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Evaluation of the electrical capacitance in biological membranes at different phospholipid to protein ratios

A study in photosynthetic bacterial chromatophores based on electrochromic effects

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Abstract. Photosynthetic chromatophores of *Rhodobacter capsulatus* were differently enriched in phospholipid content by freezing, thawing and sonicating in the presence of phospholipid vesicles. Closed vesicles, characterized by different phospholipid to protein molar ratios and increasing average radius at increasing phospholipid enrichment, were collected after sucrose density gradient sedimentation. The electrical capacitance of these systems was evaluated from the ratio of reaction center content, photooxidized by single turnover flash in the presence of antimycin, to the corresponding membrane potential difference, measured from the electrochromic red shift of the endogenous carotenoid band. The values obtained, normalized per protein content, increased at increasing phospholipid enrichment, and correlated linearly with the increasing phospholipid to protein molar ratios. The charging capacitance of chromatophores was evaluated to be $3-6\times10^{-17}$ F and was found to increase at increasing average radius of the phospholipid enriched vesicles, as predicted by the equation of the spherical shell dielectric. The carotenoid signal, elicited in the dark by imposing diffusion potentials of known extent with K^+-valinomycin pulses, significantly decreased at high phospholipid enrichment, indicating that in the presence of a large phospholipid excess, a partial displacement of the carotenoid molecules sensing the induced electric field is produced. Concomitantly, the energy transfer efficiency from carotenoids to core light harvesting complexes (B-875) was also partially affected, particularly at high phospholipid to protein molar ratio. All together, these results suggest that the reaction center complexes are dispersed within the lipid bilayer upon fusion and that carotenoids sense a delocalized light-induced transmembrane field.

Key words: Membrane fusion, membrane potential, membrane capacitance, carotenoid red shift, electrochromism

Introduction

The electrical capacitance of natural biomembranes is an important physical parameter for the evaluation and modelling of the transient behaviour of the transmembrane electric potential ($\Delta\psi$) in response to charge translocating processes. These phenomena are particularly relevant in bioenergetics for studying chemiosmotic protonic circuits in non-steady state conditions, when the electrostatic component of the protonmotive force ($\Delta p = \Delta\tilde{\mu}_{H^+}/F$) may represent the predominant driving force for ATP synthesis (see, for a survey, Junge and Jackson 1982; Ort and Melandri 1982; Nicholls 1984; Ferguson 1985).

In recent years, measurements of the electrical properties of model and natural biological membranes have been carried out in several laboratories. The values reported for the electrical capacitance range from 0.5–0.6 μF cm^{-2} for planar black lipid films to 1–2 μF cm^{-2} for biological membranes from various sources (see e.g. Pauly et al. 1960; Schwan 1963; Hanai et al. 1965; Cole 1970; Fettiplace et al. 1971; Feldberg and Delgado 1978). Generally, either inner voltage clamping experiments or dielectric dispersion measurements have been performed. More recently, the dependence on superimposed electric fields of some spectroscopic properties of an intrinsic photosynthetic pigment, such as electrochromism and photoluminescence, has been utilized as an indirect tool for evaluating the charging capacitance of photosynthetic membranes (De Grooth et al. 1980; De Grooth and

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Abbreviations: BChl, bacteriochlorophyll; $[BChl]_2$, reaction center; PL, phospholipid; cyt, cytochrome; $\Delta\psi$, transmembrane electrical potential difference; TES, 2-2-Hydroxy-1,1-*bis*-(hydroxymethyl)ethyl-amino-ethanosulfonic acid; mg_p, mg protein.

Van Gorkom 1981; Farkas et al. 1984; Arnold et al. 1985).

In this respect, membranes from some photosynthetic non-sulphur purple bacteria, such as *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, offer a unique system for the evaluation of their electrical capacitance. In chromatophore vesicles from these bacteria, the number of electrical charges translocated across the membrane can be conveniently estimated by measuring spectroscopically the concentration of reaction centers under conditions in which every turnover promotes the translocation of one electron (see, for a review, Crofts and Wraight 1983; Dutton 1983). In parallel, the electric potential difference resulting from this process is monitored from the electrochromic response of the photosynthetic pigments present in the membrane (particularly of carotenoids). This, in turn, is calibrated by means of artificial ion diffusion potentials (see, for a review, Wraight et al. 1978; Junge and Jackson 1982). This approach was first used by Packham et al., who reported for these photosynthetic membranes a capacitance of 1.1 μF cm^{-2} (Packham et al. 1978).

Recently we have set up a procedure for the specific enrichment of bacterial membranes with exogenous phospholipids and studied, in chromatophores fused with liposomes, the effect of the dilution of membrane proteins on the kinetics of intramembrane redox reactions (Casadio et al. 1984). In this paper we use the same vesicle systems for evaluating the electrical capacitance of membranes characterized by different molar ratios between phospholipids and electron transfer proteins. On the basis of the results obtained, the previous estimation of the capacitance of bacterial photosynthetic membranes is confirmed and generalized to a much broader spectrum of membrane composition. The results can also be considered as a stringent test for the validity of the electrochromic response of carotenoids as a tool for the measure of the transmembrane electric potential difference, averaged over the entire membrane surface.

Materials and methods

Photosynthetic membrane fragments (chromatophores) were obtained from a green strain of *Rhodobacter capsulatus*, grown photoheterotrophically and harvested at the end of the logarithmic phase of growth, as previously described (Baccarini Melandri and Melandri 1971).

