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Singlet and triplet absorption spectra of carotenoids bound in the reaction centers of *Rhodopseudomonas sphaeroides* R26

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The singlet and triplet state absorption spectra are reported for two carotenoids, methoxyneurosporene and spheroidene, incorporated into the reaction center protein of the photosynthetic bacterial carotenoidless mutant *Rhodopseudomonas sphaeroides* R26. The spectra for the two different carotenoid molecules are identical suggesting a strong interaction between the protein and the different chromophores. Combined effects of electrochromic band shifts and carotenoid structural changes are invoked to account for the spectral observations.

Carotenoid *Absorption spectrum* *Singlet state* *Triplet state* (Rps. sphaeroides)

1. INTRODUCTION

The absorption spectra of carotenoids bound in the reaction centers of photosynthetic bacteria are red-shifted relative to the absorption spectra of the same carotenoids in organic solvents [1]. The origin of these shifts has been attributed to the presence of external charges from amino acid residues in the protein in close proximity to the carotenoid [2]. Additional band shifts occur upon illumination in response to an interaction between the carotenoid and the electric field generated within the reaction center complex during the primary photochemical reaction [3,4].

Several groups have succeeded in incorporating carotenoids into the reaction centers of carotenoidless mutants of photosynthetic bacteria [5–7]. This ability provides an opportunity to examine different carotenoids bound in the same reaction center protein rather than in proteins isolated from different bacterial strains. Furthermore, it may provide a way to explore the molecular details of the absorption spectral band shifts. This can be accomplished by incorporating specific carotenoid molecules into the reaction center and probing the change in the carotenoid spectra which occurs upon binding to the protein and in response to the photosynthetic charge separation.

Here, we present an investigation of the optical spectroscopic properties of two carotenoids, methoxyneurosporene and spheroidene, incorporated into the reaction centers of the carotenoidless mutant *Rhodopseudomonas sphaeroides* R26. These two carotenoids have very similar molecular structures (see fig.1). Both contain a C_{40} carbon skeleton and a terminal methoxy functional group. Spheroidene, however, contains 10 conjugated carbon-carbon double bonds whereas methoxyneurosporene contains 9. Because of their similar structures these two carotenoids most likely bind to the protein in the same site.

In addition to exploring the molecular features controlling the carotenoid band shifts we were interested in observing whether carotenoids having 9 carbon-carbon double bonds (neurosporene

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chromophores) are capable of quenching the primary donor triplet state. The primary donor triplet state which is formed upon illumination of chemically reduced reaction centers is quenched by carotenoids as illustrated in scheme 1 [8]. Flash-induced absorption experiments performed by Cogdell et al. [9] on reaction centers of *Rps. sphaeroides* GA and wild type have suggested that carotenoids which contain 9 conjugated carbon-carbon double bonds (neurosporene chromophore) quench the primary donor triplet state in high yield (~100%) at room temperature as do carotenoids which contain 10 conjugated carbon-carbon double bonds (spheroidene). In contrast, flash-induced absorption experiments recently reported by Schenck et al. [10], have shown that the yield of reaction carotenoid triplet states is very low (less than 10%) in the neurosporene chromophore-containing strain *Rps. sphaeroides* GA and high (~100%) in the spheroidene-containing strain *Rps. sphaeroides* wild type.

2. MATERIALS AND METHODS

Rps. sphaeroides R26 cells were grown anaerobically and photosynthetically in modified Hutners media [11]. Chromatophores ($A_{860} = 50$) in 10 mM Tris (pH 8.0) were treated with LDAO (final concentration 1.2%). The treated chromatophores were centrifuged at 250000 × *g* for 90 min. The supernatant was diluted to approx. 5-times the original volume with 10 mM Tris and chromatographed on a DEAE-Sephacel anion-exchange column which had previously been equilibrated with 10 mM Tris (pH 8.0). The column was eluted with 10 mM Tris (pH 8.0) containing 0.15% LDAO and 40–320 mM NaCl (40 mM steps). Reaction centers were eluted at ~160 mM NaCl. Reaction center purity was assayed by comparing the protein absorbance at 280 nm with the bacteriochlorophyll absorbance at 800 nm [12]. Only those fractions with an A_{280}/A_{800} ratio of less than 1.4 were used.

