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Electron-spin resonance studies of carotenoids incorporated into reaction centers of *Rhodobacter sphaeroides* R26.1

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The carotenoids, hydroxyneurosporene, methoxyneurosporene, spheroidene, hydroxyspheroidene, spheroidenone, 3,4-dihydroanhydrorhodovibrin, and spirilloxanthin have been incorporated into reaction centers of the photosynthetic bacterium *Rhodobacter sphaeroides* (*Rhodopseudomonas sphaeroides*) R26.1 in a carotenoid-to-primary donor ratio of 1:1. These carotenoids have different functional groups and varying extents of π electron conjugation. The importance of these structural features on binding to the reaction center and quenching the primary donor triplet state was assessed. The carotenoid binding site on reaction centers of *Rb. sphaeroides* R26.1 exhibits a preference to binding carotenoids which contain polar functional groups. Electron-spin resonance spectroscopy was used to detect carotenoid triplet state formation. The zero-field splitting parameters of the reconstituted carotenoids revealed that carotenoids isomerize upon binding to the reaction center. The structure of the reaction center-bound carotenoids is one where the π electron conjugation is interrupted.

Introduction

Carotenoid molecules serve two important functions in photosynthesis. They act as accessory pigments by absorbing light in spectral regions where chlorophyll does not absorb and transfer this energy to the reaction center [1,2]. Also carotenoids protect the photosynthetic apparatus from photodestruction by either quenching the excited singlet state of oxygen [3,4], or trapping chlorophyll triplet states to prevent the sensitized formation of singlet state oxygen [3,5,6]. Both of these photoprotection mechanisms involve energy transfer to carotenoid-excited triplet states [2,7].

Little is known about the triplet state structure of carotenoids. Low quantum yields for singlet–triplet intersystem crossing make optical studies of the triplet states of carotenoids feasible only through the use of photosensitizers in conjunction with pulse radiolysis or flash photolysis techniques [8–10]. Resonance Raman spectroscopy has been used to investigate the excited state structure and configuration of carotenoids in vitro and in vivo [11–20]. To date, however, no agreement has been reached on what the configuration of the carotenoid is in the reaction centers of photosynthetic bacteria [15,19,21–22]. Also, there are conflicting reports from studies done both in vivo and in vitro as to whether the carotenoid isomerizes upon entering its triplet state [11–14,17,23], and if it does, whether the photochemistry is reversible [23].

Recently, the first observation of carotenoid triplet states by electron spin resonance (ESR) spectroscopy was reported [24]. Since that time it has been demonstrated that ESR techniques can be used to probe the structures, geometries and dynamics of carotenoids in whole cells, chromatophores and isolated pigment-protein complexes of various strains of photosynthetic bacteria [25–29].

Several workers have succeeded in incorporat-

ing carotenoids into reaction centers of the carotenoidless mutants *Rhodospirillum rubrum* G9 [6] and *Rhodobacter sphaeroides* (*Rhodopseudomonas sphaeroides*) R26 [16]. Singlet and triplet absorption and circular dichroism studies have shown that reaction centers of carotenoidless mutants reconstituted with spheroidene, spheroidenone or spirilloxanthin give rise to spectra which are very similar to those obtained from the carotenoid-containing parent preparations [6,16,23,30]. These data have been taken as evidence that the reconstituted carotenoids assume a similar structure or occupy the same environment as do the carotenoids in the parent complexes.

In this paper we present ESR spectroscopic studies of various carotenoids reconstituted into reaction centers of *Rb. sphaeroides* R26.1. The carotenoids were selected specifically to assess the effect of carotenoid structure on primary donor triplet state quenching and the efficiency of binding to the reaction center. The effects of variations in the conjugated π electron system and the presence of various functional groups on these carotenoids were evaluated. In addition, we present ESR studies on native carotenoid-containing reaction center complexes. These studies demonstrate that the reconstituted complexes provide good models for understanding the behavior of naturally occurring carotenoid-containing pigment-protein complexes.

Materials and Methods

Rhodobacter sphaeroides R26.1 cells [31,32] were grown anaerobically and photosynthetically in modified Hutners media [33], and reaction centers were prepared according to the method of Clayton and Wang [34]. *Rb. sphaeroides* wild type and GA reaction centers were prepared by the method described previously [28]. The reaction centers were alternately dialyzed against solid sucrose and 25 mM Tris buffer (pH 8.0) until they precipitated. The reaction centers were resolubilized with 25 mM Tris buffer containing 0.05% Triton X-100 and adjusted to a concentration of 0.01 mM. The reaction center solution was stored in an amber bottle at 4°C until ready for use.

The sources of the carotenoids were as follows: β-carotene (β,β-carotene) was purchased from Sigma; apo-β-carotenol (8′-apo-β-caroten-8′-ol) and zeaxanthin ((3R,3′R)-β,β-carotene-3,3′-diol) were gifts from Professor George Britton; spheroidene (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-carotene) was extracted from anaerobically grown *Rb. sphaeroides* wild type [35]; spheroidenone (1-methoxy-3,4-didehydro-1,2,7′,8′-tetrahydro-ψ,ψ-caroten-2-one) was extracted from aerobically grown *Rb. sphaeroides* wild type [2,36]; hydroxyspheroidene (1-methoxy-3′,4′-didehydro-1,2,7,8,1′,2′-hexahydro-ψ,ψ-caroten-1-ol) was extracted from *Rhodopseudomonas gelatinosa* [37]; neurosporene (7,8-dihydro-ψ,ψ-carotene) was extracted from *Rb. sphaeroides* G1C [38] and 1,2-dihydrolycopene (1,2-dihydro-ψ,ψ-carotene) was extracted from *Rhodopseudomonas viridis* [39]; spirilloxanthin (1,1′-dimethoxy-3,4,3′,4′-tetradehydro-1,2,1′,2′-tetrahydro-ψ,ψ-carotene) and 3,4-dihydroanhydrorhodovibrin (1-methoxy-1,2-dihydro-ψ,ψ-carotene) were extracted from *R. rubrum* [40–43]; rhodopin (1,2-dihydro-ψ,ψ-caroten-1-ol) was extracted from *Rhodopseudomonas acidophila* 7750 [43]; and hydroxyneurosporene (1,2,7′,8′-tetrahydro-ψ,ψ-caroten-1-ol) and methoxyneurosporene (1-methoxy-1,2,7′,8′-tetrahydro-ψ,ψ-carotene) were extracted from *Rhodopseudomonas capsulata* MT1131 [44]. Carotenoids obtained from bacterial cells [30], were purified by silica gel thin layer chromatography (1 : 1 toluene/chloroform) and adjusted to a concentration of approx. 0.3 mM in pentane. The carotenoid solutions were stored under a nitrogen atmosphere in amber bottles at −20°C.