Phospholipid enriched chromatophores were prepared following a freeze-thaw procedure described in detail elsewhere (Casadio et al. 1984). Briefly, chromatophores corresponding to approximately 1 μmole per ml of BChl were mixed with variable amounts of liposomes obtained by sonication of soybean phospholipids (100 mg ml^{-1}), to give the required protein to lipid ratio. The suspension was frozen quickly by immersion in dry ice-ethanol, then thawed at room temperature, sonicated at 0 °C for 30 s and layered onto a discontinuous sucrose gradient. Four bands were obtained after equilibrium centrifugation, characterized respectively by buoyant densities of 1.13 (Band 4), 1.12 (Band 3), 1.08 (Band 2) and 1.06 (Band 1) g cm^{-3}. Band 4 had the same buoyant density as untreated chromatophores.

The fractions were assayed for protein using a modified Lowry procedure (Lowry et al. 1951), for BChl as described by Clayton (1963) and for total phosphorus following the method of Petitou et al. (1978).

The total concentration of reaction centers, of cyt (c_1+c_2) and of cyt b_{561} were measured spectrophotometrically at controlled redox potential, with a computer-linked single beam kinetic spectrophotometer as detailed elsewhere (Venturoli et al. 1986). Measurements of the carotenoid band-shift induced by light were carried out at 503–486 nm using a dual wavelength Sigma-ZW II spectrophotometer equipped with a Xenon flashlamp (with a half width of 15 μs) for single turnover experiments. Suitable control experiments showed that this wavelength pair could be used both with native and phospholipid-enriched chromatophores, since the lipid enrichment of the membrane did not cause any distortion of the light-dark difference spectrum in the carotenoid absorption region.

The assay medium, if not otherwise specified, contained 50 m*M* TES (pH = 7.5), 5 m*M* $MgCl_2$, 0.1 m*M* Sodium succinate and 50 m*M* KCl. Generally the assay contained vesicles corresponding to 20–40 μ*M* BChl, depending on the parameters under measurement. The samples also contained sucrose at a final concentration of 10% (weight to volume); this concentration was adjusted taking into account the amount of sucrose carried over from the sucrose gradient fractions. The concentrations of antimycin and valinomycin, when added, were 5 μ*M* and 2 μ*M*, respectively.

The calibration of the electrochromic signal in the dark was performed essentially as already described (Baccarini Melandri et al. 1977): KCl was omitted from the assay medium and K^+-diffusion potentials were generated in the presence of valinomycin by adding, at constant ionic strength, variable concentrations of K^+ as a KCl-choline Cl mixture.

Absorption spectra were recorded with a Jasco Uvidec 610 spectrophotometer. Excitation and emission spectra of photosynthetic pigments were recorded using a home-made conventional fluorimeter, equipped with a S-1 photomultiplier.

The procedure for electron microscopy is detailed elsewhere (Casadio et al. 1984). Vesicle populations from different fractions of the sucrose gradient were negatively stained. Image analysis of the electron micrographs was performed using a Leitz image digitized analyzer.

Results

Characterization of the phospholipid-enriched membranes

As described in the Materials and methods section, centrifugation on a discontinuous sucrose density gradient subdivides the population of chromatophores fused with liposomes into four fractions, characterized by a different buoyant density. The four bands collected with this procedure contain closed, single-walled vesicles, as detected by electron microscopy of negative stained samples. Each vesicle population is characterized by a different phospholipid to reaction center molar ratio, while the protein to reaction center (mg to nmole) ratio and the bacteriochlorophyll to reaction center molar ratio (i.e. the size of the photosynthetic unit), are maintained relatively constant. This indicates that the fusion procedure results in a specific increase of the phospholipid content without other major alterations in the composition of the membrane. Evidence for the functional integrity of the phospholipid-enriched chromatophores has been given elsewhere (Casadio et al. 1984), together with a demonstration, based on freeze-fractured electron microscopy, of the effective increasing dispersion of the membrane protein complexes within the lipid bilayer at increasing phospholipid enrichment. The phospholipid enrichment of the chromatophore membrane causes, however, a substantial loss of soluble cyt c_2, as revealed by the decrease of the cyt $(c_1 + c_2)$ to reaction center molar ratio. A summary of the functional characteristics of the vesicle populations, used in the present study, is given in Table 1.

The distribution of the vesicle diameter in the four bands from the sucrose density gradients, has been studied by image analysis of the electron micrographs of negatively stained samples (Casadio et al. 1984). Histograms of the diameter distributions are presented in Fig. 1. The average diameter increases as the lipid to protein ratio increases. The observed distribution of the diameters is relatively narrow around the average value with the exception of the fraction most enriched in phospholipid content (Band 1), where the frequency of large diameter vesicles becomes significant.

Evaluation of the membrane capacitance in control and phospholipid-enriched chromatophores

The electrical capacitance ($C = Q/\Delta\psi$) of these vesicle populations has been calculated from the ratio of elementary charges translocated across the membrane dielectric upon photooxidation of reaction centers (Q) to the corresponding transmembrane potential difference ($\Delta\psi$).