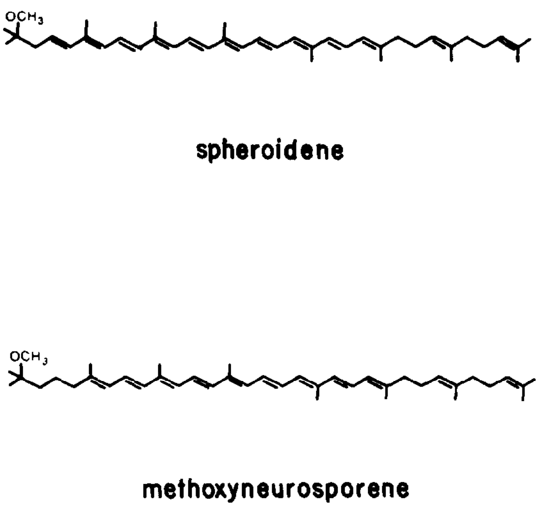
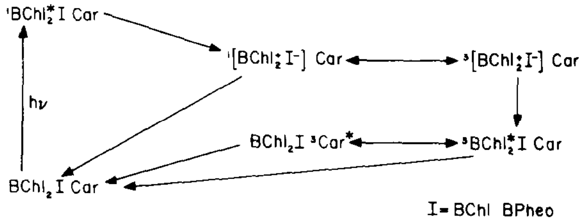


Fig.1. Chemical structures of methoxyneurosporene and spheroidene.



Scheme 1. Scheme of photochemical events in carotenoid-containing reaction centers of *Rps. sphaeroides*. Under chemically reducing conditions electron transfer beyond I is blocked and the photoinduced charge-separated state of $BChl_2^+I^-$ undergoes a back reaction either directly to the ground state or via the triplet state, $^3BChl_2^*$. This triplet may be quenched by the reaction center carotenoid triplet state, $^3Car^*$ [8].

Spheroidene was extracted from anaerobically grown *Rps. sphaeroides* wild type [13]. Methoxyneurosporene was extracted from *Rps. capsulata* MT1131 [14]. These carotenoids were obtained from bacterial cells according to the methods of Cogdell et al. [15]. Carotenoids were incorporated into reaction centers of *Rps. sphaeroides* R26 by first mixing 1.0 ml reaction center solution (10 μM) with 0.1 ml Triton X-100. The sample was gently stirred using a magnetic stir bar while a 50-fold molar excess of carotenoid in pentane solution was carefully layered onto the surface. The pentane was slowly (~20 min) evaporated under vacuum. The sample was then sealed and gently stirred at room temperature in the dark for 24 h. Excess carotenoid was removed from the reaction center solution by DEAE-Sephacel column chromatography as described above. The absorbance of the samples revealed that the carotenoids bound in a 1:1 stoichiometric ratio with the primary donor [15].

