane lipid composition of *R. sphaeroides* wild-type strain and, in particular, on the CL content.

Initially, we analyzed by TLC (**Fig. 1A**) the lipid extracts obtained from control cells and from cells exposed to high concentrations of NaCl. Individual lipid components present in the extracts were identified by comparison of their R_f values with those of authentic standard markers. The main lipid components of *R. sphaeroides* cells were identified (in R_f order) as sulfoquinovosyldiacylglycerol (SQDG), phosphatidylglycerol (PG), phosphatidylcholine (PC), phosphatidylethanolamine (PE), plus CL; the pigments run with the solvent front. It is evident that the extracts obtained from cells incubated in hyperosmotic media contained relative amounts of SQDG, PC, and PE similar to those of the cells suspended in isotonic medium (control cells), whereas the CL content changed. Figure 1B illustrates that the CL amount present in the lipid extracts of shocked cells increased in response to increasing NaCl concentrations, up to a concentration of 0.8 M NaCl. The osmoregulated neosynthesis of CL at the expense of PG has already been documented in Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis* (12–14) and in extremely halophilic microorganisms (9). The CL content dependence upon the medium osmolarity in the wild-type strain is similar to that previously observed in the R26 strain. However, it is more convenient to use the wild-type strain for the CL-based experiments because it was found that the amount of CL in the wild-type strain is always higher than that in the R26 strain, both under physiological conditions and during osmotic stress.

Figure 2 shows the time course of CL increase during osmotic shock. It can be seen that the amount of CL increased continuously in the cells resuspended in hyperosmotic medium.

Altogether, the results shown in Figs. 1 and 2 indicate that the hyperosmotic salt solutions induce neosynthesis of CL in *R. sphaeroides* cells, suggesting that bacterial CL synthase activity is stimulated under osmotic stress.

CL-enriched chromatophores

Chromatophores were prepared from both control cells and cells incubated in hypertonic 0.7 M NaCl buffer for 5 and 14 h. Lipids of control and shocked cells, together with those of corresponding chromatophore preparations, were analyzed by TLC. Quantitative CL determinations were carried out by videodensitometric analyses, and the results are illustrated in **Fig. 3**. CL of isolated chromatophores increases by raising the incubation time in hyperosmotic medium, reflecting the same behavior observed for whole cells. By comparing the CL content of chromatophores and cells, it can be seen that most of the CL

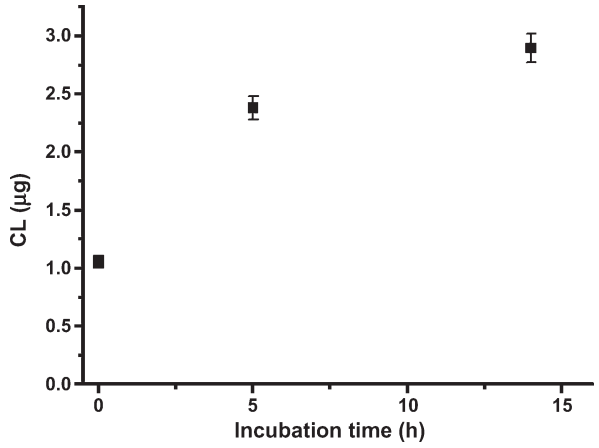

Fig. 2. Time course of the CL content increment in *R. sphaeroides* during exposure to high external osmolarity. Cells were resuspended in control buffer containing 0.7 M NaCl. Lipid extracts were obtained from equivalent cell aliquots at different incubation times in the high-salt medium. The CL content was estimated by videodensitometry. Thirty micrograms of lipid extracts was loaded onto the plate. Reported data represent mean values ± standard deviations obtained from three replicates.

increase observable in the cells is attributable to the specialized membrane domains of the chromatophores. The slightly lower CL amounts recorded for chromatophores are probably due to the loss of lipid material occurring during the chromatophore preparation procedure.