Calibration with ionic diffusion potentials of the electrochromic effects of the electric field-sensing endogenous carotenoid molecules. The values of $\Delta\psi$ have been measured from the amplitude of the spectroscopic signals of the endogenous carotenoids induced by the onset of the electric field across the membrane. The mechanism by which these signals are generated is thought to be an electrochromic effect on the carotenoid absorption bands, resulting in a shift towards longer wavelength of the absorption maximum. When measured at specific wavelength pairs, the electrochromic shift produces changes of the absorbance which are linear with the intensity of the electric field (for a complete discussion, compare De Grooth and Amesz 1977; Venturoli et al. 1987). Calibration of the amplitude of the electrochromic signal as a function of $\Delta\psi$ is usually obtained by measuring, in the dark, the absorption change produced by K^+-diffusion potentials of known amplitude in valinomycin-supplemented

Table 1. Functional analysis of phospholipid-enriched chromatophores

Band	BChl/$[BChl]_2^+$ [molar ratio]	Protein/$[BChl]_2^+$ [mg_P/nmole]	PL/$[BChl]_2^+$ [molar ratio]	cyt(c_1+c_2)/$[BChl]_2^+$ [molar ratio]
1	170±40	1.6±0.3	7000±4000	0.51±0.10
2	150±60	1.3±0.4	4000±2000	0.48±0.10
3	160±70	1.5±0.3	1900± 400	0.55±0.10
4	150±50	1.8±0.6	1300± 300	0.80±0.10
Chromatophores	150±50	1.9±0.8	1100± 500	0.80±0.10

The results are expressed as mean ± standard deviation for eight to ten experiments of fusion performed using different preparations of liposomes and chromatophores

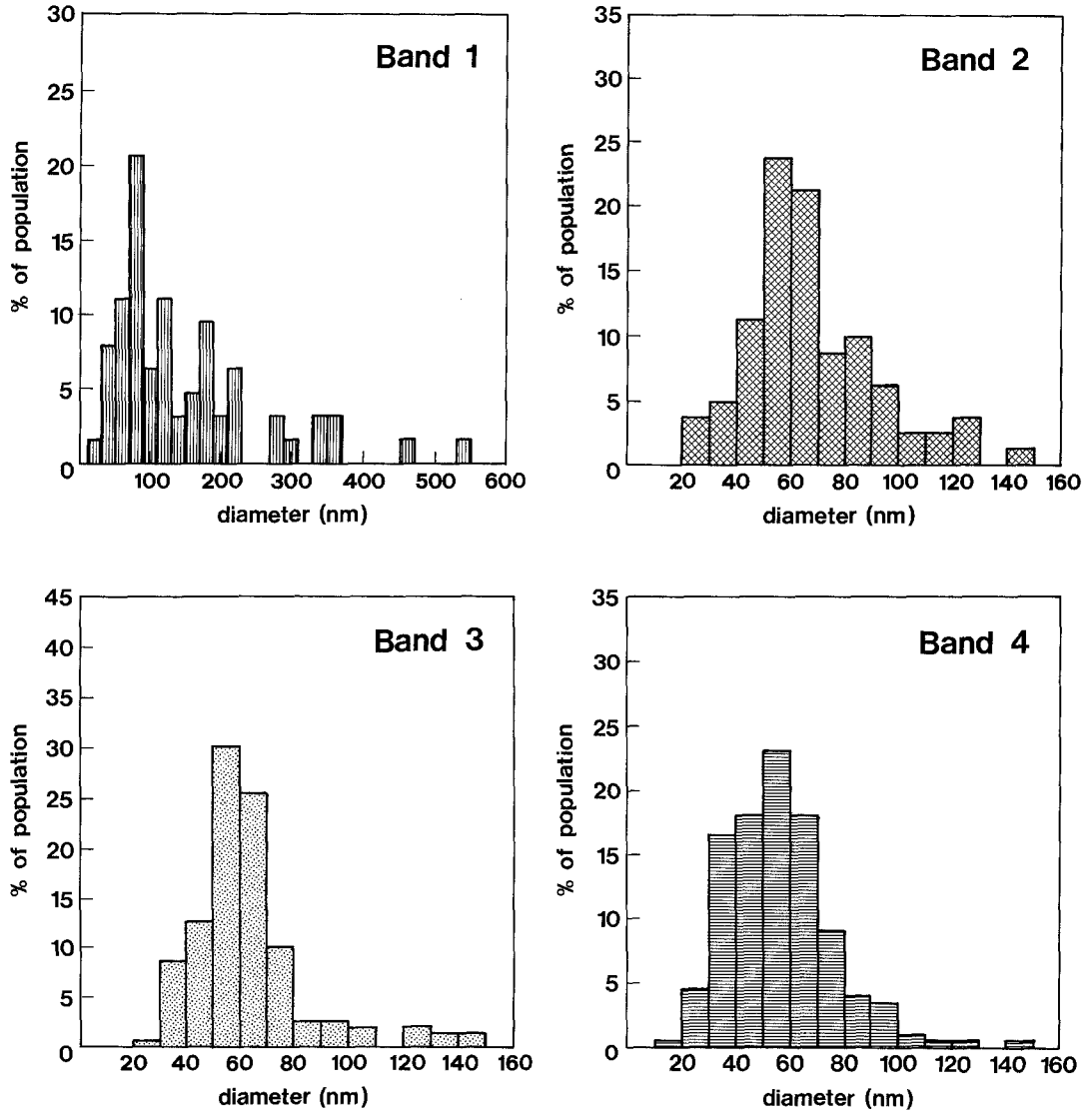

Fig. 1. Diameter distributions of phospholipid-enriched chromatophores. The phospholipid to reaction center molar ratios ($PL/[BChl]_2$) and the average diameter $\pm$ standard deviation in the four Bands are respectively: in *Band 4*, 1000 and (56 ± 18) nm; in *Band 3*, 1300 and (63 ± 23) nm; in *Band 2*, 2200 and (68 ± 23) nm; in *Band 1*, 3800 and (150 ± 107) nm. Control chromatophores, in this experiment, were characterized by a $PL/[BChl]_2$ molar ratio of 800 and an average diameter of (50 ± 20) nm (data not shown)

vesicles. The amplitude of the K^+-induced spectroscopic signals has been shown to be linear with the logarithm of the concentrations of K^+ added to generate the diffusion potential; this response is in agreement with the Nernstian dependence of $\Delta\psi$ on the K^+ concentration and confirms the linearity of the electrochromic effect with the electric field.

