# Effects of Phospholipase $A_2$ Digestion on the Carotenoid and Bacteriochlorophyll Components of the Light-Harvesting Complexes in *Rhodobacter sphaeroides* Chromatophores†

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ABSTRACT: The instantaneous electrochromic response of carotenoids associated with the B800–850 light-harvesting complex of *Rhodobacter sphaeroides* has been used widely as an intrinsic probe of membrane potential. In the present study, the structural basis for this phenomenon was examined by phospholipase $A_2$ digestion of chromatophores from *R. sphaeroides* strain NF57G, containing B800–850 as the sole pigment–protein complex. The major phospholipase-induced alterations of the overall carotenoid absorption spectrum were characterized by an absorbance loss and a blue shift that were accompanied by a decrease in absorbance at 800 nm and a red shift in the B850 absorbance band. In wild-type chromatophores, the electrochromic carotenoid response induced by both flash illumination and a $K^+$ diffusion potential was diminished by ~60% after 1 h of digestion. The initial loss of the carotenoid response was correlated specifically to the hydrolysis of phosphatidylethanolamine, and was shown to arise from effects exerted directly upon the electrochromically active carotenoid pool, possibly by alterations in the spatial relationship between the field-sensitive carotenoids and the polarizing permanent field. In phospholipase $A_2$-digested NF57G preparations in which the B800 band was diminished by nearly half and the carotenoid response was abolished, no significant changes in the efficiency of energy transfer from carotenoids to bacteriochlorophyll were detected at 77 K, suggesting that the electrochromically active carotenoids are not energetically linked to B800 bacteriochlorophyll.

The carotenoids of the intracytoplasmic membrane (ICM) of *Rhodobacter sphaeroides* exhibit a spectral response to transmembrane electric field alterations, known as the carotenoid bandshift, that has been used extensively as an intrinsic probe of membrane potential (Crielaard et al., 1988). This shift in the visible carotenoid absorption spectrum is generated by charge separation and donor re-reduction within the reaction center, as well as by electrogenic reactions of the cytochrome $bc_1$ complex (Wraight et al., 1978), and therefore occurs on a very fast time scale. The electrochromically active pigment has been suggested to arise from a red-shifted pool that comprises 20–35% of the total carotenoid (De Grooth & Amesz, 1977; Symons et al., 1977), and is associated with the peripheral B800–850 light-harvesting bacteriochlorophyll *a* (BChl)–protein complex (Holmes et al., 1980; Matsuura et al., 1980; Scolnik et al., 1980a; Webster et al., 1980).

These electrochromic absorption changes show a linear response to membrane potential, which together with the red-shifted absorption maxima of the responsive carotenoids has been explained by the existence of a large permanent field due to fixed charges within the membrane (Wraight et al., 1978). This quasi-linear character arises from the superimposition of the much smaller induced field upon the large permanent field, which shows a quadratic dependence on field strength. The recent Stark effect measurements by Gottfried et al., (1991a,b) have demonstrated that these electrochromic effects are dominated by the difference between the ground- and excited-state dipole moments caused by perturbations on carotenoid electronic structure resulting from the organized protein environment. Although the structural basis for this phenomenon has not been elucidated, model calculations by Kakitani et al. (1982) have suggested that point charges from amino acid residues in the vicinity of the carotenoids are responsible for the large dipole moment. A conserved arginine residue in the transmembrane $\alpha$-helical segment of the $\beta$-subunit of the B800–850 protein is a possible candidate, as are various charged residues in the putative cytoplasmic domain of both the $\alpha$- and $\beta$-subunits. In addition, Gottfried et al. (1991a) have proposed that helix dipoles, as well as additional polar groups, including phospholipid head groups, may contribute to the permanent electric field.

A role for the intact B800–850 protein in the electrochromic carotenoid response was first suggested from the protease digestion studies of Webster et al. (1980) in which treatment of *Rhodobacter capsulatus* ICM vesicles (chromatophores) with Pronase resulted in the disappearance of the $\beta$-subunit of the complex, as well as a parallel loss of the carotenoid band shift and the absorption of the B800 BChl. Neither light-induced electron transport nor membrane permeability was affected under these conditions. Further spectroscopic analyses by Symons and Swysen (1983) indicated that the electrochromically active pool was more sensitive to Pronase digestion than the bulk of the carotenoids; however, it was considered unlikely that these results could be explained by specific cleavage of polar residues from the B800–850 protein in the vicinity of the field-sensitive chromophores, since other proteases, as well as phospholipase $A_2$, produced similar differential effects on the two carotenoid pools. Instead, it was concluded that the portion of the carotenoid molecule which responds to electric field alterations is located at or near the polar surface of the membrane bilayer, where these degradative enzymes act. Moreover, the results with phos-

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pholipase $A_2$ suggested that the electrochromic response requires the presence of intact phospholipids within the bilayer.

Results obtained here with a mutant strain of *R. sphaeroides* lacking the B875 core antenna and reaction center complexes have provided an opportunity to examine the effects of phospholipase in membranes containing B800–850 as the sole pigment–protein complex. It is shown that these phospholipase effects are exerted directly upon the electrochromically active carotenoid pool and do not arise from changes in the activity of the field generating electron-transport components, increases in membrane permeability, or obliteration of the permanent electrical field in the vicinity of the responding chromophores. A detailed examination of the effects of phospholipase $A_2$ digestion on the spectra of the light-harvesting BChl components is also reported.

## MATERIALS AND METHODS

*R. sphaeroides* wild-type strains NCIB 8253 and 2.4.1 and the green mutants Ga and G-1-C were grown photoheterotrophically at 30 °C in 1-L Roux bottles for ~36 h at a light intensity of ~1800 lx in the medium of Cohen-Bazire et al. (1957). The other green strains were described by Hunter et al. (1988) and consisted of M21G, which lacks the B800–850 complex, and NF57G, lacking the B875 complex and reaction centers; they were grown on M22+ medium (Hunter & Turner, 1988) under photoheterotrophic and semiaerobic conditions, respectively. For preparation of phospholipids, wild-type strain NCIB 8253 was grown chemoheterotrophically in the medium of Cohen-Bazire et al. (1957) as described by Onishi and Niederman (1982).

Membrane fractions were prepared from French-pressure cell extracts by rate-zone sedimentation on sucrose density gradients as described by Reilly and Niederman (1986) and stored in 50 mM Tris (pH 7.5)/50% glycerol (v/v) at –80 °C. For pigment analysis, membranes were extracted with acetone/methanol/water (7:2:1, v/v); BChl was estimated from the absorbance at 770 nm using the extinction coefficient of Clayton (1963), while the carotenoids spheroidene and spheroidenone were determined with the extinction coefficients and spectral-crossover corrections of Cohen-Bazire et al. (1957). The levels of the green carotenoids chloroxanthin and neurosporene were estimated from the absorbance at 471 nm with the extinction coefficient given in Foppen (1971).