Reaction centers used in the room-temperature triplet-triplet absorption experiments were adjusted to give an absorbance of ~1 at 800 nm in a 1 cm cell (~3.5 μM). The reaction center carotenoid triplet state is observed only when electron transfer beyond the initial electron acceptor is blocked. In quinone-containing reaction centers this is accomplished by adding solid sodium ascorbate to a concentration of 100 mM and flashing the reaction centers with an excitation light beam to pump electrons from the $BChl_2$ donor to secondary acceptor quinones. The $BChl_2$ donor is subsequently reduced by ascorbate while the quinones remain reduced. To prevent sample cracking in the low-temperature triplet-triplet absorption measurements, concentrated reaction centers were mixed with glycerol (1:2) to give an absorbance of ~1 in a 1 mm cell at 800 nm. The low-temperature samples contained sodium ascorbate and were illuminated during freezing to reduce the secondary acceptor quinones. The triplet-triplet absorption spectra were obtained using a transient absorption spectrometer described in [16]. The reaction centers were excited by an Nd/YAG pumped dye laser (Exciton Rhodamine 640) lasing at 600 nm and having a pulse width of 3–4 ns FWHM and a pulse energy of 1.5 mJ at the sample. Absorbance changes were obtained using a single-beam spectrophotometer design with an Xe flashlamp providing the probing beam and a Tektronix 7912AD transient digitizer for capturing the signals. The transients were recorded at 5 nm increments from 400 to 560 nm.

3. RESULTS

The singlet absorption spectra of methoxyneurosporene and spheroidene in pentane are shown in fig.2. Both spectra display three well-resolved peaks at 411, 434 and 463 nm for methoxyneurosporene and 421, 447 and 475 nm for spheroidene. The fact that methoxyneurosporene absorbs at shorter wavelengths than spheroidene is due to its shorter π-electron conjugation in the all-*trans* conformations adopted in solution [17].

The incorporation of methoxyneurosporene and spheroidene into reaction centers of *Rps. sphaeroides* R26 has a dramatic effect on their singlet absorption spectra. Fig.3 shows that the absorption spectra of both methoxyneurosporene

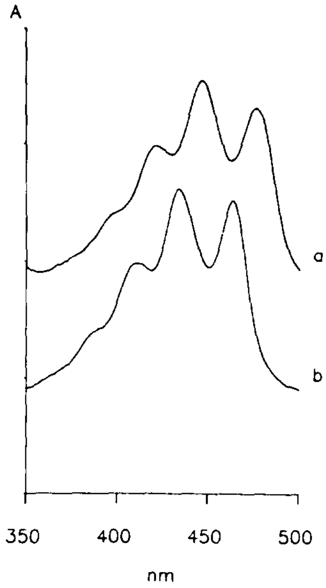


Fig.2. Singlet absorption spectra of (a) spheroidene and (b) methoxyneurosporene (~10 μM) in pentane solution. Spectra were taken on a Perkin-Elmer Lambda 3B absorption spectrophotometer.

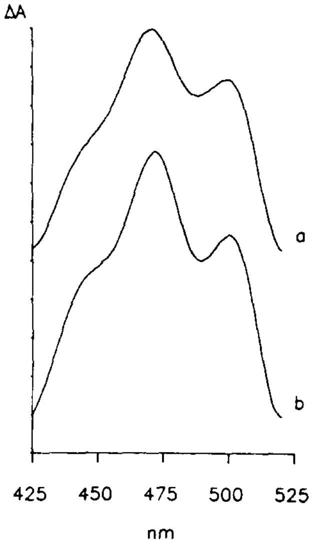


Fig.3. Singlet absorption spectra of (a) spheroidene and (b) methoxyneurosporene incorporated into the reaction centers of *Rps. sphaeroides* R26. Spectra represent the in situ absorbance of the carotenoid with the *Rps. sphaeroides* R26 reaction center contribution subtracted. Sample concentrations were ~2 μM in 20 mM Tris-HCl (pH 8.0) with 0.15% LDAO.

and spheroidene shift to essentially the same wavelength upon binding to the reaction centers. The spectra, which are differences between reconstituted and unreconstituted reaction center absorptions, display three well-resolved peaks attributable to carotenoids near 438, 470 and 499 nm. These absorption peaks are red-shifted relative to the peaks of the carotenoids in pentane solution.

