IDENTIFICATION OF THE PIGMENT POOL RESPONSIBLE FOR THE FLASH-INDUCED CAROTENOID BAND SHIFT IN *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES

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1. Introduction

A light-induced shift to longer wavelengths in the absorbance spectrum of carotenoids appears to be a consequence of electrogenic electron flow in the bacterial photosynthetic coupling membrane [1]. The carotenoid shift is also inducible by artificial diffusion potentials in the presence of valinomycin and potassium [2]. The relationship between the absorbance difference spectrum of the carotenoid shift and the absolute carotenoid absorbance spectrum has been investigated [3–5] and on the basis of these measurements it has been suggested that only a discrete pool of the carotenoid molecules within the membrane is able to respond to a transmembrane electrical field.

Two pools of light harvesting pigment–protein have been identified in *Rhodopseudomonas sphaeroides* [6,7] and are designated as B800-850 and B875 on the basis of their near-infrared absorbance maxima. It is possible to isolate both of these antenna complexes from the *R. sphaeroides* chromatophore membrane by lithium dodecyl sulfate/polyacrylamide gel electrophoresis at 4°C [8]. In the B875 preparation, two molecules each of bacteriochlorophyll and carotenoid are associated with two polypeptides [8] while in B800-850, three bacteriochlorophyll and one carotenoid are so associated [9]. The absorbance maxima attributable to carotenoids in the B875 preparation were all at shorter wavelengths relative to those of B800-850 and chromatophores [8]. Similar absorbance spectra were observed with *R. capsulata* strains containing only B800-850 or B875 as antennae [10]; studies of the diffusion-potential-induced band shift suggested that only carotenoids associated with B800-850 respond to the electrical potential [10].

On the basis of an electrochromic mechanism, the linearity of the carotenoid shift with transmembrane potential has been explained by the presence of a permanent electrical field in the membrane acting on the carotenoid molecules [11]. A further development of this hypothesis suggests that the effective permanent electrical field may be the result of an association between bacteriochlorophyll and carotenoid molecules [12]. The existence of the carotenoid molecules in the light-harvesting bacteriochlorophyll pigment–protein complexes gives some credence to this view [8,9]; however, the hypothesis in [12] assumes interaction through a hydroxyl group on the carotenoid, and cannot therefore apply to strains such as *R. sphaeroides* G1C and *R. capsulata* BY761, which show the same shifts as wild-type strains but contain only the nonhydroxylated neurosporene [3].

It is possible to alter the ratio of the B800-850 and B875 complexes of *R. sphaeroides* in vivo by alterations in the light intensity under which the cells are grown [13]. Under these circumstances, the B875/reaction centers ratio remains essentially constant, whereas increases in light intensity lead to a decreasing B800-850/B875 ratio. If only those carotenoids

Abbreviations: B800-850, B875, light-harvesting bacteriochlorophyll–protein complexes identified by their long wavelength absorbance maxima; DAD, diaminodurol; MOPS, 3-(*N*-morpholino)propane sulfonic acid; P^+, oxidized reaction center; PES, phenazine ethosulfate; PMS, phenazine methosulfate

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linked to B800-850 are responsive to the transmembrane electrical field, altering the amount of B800-850 should alter the magnitude of the carotenoid shift for a given electrical field. The measurements reported here support this hypothesis.

2. Materials and methods

R. sphaeroides NCIB 8253 was grown at 30°C as in [14] but under conditions of varying light intensity. Illumination was provided by tungsten filament lamps and controlled with a rheostat. In the case of the lowest intensity of illumination, two 40 W lamps were used; reflector spot lamps of 75 W were employed for the intermediate levels and 150 W for the highest. Light intensities were measured with a Weston model 756 illumination meter. Washed cells were disrupted in a French-pressure cell and membranes were prepared in the presence of 50 mM MOPS/100 mM KCl buffer (pH 7.0) by the differential centrifugation procedures in [15].

Absolute absorbance spectra were measured on a computer-linked scanning spectrophotometer and flash-induced difference spectra on a computer-linked single beam spectrophotometer equipped with a saturating xenon flash described in [16,17]. Saturation of the sample by the xenon flash was established by varying the concentration of the membranes in the sample cuvette. Controlled oxidation–reduction conditions were provided by an apparatus described in [16]. Reaction center concentration was estimated from the maximal ΔA_{542} in the presence of antimycin A and valinomycin, after a train of 8 xenon flashes using $\epsilon = 10.3\ mM^{-1}\ cm^{-1}$ [18]. The concentration of photo-oxidizable cytochrome c_2 was measured under similar conditions using $\Delta A_{551-542}$ and $\epsilon = 19\ mM^{-1}\ cm^{-1}$ [18]. Bacteriochlorophyll was estimated as in [15] using the extinction coefficient of [19].