Rb. sphaeroides R26.1 reaction centers were reconstituted with carotenoids by first mixing 1.0 ml of reaction center solution with 0.1 ml of Triton X-100 in a 2 cm i.d. × 8 cm vial which contained a micro magnetic stir bar. After the air bubbles dissipated, a 40- to 50-fold molar excess of carotenoid in pentane solution was carefully pipetted onto the surface of the gently stirring reaction center mixture. The pentane was then very slowly evaporated under vacuum so as to avoid mixing of the two layers. After all the pentane was removed the solution was vigorously stirred under vacuum for 5–10 s. The vial was then capped and gently stirred at room temperature, in the dark, for 16–24 h.

Samples were prepared for triplet state ESR

experiments by first deoxygenating the reaction center sample for 5 min with N_2 gas and then chemically reducing the sample with 100 mM (final concentration) sodium dithionite ($Na_2S_2O_7$). The sample mixture was placed in a quartz ESR sample tube (3 mm i.d. × 4 mm o.d.), capped and then frozen in liquid nitrogen.

Triplet state ESR spectroscopy was carried out with a Varian X-band spectrometer. The magnetic field modulation was 25 G at a frequency of 100 kHz. Excitation was accomplished by using light from a 1000 W xenon arc lamp (Kratos LH151N/1S) which was filtered through 3.5 cm of water in a Pyrex bottle. The light was then focused into a Varian TE microwave cavity fitted with a flange which allowed 100% transmission of the light. The light-induced signals were detected using a lock-in amplifier (PAR 128A) referenced to the exciting light which was modulated at 44 Hz. This modulation frequency was low enough to avoid phase shifts in the lock-in detected signals. The lock-in amplifier phase angle was tuned to maximize the overall spectral intensity. The sample temperature in the ESR experiment was 85 K. Zero-field splitting parameters were obtained from computer simulations of the experimental triplet state ESR spectra using the method reported previously by Frank et al. [45].

The extent of carotenoid reconstitution into the reaction centers was assayed after purification of the carotenoid-reaction center mixture. This was accomplished by applying 0.5 ml of the mixture to a DEAE sephacel column (5 ml bed volume), which had been equilibrated with 25 mM Tris buffer (pH 8.0). The excess carotenoid was removed by elution with approx. 100 ml of 25 mM Tris buffer (pH 8.0) which contained 10 mM sodium chloride and 0.05% Triton X-100. The reaction centers were then eluted with a minimal amount of 25 mM Tris buffer (pH 8.0) containing 1 M sodium chloride and 0.05% Triton X-100. The concentrations of the bound carotenoids and the reaction centers were calculated from the electronic absorption spectrum of the purified reaction center-carotenoid complex using the 865 nm absorption extinction coefficient for reaction centers ($1.13 \cdot 10^5\ M^{-1} \cdot cm^{-1}$) [34] and carotenoid extinction coefficients reported previously [30].

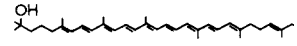
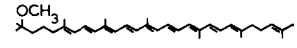
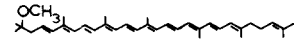
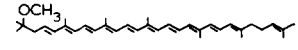
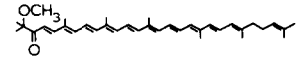
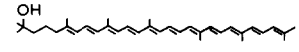
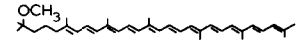
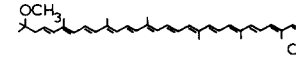
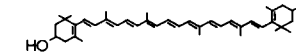
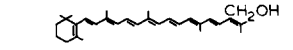
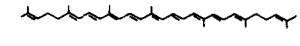
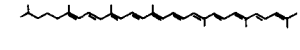
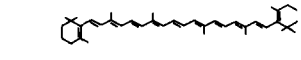
Results

Table I shows the structures of the carotenoids used in the present work. These molecules belong to three groups, hydrocarbons (carotenes), xanthophylls and apo-carotenoids. Neurosporene, 1,2-dihydrolycopene and β-carotene are hydrocarbon carotenoids. Carotenoids containing methoxy and hydroxy functional groups are classified as xanthophylls. The only apo-carotenoid studied here, apo-β-carotenol was formed by cleavage of β-carotene at the 8′ position [46].

Singlet absorption spectroscopy carried out after purification by DEAE sephacel column chromatography of the carotenoid-containing reaction center complexes revealed that all of the

TABLE I

Carotenoids used in the present study

Hydroxyneurosporene	
Methoxyneurosporene	
Spheroidene	
Hydroxyspheroidene	
Spheroidenone	
Rhodopin	
3,4-Dihydroanhydrorhodovibrin	
Spirilloxanthin	
Zeaxanthin	
Apo-β-carotenol	
Neurosporene	
1,2-Dihydrolycopene	
β-carotene	

xanthophylls, except rhodopin and zeaxanthin, bound to reaction centers of *Rb. sphaeroides* R26.1 in an approx. 1 : 1 ratio. Also, the singlet absorption spectrum of the complexes exhibited a slight red-shift (approx. 2 nm) of the bacteriochlorophyll Soret band. This is believed to be caused by interactions between the carotenoid and bacteriochlorophyll molecules [15,16], and can be taken as evidence that the carotenoid is bound in the reaction center.