For digestion with phospholipase $A_2$, membrane samples were diluted in 1 mM $CaCl_2$, 20 mM MOPS (pH 7.0), and 100 mM KCl to a concentration of 10 $\mu$g of BChl/mL. The samples were treated at 37 °C with snake (*Naja naja*) venom phospholipase $A_2$ (Sigma Chemical Co., St. Louis, MO) at concentrations of 0.001–0.002 unit/$\mu$g of BChl, unless indicated otherwise; aliquots were removed at various intervals, and measurements were made immediately. These enzyme levels resulted in limited phospholipase digestion which facilitated detailed spectroscopic analyses over periods of 1–2 h.

Absorption spectra were obtained with a Johnson Research Foundation DBS-3 double-beam spectrophotometer interfaced to an Apple II plus microcomputer; Hamamatsu R928 and R406 photomultiplier tubes were used for measurements in the visible and near-IR ranges, respectively. Continuous saturating illumination for light-minus-dark carotenoid difference spectra was provided by a Unitron tungsten microscope lamp passed through a Wratten 88A filter; the photomultiplier was protected by a 5-mm Corning 4-96 blue-glass filter. For measurements of valinomycin–KCl-induced carotenoid bandshift transients (Jackson & Crofts, 1969), the KCl in the digestion buffer was replaced by 100 mM NaCl; cuvettes were constantly mixed with a magnetic stirrer, and the reaction was started by adding a pulse of KCl. The instrument was modified for near-IR absorption and fluorescence spectra at 77 K as described by Theiler and Niederman (1991). Fluorescence polarization measurements were made with Polaroid HR filters. For studies of the flash-induced carotenoid bandshift transients, the spectrophotometer was altered to a crossed double-beam geometry and illuminated by a xenon flash-lamp through a 6-mm Schott RG-9 filter.

The decay constants of light and $K^+$-induced absorption transients were calculated by nonlinear fitting to either single or double exponentials. A program was written for this purpose by J. N. Sturgis of this laboratory using the fitting algorithm described by Press et al. (1986); solutions to the simultaneous equations in partial derivatives were obtained by Gaussian elimination. Alternatively, digitized data were transferred to a Macintosh II computer and analyzed using the fitting capabilities of Igor version 1.12 (WaveMetrics Inc., Lake Oswego, OR). This program uses the same methods described above but incorporates an additional algorithm to provide initial estimates.

For the determination of the phosphatide composition of phospholipase $A_2$-treated membranes, samples were extracted with butanol/water (1:2, v/v) (Bjerve et al., 1974) and centrifuged at 5000*g* for 10 min. The upper phase was retained and the lower phase reextracted until pigments were no longer visible. The combined butanol phases were washed twice with butanol-saturated water. The lipid extract was transferred to a tared vessel and evaporated. Phosphatides were estimated from $^{31}$P-NMR spectra as described by Sotirhos et al. (1986) using a Varian XL-400 spectrometer operating at 161.9 MHz. The extracted lipids were dissolved in deuterated chloroform/methanol (2:1); 1% triethyl phosphate (v/v) was added as an internal standard. Triethyl phosphate, deuterated chloroform, and methanol were purchased from Aldrich Chemical Co., Milwaukee, WI. The phosphatide composition was determined from the numerically integrated peaks observed in the NMR spectra, after identification of peaks by comparison to the chemical shifts of standards.

Phospholipids were extracted from aerobically grown cells for the preparation of liposomes, using the procedure of Bligh and Dyer (1959), and evaporated to dryness. The lipids (~100 mg) were dissolved in 1 mL of 0.3 mM $CaCl_2$, 20 mM MOPS (pH 7.0), and 100 mM KCl by gentle bath sonication at 20 °C for 1 h or until clear.

## RESULTS

*Effect of Carotenoid and Antenna Composition on the Electrochromic Response.* Although the electrochromically active carotenoid pool of *R. sphaeroides* has been shown to be associated with the B800–850 light-harvesting complex (Holmes et al., 1980; Matsuura et al., 1980), the availability of mutant strains with defined carotenoid compositions (Holmes & Crofts, 1977), as well as strains which lack one or more of the pigment–protein complexes (Ashby et al., 1987), has permitted a more detailed assessment of the specific requirements for the electrochromic response. In Table I, the extent of the carotenoid bandshift induced by a valinomycin–$K^+$ diffusion potential in several wild-type and green *R. sphaeroides* strains is shown. Similar responses were observed in wild-type strains 8253 and 2.4.1, containing mostly spheroidene, and in strain G-1-C, with neurosporene as the primary carotenoid. Strains Ga and NF57G, which contain neurosporene and chloroxanthin as their main colored ca-

Table I: Comparison of Carotenoid Bandshift in Various *R. sphaeroides* Strains

| strain | $\Delta A$[carotenoid]$^{-1}$ V$^{-1}$ [a] | $\Delta A$[carotenoid]$^{-1}$ [d] |
|---|---|---|
| NCIB 8253 | 0.49[b] | |
| 2.4.1 | 0.49[b] | |
| G-1-C | 0.50[c] | |
| Ga | 0.22[c] | 0.051 |
| NF57G | 0.31[c] | |
| M21G | | 0.003 |

[a] Chromatophores were suspended at 30 μg of BChl/mL in 20 mM MOPS (pH 7.0) containing 0.1 M NaCl, 1 μM antimycin A, and 1 μM valinomycin. The electrochromic response of the carotenoids ($\Delta A$-[carotenoid (mg)]$^{-1}$ V$^{-1}$) was obtained from the slope of plots of $\Delta A$[carotenoid]$^{-1}$ vs log of the KCl concentration which ranged from 7 to 100 mM; an essentially linear response was observed for each strain. The decay constants for the absorption transients following the KCl pulse fitted to a single exponential and ranged from 0.11 to 0.15 s$^{-1}$, demonstrating that intact chromatophore vesicles were obtained with each strain. [b] Measured at 523–507 nm. [c] Measured at 508–492 nm. [d] Measurements on whole cells suspended in 20 mM MOPS (pH 7.0) containing 100 mM KCl; values represent the difference between maxima and minima at 510 and 490 nm, respectively, in plots of constant illumination-induced difference spectra, measured at intervals of 2 nm; in strain Ga, this change in amplitude was approximately half that observed for the wild type.

rotenoids (Holmes & Crofts, 1977; Hunter et al., 1988), exhibited smaller responses; the somewhat greater bandshift in NF57G is a reflection of the absence of the electrochromically inactive B875 carotenoids from the chromatophores of this strain. Indeed, M21G, a green B800–850$^-$ *R. sphaeroides* mutant, did not show a light-induced carotenoid absorption change. Since M21G forms tubular rather than vesicular ICM (Hunter et al., 1988; Sturgis et al., 1990), and upon disruption gives rise to membrane fragments that are unsuitable for examination of the carotenoid bandshift, it was necessary to perform these measurements in whole cells (Table I).