A collection of such calibration curves, obtained for native chromatophores and for the four fractions of the phospholipid enriched vesicles, is presented in Fig. 2. The linearity of $\Delta A_{503-486}$ vs. log K^+ is generally observed for all samples in the whole $\Delta\psi$ range that can be tested experimentally (about 100 mV, corresponding to a fifty fold increase in K^+ added). As long as the lipid enrichment is contained within a factor of two, the slope of the response is comparable with that obtained with chromatophores, and decreases quite significantly for higher lipid to protein ratios. Since the amplitude of the electrochromic response of the chromophore depends, inter alia, on its orientation with respect to the electric field, this observation may suggest that some disorganization of the field sensing carotenoid is produced by a marked increase in the phospholipid content; this will be further examined below. Curves similar to those shown in Fig. 2 have been determined for each preparation of fused chromatophores. The constant slope of $\Delta A_{503-486}$ vs. log K^+ for each calibration curve has been used for the evaluation of the light-induced $\Delta\psi$ in that specific sample.

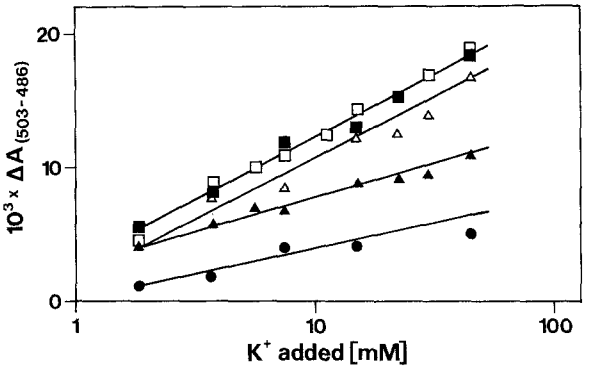

Fig. 2. Calibration of the carotenoid shift with ionic diffusion potentials in control and phospholipid-enriched chromatophores. The amplitude of the carotenoid signal induced in the dark is related to the concentration of K^+ added. For the evaluation of $\Delta\psi$, the change of $\Delta A_{503-486}$, corresponding to a ten fold increase in K^+ added, is considered to measure 59 mV of transmembrane potential difference. *Open squares:* chromatophores; *full squares:* Band 4; *open triangles:* Band 3; *full triangles:* Band 2; *full circles:* Band 1

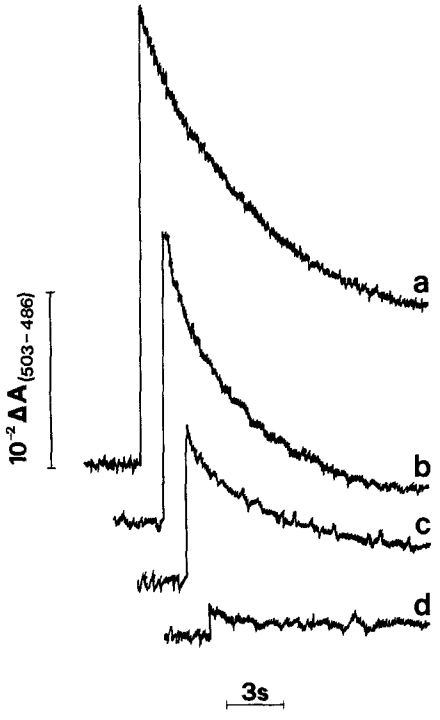

Fig. 3. Flash-induced carotenoid shift in control and phospholipid-enriched chromatophores in the presence of antimycin. *Trace a:* chromatophores, $PL/[BChl]_2 = 1000$; *Trace b:* Band 4, $PL/[BChl]_2 = 1600$; *Trace c:* Band 3, $PL/[BChl]_2 = 2000$; *Trace d:* Band 2, $PL/[BChl]_2 = 3000$. $[BChl]_2 = 0.33\ \mu M$

Light-induced $\Delta\psi$. The electrochromic signal generated by a single turnover flash of actinic light in vesicles inhibited by antimycin, has been monitored to evaluate the transmembrane electric potential difference induced by the translocation of a known number of electrical charges across the dielectric barrier. In the presence of antimycin, the secondary electron transfer is totally inhibited; one electron is transferred across the membrane for every photoactivated reaction center and delivered to the primary acceptor. Subsequently, the photooxidized electron donor, a specialized BChl pair, is reduced by the hydrophilic cyt c_2, thus completing the translocation of one electron across the whole membrane span. This latter step requires the availability of reduced cyt c_2, which in our preparations is partially lost during the fusion treatment (Table 1). In antimycin-supplemented chromatophores, a small portion of the carotenoid signal, elicited by a single turnover flash, is also associated with an electrogenic partial reaction of the ubiquinol cytochrome c_2 oxidoreductase complex, as demonstrated by Glaser and Crofts (1984). With suitable control experiments, however, we have demonstrated that in our preparations the extent and the initial rate of this phenomenon are negligible owing to the scarcity of cyt c_2 (data not shown).