Reconstitution of reaction centers with the hydrocarbon carotenoids, neurosporene and 1,2-dihydrolycopene, was inefficient. Very small amounts of these carotenoids bound to the reaction centers after purification by column chromatography. Numerous attempts to reconstitute β-carotene, rhodopin, apo-β-carotenol, and zeaxanthin into the reaction centers were unsuccessful.

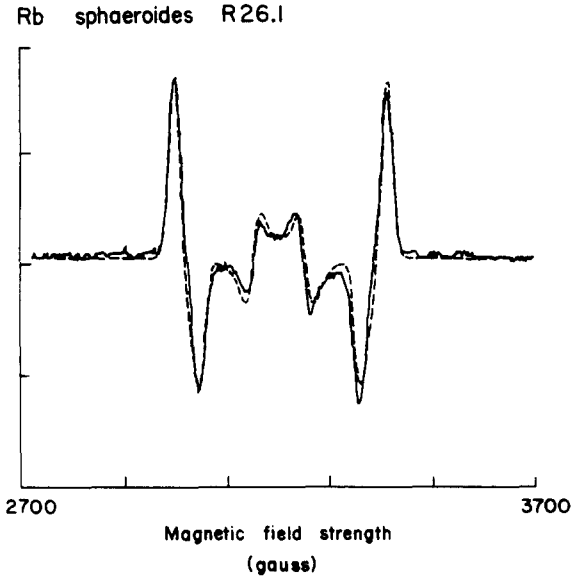


Fig. 1. Triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers. The solid line denotes the experimental spectrum taken under the following conditions: temperature, 85 K; modulation amplitude, 25 G; microwave frequency, 9.056 GHz; microwave power, 16 mW; light modulation frequency, 44 Hz; lock-in amplifier sensitivity, 10 mV; sweep rate, 30 G/min; recorder time constant, 10 s. The dashed line represents the computer-generated spectrum using the parameters, $|D| = 0.0187 \pm 0.0002$ cm^{-1} and $|E| = 0.0032 \pm 0.0002$ cm^{-1}; zero-field depopulating rate constants, $k_1 : k_2 : k_3$, 0.1 : 0.4 : 0.5 (± 0.1); relative populating rate constants, $P_{+1} : P_0 : P_{-1}$, 0.0 : 1.0 : 0.0; intrinsic linewidth parameter, 14 ± 2G.

The light induced triplet state ESR spectrum of chemically reduced reaction centers of the carotenoidless mutant *Rb. sphaeroides* R26.1 is shown in Fig. 1. This triplet state spectrum belongs to the primary donor bacteriochlorophyll dimer of the reaction center [47]. The zero-field splitting parameters which characterize this triplet state were measured to be $|D| = 0.0187 \pm 0.0002$ cm^{-1} and $|E| = 0.0032 \pm 0.0002$ cm^{-1} in agreement with previous reports [48].

Table II summarizes the triplet state zero-field splitting parameters obtained from the ESR spectra of carotenoids reconstituted into reaction centers of *Rb. sphaeroides* R26.1. The reconstituted xanthophylls exhibited triplet state ESR spectra which in most cases were indistinguishable from native reaction center complexes containing the same carotenoid. The triplet state ESR spectrum resulting from reaction centers of *Rb. sphaeroides* R26.1 reconstituted with spheroidene is shown in Fig. 2a. The triplet state ESR spectrum obtained from reaction centers of anaerobically grown *Rb. sphaeroides* wild type, which contains predominantly spheroidene [30], is shown in

TABLE II

REACTION CENTER CAROTENOID TRIPLET STATE ZERO-FIELD SPLITTING PARAMETERS

$|D|$ and $|E|$ are the triplet state zero-field splitting parameters (in cm^{-1}) which characterize the ESR spectra of the carotenoids incorporated into reaction centers of *Rb. sphaeroides* R26.1. These parameters were obtained from computer simulations of the experimental triplet state spectra. Other simulation parameters which were the same for all spectra were: zero-field depopulating rate constants, $k_1 : k_2 : k_3$, 0.4 : 0.2 : 0.4 (± 0.1); relative populating rate constants, $P_{+1} : P_0 : P_{-1}$, 0.0 : 1.0 : 0.0; intrinsic linewidth parameter, 25 ± 2G. The errors in these numbers give the range of parameters for which the simulations fell within the signal-to-noise ratio of the experimental spectra.

Carotenoid	$\|D\|$	$\|E\|$
Hydroxyneurosporene	0.0284 ± 0.0006	0.0041 ± 0.0003
Methoxyneurosporene	0.0286 ± 0.0006	0.0044 ± 0.0003
Spheroidene	0.0286 ± 0.0006	0.0044 ± 0.0003
Hydroxyspheroidene	0.0286 ± 0.0006	0.0044 ± 0.0003
Spheroidenone	0.0271 ± 0.0005	0.0042 ± 0.0003
3,4-Dihydroanhydrorhodovibrin	0.0201 ± 0.0003	0.0037 ± 0.0002
Spririlloxanthin	0.0201 ± 0.0003	0.0037 ± 0.0002
Neurosporene	0.0342 ± 0.0006	0.0035 ± 0.0003