3. Results and discussion

The near-infrared absorbance spectra of the membrane preparations isolated from *R. sphaeroides* grown under various light intensities indicated an increased B800-850/B875 ratio with decreasing levels of illumination (fig.1), in agreement with [7,13]. In table 1, it is shown that the computed B800-850/B875 ratios decreased 4-fold from 2.3–0.57 between the lowest and highest illumination levels.

If the observed differences in absorbance spectra attributable to carotenoids in the isolated light harvesting complexes ([8], table 1) are a reflection of their in vivo spectra, then a shift of carotenoid

Table 1
Absorbance maxima attributable to carotenoids in membranes and pigment–protein complexes from *R. sphaeroides*

	Light intensity (ft-candles)	B850/B875[a]	B875/reaction center[b]	Carotenoid absorbance maxima (nm)
Membranes				
	170	2.3	18	509, 477, 450
	800	1.3	22	508, 475.5, 449
	1500	0.98	20	507, 475, 449
	3000	0.57	35	506, 474, 447
B850[c]	–	–	–	507–509, 475, 448
B875[c]	–	–	–	500–505, 466–472, 439–446

[a] B850 and B875 levels were calculated from the approximations in [20] which assume a similar extinction coefficient and symmetrical absorbance bands for both antenna complexes. In these analyses, it should be noted that a single absorbance maximum is observed in the isolated B875 complex near 875 nm [8]. Although the B800-850 antenna has two absorption bands with maxima near 800 and 850 nm [9,21], the two bands are present in a fixed stoichiometry and the absolute bacteriochlorophyll concentration can be deduced from the 850 nm band alone [6]

[b] Reaction center concentrations were estimated as described in the text

[c] Antenna complexes were isolated from chromatophores by lithium dodecyl sulfate/polyacrylamide gel electrophoresis [8]. The positions of the carotenoid absorbance maxima in these preparations were detergent-sensitive as described in the text

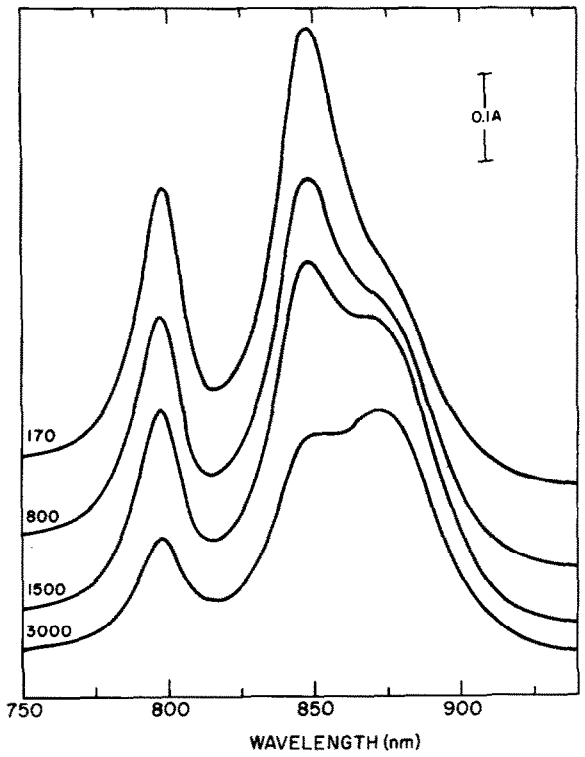

Fig.1. Near-infrared absorbance spectra of membrane preparations from *R. sphaeroides* grown at different light intensities. The light intensities in ft-candles are indicated at the left. Cuvettes contained 5.0, 4.4, 4.1 and 2.2 μM bacteriochlorophyll for the 170–3000 ft-candle preparations, respectively, in 50 mM MOPS/100 mM KCl buffer (pH 7.0).

absorbance maxima in membranes containing differing B800-850/B875 ratios would be expected. As may be seen in table 1, this expectation is fulfilled with these membrane preparations. A similar shift was observed in strains of *R. capsulata* [10]. The changes in B800-850/B875 ratios are accompanied by a corresponding shift in the position of the carotenoid absorbance maxima, from 509–506 nm in the case of the longest wavelength peak. The positions of the other carotenoid maxima also were shifted to shorter wavelengths. No obvious changes in shape of the spectrum in this region were evident. It is also shown in table 1 that although the positions of absorbance maxima attributable to carotenoids in the isolated B875 complex were always at shorter wavelengths than in isolated B800-850 preparations, the absolute values were sensitive to detergents. Both the nature of the detergent (e.g., lithium dodecyl sulfate shifted the absorbance maxima to shorter wavelengths than did cholate) and the length of exposure were important in this regard. By comparison, absorbance maxima at 511–512 nm were observed for the longest wavelength carotenoid band in a B800-850 complex isolated by the procedure in [21] and suspended in cholate.