Interestingly, by varying parameters such as the osmolite concentration or the incubation time, it is possible to obtain natural vesicles (i.e., chromatophores) containing different amounts of CL, which can be used to study the functional relationships between CL and photosynthetic enzymes. Because CL is a component of the lipid annulus of photosynthetic proteins, it is possible to test the effect of subtle changes in the lipid environment on the membrane function. **Table 1** shows that from three to eight CL molecules

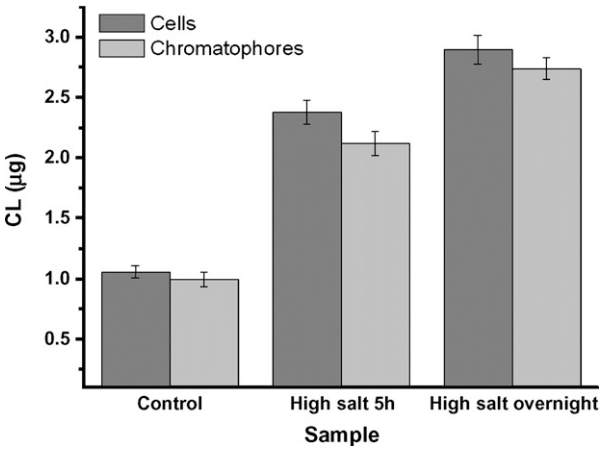

Fig. 3. CL increment in chromatophores prepared from control and cells incubated in high-salt-containing medium, as observed by TLC measurements. Quantitative CL determinations were carried out by videodensitometric analyses. Reported data represent mean values ± standard deviations obtained from three replicates.

TABLE 1. Comparison of total protein, CL, and RC concentrations of chromatophores isolated from control cells and from cells incubated in high-salt-containing medium

Sample	CL Concentration	Total Protein Concentration	RC Concentration	CL/RC Molar Ratio
	μM	*mg/ml*	*μM*	
Control	32 ± 1	5.19 ± 0.07	9.14 ± 0.25	3.5 ± 0.2
High salt 5 h	78 ± 4	5.29 ± 0.02	10.15 ± 0.42	7.7 ± 0.7
High salt overnight	107 ± 4	5.53 ± 0.14	13.14 ± 0.16	8.1 ± 0.4

CL, cardiolipin; RC, reaction center; [Bchl] = 1.025 mM.

per RC are present in the control and shocked chromatophore samples. From the Table, it can also be seen that protein or pigment content was similar in control and shocked samples; in addition, SDS-PAGE protein analyses and absorption spectroscopy analyses of pigments showed no difference in the protein and pigment profiles of control and shocked chromatophores (data not shown).

The chromatophore preparations isolated from shocked and nonshocked cells were also analyzed by means of dynamic light scattering to estimate their dimensions. Chromatophore diameter average size was approximately 80 nm and "high-salt overnight chromatophores" had a slightly smaller diameter than the "control" chromatophores (data not shown). These data indicate that chromatophores are osmotically active vesicles. The small decrease in the chromatophore size as a consequence of cellular shrinkage could be attributed at least in part to the modification of lipid composition occurring during osmotic stress. The combination of two PG molecules to give one CL molecule would increase lipid packing inside the membrane and consequently reduce the vesicle dimension.

Identification of CL molecular species

The lipid component identified as CL in the TLC analysis was also isolated by preparative TLC, starting from the lipid extract of cells incubated in high-salt-containing medium. **Figure 4A** reports the electrospray ionization mass spectrometry (ESI-MS) (negative ions) analysis of the purified CL in the 400–1,500 *m/z* range. Figure 4B shows in detail a set of bicharged peaks in the 650–750 *m/z* range,