The triplet-triplet absorption spectra of spheroidene and neurosporene in cyclohexane have been reported [18]. Spheroidene has been found to have a triplet state absorption maximum at 510 nm, whereas triplet neurosporene has its maximum absorption at 489 nm. As in the singlet spectra, the shorter wavelength of triplet absorption of neurosporene compared to spheroidene can be accounted for on the basis of its lesser extent of π-electron conjugation in solution [19].

Incorporation of methoxyneurosporene and spheroidene into the reaction centers of *Rps. sphaeroides* R26 causes a significant shifting of their triplet-triplet absorption spectra. Fig.4 shows that the spectra are red-shifted from the solution spectra and that the two different carotenoids exhibit the same wavelength of maximum absorption (545 nm). The spectra are intense suggesting efficient triplet quenching by both carotenoids if the extinction coefficients are similar to those in solution [18]. Another similarity in the spectral properties of these two different carotenoids can be observed in the carotenoid band shifts in the low-temperature time-resolved spectra. The inflections between 420 and 520 nm in the spectra probed immediately after the pulse show the same features for the two different carotenoids (see fig.5). These features are believed to be due to electrochromic band shifts of the carotenoid absorptions owing to

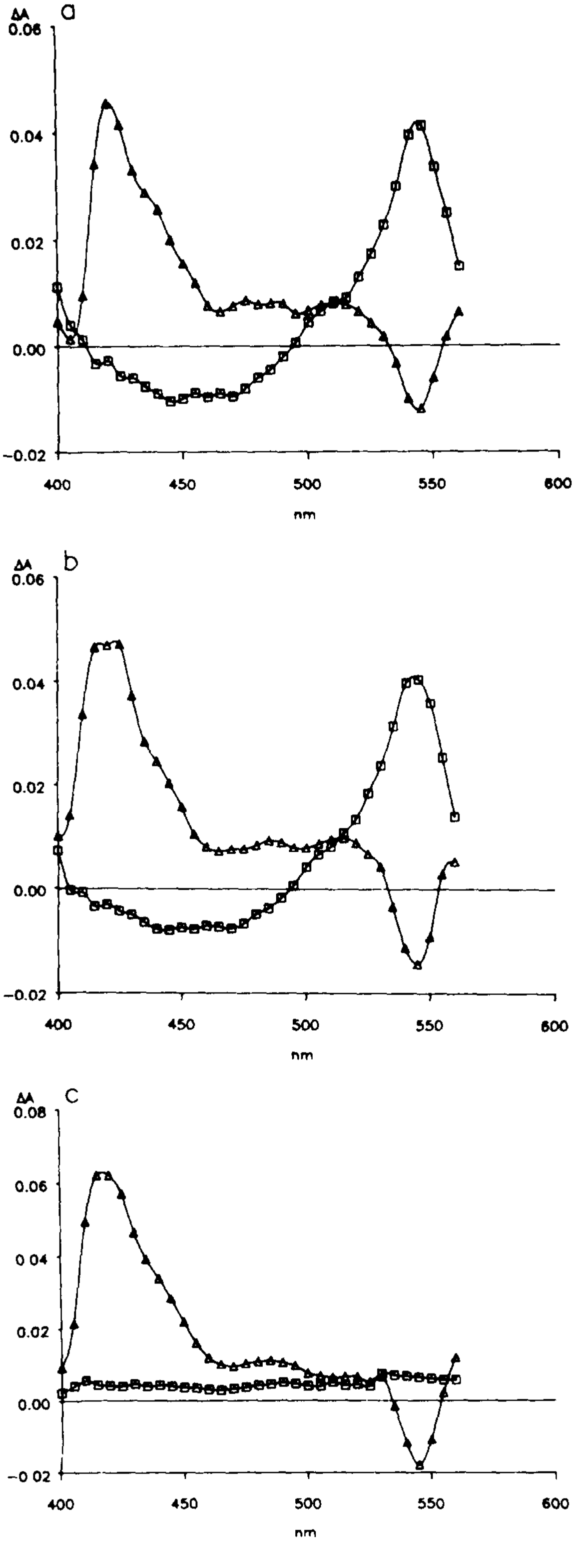


Fig.4. Room-temperature (295 K) triplet absorption spectra of (a) spheroidene and (b) methoxyneurosporene incorporated into the reaction centers of *Rps. sphaeroides* R26. The bottom trace (c) is the spectrum of *Rps. sphaeroides* R26 without carotenoids incorporated. The triangles represent the intensities of the transients measured during the laser excitation flash. The squares represent the intensities of the transients 135 ns after the flash. The reaction center concentration was 3.5 μM and the samples contained 100 mM sodium ascorbate, 0.1% Triton X-100 and 20 mM Tris-HCl, pH 8.0.