*Effect of Phospholipase $A_2$ Digestion on Carotenoid Spectra and the Electrochromic Response.* In studies on the effects of Pronase digestion on the carotenoid bandshift, Symons and Swysen (1983) and Swysen et al. (1984) noted that the electrochromically active carotenoid pool was also more sensitive to phospholipase $A_2$ treatment than the bulk of the carotenoids. To further elucidate the effects of perturbations of the phospholipid bilayer on the pigment components of the B800–850 complex, chromatophores of strain NF57G were digested with phospholipase $A_2$, and absorbance changes in the carotenoid (410–510 nm) and BChl $Q_x$ bands (590 nm) were examined (Figure 1A). Alternations in the carotenoid absorption spectrum were characterized by an absorbance loss followed by a blue shift; the amplitude of the overall change in these difference spectra of treated-minus-untreated preparations was similar in magnitude to that of the continuous illumination-induced carotenoid change in the wild type (see Figure 2). An analysis of the changes in absorbance vs time at various wavelengths in the carotenoid region fitted a double exponential and revealed that the major change was characterized by a loss of absorption with a concomitant shift to the blue (Figure 1B, curve 1). A smaller, more rapid absorbance loss characterized the earlier stages of digestion (Figure 1B, curve 2).

The apparent phospholipase $A_2$-induced effects on the electrochromically active carotenoid pool were investigated directly in constant illumination-minus-dark difference spectra of wild-type chromatophores (Figure 2). Between 10 and 60 min, marked changes in the amplitude differences between the maxima were observed without appreciable shifts in their

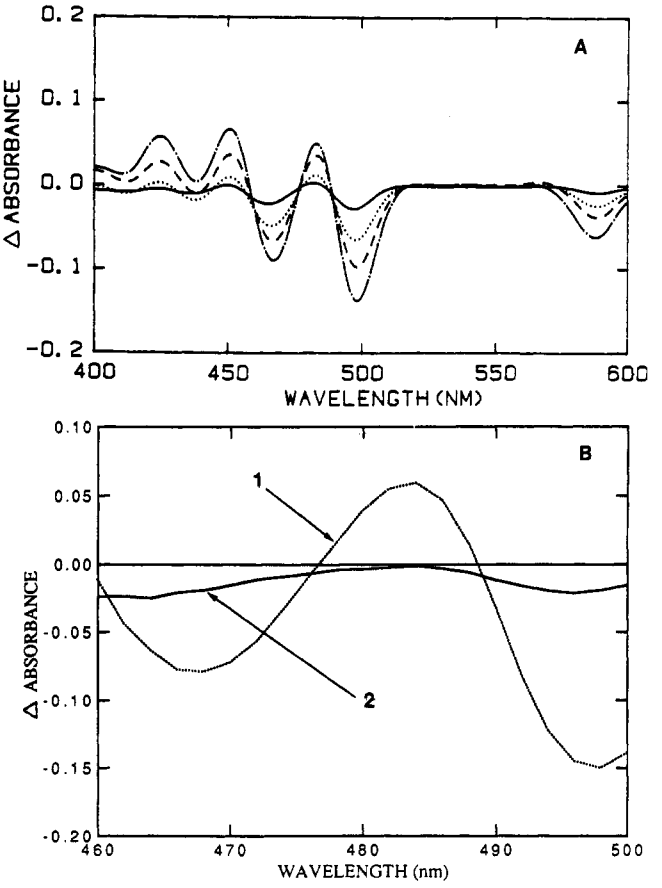


FIGURE 1: Effect of phospholipase $A_2$ on the visible absorption spectrum of *R. sphaeroides* NF57G chromatophores. (A) The chromatophores (10 μg of BChl/mL) were suspended in 20 mM MOPS (pH 7.0), containing 1 mM $CaCl_2$ and 100 mM KCl, and incubated with phospholipase $A_2$ (0.001 unit/μg of BChl) at 37 °C. Aliquots were removed at various intervals, and digested-minus-undigested difference spectra were obtained. No significant absorption changes were observed in the undigested controls which were incubated for the same time periods in the absence of enzyme. (—) 5 min-minus-control; (···) 30 min-minus-control; (- - -) 60 min-minus-control; (– · –) 120 min-minus-control. (B) Kinetic analysis of the phospholipase $A_2$-induced alterations in the carotenoid absorption spectra of *R. sphaeroides* NF57G chromatophores. The changes in absorbance at each wavelength were fitted to a set of double exponentials with wavelength-dependent amplitudes using Igor version 1.12 as described in the text. The rate constants calculated under these conditions were 0.013 and 0.385 min$^{-1}$ for the spectral components in curves 1 and 2, respectively. An additional, minor component, that arose more slowly, was also detected. The loss of absorption in the BChl $Q_x$ band, which accompanied the absorbance changes in the carotenoid spectrum, reflected alterations in the BChl $Q_y$ band described below.

positions. Although a slight increase in amplitude was seen at 10 min (not shown), this was followed by a gradual decline which reached about one-fourth the control value at the end of 2 h. Possibilities to explain this observed decrease in the electrochromic carotenoid response include (i) an increase in the permeability of the membrane to ions, (ii) a decrease in the activity of the light-dependent field-generating apparatus, (iii) an effect upon the local membrane environment, such that the polarizing charges on the B800–850 protein are removed from the electrochromically active carotenoids, rendering these chromophores insensitive to alterations in the transmembrane electric field.