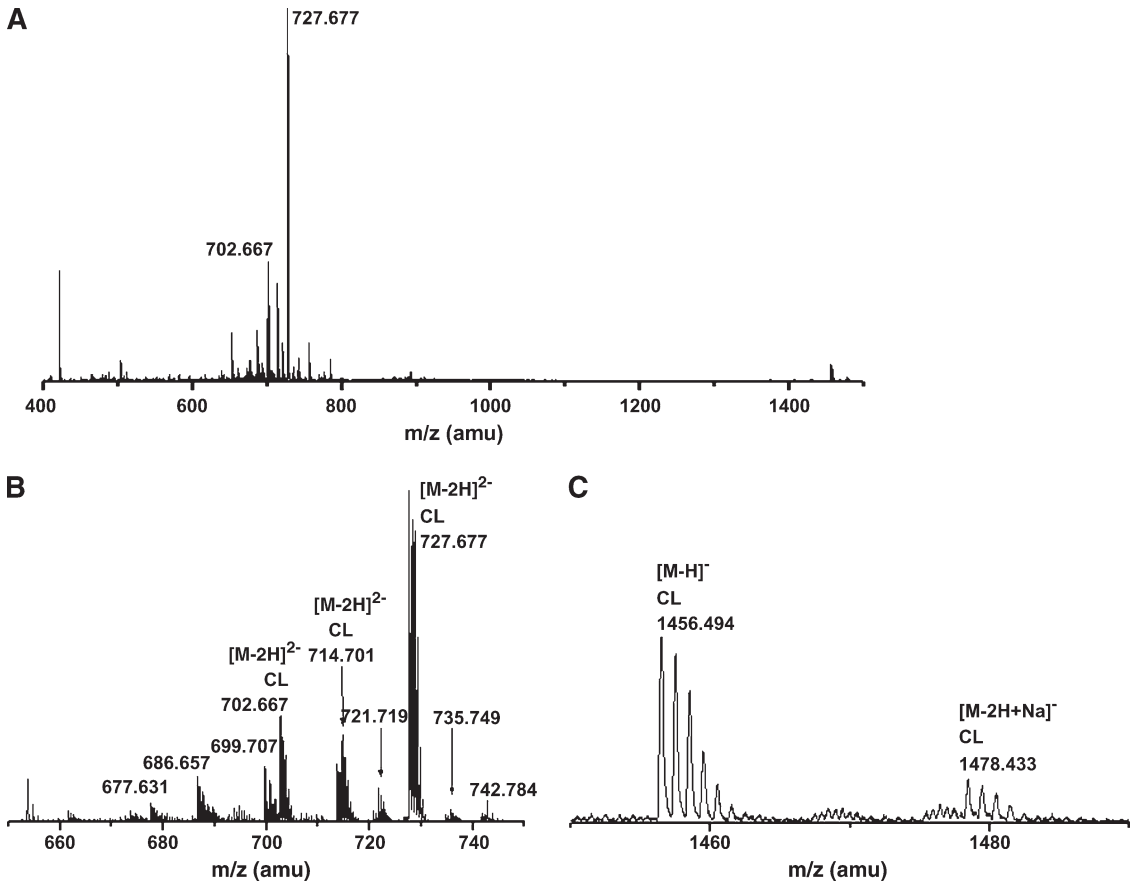

Fig. 4. Electrospray ionization mass spectrometry (negative ions) of the CL isolated from *R. sphaeroides* cells. A: The 400–1,500 *m/z* range. B: Details of the 650–750 *m/z* range, showing bicharged CL ions. C: Zoom image of the 1,450–1,500 *m/z* range, where monocharged CL ions can be observed.

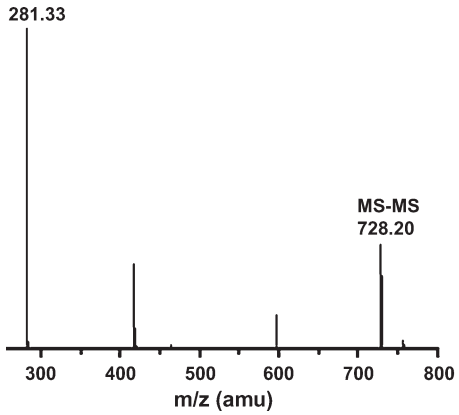

Fig. 5. Daughter fragments of the peak at 728.20 *m/z* (tandem mass spectrometry analysis).

originating after the loss of two protons, all assigned to CL. In particular, the cluster at 727.67 *m/z* is ascribed to CLs with four C18 acyl chains and variable degrees of unsaturation. The CL at 727.67 *m/z* (the most-intense peak) corresponds to a species with four degrees of unsaturations; in the same cluster, differences in one unsaturation generate peaks that differ by one *m/z* unit.

The clusters located at 714.70 and 702.66 *m/z* were found to be less intense; they can be attributed to CLs showing variable degrees of unsaturation and having three C18 and one C16 acyl chains in the first case, and two C18 and two C16 acyl chains in the second case.

Another cluster is present at 721.72 *m/z*, even if it is less intense with respect to the other clusters in the studied *m/z* range; this family of CL peaks has also recently been found in association with spinach Photosystem II complexes (20), and it must be assigned to CLs with four C18 acyl chains and variable degrees of unsaturation (from a total of 8 to 12 unsaturations).

