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Carotenoid triplet state formation in *Rhodobacter sphaeroides* R-26 reaction centers exchanged with modified bacteriochlorophyll pigments and reconstituted with spheroidene

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Abstract

Triplet state electron paramagnetic resonance (EPR) experiments have been carried out at X-band on *Rb. sphaeroides* R-26 reaction centers that have been reconstituted with the carotenoid, spheroidene, and exchanged with 13^2-OH-Zn-bacteriochlorophyll *a* and [3-vinyl]-13^2-OH-bacteriochlorophyll *a* at the monomeric, 'accessory' bacteriochlorophyll sites $B_{A,B}$ or with pheophytin *a* at the bacteriopheophytin sites $H_{A,B}$. The primary donor and carotenoid triplet state EPR signals in the temperature range 95 – 150 K are compared and contrasted with those from native *Rb. sphaeroides* wild type and *Rb. sphaeroides* R-26 reaction centers reconstituted with spheroidene. The temperature dependencies of the EPR signals are strikingly different for the various samples. The data prove that triplet energy transfer from the primary donor to the carotenoid is mediated by the monomeric, $BChl_B$ molecule. Furthermore, the data show that triplet energy transfer from the primary donor to the carotenoid is an activated process, the efficiency of which correlates with the estimated triplet state energies of the modified pigments.

Abbreviations: BChl – bacteriochlorophyll; BPhe – bacteriopheophytin; Chl – chlorophyll; EPR – electron paramagnetic resonance; LDAO – lauryl-dimethylamine-*N*-oxide; Phe – pheophytin

Introduction

The elucidation by X-ray crystallography of the structures of the photosynthetic bacterial reaction centers from *Rps. viridis* and *Rb. sphaeroides* has revealed two monomeric, 'accessory', bacteriochlorophyll molecules lying between the dimeric primary donor and the two bacterioheophytins (Deisenhofer et al. 1985, Chang et al. 1986, Michel et al. 1986, Allen et al. 1988, Deisenhofer and Michel 1988, Yeates et al. 1988, Arnoux et al. 1989, El-Kabbani et al. 1991). The accessory bacteriochlorophylls (denoted $BChl_A$ and $BChl_B$) and the bacteriopheophytins (denoted $BPhe_A$ and $BPhe_B$) are non-covalently bound to two protein subunits (L and M). These molecules and the protein subunits are related geometrically by an approximate C_2 symmetry transformation. Recent work has shown that only the pigments on the so-called A-side of the reaction center participate in the photoinduced electron transfer from the primary donor to the BPhe at site H_A (Kirmaier et al. 1985, Zinth et al. 1985). The precise role of the bridging $BChl_A$ in the primary electron transfer photochemistry is the subject of some controversy and intense scrutiny (Finkele 1992).

Also uncertain in the *Rb. sphaeroides* wild type reaction center is the role of the $BChl_B$ at site B_B in promoting triplet energy transfer from the primary donor to a bound carotenoid (Frank 1993). This reaction is important in preventing the formation of excited singlet state ($^1\Delta_g$) oxygen via sensitization from the primary donor triplet state (Cogdell and Frank 1987). Singlet oxygen is a powerful oxidizing agent. The X-ray analyses of *Rb. sphaeroides* and *Rps. viridis* have revealed that the carotenoid is located on the B-side of the reaction center which is thought to be inactive in electron transfer (Deisenhofer et al. 1985, Michel et al. 1986, Allen et al. 1988, Deisenhofer and Michel 1988, Yeates et al. 1988, Arnoux et al. 1989). The carotenoid resides ~4 Å from $BChl_B$ and ~10.5 Å away from the primary donor. A role for $BChl_B$ in triplet energy transfer is suggested but not proven by the X-ray structure which locate the $BChl_B$ molecule on a direct path between the primary donor and the carotenoid within ~4 Å of both cofactors.

The locations of the carotenoids in *Rb. sphaeroides* and *Rps. viridis* reaction centers are very similar, but their triplet energy transfer properties are profoundly different. The carotenoid, 1,2-dihydroneurosporene, in *Rps. viridis* reaction centers does not enter its triplet state upon photoexcitation of the reaction center at any temperature (Holten et al. 1978, Frank et al. 1980). Spheroidene, in reaction centers of *Rb. sphaeroides* wild type strain 2.4.1, however, quenches the primary donor triplet state with very high quantum yield at temperatures above 35 K (Parson and Monger 1976). Because transfer of the triplet energy from the primary donor to the carotenoid is important in protecting the photosynthetic apparatus, two questions should be asked: (1) Why is spheroidene an efficient triplet quencher in the BChl *a*-containing *Rb. sphaeroides* reaction center, whereas 1,2-dihydroneurosporene in the BChl *b*-containing *Rps. viridis* complex is not; (2) What is the role of the $BChl_B$ molecule in the transfer of triplet energy from the primary donor to the carotenoid in *Rb. sphaeroides*?