The decay kinetics of the flash illumination-induced carotenoid bandshift were examined to assess whether phospholipase $A_2$ digestion had altered the ion permeability of the membrane (Figure 3A). A gradual decrease in the amplitude

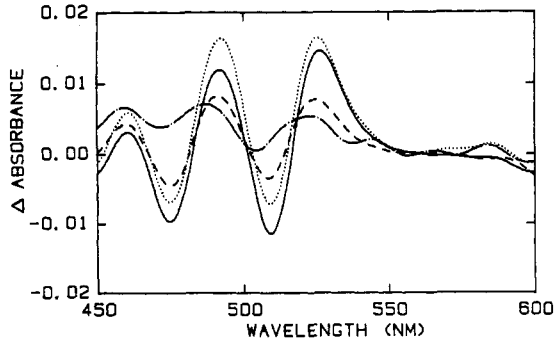


FIGURE 2: Effect of phospholipase $A_2$ on light-minus-dark carotenoid difference spectra of *R. sphaeroides* wild-type chromatophores. Treated with phospholipase $A_2$ as described in Figure 1. Digitized spectra were filtered using a Fourier-transform filtering program described in Sturgis et al. (1988). (—) Undigested control; (···) digested 30 min; (- - -) digested 60 min; (– · –) digested 120 min.

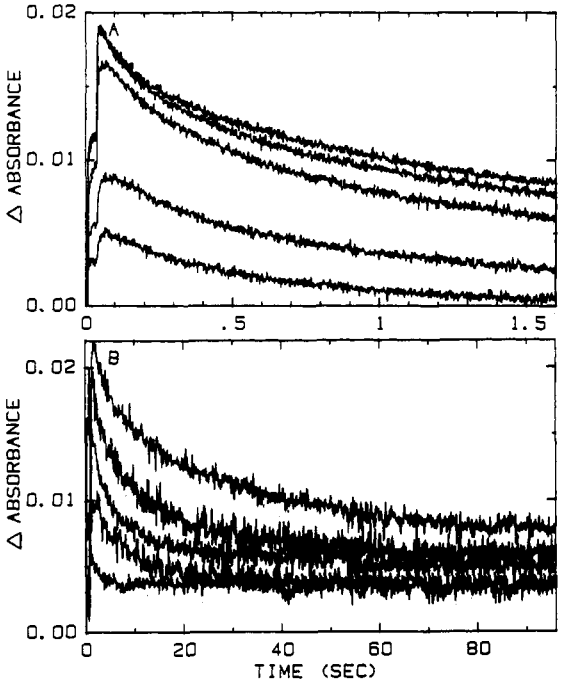


FIGURE 3: Effect of phospholipase $A_2$ on carotenoid bandshift transients. (A) Flash-induced carotenoid bandshift of *R. sphaeroides* wild-type chromatophores. Measurements were performed at 523–507 nm with a xenon flash lamp as the actinic light source. Two flashes 32 ms apart were used to generate the membrane potential. Treated with phospholipase $A_2$ as described in Figure 1. Top to bottom: 0, 10, 30, 60, and 120 min. (B) KCl–valinomycin-induced carotenoid bandshift of *R. sphaeroides* wild-type chromatophores. Treated with phospholipase $A_2$ as described in Figure 1, except that KCl was replaced by NaCl, and 1 μM antimycin A together with 1 μM valinomycin was also present. KCl was added to each sample to a final concentration of 12.5 mM and the KCl-induced response at 523–507 nm measured. Top to bottom: 0, 10, 30, 60, and 120 min. These transients were not corrected for dilution caused by the addition of the KCl solution which accounted for the bulk of the absorption difference after ~10 s in the 120-min trace.

of these transients was observed, which reached 39% of the control value at 60 min and 25% at 120 min. Their decay kinetics fit a double exponential with a fast phase ($k_1$) of ~4.5 $s^{-1}$ and a slower decay ($k_2$) of ~0.5 $s^{-1}$. Both rate constants were essentially stable for the first 30 min of digestion, with the amplitude attributable to the fast phase accounting for ~40% of the total decay; thereafter, the two phases were no longer separable, and single new rate constants of 2.9 and 4.3 $s^{-1}$ were determined at 60 and 120 min, respectively. Although these decay constants appear to be consistent with the maintenance of membrane integrity during

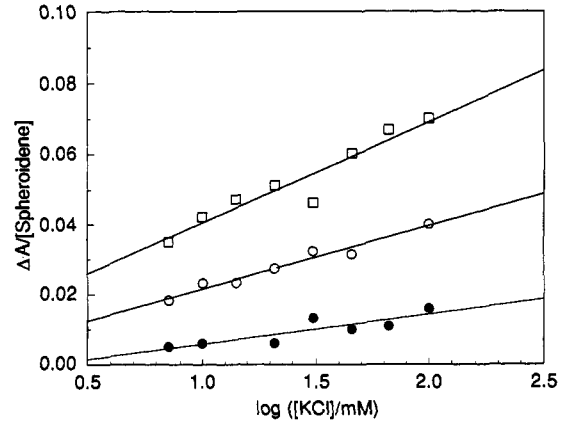


FIGURE 4: Relation between amplitude of carotenoid bandshift and applied $K^+$ gradient. Chromatophores (30 μg of BChl/mL) were incubated with phospholipase $A_2$ (0.0002 unit/μg of BChl) at 37 °C in 20 mM MOPS (pH 7.0) containing 1 mM $CaCl_2$, 0.1 M NaCl, 1 mM antimycin A, and 1 mM valinomycin. The final concentration of KCl ranged from 7 to 100 mM. (□) Untreated control; (○) digested 10 min; (●) digested 60 min.

the early states of digestion, it was not possible to distinguish if the reduction in the amplitude of the bandshift was a consequence of a decreased carotenoid sensitivity or reduced activity of the electrogenic components of light-driven electron flow.

Possible contributions to the digestion results from changes in the activity of the field-generating components were bypassed by determining the effect of phospholipase $A_2$ treatment on the carotenoid bandshift induced by a valinomycin–$K^+$ diffusion potential (Figure 3B). As with the light-induced transients, the amplitude of the bandshift decreased gradually, reaching 80%, 36%, and 27% of the control value after 30, 60, and 120 min, respectively. The decay kinetics of these transients were less complex than those of the flash-induced carotenoid changes and fitted well to a single exponential; the rate constants derived from these data remained stable at about 0.13 $s^{-1}$ for the first 60 min of digestion, confirming that during this period the permeability of the membrane to cations remained unaltered. After 120 min, however, the decay became more rapid, and the rate constant was increased to ~0.80 $s^{-1}$. These results suggest that the decrease in the electrochromic response during the early stages of digestion is not due to diminished activity of the electrogenic components, but is instead a consequence of alterations in the local carotenoid environment.