A series of electrochromic signals induced by one single turnover flash (in the presence of antimycin) in control chromatophores and in three fractions of phospholipid-enriched vesicles is shown in Fig. 3. The concentrations of BChl and photosynthetic reaction center are comparable in all the assays. At increasing phospholipid content, a very pronounced decrease in the amplitude of the electrochromic signals is noticeable. A kinetic analysis of the decay in the dark of the electrochromic traces, obtained using the different vesicle populations, was performed. The results (not shown) indicate that the traces depicted in Fig. 3 cannot be accurately fitted by a single exponential decay, but rather by the convolution of several exponential decays, with slightly different relaxation times. Moreover, it is evident that the freezing and thawing procedure causes an approximately two fold acceleration of the decay of the electrochromic signal as compared to control chromatophores (compare trace *a* and *b* of Fig. 3); no further change in the decay rate is observed at increasing phospholipid-enrichment. In no case, however, are the changes in the decay rates large enough to account for the decreases in the initial amplitude of the flash induced signals. This observation rules out the possibility that the progressive decrease of the amplitude of the carotenoid shift might be due to an increase of the passive permeability of the membrane after phospholipid-enrichment. The progressive decrease of the signal amplitude, shown in Fig. 3, can only be partially accounted for by the decreased slope of the $\Delta\psi$ calibration curves in the phospholipid-enriched vesicles.

Evaluation of the membrane capacitance. A complete set of data for the evaluation of the electrical capacitance in chromatophores and in phospholipid en-

Table 2. Evaluation of the electrical capacitance in control and phospholipid-enriched chromatophores

Band	PL/Protein [nmoles/mg_P]	$[BChl]_2^+$/Protein [$10^{12} \times$ moles/mg_P]	f_{Cal}*	$\Delta\psi$ [mV]	Q/Protein [$10^7 \times$ C/mg_P]	Capacitance/Protein [$10^4 \times$ F/mg_P]
1	3200	708	0.15	25	682.8	27.3
2	2700	770	0.17	30	742.5	24.8
3	1100	670	0.42	45	646.1	14.4
4	800	560	0.44	60	540.0	9.0
Chromatophores	800	530	0.30	59	511.1	8.7

* Calibration factor (f_{Cal}) measured from experiments similar to those described in Fig. 2; f_{Cal} is expressed as $10^3 \times \Delta A_{(503-486)}$ μM^{-1} BChl 59 mV^{-1}. BChl concentration is 50 μM

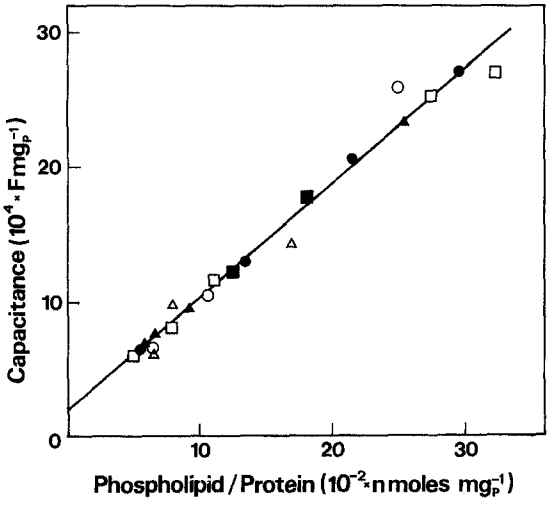

Fig. 4. Dependence of the specific electrical capacitance on the phospholipid to protein ratio of control and phospholipid enriched-chromatophores. The different symbols indicate different experiments of phospholipid-enrichment

riched vesicles is presented in Table 2. In this table the values of $\Delta\psi$ generated by a single turnover flash are related to the amount of reaction centers photooxidized under the same experimental conditions, but after addition of 2 μM valinomycin, in order to abolish the electrochromic effect. The corresponding electrical capacitance is thereby calculated. It is quite evident from these results that, at increasing phospholipid to reaction center ratios, $\Delta\psi$ is progressively diminished, demonstrating that the phospholipid enrichment of the chromatophore membrane causes a marked increase of the membrane charging capacitance.

The apparent electrical capacitance of native and phospholipid-enriched chromatophores is correlated in Fig. 4 with the phospholipid content of the vesicles. Both parameters are normalized to the total proteins contained in every single preparation, in order to compensate for a possible protein loss during the fusion process. The increase in the apparent capacitance is linear with the phospholipid to protein ratio up to a seven fold enrichment of the membrane with phospholipid. The constant slope of the correlation pattern corresponds to a specific capacitance increase of about 0.84 μF nmole PL^{-1} (or 1.4×10^{-15} μF per PL molecule). Assuming an average area of 0.5–0.6 nm^2 for a phospholipid molecule in a bilayer arrangement, the specific capacitance per phospholipid molecule corresponds to an average increase of the membrane capacitance per surface area of the bilayer, of about 0.5–0.6 $\mu F\ cm^{-2}$. This result is in good agreement with the electrical capacitance values measured in black phospholipid films (see e.g. Hanai et al. 1965; Fettiplace et al. 1971; Feldberg and Delgado 1978). The extrapolation to zero phospholipid content of the correlation line of Fig. 4, suggests that the contribution to the overall capacitance of the protein content of the membrane is very limited and can become negligible in the most phospholipid-enriched vesicles. When the reasonable assumption is made that only about 50% of the total chromatophore proteins are intrinsic membrane proteins and contribute to the membrane capacitance, the intercept in Fig. 4 (1.76×10^{-4} F mg_P^{-1}) would correspond to a capacitance per unit area of the membrane protein component of about 0.2–0.4 $\mu F\ cm^{-2}$, similar to that of the lipid. These values have been calculated by assuming for the density of a membrane protein the value of 1.24 g cm^{-3} (as determined for delipided bacteriorhodopsin by Hwang and Stoeckenius 1977), and limiting values for the thickness of the protein assembly of 5 and 10 nm, respectively. The average dielectric constant of the membrane protein component can be thereby calculated to range from 1 to 5, when a charge separation distance of 5 or 10 nm is assumed, respectively. It is therefore rather close to that of the lipid component and smaller than expected when the high polarizability of some of the membrane proteins (e.g. that of the reaction center measured by Lockart and Boxer 1987) is considered. In fact, the capacitance per unit area of biological membranes (1–2 $\mu F\ cm^{-2}$), as compared to that of black lipid membranes (0.5–0.6 $\mu F\ cm^{-2}$), indicates that the contribution of the