Several researchers have attempted to explain how the triplet energy is transferred from the primary donor to the carotenoid and which energy states are involved in the process (Parson and Monger 1976, Frank et al. 1983, Schenck et al. 1984, Lous 1988, Takiff and Boxer 1988a,b, Kolaczkowski 1989). Recent spectroscopic studies of reaction centers from the carotenoidless mutant *Rb. sphaeroides* R-26 reconstituted with spheroidene and treated with sodium borohydride provided direct evidence for the involvement of the $BChl_B$ molecule in triplet energy transfer (Frank and Violette 1989). However, it was previously thought that sodium borohydride completely removed the $BChl_B$ molecule from the reaction center (Ditson et al. 1984, Maróti et al. 1985). Subsequent quantitative pigment analysis by high performance liquid chromatography (HPLC) indicated that the $BChl_B$ molecule is not actually removed by the borohydride treatment, but may be dislocated from the position it occupied in the native, untreated complex (Struck et al. 1991). Uncertainties in the structure of the borohydride-treated reaction center have prompted the search for more specific ways to probe the nature of the involvement of the $BChl_B$ molecule in the triplet energy transfer reaction.

A specific manner in which the role of $BChl_B$ in the transfer of triplet energy from the primary donor to the carotenoid can be probed is derived from the fact that incubation of photosynthetic reaction centers from the carotenoidless mutant, *Rb. sphaeroides* R-26, in the presence of modified bacteriochlorophyll pigments results in the exchange of the modified pigment for the endogenous $BChl_A$ and $BChl_B$ molecules (Struck and Scheer 1990, Struck et al. 1990a,b). The reaction centers that have been exchanged with different modified bacteriochlorophylls may then be reconstituted with carotenoids and the triplet energy transfer reaction probed (Frank and Violette 1989). In this work, we present high-field, X-band, triplet state electron paramagnetic resonance (EPR) experiments on reaction centers from *Rb. sphaeroides* R-26 that have been exchanged with modified bacteriochlorophylls and reconstituted with spheroidene. The temperature dependence of the carotenoid triplet state signals indicate that the triplet energy transfer reaction is an activated process, the efficiency of which is correlated with the triplet state energies of the modified pigments.

Materials and methods

Reaction center preparations

For the *Rb. sphaeroides* R-26 control experiments, the cells were grown anaerobically in modified

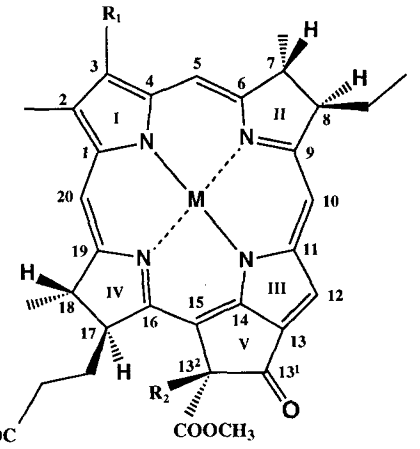
Hutners media. Chromatophores were obtained by French pressure disruption at 20 000 psi of whole cells followed by ultracentrifugation at 250 000 × g for 90 min. The chromatophore membranes were incubated in 15 mM Tris buffer (pH 8.0), 150 mM NaCl, 1 mM EDTA and 0.6% LDAO (Fluka) at room temperature in the dark for 30 min. The mixture was then centrifuged at 250 000 × g for 90 min at 4 °C. The supernatant, enriched in reaction centers was diluted with 15 mM Tris buffer (pH 8.0) and 1 mM EDTA to an absorbance of 50 at 865 nm. LDAO was added to a final concentration of 1% (v/v). Solid ammonium sulfate (Sigma) was added in the amount 0.3 g/ml resulting in a 30% (w/v) ammonium sulfate solution. The pH of the solution was maintained at 7.5–7.8 during ammonium sulfate addition. The mixture was centrifuged at 12 000 × g for 10 min at 4 °C. The reaction center levitate was then resuspended in Tris buffer solution, and several high and low ammonium sulfate concentration precipitations were performed to isolate the reaction centers.

The reaction centers were then suspended in 15 mM Tris, 1 mM EDTA buffer, pH 8.0 containing 0.06% LDAO and loaded onto a 3 × 20 cm DEAE Sephacel (anion exchange, Sigma #I-6505) column which was previously equilibrated with 1 L of 15 mM Tris buffer, pH 8.0, containing 0.03% LDAO. The protein fractions were eluted from the column by a step gradient elution using 15 mM Tris buffer, pH 8.0, containing 0.06% LDAO and 0.04 – 0.20 M NaCl in 0.02 M NaCl concentration steps. The reaction center protein fractions were obtained at 0.18 M NaCl concentration. Fractions having an absorbance ratio A_{280}/A_{800} = 1.4 – 1.6 were combined and diluted 1:5 with 15 mM Tris buffer, pH 8.0, containing 0.06% LDAO and loaded onto a 1 × 8 cm DEAE Sephacel column. The reaction centers were washed with 15 mM Tris, pH 8.0, containing 0.06% LDAO and 0.06 M NaCl until the remaining free protein was removed. The purified reaction centers (A_{280}/A_{800} = 1.3) were obtained by elution with 15 mM Tris buffer, pH 8.0, containing 0.06% LDAO and 0.40 M NaCl. The purified reaction centers were diluted 1:5 with Tris buffer, loaded onto a third 1 × 8 cm DEAE Sephacel column and washed with 15 mM Tris, pH 8.0, containing 0.1% Triton X-100 (Sigma) in order to exchange the detergent. The purified reaction centers in Triton X-100 were eluted in 15 mM Tris buffer, pH 8.0, in 0.1% Triton X-100, containing 0.40 M NaCl. The reaction centers were dialyzed overnight in Spectrapor standard cellulose dialysis tubing (25 mm, m.w. cutoff 12 000 – 14 000) against 15 mM Tris buffer, pH 8.0, containing 0.1% Triton X-100 and then concentrated against a slurry of Aquacide I (Calbiochem).