If the pool of pigment responsible for the carotenoid shift is indeed that linked to the B800-850 pigment–protein complex one may make two predictions concerning the carotenoid shift measured in this series of membrane preparations:

(i) The absorbance difference spectrum of the carotenoid shift should not alter, even though the absolute absorbance spectrum in the carotenoid region shifts to the blue with decreasing B800-850/B875 ratio;

(ii) The amplitude of the carotenoid absorbance change should be related to the amount of B800-850 in the membrane.

In order to test this idea we have measured the carotenoid absorbance change induced by a single saturating flash for each membrane preparation over the region of the spectrum covering the longest wavelength maximum of the carotenoid absorbance. These measurements were made in the presence of the antibiotics nigericin and antimycin A. Nigericin effectively restricts the light-induced protonmotive force to a transmembrane electrical gradient [22] and antimycin A eliminates the electrogenic ubiquinone–cytochrome b/c_2 oxidoreductase step of cyclic electron flow [23] which accounts for the third phase of the carotenoid band shift [24]. The carotenoid shift seen would therefore be due only to the first two phases, namely the initial charge separation and consequent re-reduction of P^+ by cytochrome c_2 [1]. Furthermore, the use of antimycin A causes the amplitude of the carotenoid change to be essentially independent of redox potential over 100–250 mV [25] and therefore minimizes the possibility of any artifact arising as a result of small shifts of oxidation–reduction potential over the course of the experiment. Flash-induced absorbance spectra in the presence of valinomycin which collapses the membrane potential, were subtracted from the changes seen in its absence. The resulting absorbance difference spectra normalized to reaction center concentration are shown in fig.2.

As can be seen from fig.2, there is no major difference in the shape of the carotenoid shift spectrum in the four membrane preparations examined. If an electrochromic mechanism is assumed, then the zero point of the spectrum (i.e., the wavelength at which there is no change) corresponds to the wavelength of

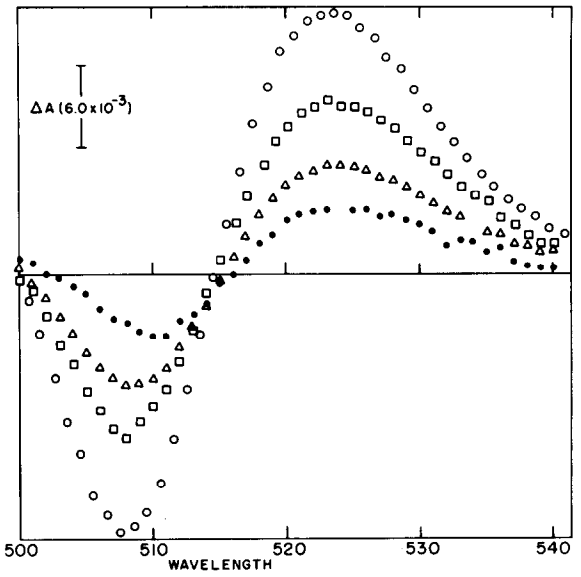

Fig.2. Spectra of flash-induced absorbance changes due to transmembrane electrical potential. The membrane preparations were from cells grown at different light intensities: (○) 170; (□) 800; (△) 1500; (●) 3000 ft-candles. The spectra include the region of the longest wavelength band attributable to carotenoids. The changes were measured 50 ms after a single saturating flash in the presence of 1 μM nigericin, 2 μM antimycin A, and with 3 μM PMS, 3 μM PES and 10 μM DAD as redox mediators at an oxidation–reduction potential of 140 ± 20 mV. Signals were averaged to obtain each point. The changes are normalized to 0.3 μM reaction centers. The change under identical conditions but with the addition of 2 μM valinomycin has been subtracted. The maximal positive absorbance change in the presence of valinomycin was near 530 nm and the percentage values for these changes relative to those in the absence of valinomycin were 6.6, 7.4, 13.6 and 17.9% in the 170, 800, 1500 and 3000 ft-candle preparations, respectively.

maximum absorbance of the species undergoing the shift, provided the extent of the shift is small [12]. In these samples, the measured zero points were 514.6, 514.8, 515.3 and 516.5 nm for the 170, 800, 1500 and 3000 ft-candle grown preparations, respectively*. The actual extent of the shift would be expected to be a function of the membrane potential generated on the single flash, and the value of this would depend on the number of centers, and the capacitance per unit area of chromatophore membrane. The small differences in zero point may therefore indicate differences in these latter parameters between preparations from cells grown at different light intensities. Furthermore, the capacitance could be related directly to the ratio of hydrophobic antenna proteins to phospholipids in the membrane bilayer. It has been noted [1,26] that the anomalous displacement of the isosbestic point to the red such as that seen in fig.2 can be explained in part by an electric field-induced hypochromicity underlying a carotenoid band shift. Although the difference in zero point is greatest in membranes enriched in B875, no such hypochromicity has been demonstrated directly in the B875 carotenoids. It is noteworthy that there is no shift to the blue in the zero point which would correspond with the change to the blue in the absorption maxima of the carotenoid spectra. It is clear from these spectra that the total amplitude of the light-induced changes vary with the variation in B800-850/B875 ratio. Fig.3