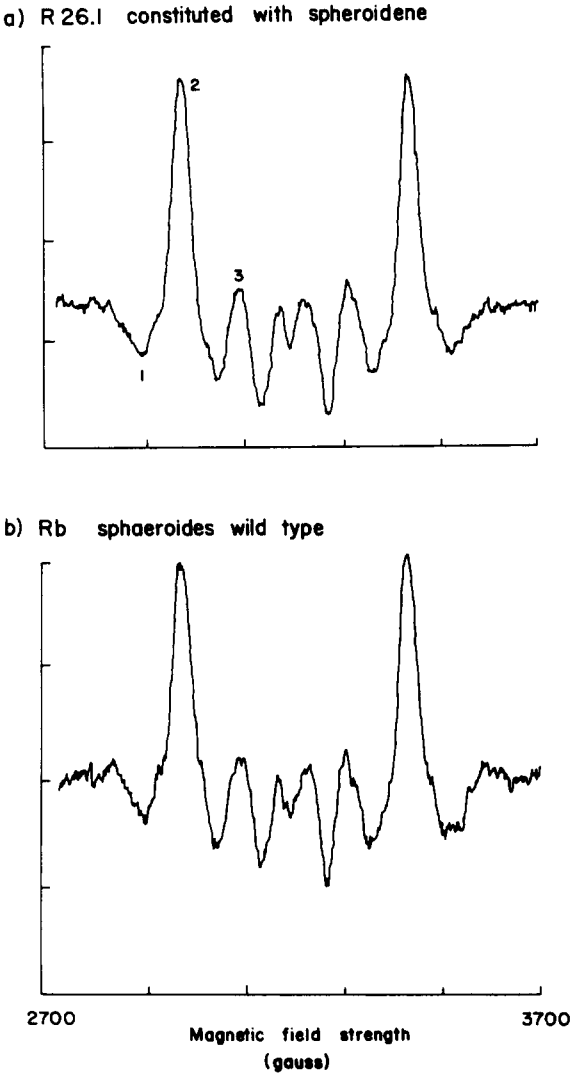


Fig. 2. (a) Triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers reconstituted with spheroidene. The peak assignments 1 (emission), 2 and 3 (absorption) denote the triplet canonical field positions. The spectrum was taken under the same conditions as Fig. 1, except for the temperature which was 90 K. (b) Triplet state ESR spectrum of *Rb. sphaeroides* wild-type reaction centers. The spectrum was taken under the same conditions as Fig. 1.

Fig. 2b. These spectra are strikingly similar and exhibit the same zero-field splitting parameters and relative peak intensities. Incorporation of hydroxyspheroidene into reaction centers of *Rb. sphaeroides* R26.1 resulted in a triplet state ESR spectrum (data not shown) which was virtually identical to those observed from reaction centers reconstituted with spheroidene or from reaction centers of *Rb. sphaeroides* wild type (Fig. 2). Reconstitution of spheroidenone, which is the most abundant carotenoid in aerobically grown *Rb. sphaeroides* wild type [2,36], into reaction centers of *Rb. sphaeroides* R26.1 yielded a triplet state ESR spectrum which was indistinguishable from the ESR spectrum of the aerobically grown, chemically reduced whole cells [25]. The triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers reconstituted with spirilloxanthin (Fig. 3) exhibited the same zero-field splitting parameters as *R. rubrum* S1 [24], which contains spirilloxanthin as the reaction center carotenoid [49]. The zero-field splitting parameters reported here for spirilloxanthin differ from those reported previously for *R. rubrum* S1 [24]. A contribution from the primary donor triplet state was not accounted for correctly in the previous measurement of the zero-field splitting parameters. This has been done correctly in the present analysis.

The triplet state ESR spectrum resulting from

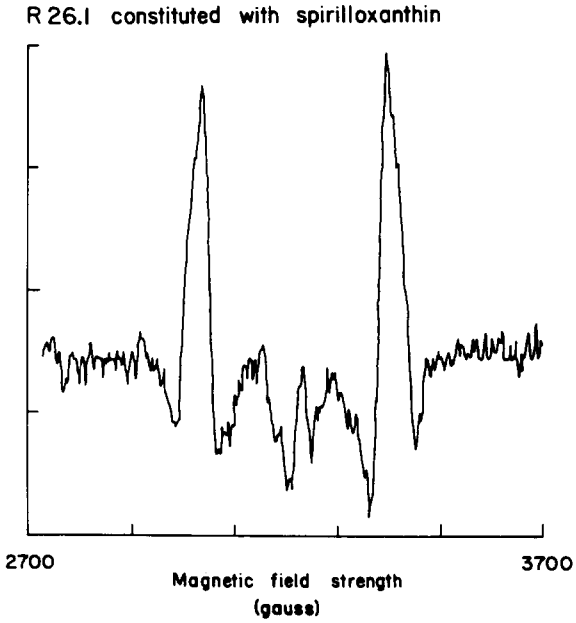


Fig. 3. Triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers reconstituted with spirilloxanthin. The spectrum was taken under the same conditions as Fig. 1, except for the temperature (107 K) and the lock-in amplifier sensitivity (2.5 mV).