In Fig. 4C, a zoom of the region at high *m/z* value is reported: the monocharged peak at 1,456.49 *m/z* corresponds to the bicharged peak observable at 727.67 *m/z*, and the 1,478.43 *m/z* signal is due to the monocharged sodium salt of the same CL molecule.

To gain more-detailed information about the most representative CL structure, MS/MS analyses were performed, as reported in **Fig. 5**. The signal at 281.33 *m/z* confirmed that the CL generating the peak at 727.67 *m/z* consisted of four C(18:1). The cluster of CL bicharged peaks described above could also be seen in the ESI-MS profile of the total lipid extract of control cells, indicating that no difference in CL species is introduced by osmotic shock.

In the following, the term CL will indicate all the different molecular species of CL.

Role of CL in RC thermal stability and photodegradation

A protein structure results from a critical balance between the attractive and the repulsive forces involving the biopolymer and the aqueous or lipid surroundings. If this balance is disturbed by chemical or physical factors, denaturation can take place and the structural details can be partially or completely lost, including details from the quaternary to the secondary protein structure (21).

One of the simplest ways to examine protein stability is to observe the effects due to the rise in temperature; usually, thermal denaturation is studied by investigations into kinetics or thermodynamics, which give indications about the loss of functional or structural integrity.

In the literature, numerous works about hydrophilic protein thermal stability are reported, whereas less information is available about transmembrane proteins, which are generally studied in detergent micellar or in lipid reconstituted systems. It appears unexpected that the study of the stability of many interesting protein systems in native lipid bilayers has been neglected until now (22).

A number of authors (4, 6, 23) have also pointed out the lack of specific studies involving the effects of the interaction CL/RC on protein activity and stability. Some works have investigated the RC thermal stability of *Thermochromatium tepidum* (24), *Chloroflexus aurantiacus* (25), and *R. sphaeroides*, but in most of them, the authors have reported only the

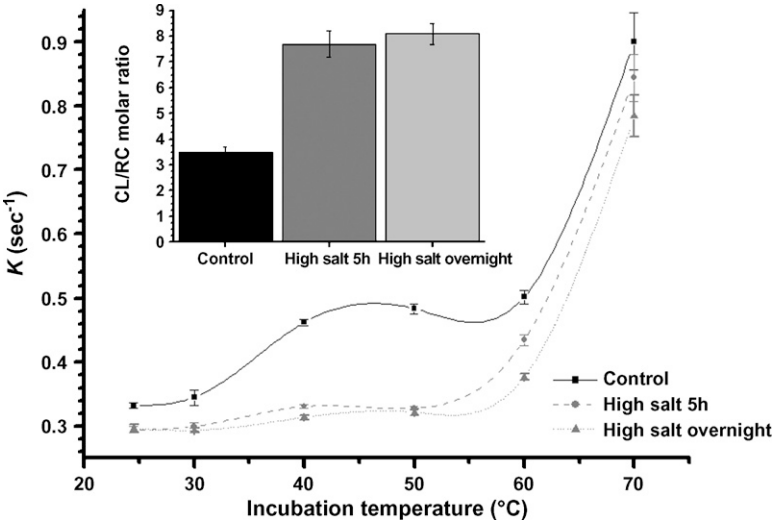

Fig. 6. Charge recombination (CR) kinetic constant values as a function of temperature of chromatophores with increasing CL/reaction center (RC) molar ratio (inset). The incubation time was 30 min; lines are only guides for reading the graph. Reported data represent mean values ± standard deviations obtained from 15 replicates.

temperature at which the native RC spectrum is completely or 50% lost; the possibility that CL presence could confer stability to RC has only been vaguely hypothesized.

In a previous article on a mutant *R. sphaeroides* strain, X-ray crystallography and differential scanning calorimetry data suggested that CL may contribute to preserving the purified RC from thermal denaturation (26).