BChl *a* and Chl *a* were extracted from *Rb. sphaeroides* and *Spirulina geitleri*, respectively. Phe *a* and [3-vinyl]-13[2]-OH-BChl *a* were prepared as before (Struck et al. 1992). 13[2]-OH-Zn-BChl *a* was obtained by metalation of the respective bacteriopheophytin with zinc acetate in acetic acid (Fiedor et al. 1993, unpublished results), or by transmetalation of the cadmium complex (Hartwich et al. 1993).

Reaction centers of *Rb. sphaeroides* R-26 for the pigment exchange experiments were prepared according to standard procedures (Struck and Scheer 1990, Scheer and Struck 1993). Reaction centers containing the 13[2]-OH-Zn-BChl *a*, [3-vinyl]-13[2]-OH-BChl *a* and Phe *a* pigments (Table 1) were prepared according to the methods previously published (Struck and Scheer 1990, Struck et al. 1990a). The extent of pigment exchange was nearly 100% in all cases as evaluated by HPLC except for Phe *a* where it was ~90%.

Table 1. Pigments used in the present experiments



Pigment	R_1	R_2	M
BChl *a*	$COCH_3$	H	2H
Phe *a*[a]	$CHCH_2$	H	2H
13[2]-OH-Zn-BChl *a*	$COCH_3$	OH	Zn
[3-vinyl]-13[2]-OH-BChl *a*	$CHCH_2$	OH	Mg

[a] Phe *a* has a double bond at C_7–C_8.

Spheroidene extraction, purification and incorporation into the reaction centers

Spheroidene was obtained from anaerobically grown *Rb. sphaeroides* wild type strain 2.4.1 cells by acetone extraction and pentane partitioning. The spheroidene was purified by alumina column chromatography using 0.25, 0.5 and 1% ethyl acetate in petroleum ether. The purified spheroidene was stored in 1% ethyl acetate in petroleum ether at 4 °C. For the reconstitution a 15-fold molar excess (relative to the reaction center concentration) of spheroidene was put into a small (8 ml) vial and the solvent evaporated with a stream of N_2 gas to deposit the carotenoid as a thin film on the sides of the vial. ~1.5 ml of 15 mM Tris buffer (pH 8.0) containing 1.0% Triton X-100 was added to the vial and vortexed for approximately 2 min. 0.5 ml of the reaction centers (A_{800} = 4.0 in a 1 cm path) was added to the carotenoid solution. The vials were then sonicated at 4 °C in the dark for 30 min. Following this, an additional 15-fold molar excess of spheroidene in petroleum ether was added. The petroleum ether that settled on the top of the solution was evaporated using a stream of N_2 gas and the mixture was sonicated for one hour.

Preparation of samples for the spectroscopic experiments

For most of the EPR experiments, the spectra were taken prior to removing the excess carotenoid. Because carotenoids do not form triplet states unless they are bound in the reaction center and involved in energy transfer with the primary donor, the presence of excess carotenoids in the sample poses no particular problem to the EPR experiments. Also, a control experiment using native *Rb. sphaeroides* R-26 reaction centers with spheroidene reconstituted revealed no difference in the EPR spectra before or after removal of the excess carotenoid. EPR samples were prepared by degassing the solutions with N_2 for 5 min, followed by the addition of sodium dithionite (10 mM final concentration) and ethylene glycol to a final concentration of 10% (v/v). The samples were quickly pipetted into quartz EPR tubes, capped and frozen in liquid nitrogen. The triplet state EPR spectra were obtained with a Varian X-band spectrometer equipped with a variable temperature, liquid nitrogen flow cryostat as described previously (Chadwick and Frank 1986). The EPR spectra at each temperature were obtained by averaging 3 or 4 scans between 2700 – 3700 G using a sweep time of 30 min and a time constant of 10 s. Temperature fluctuations during the scans were within ± 2 degrees.

After the EPR experiments, the samples were recovered, and the excess carotenoid removed from the solutions by DEAE Sephacel column chromatography as described above for the reaction center purification. The extent of carotenoid incorporation was measured by absorption spectroscopy using a Milton-Roy (SLM) single-beam diode array spectrometer.