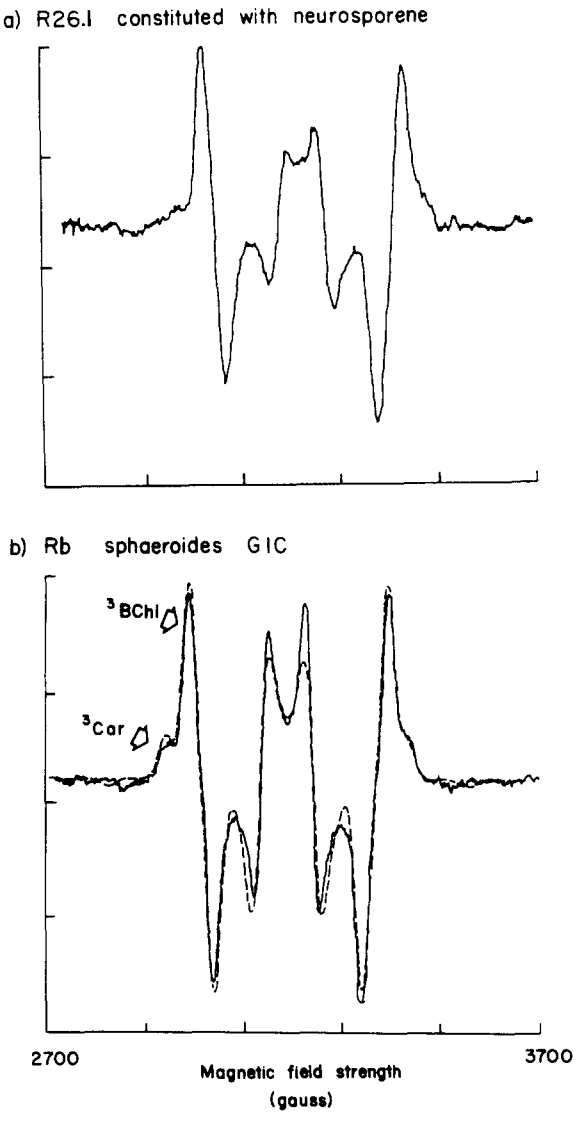


Fig. 4. (a) Triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers reconstituted with neurosporene. The spectrum was taken under the same conditions as Fig. 1. (b) Triplet state ESR spectrum of *Rb. sphaeroides* G1C whole cells. The solid line denotes the experimental spectrum taken under the same conditions as Fig. 1 except for the temperature (90 K), the microwave frequency (9.055 GHz), the lock-in amplfier sensitivity (2.5 mV) and the. sweep rate (33 G/min). The dashed line represents the computer-generated spectrum obtained by adition of a computer-generated primary-donor triplet-state ESR spectrum using the zero-field splitting parameters given in Fig. 1 with a computer-generated carotenoid triplet-state ESR spectrum, using the parameters given in Table II. The dominant features of both triplets are indicated.

reconstitution of reaction centers with neurosporene (Fig. 4a) was characterized by the same zero-field splitting parameters and relative peak intensities as that of *Rb. sphaeroides* G1C (Fig. 4b). Although reconstitution with neurosporene was inefficient there are features in the ESR spectrum attributable to neurosporene. These appear as small peaks and shoulders superimposed on the primary donor triplet state signals [25] (see Fig. 4). In this case the zero-field splitting parameters of the carotenoid were obtained by the summation of two computer generated spectra, one belonging to the primary donor whose parameters are known and the other from the carotenoid (compare Fig. 4 with Fig. 1). Thin-layer chromatography (TLC) of the pigments extracted from *Rb. sphaeroides* G1C showed only one carotenoid, neurosporene, in agreement with earlier work by Cogdell and Crofts [38]. Attempts to reconstitute 1,2-dihydrolycopene into *Rb. sphaeroides* R26.1 reaction centers resulted in a triplet state ESR spectrum showing the same small peaks as the spectrum attributable to reconstituted neurosporene (Fig. 4). However, in this case the poor signal-to-noise ratio of the small carotenoid peaks in the spectrum precluded a determination of the zero-field splitting parameters.

Rb. sphaeroides R26.1 reaction centers reconstituted with 3,4-dihydroanhydrorhodovibrin gave rise to a triplet state ESR spectrum which exhibited the same zero-field splitting parameters as the spectra obtained from chemically reduced whole cells of *R. rubrum* S1 and *Rb. sphaeroides* R26.1 reconstituted with spirilloxanthin (see Table II). Reconstitution of *Rb. sphaeroides* R26.1 reaction centers with hydroxyneurosporene or methoxyneurosporene (Fig. 5) yielded identical triplet state ESR spectra and zero-field splitting parameters as reaction centers reconstituted with spheroidene (see Figs. 2 and 5 and Table II). The triplet state ESR spectrum of chemically reduced whole cells of *Rb. sphaeroides* GA, where the reaction center carotenoid has been reported to be either hydroxyneurosporene [30,50] or methoxyneurosporene [17], revealed spectral features quite different from those of reaction centers of *Rb. sphaeroides* R26.1 reconstituted with those two carotenoids. The zero-field splitting parameters and relative peak intensities of the *Rb. sphaeroides*

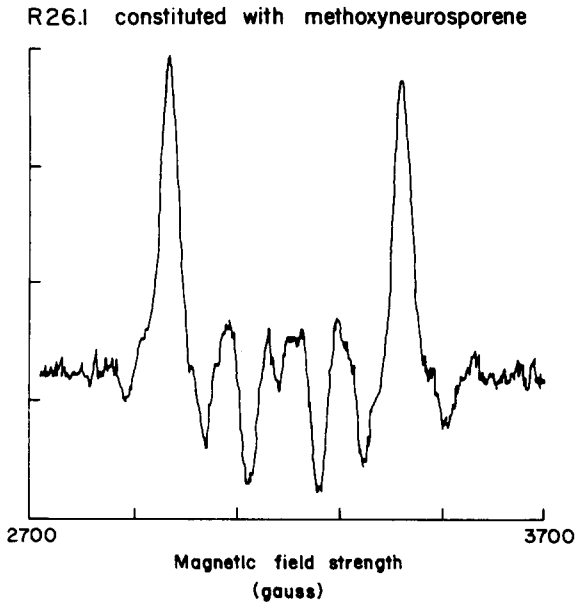


Fig. 5. Triplet state ESR spectrum of *Rb. sphaeroides* R26.1 reaction centers reconstituted with methoxyneurosporene. The spectrum was taken under the same conditions as Fig. 1, except for the temperature which was 90 K.

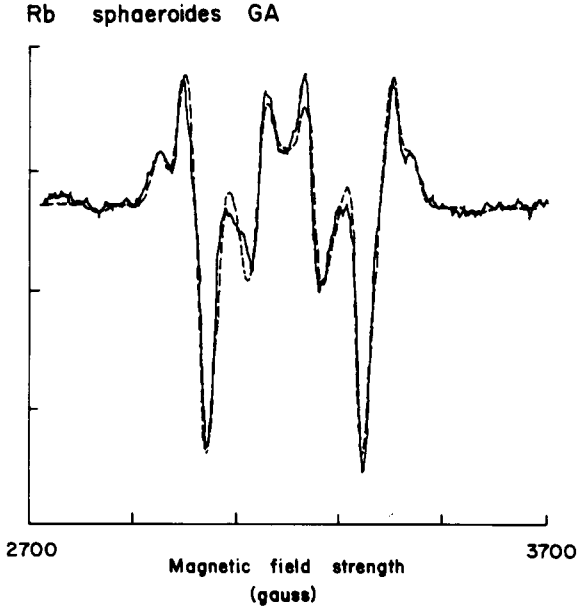


Fig. 6. Triplet state ESR spectrum of *Rb. sphaeroides* GA whole cells. The solid line denotes the experimental spectrum taken under the same conditions as Fig. 1, except for the temperature (90 K), the microwave frequency (9.057 GHz), the sweep rate (50 G/min) and the recorder time constant (3 s). The dashed line represents the computer-generated spectrum obtained using the same procedure and zero-field splitting parameters given for Fig. 4b.