Figure 4 shows that while phospholipase $A_2$ treatment caused a loss in the response of the carotenoids to $K^+$-induced diffusion potentials, the linear relationship between the amplitude of the residual carotenoid bandshift and the $K^+$ concentration was not altered. The response of the undigested preparation was 0.49 $\Delta A \cdot V^{-1} \cdot mL \cdot mg^{-1}$, but decreased to 0.30 $\Delta A \cdot V^{-1} \cdot mL \cdot mg^{-1}$ after 10 min and 0.14 $\Delta A \cdot V^{-1} \cdot mL \cdot mg^{-1}$ after 60 min of treatment. As will be discussed further below, a decrease in the extent of the polarizing permanent field is among the possible ways in which phospholipase-induced alterations in the membrane environment could result in reduced carotenoid sensitivity. The observed linear dependence of the residual bandshift on $\Delta\psi$, however, suggests that despite a 3–4-fold reduction in the effective local field strength, it is still sufficient to far exceed the magnitude of the applied transmembrane field.

*Effect of Phospholipase $A_2$ Digestion on Near-IR Absorption Spectra of Antenna Complexes.* The apparent relationship between the loss of the carotenoid band shift and

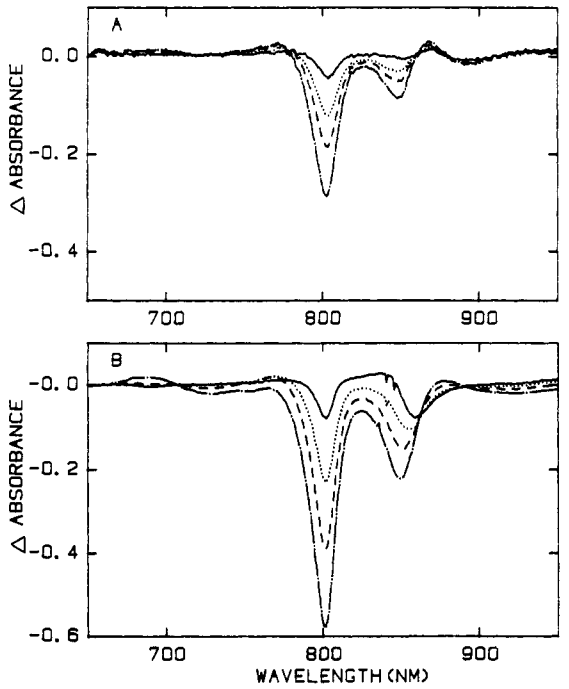


FIGURE 5: Effect of phospholipase $A_2$ on the near-IR absorption spectrum of *R. sphaeroides* chromatophores. (A) Wild-type chromatophores (10 μg of BChl/mL) were treated with phospholipase $A_2$ as described in Figure 1 to obtain digested-minus-undigested difference spectra. No significant absorbance changes were observed in the undigested controls. (—) 5 min-minus-control; (···) 30 min-minus-control; (- - -) 60 min-minus-control; (– · –) 120 min-minus-control. (B) Digested-minus-undigested near-IR absorption difference spectra of strain NF57G chromatophores. Conditions of phospholipase $A_2$ treatment as described above.

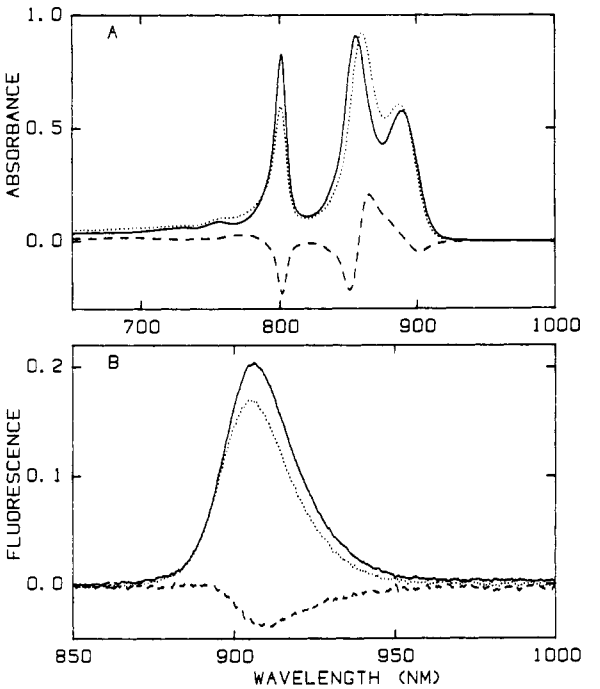


FIGURE 6: Absorption and fluorescence emission spectra of phospholipase $A_2$-treated *R. sphaeroides* wild-type chromatophores at 77 K. Treated with phospholipase $A_2$ as described in Figure 1; samples were suspended in 60% (v/v) glycerol prior to freezing. (A) Absorption spectra. (B) Fluorescence emission spectra; excitation was at 800 nm, and spectra were normalized on the basis of the relative number of quanta absorbed, using $1 - T_{800nm}$. (—) Undigested control; (···) digested 10 min; (- - -) digested-minus-control. Fluorescence is expressed in arbitrary units.

changes in near-IR absorption spectra reported by Webster et al. (1980) was investigated further. Figure 5 shows difference spectra of phospholipase $A_2$ digested-minus-undigested chromatophores in the region of the BChl $Q_y$ bands. In the wild type, a substantial loss of absorbance at 800 nm, and to a lesser extent at 850 nm, was observed, as well as a positive lobe at ~868 nm and a small absorbance decrease at ~896 nm. Analyses of changes in the B800 and B850 bands were simplified with NF57G chromatophores; nonlinear fitting showed that a single exponential resulted in the best fit and that the changes at 800 and 850 nm occurred with kinetics similar to those of the major changes in the overall carotenoid spectrum (Figure 1). In contrast to the wild type, no alterations were observed at 896 nm in this strain, which can therefore be attributed to the B875 complex. Figure 5 also revealed that the absolute losses in absorption at 800 and 850 nm were greater in NF57G than in the wild type, which further reflects the absence of B875 in the former.

Near-IR absorption spectra were performed at 77 K in order to enhance the analysis of the phospholipase $A_2$-induced changes in the BChl $Q_y$ bands of the wild-type chromatophores (Figure 6A). The results indicated that the loss of absorption was confined to the 800-nm band; a red shift and an absorption increase were observed for the B850 band, while the B875 band showed a slight blue shift. Treatment of the B800–850 complex with lithium dodecyl sulfate also results in diminution of the B800 band, which like the phospholipase-induced change observed here is not countered by the obvious appearance of a new absorbance band (Clayton & Clayton, 1981). A resonance Raman study of the B800–850 complex of *Rhodopseudomonas acidophila* by Robert and Frank (1988) suggested that during lithium dodecyl sulfate treatment, the B800 BChl remains bound to the apoprotein via its central magnesium atom and that the spectral attenuation results from major conformational perturbations of the protein.