an interaction of the chromophores with the electric dipole resulting from the photosynthetic charge separation process [4].

4. DISCUSSION

The most striking feature of these data is summarized in table 1. Both the singlet and triplet absorption spectra of the two different carotenoid molecules shift to the same wavelength upon binding to the protein. These carotenoids are bound to the reaction center in a carotenoid-to-primary donor ratio of 1:1. They also have the polar methoxy group that allows strong binding to the reaction center [7]. This suggests that there exists a single, well-defined binding site for these carotenoids in the protein.

The red shift in absorption spectra of carotenoids in vivo relative to their absorption in vitro has been attributed to an interaction between the chromophore and a strong local field induced by charged residues in the protein [2]. Molecular orbital calculations have shown that the magnitude of the shift is a sensitive function of the distance and orientation of charges (or dipoles) relative to the chromophore [2]. Bovine rhodopsin and bacteriorhodopsin reconstituted with various retinal analogs are examples of charge-induced spectral shifts in linear polyenes [20,21]. The present results show that methoxyneurosporene displays a more pronounced red shift of the carotenoid absorption upon binding to the reaction center protein than does spheroidene. It is unlikely that a single distribution of charges and dipoles would shift both the singlet and triplet absorption spectra of spheroidene and methoxyneurosporene to exactly the same wavelength.