Results

Figure 1 shows the absorption spectra of reaction centers from *Rb. sphaeroides* (a) wild type strain 2.4.1; (b) R-26 reconstituted with spheroidene; (c) R-26 exchanged with Phe *a* and reconstituted with spheroidene; (d) R-26 exchanged with 13^2-OH-Zn-BChl *a* and reconstituted with spheroidene; and (e) R-26 exchanged with [3-vinyl]-13^2-OH-BChl *a* and reconstituted with spheroidene. The reaction center sample incubated with Phe *a* substitutes two Phe *a* molecules for the BPhe pigments at sites H_A and H_B. Incubation of the reaction center samples with either 13^2-OH-Zn-BChl *a* or [3-vinyl]-13^2-OH-BChl *a* results in an exchange of those molecules for the native, accessory $BChl_A$ and $BChl_B$ bacteriochlorophylls. The most dramatic alterations in the absorption spectra are found in the samples from *Rb. sphaeroides* R-26 that have been reconstituted with spheroidene and exchanged with either Phe *a* or [3-vinyl]-13^2-OH-BChl *a*. In these samples profound differences in the Q_x and Q_y regions are apparent and indicate changes in the singlet state transition energies of those pigments.

The absorption spectra in the carotenoid region (450 – 500 nm) allow a calculation of the extent of spheroidene incorporation in each sample. This was done as follows: The extent of spheroidene incorporation in every sample was calculated by dividing the value of the maximum absorption of spheroidene at 475 nm by the value of the primary donor Q_y absorption at 865 nm in the near-IR region of the reaction center spectrum. These values were then compared with the same calculation for the *Rb.*

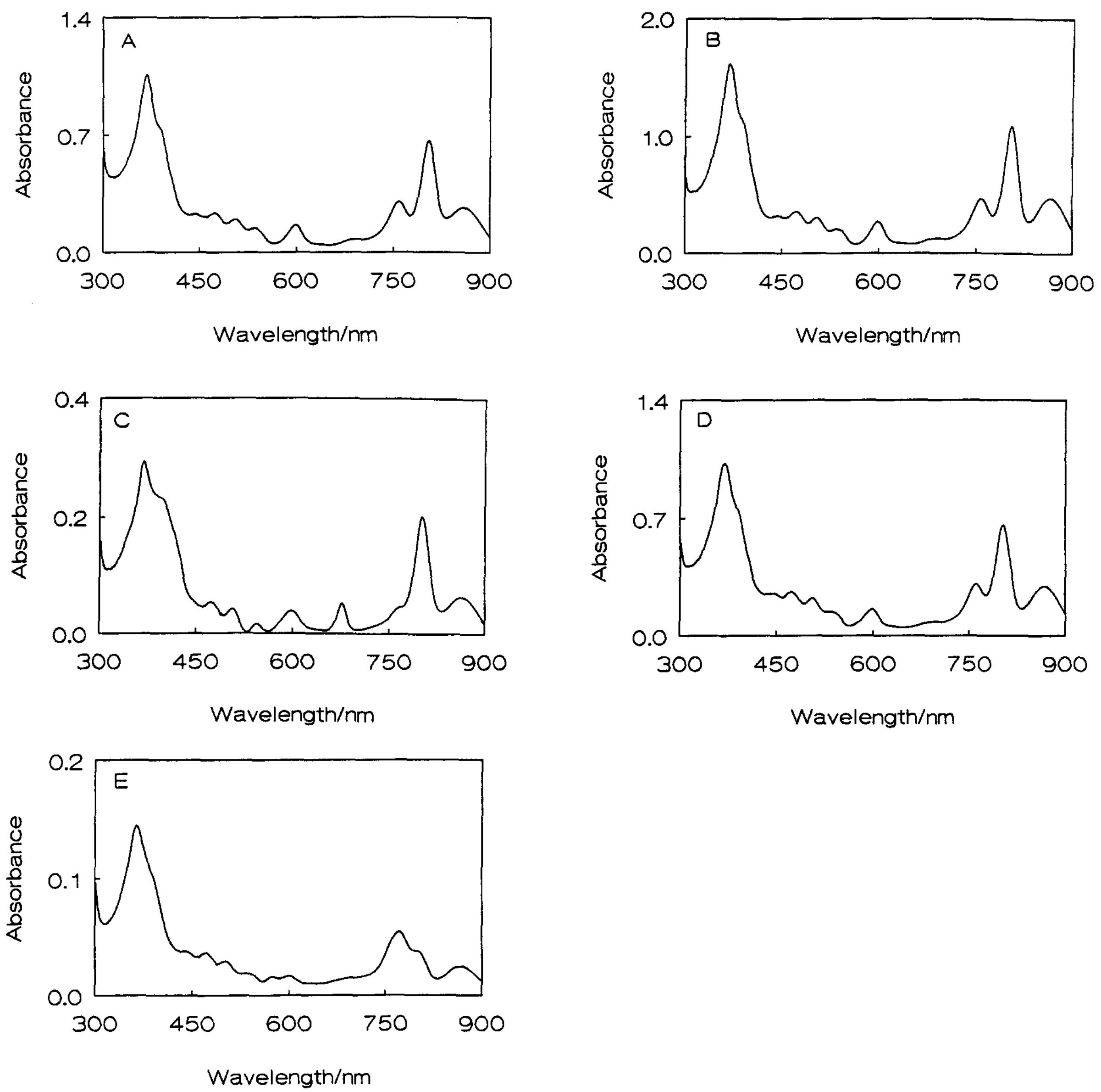


Fig. 1. The absorption spectra of reaction centers from *Rb. sphaeroides* (a) wild type strain 2.4.1; (b) R-26 reconstituted with spheroidene; (c) R-26 exchanged with Phe *a* and reconstituted with spheroidene; (d) R-26 exchanged with 13^2-OH-Zn-BChl *a* and reconstituted with spheroidene; and (e) R-26 exchanged with [3-vinyl]-13^2-OH-BChl *a* and reconstituted with spheroidene.