In this work, thermal denaturation was studied in control and CL-enriched chromatophores. **Figure 6** shows the CR constant values of the RC in control and CL-enriched chromatophores. It can be observed that even at room temperature, before starting heating, CL-enriched chromatophore membranes were found to have CR constant values significantly lower than those of control chromatophores. This is consistent with the data in the literature (27), which confirm that CL addition to isolated RCs in suspension with detergent caused the CR $P^+Q_B^-$ to significantly slow down. Because the main path for the CR is via $P^+Q_A^-$, this slowing down effect indicated a high equilibrium constant value for the monoelectronic transfer

$$Q_A^- \; Q_B \rightleftarrows Q_A \; Q_B^-$$

suggesting an increase in the functional occupation of the Q_B site. Moreover, it is evident that RCs in CL-enriched chromatophores preserve their original functional characteristics in a wider temperature range, showing clear differences from the control even at 25°C. These differences became of no importance at temperatures higher than 50°C, because denaturation started to be too pronounced.

It is likely that the changes in CR values with the rise in temperature are caused by variations in the distances between cofactors inside RCs, which occurred as an obvious consequence of the structural collapse of the protein scaffold (22).

As a matter of fact, the RC binding sites of cofactors [Bchls and bacteriopheophytins (Bpheos)] are located at the interface between the L and M protein subunits, and every cofactor is in contact with both subunits. According to Hughes et al. (22), the thermal denaturation process could occur by separation of the L and M polypeptides and consequent destruction of the binding sites with their cofactors. Then the cofactors would be released from the protein scaffold when the denaturation was advanced. This hypothesis was verified by fluorescence measurements for purified RCs that were embedded into liposomes. Usually, cofactors do not exhibit a strong fluorescence when they are bound in the RC, whereas free Bchls and Bpheos have to give the absorbed energy back radiatively because they cannot transfer this excitation to close pigments.

In the case of chromatophores, because they also contain antenna complex pigments, the fluorescence spectra were more complex. When the three chromatophore preparations were subjected to high temperature for variable periods of time, excitation at 531 nm caused fluorescence emission at 760 nm (attributable to free Bpheo) to increase linearly with time and this increase was faster for control than for shocked samples.

The graph shown in **Fig. 7** refers to a chromatophore denaturation that was conducted at 60°C, even though

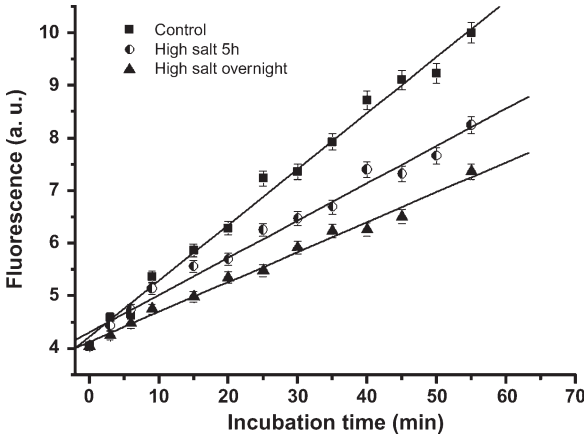

Fig. 7. Fluorescence emission values for chromatophores containing variable CL amounts, as a function of the denaturation time at 60°C. Similar results were obtained at 70°C and 80°C (data not shown). The observable lines were obtained by linear regressions. Reported data represent mean values ± standard deviations obtained from three replicates.

analogous results were obtained at 70°C and 80°C. Therefore, all the recorded data confirm that in the CL-enriched membrane, the photechemical apparatus was more resistant against thermal denaturation both from a functional and from a structural point of view. The stabilization effect of CL with respect to thermal denaturation was also confirmed by absorption measurements (data not shown).

Furthermore, in this work, the response of different chromatophore preparations with respect to photooxidative damage was studied. When *R. sphaeroides* cells undergo strong and continuous illumination with white light in the presence of oxygen, bleaching of the protein pigments occurs. Under such conditions, the RCs stay in a charge separation state that is unstable, and consequently, P^+ and Q^- are exposed to external oxidant agents for a long period of time (28). This unstable redox state, together with a