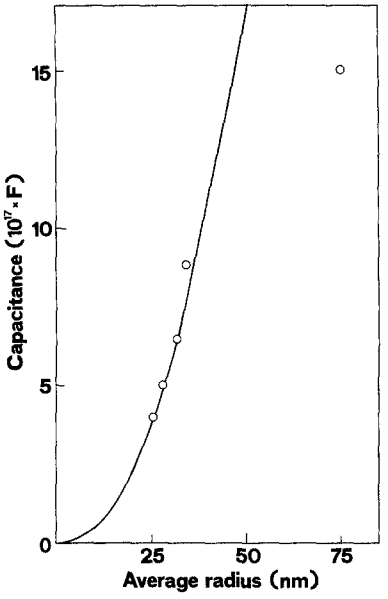

Fig. 5. Correlation between the electrical capacitance of control and phospholipid-enriched chromatophores and their average diameter. The capacitance values, at a given phospholipid to protein molar ratio, are obtained from Fig. 4. The diameters, for the same vesicle preparations, are the average values obtained from the histograms of Fig. 1. The capacitance value of 4×10^{-17} F for a single chromatophore is calculated from the correspondent C mg_P^{-1}, by assuming that a single vesicle contains 3×10^3 molecules of BChl, and by considering that in this preparation the protein to reaction center ratio was 1.7 mg_P $nmoles^{-1}$ $[BChl]_2$, and that the BChl to reaction center molar ratio was 150. The capacitance values of the phospholipid-enriched vesicles was similarly determined. By utilizing the equation of the spherical shell dielectric ($C = 4\pi\,\varepsilon_m\,\varepsilon_0\,R(R-d)/d$; with ε_0, vacuum dielectric constant, 8.81×10^{-12} Fm^{-1}; ε_m, membrane dielectric constant; R, average vesicle radius; d, dielectric thickness) the value of ε_m is calculated to be 2.5 when d is considered equal to 37.5 nm (for the evaluation of the dielectric thickness, see Discussion). The theoretical curve indicates the expected dependence of the capacitance on the vesicle radius

protein component to the overall capacitance should be more consistent. It should be noticed, however, that the linear extrapolation to zero phospholipid content in Fig. 4 might be unjustified, since it postulates an unrealistic model of a membrane totally devoid of lipid.

Assuming an average content of $3-5 \times 10^3$ BChl molecules per chromatophore (Saphon et al. 1975; Packham et al. 1978), and by considering that the average size of the photosynthetic unit, in our chromatophore preparations, is about 150 BChl per reaction center molecule (see Table 1), the measured capacitance per mg protein would correspond to an electrical capacitance per chromatophore of $4-7 \times 10^{-17}$ F. These values are in good agreement with previous estimates (Packham et al. 1978). On a similar basis, the average electrical capacitance of the different vesicle populations can also be evaluated, by assuming that, during the phospholipid-enrichment, the average number of BChl molecules present in the control vesicle is maintained, and considering that the average size of the photosynthetic unit is conserved (as shown in Table 1). This is equivalent to the assumption that the relative increase in capacitance is proportional to the actual increase in surface area. The capacitance values, obtained from the straight line of Fig. 4 for the experimental values of the lipid to protein ratios, are plotted in Fig. 5 as a function of the average radius of the vesicle population (as determined by electron microscopy and shown in Fig. 1). The electrical capacitance increases at increasing average radius of the vesicle population, as predicted by the equation of the spherical shell dielectric. A marked deviation from the theoretical curve is observed only for the fractions most enriched in phospholipid. This may be interpreted by considering that the dispersion towards large diameters in the distribution frequency of the vesicle sizes of Band 1 (see Fig. 1) includes a fraction of inactive vesicles; the theoretical curve would predict for the active population an average diameter of about 90 nm, coinciding with the frequency peak in the size distribution. It is noteworthy that, on the basis also of the other functional tests [$\Delta\psi$ calibration with K^+-diffusion potentials and measurements of energy transfer (described below)], Band 1 appears to contain the most damaged vesicles. The fair agreement of the experimental and theoretical behaviour suggests that the phospholipid-enrichment process originates from fusion of one chromatophore with one or more phospholipid vesicles and that no subsequent fragmentation of the fused vesicle occurs.

Energy transfer in phospholipid-enriched chromatophores

In order to investigate the possible causes of the decrease in the slopes of the $\Delta\psi$ calibration observed in fused vesicles at high phospholipid enrichment, energy transfer between the photosynthetic pigments has been studied. The rationale of these measurements rests on the notion that the electrochromic linear response of carotenoids is due to pigments located in the most peripheral part of the antenna, which is formed by the B-800–850 light harvesting complexes (or LH_{II}) (Cogdell and Thornber 1980). In principle, a possible dissociation of this complex from a supramolecular organization of the antenna might induce a random orientation of the chromoproteins and, consequently, a decrease in the amplitude of the electrochromic response. This phenomenon is, in fact, strictly dependent on the mutual orientation of the light-induced electric field and of the difference in the electric dipole moment of the excited minus ground state of the chromophore transition.