sphaeroides wild type reaction center sample in which the primary donor is known to exist in a 1:1 stoichiometric ratio with the bound carotenoid; i. e. 100% carotenoid incorporation is assumed present in the *Rb. sphaeroides* wild type sample. Table 2 summarizes the extents of spheroidene incorporation in each of the samples.

The triplet state EPR signals from *Rb. sphaeroides* wild type in the 105 – 150 K range are indicative of the formation of carotenoid triplet states (Fig. 2). The signals are characterized by the spin Hamiltonian parameters $|D| = 0.0286\ cm^{-1}$ and $|E| = 0.0044\ cm^{-1}$ which are consistent with an assignment of the signals to spheroidene (Chadwick and Frank 1986). The signals are also characterized by a non-Boltzmann population distribution in the spin

Table 2. Extent of spheroidene incorporation in the reaction center samples. The values are measured relative to the amount of spheroidene in *Rb. sphaeroides* wild type strain 2.4.1 which is assumed 100% carotenoid-reconstituted. The uncertainties are based on the error in measuring the absorption intensities of the spectral features

Sample of *Rb. sphaeroides* R-26 reaction centers	% Spheroidene incorporation
Native	91 ± 2
Exchanged with Phe *a*	76 ± 5
Exchanged with 13^2-OH-Zn-BChl *a*	100 ± 5
Exchanged with [3vinyl]-13^2-OH-BChl *a*	100 ± 5

sublevels of the triplet. These 'spin polarized' EPR signals are observed as either emission or enhanced absorption EPR lines. The particular pattern of the lines observed here, *eaa eea* where *e* denotes a signal in emission and *a* denotes a signal in enhanced absorption indicates that the triplet was formed via the radical pair mechanism, or has accepted triplet state energy from a donor born via that mechanism. This is consistent with the carotenoid quenching the triplet state formed on the primary donor after charge recombination from the BPhe at site H_A. In Fig. 2 there is little, if any, evidence for the presence of primary donor triplet state signals. These would appear as shoulders inside the major positive features of the carotenoid signals (Chadwick and Frank 1986). The primary donor triplet state signals are observed either as one lowers the temperature to below 50 K where the triplet transfer to the carotenoid is inhibited (Frank et al. 1980, 1983), or in samples where there is less than a 1:1 carotenoid-to-primary donor stoichiometric ratio (Chadwick and Frank 1986). An important concern in triplet state EPR experiments is that the signal amplitudes are not simply related to the concentration of the triplets. However, the changes in the EPR signal intensities with temperature do correlate with the changes observed in optical triplet-triplet absorption experiments (Frank et al. 1980). Because only trends are sought in the present work, the use of the EPR signal amplitudes as a measure of triplet concentration is justified. From the experiment represented by Fig. 2 one can conclude that the carotenoid has been ~100% efficient at quenching the primary donor triplet state. The temperature dependence of these carotenoid triplet state EPR signals in this temperature range consists solely of a uniform decreasing

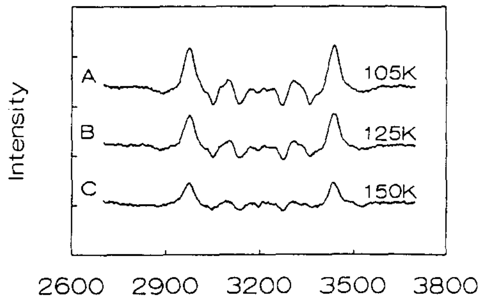


Fig. 2. The temperature dependence of the triplet state EPR signals from *Rb. sphaeroides* wild type reaction centers.

of the EPR intensities with increasing temperature. This is due to spin-lattice relaxation which sets in at these higher temperatures and tends to equilibrate the spin sublevel populations.