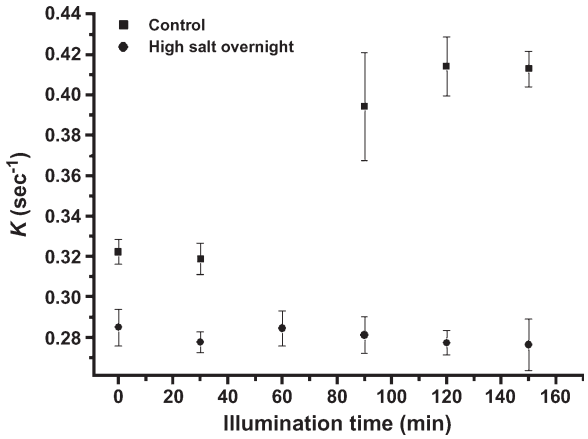

Fig. 8. CR kinetic constant values as a function of the illumination time for control and CL-enriched chromatophores. Every 30 min, the RC functionality was evaluated by sampling the CR constant values. Reported data represent mean values ± standard deviations obtained from 15 replicates.

concomitant protein conformational change, is responsible for the propensity of the RC to be irreversibly damaged. The loss of pigments by RC determines a lower efficiency of the light energy absorption, which is the first requisite for photochemical activity (29). From the graph shown in **Fig. 8**, it is evident that CR constant values in shocked chromatophores are not modified during sustained illumination but that the control sample showed a tendency to speed up its kinetics. This is in accordance with the literature; in fact, it was verified that under continuous and intense illumination, the RC slowly reaches a conformational state that has a faster CR (28).

DISCUSSION

Most purple photosynthetic bacteria develop an extensive internal cytoplasmatic membrane system under photoheterotrophic growth conditions. In *R. sphaeroides*, the interconnected vesicular structures that originate from the cytoplasmic membrane and that are continuous with it can be isolated as "inside-out" vesicles, called chromatophores; these consist of about 25% phospholipids, 5% pigments, and 64% proteins, which, for the most part, belong to the photosynthetic apparatus (30).

We have shown that it is possible to selectively change the phospholipid composition of isolated chromatophores without introducing modification of either pigments or protein membrane profiles. In particular, we have documented that chromatophores appear to be osmotically active membrane vesicles showing a progressive increase in CL levels when isolated from cells exposed to high-salt-containing media for increasing incubation times, this adding new information on the role of CL in osmoadaptation. As well as *Escherichia coli* minicells (31), chromatophores of *R. sphaeroides* represent specialized membrane domains where the PG/CL ratio can be shifted toward the CL formation by osmotic stress. Changes in the amount of this minor lipid in the membrane exert profound effects on the structure and functions of chromatophores.

In previous studies, it was suggested that the CL increase occurring during cell shrinkage (or swelling) may represent a general physiological response of microorganisms to osmotic stress. A higher CL membrane content could protect cells from lysis and confers an increased resistance to mechanical stress. On the other hand, it is widely recognized that CL influences the properties of the proteins associated with it (4–7, 31).

In the past, the role of CL in transmembrane protein functions has been examined by measuring retained phospholipids and phospholipids binding to purified proteins and by reconstituting proteins in artificial membranes. Here, for the first time, we have demonstrated that it is possible to raise CL levels in chromatophores by imposing an osmotic imbalance between the intra- and extracellular medium of the cells. The novel chromatophore preparations used in the present study offer the advantage of introducing changes in the lipid composition that avoid the use of exogenous lipid or detergent molecules that can alter the real and delicate equilibrium existing in vivo between the photosynthetic proteins and the lipid membrane bilayer.

In this work, control and CL-enriched chromatophores have been used as models to test the effect of raising CL membrane levels on the stability of photosynthetic enzymes. The thermal stability of photosynthetic RC, together with its resistance to photodegradation or photooxidation damage, is significantly increased in CL-enriched membranes. The CL stabilizing and activating effects on *R. sphaeroides* RC could be ascribed to the formation of functional molecular complexes involving photosynthetic proteins and surrounding lipids. In fact, RC-CL complexes, coated by an annulus of motionally restricted lipids, have been evidenced by electron paramagnetic resonance spectroscopy experiments (32, 33). It is also reported that CL molecules fill the crevices between subunits or adjacent RC monomers and appear to be important in providing a tight insertion in the membrane lipid matrix (7, 34); therefore, CL protein binding domains may be responsible for the stimulation of enzymatic activity and the stabilizing effect.

The authors thank Dr. Giuseppe Mascolo (CNR-IRSA, Bari, Italy) for the mass spectrometry analyses.

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