The absorption difference spectrum of the digested-minus-undigested preparations (Figure 6A) revealed that the change in the B875 band was manifested by a small minimum at ~900 nm. The fluorescence emission spectra at 77 K (Figure 6B) showed no changes on the blue side of the emission band in the digested preparation, which demonstrates that energy transfer between the B850 and B875 complexes was unaltered. Some fluorescence quenching occurred that was manifested as a minimum near 910 nm in the digested-minus-undigested spectrum. As will be discussed further below, both the treated and untreated samples exhibited an increase over the red wing of the B875 $Q_y$ band in their 77 K fluorescence polarization spectra, thought to arise from an anisotropic long-wavelength component (not shown).

The phospholipase $A_2$-induced, parallel reduction in the B800 absorption band and the carotenoid bandshift provided an opportunity to test whether the red-shifted, electrochromically active carotenoids, thought to comprise about one-third of the total (De Grooth & Amesz, 1977; Symons et al., 1977), are the same as those which form a putative, functionally distinct B800 carotenoid pool proposed to transfer excitation energy directly to B800 BChl (Kramer et al., 1984a). Accordingly, fluorescence excitation spectra were performed at 77 K on phospholipase $A_2$-treated NF57G chromatophore preparations to assess whether energy transfer from the B800–850 carotenoids was also effected by the enzymatic digestion (Figure 7). Despite a diminution of nearly half in the 800-nm band and the complete disappearance of the KCl–valinomycin-induced carotenoid bandshift in the phospholipase-treated preparation (not shown), the efficiency of energy transfer from the redmost carotenoid band to the B850 band remained

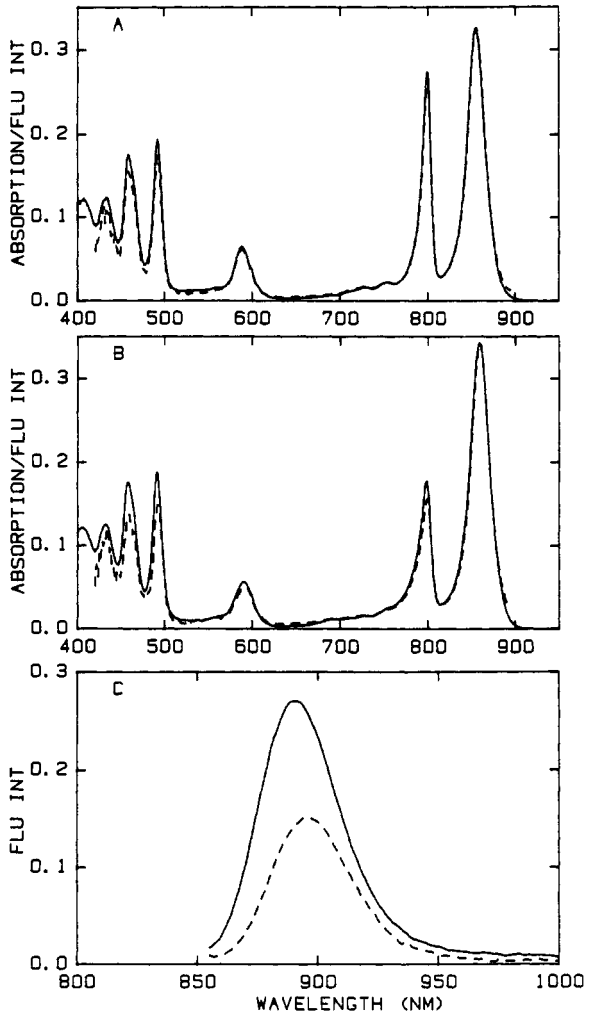


FIGURE 7: Fractional absorption and fluorescence excitation and emission spectra of phospholipase $A_2$-treated *R. sphaeroides* NF57G chromatophores at 77 K. (A) Untreated control: fractional absorption (—) and fluorescence excitation spectra (- - -); emission was detected at 910 nm. Excitation spectra were normalized to the fractional absorption at the maxima of the B850 bands. FLU INT, fluorescence intensity expressed in arbitrary units. (B) Treated with phospholipase $A_2$ for 30 min as described in Figure 1, except that KCl was replaced by NaCl and the phospholipase $A_2$ concentration was 0.02 unit/$\mu$g of BChl. Under these conditions, the KCl–valinomycin-induced carotenoid bandshift, measured at 510–490 nm as described in Figure 3, was abolished (not shown), and reduction of the B800 band was maximized. Absorption spectrum corrected for light scattering by subtracting a linear base line to obtain a B850/650-nm absorption ratio equal to that in the spectrum of the untreated control. (C) Fluorescence emission spectra: (—) untreated control; (- - -) treated with phospholipase $A_2$ for 30 min. Excitation was at 840 nm; spectra were normalized according to the number of quanta absorbed at this wavelength and corrected for the wavelength dependence of the detection system. Similar results were obtained with 590-nm excitation, but with a higher signal-to-noise ratio.

at 87.7%, compared to 95.5% in the untreated chromatophores and 89.7% in a preparation held at 37 °C in the absence of enzyme. Essentially identical fluorescence excitation spectra were obtained with detection at 880 nm (not shown), the emission wavelength used by Kramer et al. (1984a); here the efficiencies of energy transfer from the redmost carotenoid to the B850 band were 91.7 and 89.5%, respectively, in the untreated and phospholipase-treated preparations. Therefore, no significant decreases in the transfer of excitation energy from carotenoids to B850 BChl can be ascribed to the action of the phospholipase on the chromatophore membrane bilayer.

The ~4-nm red shift in the absorption band of the treated chromatophores was also reflected in the emission spectrum

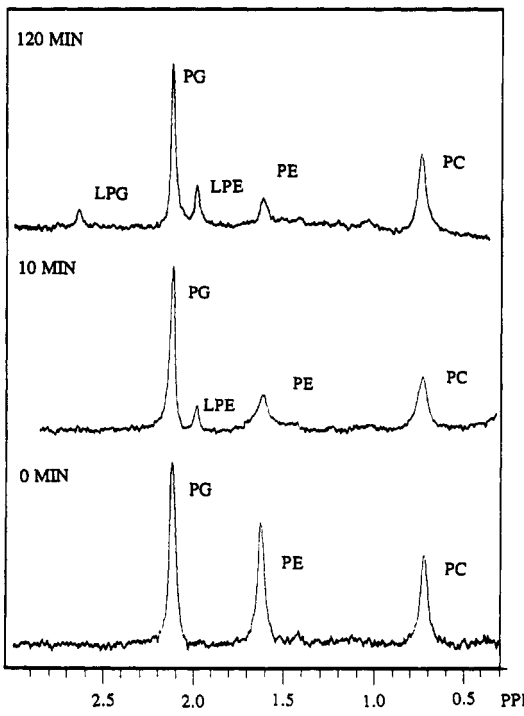


FIGURE 8: $^{31}$P-NMR spectra of lipids extracted from *R. sphaeroides* chromatophores during limited digestion with phospholipase $A_2$. Wild-type chromatophores were incubated with phospholipase $A_2$ under the conditions described in Figure 1. Phosphatides were extracted in 1-butanol, and samples were prepared for NMR as described by Sotirhos et al. (1986). The peaks correspond to PC (0.69 ppm), PE (1.62 ppm), PG (2.12 ppm), LPE (2.02 ppm), and LPG (2.59 ppm); they were identified by comparison of their chemical shifts to those of phosphatide standards.