The triplet state EPR signals in the same temperature range from *Rb. sphaeroides* R-26 reaction centers that have been reconstituted with spheroidene show pronounced shoulders on the inside of the major features attributable to the carotenoid (Fig. 3). These shoulders are consistent with a triplet state having zero-field splitting parameters $|D| = 0.0187$ cm^{-1} and $|E| = 0.0032$ cm^{-1} and belonging to the primary donor (Chadwick and Frank 1986). Because this particular sample has only 91% carotenoid incorporation, some triplet state signals from the primary donor are observed in addition to the carotenoid signals. It is significant, however, that upon increasing the temperature from 105 to 148 K, both the carotenoid and primary donor signals are reduced uniformly and at the same rate owing to the

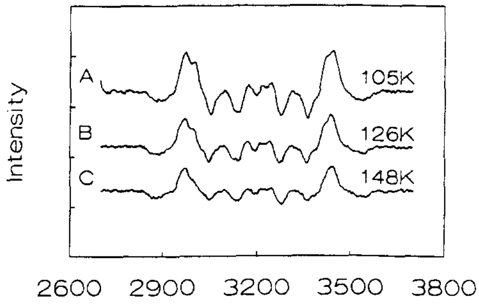


Fig. 3. The temperature dependence of the triplet state EPR signals from *Rb. sphaeroides* R-26 reaction centers that have been reconstituted with spheroidene.

onset of spin-lattice relaxation. There is no change in the relative signal intensities of the carotenoid compared to the primary donor upon increasing the temperature. This behavior indicates that these signals represent different populations of reaction centers, some which have carotenoids incorporated and some which do not. The temperature dependence of triplet energy transfer from the primary donor to the carotenoid in these samples reconstituted with spheroidene has been shown to be identical to that observed for the *Rb. sphaeroides* wild type sample and discussed above; i. e. primary donor signals are observed from reaction centers that have been reconstituted with spheroidene only at temperatures below 50 K (Frank et al., unpublished data).

The triplet state signals in the *Rb. sphaeroides* R-26 reaction centers that have been exchanged with Phe *a* are very weak (Fig. 4), despite the fact that this sample had a similar concentration to the reaction center sample exchanged with [3-vinyl]-13^2-OH-BChl *a* and discussed below which displays very strong signals. It could be that the triplet yield associated with the reaction center exchanged with Phe *a* may be smaller than the other samples, although, as stated above, the EPR experiments do not explicitly measure the triplet yield. This is the only sample studied here where the primary electron acceptor, $BPhe_A$, is modified. Similar to the *Rb. sphaeroides* R-26 reaction centers that have been reconstituted with spheroidene, the *Rb. sphaeroides* R-26 reaction centers having been exchanged with Phe *a* exhibit shoulders belonging to the primary donor on the inside of the major features belonging to the carotenoid triplet state. These shoulders arise because the carotenoid incorporation in this sample is 76%. Once again the temperature dependence in the 105 to 150 K range show that these signals are uniformly reduced upon raising the temperature. This is consistent with the observation that an exchange of Phe for BPhe has only little effect on the neighboring pigments in the B-pockets (Scheer et al. 1992). Indeed, one would not expect any significant change in the rate of energy transfer from the primary donor to the carotenoid upon exchange of Phe *a* for the native BPhe molecules at sites H_A and H_B.

In contrast to all previously discussed samples, the *Rb. sphaeroides* R-26 reaction centers exchanged with 13^2-OH-Zn-BChl *a*, at 98 K, exhibit very strong primary donor triplet state signals with features of the carotenoid triplet appearing as shoulders on the outside of the major primary donor peaks (Fig. 5). The very strong primary donor signals appear despite this sample having 100% carotenoid incorporation. Raising the temperature to 125 K results in an increase in the carotenoid triplet state features while the primary donor signals are starting to attenuate. At 150 K the features belonging to the primary donor have almost completely disappeared and the spectrum resembles that of *Rb. sphaeroides* wild type shown in Fig. 2. The temperature at which one sees equal amounts of the primary donor and carotenoid triplet state signals occurs at ~35 K in the *Rb. sphaeroides* wild type (Frank et al. 1983) or spheroidene-reconstituted *Rb. sphaeroides* R-26 samples (Frank et al., unpublished data), whereas in the *Rb. sphaeroides* R-26 reaction centers that have been exchanged with 13^2-OH-Zn-BChl *a* and recon-

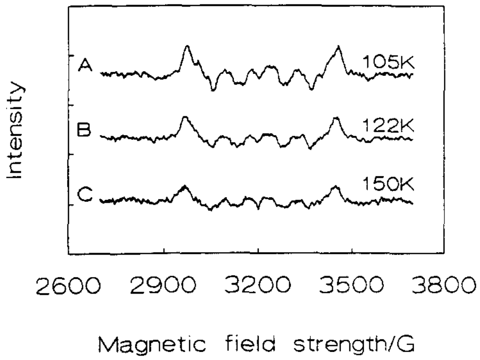


Fig. 4. The temperature dependence of the triplet state signals in *Rb. sphaeroides* R-26 reaction centers that have been exchanged with Phe *a* and reconstituted with spheroidene.

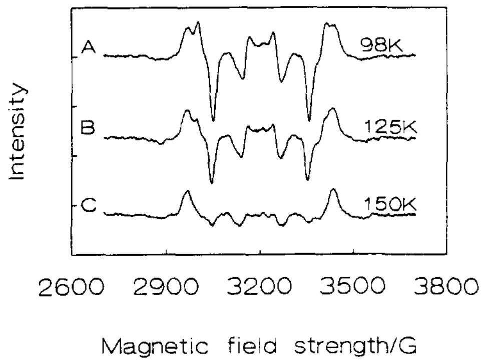


Fig. 5. The temperature dependence of the triplet state signals in *Rb. sphaeroides* R-26 reaction centers that have been exchanged with 13^2-OH-Zn-BChl *a* and reconstituted with spheroidene.

stituted with spheroidene, the temperature is ~100 K. These data indicate that the activation energy for the transfer of triplet energy from the primary donor to the carotenoid in this sample is larger than that seen in the native reaction center samples.