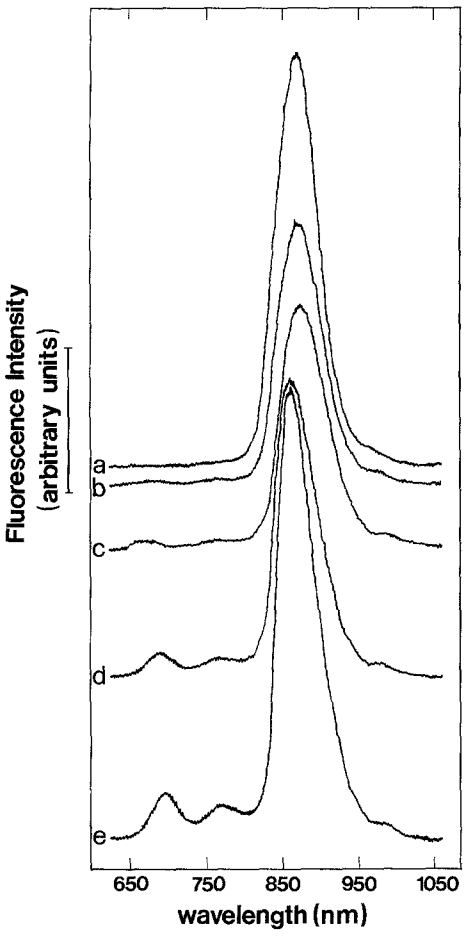

Fig. 6. Fluorescence emission spectra of control and phospholipid-enriched chromatophores. Excitation wavelength = 510 nm; $T = 300$ K. Each sample was adjusted so as to have an identical absorbance of 0.38 at 855 nm, corresponding approximately to 4 μM BChl. For trace symbols see text

Dissociation phemomena within the photosynthetic antenna can be conveniently monitored by studying the fluorescence emission of chromatophores. In Fig. 6, the fluorescence emission spectrum of chromatophores is compared to that of the four enriched fractions. The spectra have been recorded at room temperature, exciting, at a wavelength of 510 nm, samples rigorously adjusted so as to have a constant absorbance at 855 nm. The wavelength chosen for fluorescence excitation falls in the absorption band of carotenoids. Under normal conditions of growth, the amount of BChl contained in the *B*-800–850 complex is approximately five fold more abundant than that in the *B*-875 complex (the inner antenna complex directly associated with the reaction center). The BChl-carotenoid molecular ratios in the *B*-800–850 and *B*-875 complexes are 2:1 and 1:1, respectively (Niedermann and Gibson 1978). It is therefore possible to estimate that at least two thirds of the excitation energy at 510 nm is captured by the *B*-800–850 complexes. Under these experimental conditions, the emission spectrum of chromatophores (trace *a*) is characterized by a single band centered at 890 nm, which, at 300 K, results from the convolution of the *B*-800–850 and *B*-875 emission bands, centered respectively around 865 and 900 nm (Van Grondelle et al. 1982). Upon freezing and thawing, in the less enriched fractions (trace *b* and *c*), a 10 nm blue shift and a considerable decrease of the fluorescence emission are detectable. It can be excluded, on the basis of absorbance spectra (data not shown), that a loss of the carotenoid content occurs during phospholipid enrichment of the chromatophore membrane. A possible explanation of the observed decrease in the fluorescence emission is, therefore, that the freezing and thawing procedure causes a reorganization within the antenna complexes in the chromatophore membrane, resulting in non-radiative dissipation phenomena. At higher phospholipid content (traces *d* and *e*), a more pronounced blue shift and an enhancement of the fluorescence intensity in the emission spectra are noticeable. Furthermore, a second weak emission band, with a maximum at 675 nm, becomes evident. These data are consistent with an increasing predominance of the direct fluorescence emission from the peripheral *B*-800–850 (emission at 865 nm) as it would occur upon dissociation from the *B*-875 core complex, and consequent loss in the efficiency of energy transfer between the two types of complexes. The conclusion that *B*-800–850 dissociates from the antenna as a consequence of a large phospholipid-enrichment has been reached also by Westerhuis et al. (1987) on the basis of fluorescence yield and singlet-singlet annihilation measurements.

Discussion

Two basic assumptions are the basis of the present study: *a*) the photosynthetic electrogenic reaction within the reaction center results in an effective translocation of one electron per turnover across the entire thickness of the membrane dielectric, so that a truly delocalized electric potential difference is generated; *b*) the electrochromic phenomena used for the evaluation of $\Delta\psi$ do monitor a potential difference between the membrane opposite faces, which are in electrical equilibrium with the aqueous bulk phases. Consequently, the generation of valinomycin mediated K^+-diffusion potentials is a reliable method for the calibration of $\Delta\psi$ generated by transmembrane charge separating processes. These assumptions will be discussed separately in the following.

It is generally believed, on the basis of experimental observations, that, in the presence of inhibitors of the secondary electron transfer (in our case antimycin),

the excitation of the electron donor in the reaction center promotes the photochemical reduction of a chain of electron acceptors, until one electron is delivered to a ubiquinone 10 molecule (Q_B), which can exchange with the pool of ubiquinone freely diffusable with the membrane lipid. The structure of the reaction center complex, recently elucidated by X-ray crystallography in the related species *Rb. viridis* and *Rb. sphaeroides* (Deisenhofer et al. 1984; Allen et al. 1986), is such as to locate the primary donor inside the hydrophobic core of the membrane (about 25% of the length of the transmembrane protein helices from the inside of chromatophores) and the quinone acceptor molecule Q_B close to the external surface of the chromatophores. From these structural data, it can be evaluated that the primary donor and Q_B are about 3 nm apart when a 4 nm length is assumed for the protein helices. The primary electron transfer step between these two electron carriers occurs in about 150 μs and induces a very fast electrochromic signal of the electric field-sensing carotenoid molecules (kinetic phase I). Re-reduction of the photooxidized donor is performed by the hydrophilic, soluble cyt c_2, present inside the lumen of the chromatophores. The electron delivery by the reduced cyt c_2 to the reaction center promotes, therefore, the translocation of one electron across part of the membrane dielectric, and corresponds to a second phase of the electrochromic signal (kinetic phase II). Thus, only the total amplitude of phase I+II is related to the movement of one electron across the entire membrane span (see Junge and Jackson 1982; Crofts and Wraight 1983).