(Figure 7); a substantial fluorescence quenching was observed in this preparation which may be a consequence of the higher enzyme levels used in this experiment. Again, low-temperature fluorescence polarization spectra (not shown) exhibited the inhomogeneity over the red wing of the B850 band described by van Dorssen et al. (1988), with that of the phospholipase-treated preparation shifted overall to the red.

*Phosphatide Composition of Digested Membranes.* The lysophospholipids and free fatty acids formed during the hydrolytic cleavage of phospholipids by phospholipase $A_2$ at the *sn*-2 position would be expected to cause an increase in membrane fluidity and thereby affect the behavior of other membrane components. In order to assess the extent of degradation, the phospholipase-treated chromatophore preparations were subjected to $^{31}$P-NMR (Sotirhos et al., 1986) which permitted quantitation of both phospholipids and their lysophospholipid derivatives without prior chromatographic separation. Phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and phosphatidylcholine (PC), the three major phospholipid species of the *R. sphaeroides* chromatophore membrane, were readily observable in the NMR spectrum of the undigested samples (Figure 8); integration of their peaks (Table II) resulted in a composition in agreement with that determined after separation of the $^{32}$P-labeled species in thin-layer chromatography (Onishi & Niederman, 1982; Donohue et al., 1982). After 10 min of digestion, 24% of the PE was degraded to lysophosphatidylethanolamine (LPE), but no other digestion products were detected (Table II); at 2 h, 39% of the PE appeared as LPE, and 11% of the PG was converted to lysophosphatidylglycerol (LPG). Although PC is a preferred substrate for the *Naja naja* phospholipase $A_2$ (Roelofson, 1982; Fedarko et al., 1978), no lysophosphatidylcholine (LPC) was detected in any of these spectra.

Table II: Phosphatide Composition of Membrane Fractions during Limited Phospholipase $A_2$ Digestion[a]

| membranes | incubation time (min) | composition[b] PC | LPC | PE | LPE | PG | LPG |
|---|---|---|---|---|---|---|---|
| chromatophore | 0 | 31 | 0 | 31 | 0 | 38 | 0 |
| | 10 | 31 | 0 | 26 | 8 (24)[c] | 36 | 0 |
| | 120 | 35 | 0 | 17 | 11 (39) | 33 | 4 (11) |
| upper pigmented band | 0 | 20 | 0 | 43 | 0 | 37 | 0 |
| | 60 | 17 | 3 (15) | 22 | 21 (48) | 21 | 16 (43) |
| | 120 | 18 | 2 (10) | 0 | 45 (100) | 12 | 25 (72) |

[a] Wild-type chromatophore and upper pigmented band fractions were digested with phospholipase $A_2$, and the resulting phosphatides were extracted as described in the text. [b] Expressed as percentage of phosphatides determined by integration of peak areas in $^{31}$P-NMR spectra. [c] Values in parentheses represent percent conversion of phospholipid species to respective lysophospholipid and were calculated from the total phospholipid + lysophospholipid determined at each time point.

The possibility that *R. sphaeroides* PC is refractory to hydrolysis by phospholipase $A_2$ was assessed further by using as a substrate unilamellar liposomes prepared from the native phospholipids of aerobically grown cells. $^{31}$P-NMR spectra revealed that after 60 min of digestion, approximately 75, 60, and 25% of the PE, PC, and PG, respectively, were digested to form their corresponding lysophospholipids (not shown). Since this suggested that chromatophore proteins may be responsible for modulating the activity of phospholipase $A_2$ toward PC, enzymatic digestion was performed on the membrane fraction isolated as an upper pigmented band, which has a protein composition largely distinct from that of chromatophores (Niederman et al., 1979) and consists of open fragments (Inamine et al., 1984). The lipid composition of the upper pigmented band was found to be 20% PC, 43% PE, and 37% PG (Table II). Digestion of the membranes for 2 h resulted in complete hydrolysis of PE to LPE and the degradation of most of the PG to LPG; however, only small amounts of LPC were detected at 60 min, and no further hydrolysis of PC was observed thereafter.

## DISCUSSION

The electrochromic carotenoid response was found to vary among several *R. sphaeroides* strains, which is apparently attributable to differences in carotenoid composition. Strain G-1-C, in which neurosporene comprises virtually all of the carotenoid (Holmes & Crofts, 1977), exhibited a response similar to that of the wild type, while strain Ga, in which about one-third of the neurosporene is replaced by chloroxanthin (hydroxyneurosporene) and methoxyneurosporene (Scolnik et al., 1980b), showed a much smaller electrochromic response. This may therefore result from an inability of the hydroxy and methoxy derivatives of neurosporene to sense electric field alterations. Chromatophores with large amounts of spheroidenone also showed a smaller electrochromic response than membranes with spheroidene or neurosporene as their main carotenoids (not shown). This is apparently a consequence of the very broad peaks in the visible spectrum of spheroidenone, since recent Stark effect measurements with isolated carotenoids have indicated that spheroidenone is actually more field-sensitive than spheroidene (Gottfried et al., 1991b), consistent with the less symmetric extended $\pi$-system resulting from introduction of a conjugated carbonyl group.