GA ESR spectrum (Fig. 6) were more similar to those of *Rb. sphaeroides* G1C (Fig. 4b) than to reaction centers reconstituted with hydroxyneurosporene or methoxyneurosporene (Fig. 5).

Thin layer chromatography of the carotenoids extracted from reaction centers of *Rb. sphaeroides* GA having a carotenoid-to-primary donor ratio of 1 : 1, revealed $70 \pm 5\%$ neurosporene, $20 \pm 5\%$ methoxyneurosporene and $10 \pm 5\%$ hydroxyneurosporene. This is close to the findings of Cogdell and Crofts [38] who reported that chromatophores of *Rb. sphaeroides* GA contain approx. 60% neurosporene. The fact that reaction centers of *Rb. sphaeroides* GA contain primarily neurosporene accounts for the similarity of its triplet state ESR spectrum with those of *Rb. sphaeroides* G1C and R26.1 reaction centers reconstituted with neurosporene (Fig. 4).

Discussion

Virtually all carotenoids containing polar functional groups (xanthophylls) bound to the reaction center in a carotenoid-to-primary donor ratio of approx. 1 : 1 and gave rise to strong reaction center carotenoid triplet state ESR signals. Previous workers have also shown that the reaction center complex exhibits a preference for binding xanthophylls over carotenes [6,16,30]. The polar functional groups must be aiding in the binding of these molecules in a specific site on the reaction center protein where the carotenoids can function efficiently in quenching the triplet state energy of the primary donor. Rhodopin, zeaxanthin and apo-β-carotenol which contain polar functional groups were unable to be incorporated, however. For zeaxanthin this is likely to be because the carotenoid binding site is sterically hindered. The presence of the polar hydroxy functional groups on the cyclohexene rings of zeaxanthin is insufficient to overcome the steric hindrance caused by the rings. β-Carotene also showed no trace of incorporation into reaction centers, probably because of the compound effects of steric inhibition to binding and the lack of a polar functional group. Rhodopin and apo-β-carotenol were observed to have a much greater solubility in the aqueous detergent phase than the other carotenoids. Other workers reported that reconstitution

of spheroidene, spheroidenone, spirilloxanthin and β-carotene were unsuccessful when LDAO was used as the detergent indicating that detergent type may influence the efficiency of reconstitution [16]. Variation of detergent and carotenoid concentration did not lead to successful reconstitution of rhodopin, apo-β-carotenol, β-carotene or zeaxanthin.

The triplet state zero-field splitting parameters, $|D|$ and $|E|$, which were obtained by a computer simulation of the ESR spectral lineshape, assess the dipolar interaction as a spatial average over the electronic part of the triplet state wave function [51,52]. For $\pi\pi^*$ triplet states $|D|$ is a measure of the extent of π electron delocalization [52]. As the extent of π electron delocalization increases the magitude of $|D|$ decreases. The $|E|$ parameter measures the deviation of the triplet state spin system away from axial symmetry and also assesses the extent of π electron delocalization. The triplet state zero-field splitting parameters of several of the reconstituted carotenoids follow the trend of having the $|D|$ value decrease as the extent of π electron delocalization increases. For example, the value of $|D|$ decreases in the order spheroidene > spheroidenone > spirilloxanthin (see Table II). Although less compelling than the trends in $|D|$, the magnitude of $|E|$ for these xanthophylls appear to follow the same trend. This suggests that these reaction center-bound carotenoids have the same symmetry and that the observed variation in the zero-field splitting parameters is a result of a change in the extent of π electron delocalization. There is precedent for this behavior in that similar trends in zero-field splitting parameters have been observed for the linear polyacenes, naphthalene, anthracene and tetracene. These molecules have the same symmetry (D_{2h} point group), but differ in their extent of π electron conjugation. The magnitudes of both $|D|$ and $|E|$ were found to decrease as the extent of π electron delocalization increases [52,53]. The zero-field splitting data on spheroidene, spheroidenone and spirilloxanthin suggest that they adopt the same configuration and/or conformation when bound to reaction centers of *Rb. sphaeroides* R26.1.

As stated in the Results section the triplet state ESR spectra of reaction centers reconstituted with hydroxyneurosporene and methoxyneurosporene are identical to the spectra of reaction centers reconstituted with spheroidene. Hydroxyneurosporene and methoxyneurosporene have one less carbon–carbon double bond than spheroidene (Table I). In fact, the singlet and triplet absorption spectra of reaction center-bound methoxyneurosporene and spheroidene were also found to be identical despite the fact that the absorption spectra in pentane solution were different (Frank, H.A., Chadwick, B.W., Taremi, S., Kolaczkowski, S. and Bowman, M.K., unpublished data). A simple point dipole calculation suggests that a reduction in the extent of π electron conjugation of a linear polyene from 10 (spheroidene) to 9 (neurosporene chromophore) carbon–carbon double bonds would lead to an increase in the magnitude of the zero-field splitting parameter $|D|$ by a factor of approx. 1.8 (Chadwick, B.W. and Frank, H.A., unpublished results). Similarly, 3,4-dihydroanhydrorhodovibrin and spirilloxanthin exhibit identical triplet state zero-field splitting parameters despite the fact that they differ in their extent of π electron delocalization by two carbon–carbon double bonds. The most likely interpretation for the similarity in the triplet state ESR zero-field splitting parameters between reaction center-bound methoxyneurosporene and spheroidene and between 3,4-dihydroanhydrorhodovibrin and spirilloxanthin is that these carotenoids isomerize upon binding to the reaction center. Resonance Raman studies by Lutz et al. [15] have confirmed that carotenoids rapidly isomerize from a strained cis isomer to the all-*trans* form upon extraction from reaction centers. Koyama et al. [19] concluded, based on a comparison of the resonance Raman spectra of the carotenoid in reaction centers of *Rb. sphaeroides* G1C (neurosporene) and wild type (spheroidene) with the spectra of 14 different configurational isomers of β-carotene, that the reaction center-bound carotenoid assumes a 15,15′-*cis* configuration.