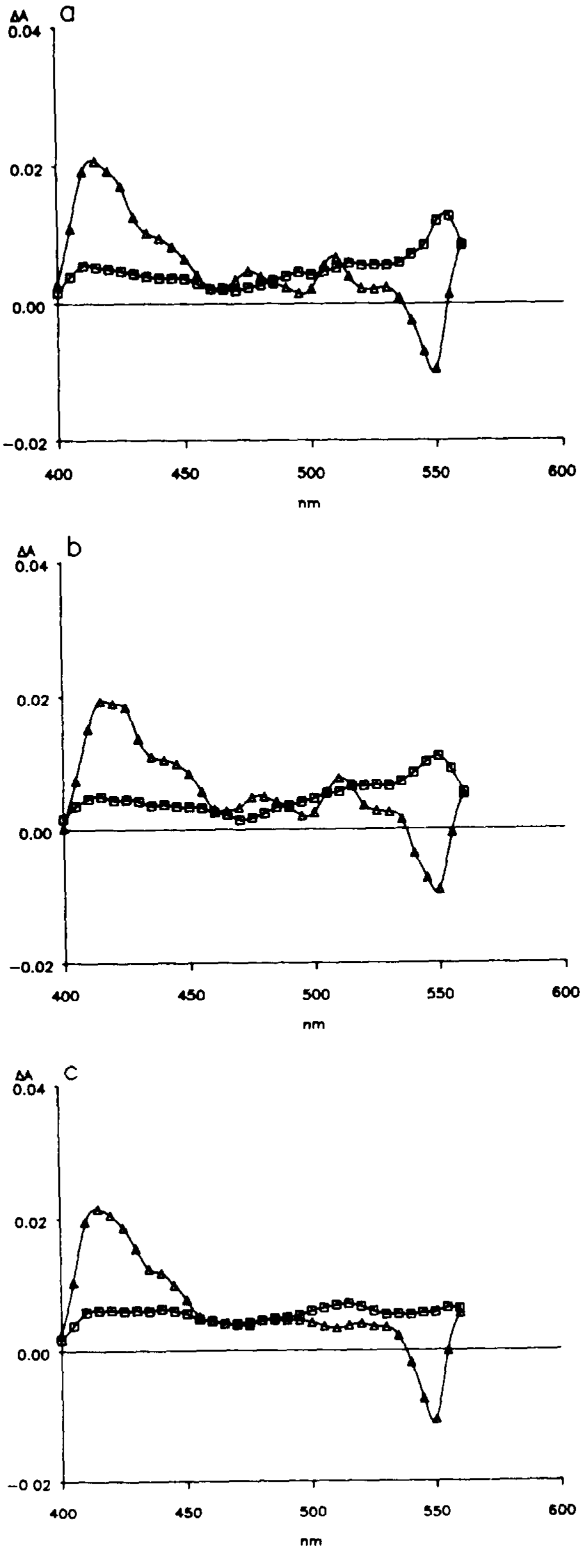


Fig.5. Low-temperature (12 K) triplet absorption spectra of (a) spheroidene and (b) methoxyneurosporene incorporated into the reaction centers of *Rps. sphaeroides* R26. The bottom trace (c) is the spectrum of *Rps. sphaeroides* R26 without carotenoids incorporated. The triangles represent the intensities of the transients measured during the laser excitation flash. The squares represent the intensities of the transients 125 ns after the flash. The reaction center concentration was 35 μM and the samples contained 100 mM sodium ascorbate, 0.1% Triton X-100, 20 mM Tris-HCl, pH 8.0, and 66% (v/v) glycerol.

Table 1

Absorption spectral features of spheroidene and methoxyneurosporene (nm units)

	Solution		Bound to reaction center	
	Spheroidene	Methoxy-neurosporene	Spheroidene	Methoxy-neurosporene
Singlet spectral absorbance peaks	475	463	498	500
	447	454	470	471
	421	411	437	438
Triplet spectral absorbance peaks	510[a]	489[a]	545	545

[a] Values reported for spheroidene and neurosporene in [18]

The singlet spectral wavelengths are reported to ± 1 nm. The triplet spectral wavelengths are reported to ± 2.5 nm

While charges will shift the spectra, there must be a different reason for why the spectra are the same.

Agalidis et al. [6] have performed resonance Raman experiments on spheroidene incorporated into reaction centers of *Rps. sphaeroides* R26. They have shown that the spectra are consistent with the carotenoid adopting a folded and twisted *cis* conformation upon binding to the protein, despite an all-*trans* conformation in solution [6]. These resonance Raman studies indicate that the carotenoid isomerizes upon binding to the reaction center protein. A plausible interpretation for our results is that strong binding of the carotenoid to the reaction center is accompanied not only by an electrochromic absorption spectral band shift to the red, but also by a twisting of the carotenoids in a manner that interrupts their π-electron conjugation. This could result in effective π-electron conjugation lengths which are the same in both molecules rendering the visible absorption spectra nearly identical. Configurational changes alone could not account for the present results since interrupting the π-electron conjugation would cause blue shifts of the absorption spectra. The net red shifts which are observed are the result of charge-induced red shifts of chromophores which have the same conjugation length owing to the twisted conformations.

Finally, this work demonstrates that methoxyneurosporene and spheroidene, when bound to the reaction center protein, are very efficient quenchers of the primary donor triplet state at room temperature, in agreement with the conclusions of Cogdell et al. [9]. The present results suggest that this carotenoid function is regulated by the reaction center protein. Primary donor triplet quenching appears to be more strongly influenced by how the carotenoid binds to the reaction center and what conformation it adopts (i.e. its effective conjugation length) than by its total number of carbon-carbon double bonds.

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