In the studies of Swysen et al. (1984) on the effects of phospholipase $A_2$ digestion on the absorption spectrum and electrochromic response of the carotenoids of wild-type *R. sphaeroides* chromatophores, short incubations resulted in a small blue shift in the isosbestic point of the constant illumination-induced difference spectrum, without shifts in overall carotenoid absorption maxima. In contrast, the present analysis of phospholipase $A_2$-induced effects on the carotenoid absorption spectrum in *R. sphaeroides* NF57G, in which B800–850 is the sole pigment–protein complex, revealed that the major change was characterized by a loss in overall carotenoid absorption with a concomitant blue shift. The corresponding diminution in the amplitude of the electrochromic carotenoid response did not result from a reduction in the ability of the membrane to sustain an electric field, since an analysis of the decay rate of both the flash illumination and $\Delta\psi$-induced transients indicated that membrane permeability was not affected until the latter stages of digestion. Instead, the decrease in the carotenoid bandshift must be caused by effects exerted directly upon the electrochromically active carotenoid pool. The observed blue shift in the carotenoid spectrum, which is also indicative of an alteration in the local carotenoid environment, occurred at approximately the same rate, which is consistent with the proposal of De Gooth and Amesz (1977) that the capacity of these chromophores to respond to a transmembrane field is correlated to their red shift. It is not possible on the basis of the present results, however, to distinguish whether all chromophores undergo a simultaneous, gradual transition via states of reduced sensitivity to a nonresponding state, or if the field sensitivity is lost in a single rapid step such that the kinetics of the effects induced by enzymatic digestion reflect the rate at which the responding pool is diminished. The second possibility, however, is favored by the lack of significant movement of the isosbestic point in the spectra for both the absorption blue shift and the carotenoid bandshift. In either case, the magnitude of the polarizing permanent field sensed by the remaining active pigments is sufficiently large to preserve the quasi-linear character of the response.

Since the amplitude of the linear response generated by a given transmembrane potential depends both upon the orientation and upon the size of the permanently induced dipole moment on the carotenoid, the observed reduction of the extent of the bandshift may result either from a reorientation of the chromophore or from a decrease in the magnitude of the dipole. The concomitant blue shift in the absorption spectrum, however, suggests that such a reorientation is probably not a major factor and it is more likely that alterations have occurred in the spatial relationship between the field-sensitive carotenoids and nearby charge(s), responsible for the transmembrane field sensitivity. Thus, the phospholipase-induced degradation of the lipid bilayer could conceivably lead to local alterations in the conformation of the B800–850 complex, resulting in removal of carotenoid from its binding site or dislocation from the carotenoid of the portions of the protein carrying the relevant charge(s). In the theoretical studies of Kakitani et al. (1982), it was postulated that moving a charge from near the end of a carotenoid to the center of the molecule

would result in the loss of (induced) dipolar character in concert with a significant spectral blue shift. The more limited blue shift observed here could be a consequence of differential dislocations among a constellation of polarizing point charges, resulting in the gradual reduction in the amplitude of the electrochromic response. The removal of these portions of the protein from the carotenoid is also favored by the lack of any significant effect of digestion upon energy transfer from carotenoids to B850 BChl, which indicates that no major increases in the distance between these chromophores had occurred.

During phospholipase $A_2$ digestion, LPE was the first lysophospholipid observed, as well as the major species present while the decay rate of the carotenoid bandshift still remained stable. LPG was seen after 2 h, at which time the integrity of the membrane had been affected sufficiently to eliminate the field normally sensed by the carotenoids. No LPC appeared, despite the report by Fedarko et al. (1978) that in mixed micelles, *Naja naja* phospholipase $A_2$ preferentially hydrolyzed PC, while PE was a 10-fold poorer substrate. When liposomes prepared from native *R. sphaeroides* phospholipids were treated with phospholipase $A_2$, both PE and PC were hydrolyzed to comparable extents, suggesting that the inactivity toward PC in the native membranes could be due to a lack of enzyme accessibility. PC was also refractory to phospholipase $A_2$ digestion of the upper pigmented band, even though both sides of the bilayer are exposed in this membrane fraction (Inamine et al., 1984). These results imply that protein components of both the upper pigmented band and chromatophores somehow protect PC from digestion. Such an affect has been reported for *Mycoplasma hominis* in which phospholipids were resistant to phospholipase C digestion unless the membranes were pretreated with proteases (Rottem et al., 1973). Because the penetration of phospholipase $A_2$ into phospholipid bilayers is dependent upon the distance between lipid molecules (Op van den Kamp et al., 1975), the protection from enzymatic hydrolysis could also reflect phase separation or crystallization, thereby hindering enzyme binding.

Treatment of *R. sphaeroides* membranes with phospholipase resulted in a number of changes in the near-IR absorption spectra of the B800–850 and B875 complexes. While room temperature absorption measurements suggested decreases in the extinction for both the B800 and B850 bands, low-temperature spectra revealed that the absorbance of the latter band was actually increased slightly and red-shifted. The small blue shift of the B875 band was apparently confined to the anisotropic spectral component, designated as B896, that was thought to funnel excitations to the reaction center (Kramer et al., 1984b). It has recently been proposed, however, that due to extended pigment–pigment interactions, this redmost species of B875 arises as a consequence of the oligomerization of the complex (Westerhuis and Niederman, unpublished results). Thus, the blue shift observed here may reflect some disaggregation of the B875 complex during phospholipase degradation of the membrane bilayer. Since these spectral changes were complete within 10 min, they may be related to the rapid formation of LPE, possibly to its detergent role, or to increases in the fluidity of the membrane.

The observed loss of B800, together with a blue shift of the carotenoids and a red shift in B850 absorption, resembles the effect of lithium dodecyl sulfate upon the isolated B800–850 complex (Kramer et al., 1984a). On the basis of the attenuation by this detergent of both B800 absorption and energy transfer from the carotenoids to B850 it was proposed that one-third of these chromophores transfer excitations directly to B800 while the remainder is linked energetically to the B850 BChls. The recent report, that in *R. sphaeroides* chromatophores the low-temperature carotenoid excitation spectrum for fluorescence emission from B800 is red-shifted relative to that for B850 emission (Crielaard et al., 1992), has provided further support for the existence of separate B800 and B850 carotenoid pools. The possibility that the electrochromically active carotenoid pool (De Grooth & Amesz, 1977; Symons et al., 1977) transfers excitation energy directly to B800 BChl, however, was not supported by treatment of NF57G chromatophores with phospholipase under conditions where the carotenoid bandshift was abolished completely. Although the absorption of the B800 band was diminished by nearly 50%, the decrease in energy transfer efficiency from carotenoid to B850 attributable to enzymatic digestion was less than 3%. In this connection, it is noteworthy that the recent Stark effect measurements on the isolated B800–850 complex by Gottfried et al. (1991b) suggested that the structure in the vicinity of all B800–850 carotenoids is similar. Moreover, lithium dodecyl sulfate treatment appeared to affect the Stark spectra of all carotenoids equivalently, presumably as result of a major change in the local electronic environment, which abolished the features indicative of a significant permanent field. The reasons for these discrepancies in the results of the Stark effect measurements and the spectroscopic studies involving detergent attenuation and phospholipase digestion are unknown and require further investigation.

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