For one to observe the same triplet state ESR spectra for carotenoids of different π electron structure, the carotenoids must be twisted in such a way that the extent of π electron conjugation in the polyene chain is inhibited. One example of this kind of isomerization might be a structure where out-of-plane twists of the polyene chain

occur at the 6,7 and 6′,7′ carbon positions of the carotenoids (see Fig. 7). In this example the π electron conjugation would be interrupted at these positions and hydroxyneurosporene, methoxyneurosporene, spheroidene and hydroxyspheroidene would give rise to spectra consistent with having eight conjugated carbon–carbon double bonds. Spirilloxanthin and 3,4-dihydroanhydrorhodovibrin would give rise to spectra consistent with having nine conjugated carbon–carbon double bonds. Spheroidenone would appear to contain slightly greater than eight conjugated carbon–carbon double bonds owing to the additional effect of the carbonyl group in that molecule. These effective lengths of π electron conjugation are consistent with the observed trends in the triplet state zero-field splitting parameters; i.e., $|D|$ and $|E|$ are greater for hydroxyneurosporene, methoxyneurosporene, spheroidene and hydroxyspheroidene than for 3,4-dihydroanhydrorhodovibrin and spirilloxanthin. Also, twists at the 6,7 and 6′,7′ carbon positions are reminiscent of the position of ring closure and twisting in the β-carotene molecule. Although this is just an example, structures in which the twists in the π electron system are close to the center of the polyene chain can be discounted by the fact that such twists would form short segments of π electron conjugation in the carotenoids and give rise to ESR spectra characterized by much larger zero-field splitting parameters than are observed here. Furthermore, if the interruption of the π electron conjugation occurred toward the middle of the polyene chain, the optical triplet–triplet absorption spectra would be significantly blue-shifted from their observed values of approx. 545 nm (Frank et al., unpublished results).

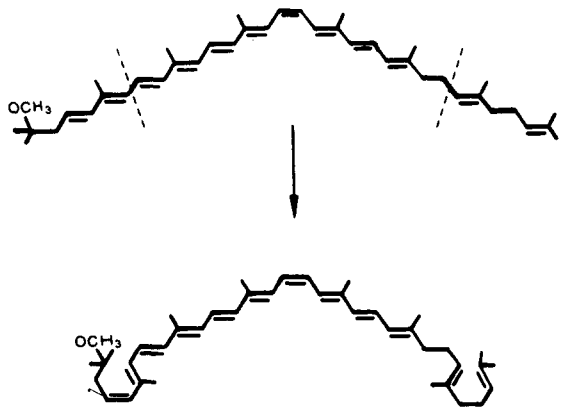


Fig. 7. The isomerization of 15,15′-*cis*-spheroidene which may occur upon its binding to the reaction center protein. The dotted lines across the polyene chain represent the positions at which this and more extensively conjugated molecules could be twisted and their π electron conjugation interrupted. This example is consistent with the ESR spectral features of the carotenoids studied here. The 15,15′-*cis*-isomer as the initial configuration is based on previous optical and resonance Raman studies [6,19].

Neurosporene in reaction centers of *Rb. sphaeroides* R26.1, G1C or GA displays different ESR properties than the xanthophyll-containing systems. In all cases where neurosporene is present in the reaction center it gives rise to very weak ESR spectra and displays very large zero-field splitting parameters (see Table II). The latter result suggests less effective π electron delocalization for neurosporene than for the xanthophylls bound to the reaction center. Invoking isomerization behavior different from the xanthophylls suggests, however, that the photophysical properties of the carotenoid are affected by the nature of the binding to the protein. Studies of the triplet states of carotenoids in different proteins would be valuable in examining whether environmental features play a role in determining the structural properties of these molecules.

The present analysis demonstrates that polar functional groups on the carotenoid are necessary to bind the molecule in a position or geometry conducive to efficient triplet energy transfer. This is the most likely interpretation of these data because large triplet state ESR signals are observed from reaction centers reconstituted with carotenoids which have polar functional groups, but not from carotenes which have the same number of carbon–carbon double bonds, but lack polar functional groups. The effectiveness of carotenoids in quenching the primary donor triplet state is not only a function of its number of conjugated carbon–carbon double bonds, but also depends on its ability to obtain the proper geometry and orientation relative to the primary electron donor.