In our preparations, control untreated chromatophores contain about 0.8 cyt ($c_1 + c_2$) per reaction center molecule, about 0.5 per reaction center of which should be cyt c_1 (Crofts and Wraight 1983): only about one third of the reaction center seems, therefore, to be associated with its direct electron donor. Moreover, cyt c_2, being soluble, is progressively lost in the lipid enriched fractions. Correspondingly, an increasing proportion of BChl (from about 65% to 100%) is not promptly reduced and generates, in the time scale of our measurements, a relatively stable fixed positive charge inside the lipid bilayer. In parallel the amplitude of the corresponding carotenoid signal is reduced from that of phase I + II to that of phase I, monitoring the movement of one electron across only part of the membrane dielectric. This thickness can be estimated to range from 75% to 60% of the total membrane span, from the structural (Deisenhofer et al. 1984) and spectroscopic data (Takamiya and Dutton 1977), respectively. In the present study, this conclusion has been confirmed experimentally, by measuring the electrochromic signal at high redox potential (higher than 400 mV), when cyt c_2 is totally oxidized before the flash. Under these experimental conditions, kinetic phase II is abolished and only phase I contributes to the electrochromic effect of the carotenoid molecules (Takamiya and Dutton 1977). On this basis, we have determined that in control chromatophore preparations the kinetic phase II is only 15%–20% of the total kinetic phase I+II (data not shown), indicating that in these membrane vesicles a majority of reaction center molecules are not associated with cyt c_2. The slope of the linear dependence of the membrane capacitance on the increasing phospholipid content, shown in Fig. 4, includes also, in the capacitance values, the effects of the progressive loss of cyt c_2. Corrections for this phenomenon, are, however, hardly significant, since, as previously discussed, control chromatophores are already partially depleted of soluble cyt c_2.

From the previous observations, it follows that the values of the membrane electrical capacitance, shown in Fig. 4 as a function of the phospholipid to protein ratio, should be related with 75%–60% of the membrane thickness when evaluating the dielectric constant of the membrane bilayer. On this basis, and by using the equation for the spherical shell dielectric, the dielectric constant of the chromatophore membrane is calculated to be rather similar to that of a pure lipid bilayer and to range from about 2 to 4, depending on the assumed thickness of the dielectric barrier (from 3 to 5 nm, Fettiplace et al. 1971) and the number of bacteriochlorophyll molecules per chromatophore membrane (Saphon et al. 1975). The scarcity of cyt c_2 can also be associated with the presence of fixed positive charges in the membrane core. However, our experimental finding that all the capacitance values correlate linearly with the phospholipid content of the membrane, i.e. with the average surface area per reaction center, indicates that the electrostatic effects of these fixed charges are effectively delocalized, possibly by a fast rearrangement of counterions at the membrane interface. The same phenomenon may occur at the opposite face of the membrane, where some of the electrons transferred could be stabilized as ubiquinone anionic radicals.

As to the assumption in *b*), criticism has been raised against the use of electrochromic signals for the absolute measure of electrical potential difference between the two aqueous compartments at the opposite sides of the membrane. It has been argued that quite significant distortions in the electrical potential profile might be caused by the presence of fixed charges (present in the dark, or generated by light-driven processes) and might be sensed differently by the field-responding pigments (see, for review, Wraight et al. 1978; Baccarini Melandri et al. 1981; Junge and Jackson 1982). Consequently, the use of a calibration technique based on diffusion potentials and performed in the presence of high concentration of a lipophilic cation (the valinomycin-K^+ complex), could cause an overestimation of

the average potential difference generated in the light, not taking into account the local short range electrochromic responses. Our results do not support this rather pessimistic conclusion: in fact, the values of the capacitance determined experimentally are quite in line with those obtained for other biological membranes with different experimental approaches and do not suggest any overestimation of $\Delta\psi$. Particularly convincing is the specific increase in capacitance due to the phospholipid-enrichment, obtained from the slope of the correlation line in Fig. 4. The value of 0.5–0.6 $\mu F\ cm^{-2}$, which should be related only to the specific contribution of the membrane lipids, coincides with that measured directly in pure phospholipid planar membranes.

The linear electrochromic response of carotenoids is specifically due to the pigments associated with the *B*-800–850 complexes, i.e. to the outmost components of the antenna structure. The phospholipid-enrichment procedure has been shown to decrease the efficiency of energy transfer between *B*-800–850 and the *B*-875-reaction center system. Westerhuis et al. (1987) reported that the loss in efficiency is due to a progressive dissociation of *B*-800–850 from the antenna following dilution with phospholipid. This conclusion is suggested also by our fluorescence emission data (Fig. 6). It is quite indicative that in the vesicle preparations characterized by the same high lipid to protein ratio, the slope of the calibration curves of the electrochromic signal vs. diffusion potentials decreases markedly. We suggest, therefore, that, following the dissociation from the antenna, the *B*-800–850 complexes are released from the structural constraints which keep their orientation with respect to the membrane plane. Consequently the dissociated complexes become randomly oriented and silent in the overall linear electrochromic response of the membrane pigments. The field-sensitive carotenoids are, therefore, only those present in an organized antenna structure and remaining at a constant average distance from the reaction centers. These considerations strengthen further the conclusion that the electrochromic signals monitor a truly delocalized transmembrane potential difference.

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