* This is in reasonable agreement with the analyses in [4] which indicated that the carotenoid pool that shows an electrochromic response to illumination has a long wavelength absorption maximum at 515 nm

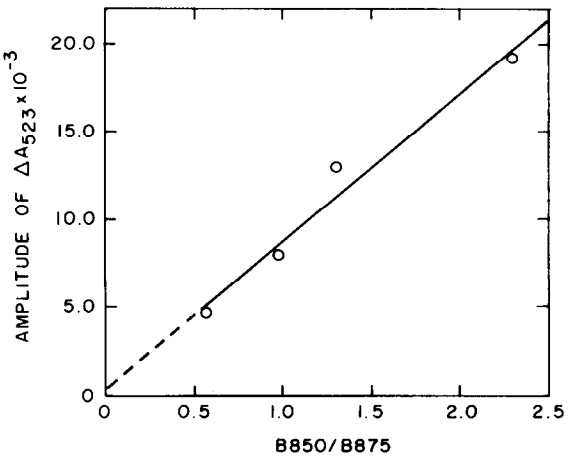

Fig.3. Relation between the amplitude of the carotenoid absorbance changes and the levels of antenna species in membrane preparations from cells grown at different light intensities. The extent of the absorbance change was measured at 523 nm and normalized to reaction center concentration as described in fig.2. The line through the points was constructed by linear regression analysis; the correlation coefficient was 0.986. In relating the amplitude of the carotenoid change/reaction centre to the B850/B875, the assumption is made that the reaction centre/B875 ratio is constant under the different growth conditions [13]. Our measurements suggest that this is not necessarily the case (table 1). When this normalized amplitude of the absorbance change was corrected for the estimated B875 levels in these membrane preparations and plotted against B850/B875, the line through the points gave a higher correlation coefficient but extrapolated somewhat below the zero point on the y-axis. The reason for this anomaly is not understood but could possibly reflect a negative absorbance change associated with the carotenoids of B875 which underlies the positive change normally observed at this wavelength.

shows a linear relationship between the amplitude of the carotenoid absorbance change and the amount of B800-850 in the membrane. The proximity of the extrapolated line to the zero point of the graph suggests that upon flash illumination, only carotenoids associated with the B800-850 complex respond with a positive absorbance change at this wavelength.

Since the carotenoid shift is a result of a transmembrane electrical gradient, any change in permeability of the membrane might manifest itself as a decrease in the amplitude of the carotenoid absorbance change seen on the time scale of these measurements. We therefore measured the decay of the change in the absence of nigericin but in the presence of 1.0 μM PMS/1.0 mM ascorbate, to poise the oxidation–reduction potential. The half decay times measured were comparable for all 4 samples (560, 700, 700 and 470 ms for 170–3000 ft-candle preparations, respectively). The total photo-oxidizable cytochrome c_2/reaction center ratio was also measured, since at least some of the cytochrome c_2 may be lost from chromatophores upon mechanical disruption of the cells. The ratio increased from 0.7 in the 170 ft-candle preparation to 1.2 in the 3000 ft-candle preparation. It therefore seems clear that the effect described here cannot be interpreted simply as an increase in permeability or a decrease in the integrity of the vesicle preparations, nor can they be attributed to an increased photo-oxidizable cytochrome c_2 level at the lower light intensities.

From our measurements, it has also been possible to calculate the ratio of B875/reaction centers. This ratio varied between 18 and 35 (table 1) in broad agreement with [13,27]. Because in the mature membrane, reaction centers are interconnected by B875 with B800-850 arranged peripherally [28] this is not a discrete 'photosynthetic unit'. During the induction of photosynthetic membrane development, however, B875 in association with reaction center is apparently inserted at discrete sites prior to B800-850 incorporation [15,29,30]. This forms a minimal functional unit [29,30] and only interlinks at later developmental stages.

In conclusion, the carotenoid molecules able to respond to the transmembrane electrical potential do not appear to be those of the B875 light-harvesting complex but are instead solely associated with the B800-850 complex. The reason for this selectivity may be related to an unfavorable orientation of the B875-associated carotenoids within the membrane, or alternatively, the lack of the permanent electrical field necessary for a detectable shift in the absorbance spectrum.

Similar conclusions have been arrived at recently by the laboratories of M. Nishimura, R. K. Clayton, and R. J. Cogdell from studies of illumination and diffusion potential induced band shifts in *R. sphaeroides* and *R. capsulata* membranes (personal communications).

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