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References

1 Sauer, K. (1978) Acc. Chem. Res. 11, 257–264
2 Cogdell, R.J., Hipkins, M.F., MacDonald, W. and Truscott, T.G. (1981) Biochim. Biophys. Acta 634, 191–202
3 Krinsky, N.I. (1971) in Carotenoids (Isler, O., Gutmann, H. and Solms, U., eds.), pp. 48–51, Birkhauser, Basel
4 Foote, C.S., Chang, Y.C. and Denny, R.W. (1970) J. Am. Chem. Soc. 92, 5216–5217
5 Renger, G. and Wolff, C. (1977) Biochim. Biophys. Acta 460, 47–57
6 Boucher, F., Van der Rest, M. and Gingras, G. (1977) Biochim. Biophys. Acta 461, 339–357
7 Rademaker, H., Hoff, A.J., Van Grondelle, R. and Duysens, L.N.M. (1980) Biochim. Biophys. Acta 592, 240–257
8 Chessin, M., Livingston, R. and Truscott, T.G. (1966) Trans. Farad. Soc. 62, 1519–1524
9 Truscott, T.G., Land, E.J. and Sykes, A. (1973) Photochem. Photobiol. 17, 43–51
10 Bensasson, R., Land, E.J. and Maudinas, B. (1976) Photochem. Photobiol. 23, 189–193
11 Jensen, N.H., Wilbrandt, R., Pagsberg, P.B., Sillesen, A.H. and Hansen, K.B. (1980) J. Am. Chem. Soc. 102, 7441–7444
12 Jensen, N.H., Nielsen, A.B. and Wilbrandt, R. (1982) J. Am. Chem. Soc. 104, 6117–6119
13 Dallinger, R.F., Guanci, J.J., Woodruff, W.H. and Rodgers, M.A.J. (1979) J. Am. Chem. Soc. 101, 1355–1357
14 Dallinger, R.F., Farquharson, S., Woodruff, W.H. and Rodgers, M.A.J. (1981) J. Am. Chem. Soc. 103, 7433–7440
15 Lutz, M., Agalidis, I., Hervo, G., Cogdell, R.J. and Reiss-Husson, F. (1978) Biochim. Biophys. Acta 503, 287–303
16 Agalidis, I., Lutz, M. and Reiss-Husson, F. (1980) Biochim. Biophys. Acta 589, 264–274
17 Lutz, M., Chinsky, L. and Turpin, P.Y. (1982) Photochem. Photobiol. 36, 503–515
18 Koyama, Y., Kito, M., Takii, T., Saiki, K., Tsukida, K. and Yamashita, J. (1982) Biochim. Biophys. Acta 680, 109–118
19 Koyama, Y., Takii, T., Saiki, K. and Tsukida, K. (1983) Photobiochem. Photobiophys. 5, 139–150
20 Kito, M., Yamashita, J. and Koyama, Y. (1983) Photobiochem. Photobiophys. 5, 209–217
21 Lutz, M. and Kleo, J. (1976) Biochim. Biophys. Acta 69, 711–717
22 Agalidis, I., Lutz, M. and Reiss-Husson, F. (1984) Biochim. Biophys. Acta 766, 188–197
23 Boucher, F. and Gingras, G. (1984) Photochem. Photobiol. 40, 277–281
24 Frank, H.A., Bolt, J.D., De B. Costa, S.M. and Sauer, K. (1980) J. Am. Chem. Soc. 102, 4893–4898
25 Frank, H.A., Machnicki, J. and Felber, M. (1982) Photochem. Photobiol. 35, 713–718
26 Frank, H.A., Machnicki, J. and Friesner, R. (1983) Photochem. Photobiol. 38, 451–455
27 Frank, H.A., Machnicki, J. and Toppo, P. (1984) Photochem. Photobiol. 39, 429–432
28 McGann, W.J. and Frank, H.A. (1985) Biochim. Biophys. Acta 807, 101–109
29 McGann, W.J. and Frank, H.A. (1985) Chem. Phys. Lett. 121, 253–261
30 Cogdell, R.J., Parson, W.W. and Kerr, M.A. (1976) Biochim. Biophys. Acta 430, 83–93
31 Davidson, E. and Cogdell, R.J. (1981) FEBS Lett. 132, 81–84
32 Theiler, R., Suter, F., Zuber, H. and Cogdell, R.J. (1984) FEBS Lett. 175, 231–237
33 Bolt, J.D. (1980) Ph.D. Thesis, University of California, Berkely, CA
34 Clayton, R.K. and Wang, R.T. (1971) Methods Enzymol. 23, 696–704
35 Goodwin, T.W., Land, D.G. and Osman, H.G. (1955) Biochem. J. 59, 491–496
36 Schneour, E.A. (1962) Biochim. Biophys. Acta 62, 534–540
37 Jackman, L.M. and Liaaen-Jensen, S. (1964) Acta Chem. Scand. 18, 1403–1411
38 Cogdell, R.J. and Crofts, A.R. (1976) Biochim. Biophys. Acta 502, 409–416
39 Thornber, J.P. (1971) Methods Enzymol. 23, 688–691
40 Liaaen-Jensen, S., Cohen-Bazire, G., Nakayama, T.O.M. and Stanier, R.Y. (1958) Biochim. Biophys. Acta 29, 477–498
41 Sistrom, W.R. and Clayton, R.K. (1964) Biochim. Biophys. Acta 88, 61–63
42 Davies, B.H. (1970) Biochem. J. 116, 101–110
43 Schmidt, K. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds.), pp. 729–750, Plenum Press, New York, NY
44 Scolnik, P.A., Zannoni, D. and Marrs, B.L. (1980) Biochim. Biophys. Acta 593, 230–240
45 Frank, H.A., Friesner, R., Nairn, J.A., Dismukes, G.C. and Sauer, K. (1979) Biochim. Biophys. Acta 547, 484–501
46 Weedon, B.C.L. (1971) in Carotenoids (Isler, O., Gutmann, H. and Solms, U., eds.), pp. 48–51, Birkhauser, Basel
47 Dutton, P.L., Leigh, J.S. and Seibert, M. (1972) Biochim. Biophys. Res. Commun. 46, 406–413
48 Levanon, H. and Norris, J.R. (1978) Chem. Rev. 78, 185–198
49 Van der Rest, M. and Gingras, G. (1974) J. Biol. Chem. 249, 6446–6453
50 Scolnik, P.A., Walker, M.A. and Marrs, B.L. (1980) J. Biol. Chem. 255, 2427–2432
51 Carrington, A. and McLachlan, A.D. (1967) Introduction To Magnetic Resonance, pp. 116–118, Harper and Row, New York, NY
52 McGlynn, S.P., Azumi, T. and Kinoshita, M. (1969) Molecular Spectroscopy Of The Triplet State, pp. 329–366, Prentice-Hall, Englewood Cliffs, NJ
53 Yarmus, L., Rosenthal, J. and Chopp, M. (1972) Chem. Phys. Lett. 16, 477–481