This activation energy is even larger in the *Rb. sphaeroides* R-26 reaction center sample that has been exchanged with [3-vinyl]-13^2-OH-BChl *a*. At 95 K the sample is completely dominated by primary donor triplet signals (Fig. 6). Very small carotenoid peaks appear on the outside of the major primary donor triplet features. This dominance of the primary donor triplet is observed despite this sample being 100% reconstituted with spheroidene. As the temperature is raised, the carotenoid signals begin to grow in at the expense of the primary donor triplet. At ~135 K equal amounts of the carotenoid and primary donor triplet state signals can be observed.

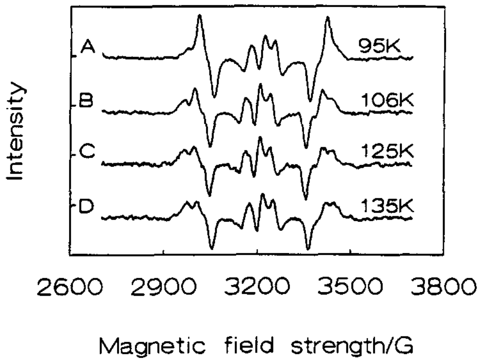


Fig. 6. The temperature dependence of the triplet state signals in *Rb. sphaeroides* R-26 reaction centers that have been exchanged with [3-vinyl]-13^2-OH-BChl *a* and reconstituted with spheroidene.

Discussion

These data provide direct experimental evidence that the monomeric $BChl_B$ is involved in triplet energy transfer from the primary donor to the carotenoid. Strictly speaking both the B_A and B_B sites are occupied by the modified pigments. However, the distance of B_A to the carotenoid is so large that the following discussion assumes that its influence on triplet energy transfer to the carotenoid is negligible. The experiments on the reaction centers exchanged with either 13^2-OH-Zn-BChl *a*- or [3-vinyl]-13^2-OH-BChl *a* clearly show that changing the nature of the $BChl_B$ molecule, which bridges the distance between the primary donor and the carotenoid, has a profound effect on the temperature dependence of triplet energy transfer. Principally, both structural and energetic reasons can be responsible for this. Although no crystal structure has been determined at present for reaction centers with modified pigments, several spectroscopic and dynamics experiments have been carried out that suggest that the structure changes only very little, if at all (Scheer and Struck 1993).

The triplet energies of the modified pigments described herein have not yet been measured by phosphorescence techniques. Indeed, it would be useful for someone to carry out these measurements. For the present analysis, it is possible to estimate the triplet state energies of the modified pigments in the following manner.

The lowest energy singlet states associated with the Q_y transitions of BChl *a*, 13^2-OH-Zn-BChl *a* and [3-vinyl]-13^2-OH-BChl *a* are estimated from their in vitro absorption and fluorescence spectra to be at 12 910 cm^{-1}, 13 060 cm^{-1} and 13 330 cm^{-1}, respectively (Fiedor et al. 1993, Struck et al. 1990a). It is known from absorption, fluorescence and phosphorescence experiments on Chlorophyll *a*, Chlorophyll *b*, Pheophytin *a* and Pheophytin *b* in various solvents that the singlet-triplet splittings of these pigments are relatively constant around 4500 ± 450 cm^{-1} (Krasnovskii et al. 1973, 1974). The singlet-triplet splitting of BChl *a* has been estimated from fluorescence and phosphorescence experiments to be 4610 cm^{-1} (Takiff and Boxer 1988b, Losev et al. 1990). An environment-induced red-shift corresponding to ~480 cm^{-1} for the Q_y transitions has been found for the native and modified pigments in the B_A and B_B sites (Struck et al. 1990a, Scheer and Struck 1993). This allows us to approximate the energies of the lowest excited triplet states of the 13^2-OH-Zn-BChl *a* and [3-vinyl]-13^2-OH-BChl *a* molecules bound in the reaction center. Using the red-shifted excited singlet state energies of 12 430 cm^{-1} for BChl *a*, 12 580 cm^{-1} for 13^2-OH-Zn-BChl *a* and 12 850 cm^{-1} for [3-vinyl]-13^2-OH-BChl *a* and singlet-triplet splittings of 4,610 cm^{-1} for BChl *a* (5-coordinate, Takiff and Boxer 1988b), 4600 cm^{-1} for 13^2-OH-Zn-BChl *a* (here the value for 5-coordinate Zn-BChl *a* from Takiff and Boxer (1988b) is used) and 4570 cm^{-1} (the median value between Chlorophyll *a* and BChl *a*, Krasnovskii et al. 1973, 1974, Takiff and Boxer 1988b) for

[3-vinyl]-13^2-OH-BChl *a*, the lowest excited triplet state energies of the BChl *a*, 13^2-OH-Zn-BChl *a* and [3-vinyl]-13^2-OH-BChl *a* molecules are found to be approximately 7820 cm^{-1}, 7980 cm^{-1} and 8280 cm^1, respectively (Fig. 7). These compare to the 8240 cm^{-1} triplet state energy of 5-coordinate BChl *a* measured by phosphorescence techniques (Takiff and Boxer 1988b).

With these triplet state energies it is possible to rationalize the differences in the temperature dependencies of triplet state energy transfer from the primary donor to the carotenoid in these various samples. It has been estimated that the uphill energy barrier is ~200 cm^{-1} for transfer of the triplet energy

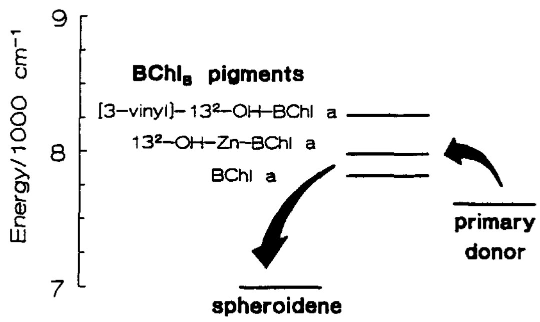


Fig. 7. The energy levels of the triplet states of the primary donor from *Rb. sphaeroides* R-26 (Takiff and Boxer 1988b), the modified $BChl_B$ pigments occupying the B_B site in reaction centers of *Rb. sphaeroides* R-26, and the carotenoid spheroidene. The arrows indicate the direction of triplet state energy transfer from the primary donor through an activation barrier determined by the triplet energies of the $BChl_B$ pigments and to the carotenoid, spheroidene. Please see the text for a discussion of how the triplet state energies of the modified $BChl_B$ pigments were determined. The triplet state energy of spheroidene at ~7000 cm^{-1} was derived from two sources: (1) From the extrapolation to carotenoids by Bensasson et al. (1976) of the data from Evans (1960, 1961) who used magnetic perturbation by oxygen at high pressures to determine the triplet state energies of several short polyene triplets; and (2) By the rule-of-thumb that the triplet state energy of polyenes is approximately one-half the energy of its lowest lying singlet state (Hudson et al. 1982). The lowest lying singlet state of spheroidene has been determined to be 14 100 cm^{-1} (Frank et al. 1993) placing the triplet state energy of spheroidene at ~7000 cm^{-1}.

from the primary donor to the carotenoid via the native $BChl_B$ (Takiff and Boxer 1988b). Comparing the triplet state energies of the modified pigments given above to that of BChl *a*, this energy barrier would climb to ~360 cm^{-1} for the *Rb. sphaeroides* reaction centers exchanged with 13^2-OH-Zn-BChl *a* and ~660 cm^{-1} for the reaction centers exchanged with [3-vinyl]-13^2-OH-BChl *a* (Fig. 7). These values are consistent with the trend of increasing temperatures at which equal amounts of primary donor and carotenoid triplet state signals are observed. A simple Boltzmann population analysis of the crossover temperatures of 35 K for the native *Rb. sphaeroides* wild type or spheroidene-reconstituted R-26 reaction centers, ~100 K for the reaction centers exchanged with 13^2-OH-Zn-BChl and reconstituted with spheroidene, and ~135 K for the reaction centers exchanged with [3-vinyl]-13^2-OH-BChl *a* and reconstituted with spheroidene, would predict activation barriers of 571 cm^{-1} and 771 cm^{-1}, respectively, for the reaction centers that have been exchanged with modified pigments. This is based on a 200 cm^{-1} activation energy for the native *Rb. sphaeroides* wild type or spheroidene-reconstituted, R-26 reaction centers. The differences between these activation energies and those determined from the spectroscopic data stem from inaccuracies in carrying out a Boltzmann population analysis without concrete dynamics data and from inherent difficulties in knowing the singlet and triplet transition energies for the pigments bound in the reaction center protein. Nevertheless, the present data prove that triplet state energy transfer from the primary donor to the carotenoid is an activated process and attest to the direct involvement of $BChl_B$ in the mechanism.

These data are also consistent with the hypothesis by Takiff and Boxer (1988b) that a large energy barrier (~1000 cm^{-1}) between the $BChl_B$ and the primary donor in the BChl *b*-containing *Rps. viridis* reaction center inhibits the transfer of triplet energy to the carotenoid, 1,2-dihydroneurosporene, in that system. The most likely mechanism that explains how $BChl_B$ participates in triplet state energy transfer in the *Rb. sphaeroides* reaction center complex involves a stepwise hopping of the triplet energy from the primary donor to the $BChl_B$ molecule and onto the carotenoid (Fig. 7). The rate of the transfer would be determined by the activation barrier in the primary donor-to-$BChl_B$ step. If the subsequent $BChl_B$-to-carotenoid transfer step were activationless, its very fast nature would explain why no high-field EPR signals associated with the build-up of the $BChl_B$ triplet state have ever been observed. This mechanism is simple to visualize and should be able to be confirmed by detailed dynamics measurements on these and other reaction center samples.

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