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CAROTENOID TRIPLET YIELDS IN NORMAL AND DEUTERATED *RHODOSPIRILLUM RUBRUM*

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Summary

Quantum yields of carotenoid triplet formation in *Rhodospirillum rubrum* wild type and fully deuterated cells and chromatophores were determined in weak laser flashes for excitation wavelength λ_i = 530 nm (mainly absorbed by the carotenoid spirilloxanthin) and for λ_i = 608 nm (mainly absorbed by bacteriochlorophyll) in the presence and absence of magnetic fields. All experiments were performed at room temperature and in the absence of oxygen.

The quantum yield of reaction center bacteriochlorophyll oxidation in wild type preparations, in which all reaction centers are in state P I X, at λ_i = 608 nm is close to unity, whereas the quantum yield of antenna carotenoid triplet formation is low (about 5%); P is the primary electron donor, a bacteriochlorophyll dimer, I the primary acceptor, a bacteriopheophytin, and X the secondary acceptor, an iron-ubiquinone complex. In cells in which the reaction centers are in the state $P^+I\,X^{(-)}$, the antenna carotenoid triplet yield is about 0.2. In contrast, at λ_i = 530 nm, the quantum yield of P^+ formation is relatively low (0.3) and the yield of the antenna carotenoid triplet state in state P I X unusually high (0.3).

At increasing light intensities of 530 nm only about 3 carotenoids per reaction center of the 15 carotenoids present are efficiently photoconverted into the triplet state, which indicates that there are two different pools of carotenoids, one with a low efficiency for transfer of electronic excitation to bac-

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Abbreviation: BChl, bacteriochlorophyll.

teriochlorophyll and a high yield for triplet formation, the other with a high transfer efficiency and a low triplet yield.

The absorption difference spectrum of the antenna carotenoid triplet, excited by 608 or 530 nm light in the state $P^+I\,X^{(-)}$ does not show the peak at 430 nm, that is present in the difference spectrum of the reaction center carotenoid triplet, mainly observed at λ_i = 608 nm with weak flashes.

The yield of the reaction center carotenoid triplet, generated in chromatophores in the state $P\,I\,X^-$, is decreased by about 10% by a magnetic field of 0.6 T. In a magnetic field of 0.6 T the yield of the carotenoid triplet, formed by 530 nm excitation in chromatophores at ambient redox potential, is decreased by about 45%.

The high quantum yield of formation and the pronounced magnetic field effect for the carotenoid triplet generated by direct excitation at 530 nm can be explained by assuming that this triplet is not formed by intersystem crossing, but by fission of the singlet excitation into two triplet excitations and subsequent annihilation (triplet pair mechanism), or by charge separation and subsequent recombination (radical pair mechanism).

Fully deuterated bacteria give essentially the same triplet yields, both in the reaction center and in the antenna carotenoids and show the same magnetic field effects as non-deuterated samples. This indicates that hyperfine interactions do not play a major role in the dephasing of the spins in the radical pair P^+I^- nor in the formation of the antenna carotenoid triplet.

Introduction

In photosynthetic bacteria light is absorbed by antenna pigments which transfer the energy to the reaction center, where photochemistry takes place. The antenna system consists mainly of bacteriochlorophyll (BChl) molecules that transfer singlet electronic excitation to the reaction center with an efficiency not far from unity [1,2] and of carotenoids which, in *Rhodospirillum rubrum*, transfer their energy to the BChl with an efficiency of about 0.3 [3,4]. In *R. rubrum* the main carotenoid is spirilloxanthin [5], but also other carotenoids may be present, depending on the age of the bacteria [3]. In the reaction center, the energy is trapped by transfer of an electron from the primary donor P (reaction center bacteriochlorophyll dimer) to the primary acceptor I (bacteriopheophytin) in less than 10 ps. The charge is then transferred from I to the acceptor X (an iron-ubiquinone complex) in about 160 ps. If this transfer is blocked by chemical reduction of X, the radical pair P^+I^- has a lifetime of about 50 ns [6] (for a description of the primary processes, see e.g. Ref. 7). During this time the radical pair, which is formed from singlet P^* in a singlet configuration ($S = 0$; S is the total spin), can acquire some triplet character ($S = 1$) through the influence of magnetic interactions, which differ for the two radicals. From the singlet configuration the radical pair may recombine to form the singlet excited or ground state. Recombination to the excited singlet state is thought to be responsible for the increased emission yield in the presence of reduced X [8—10]. From the triplet configuration the radical pair may recombine to the triplet state of the primary donor. If carotenoids are present, the

reaction center BChl triplet P^1 cannot be detected. Instead, a carotenoid triplet is observed [6,11]. The reaction center triplet yield under reducing conditions at room temperature is about 0.1—0.2 [12]. The triplet state of the radical pair consists of three substates ($m_s = 0, \pm 1$; m_s is the magnetic quantum number). In the absence of an external magnetic field these three levels lie close to the singlet level. In a high magnetic field B, however, the triplet sublevels are separated by an energy γB (γ is the gyromagnetic ratio) and the singlet level can mix only with the $m_s = 0$ triplet level (For an introduction to the radical pair mechanism, see Ref. 13). Hence, a magnetic field decreases the reaction center triplet yield and, because the probability of recombination to the excited singlet state is now enhanced, the emission yield will increase. These effects have been demonstrated in various preparations of *Rhodopseudomonas sphaeroides* [9,14—16].

Up to now it was generally believed that dephasing of the spins in the radical pair is governed by interactions of the electrons with the nuclear spins on P and I [14,15,17,18]. If hyperfine interactions indeed play an important role in the singlet-triplet conversion, then deuteration of the bacteria would significantly decrease the conversion rate, as hyperfine interactions with deuterons are a factor of 6.5 less than those with protons, making the root mean square value of the hyperfine interactions in deuterated reaction centers about half of that in protonated centers (see Ref. 18, and Appendix). This prompted us to study the influence of deuteration on the reaction center triplet yield and on the magnetic field effect of whole cells and chromatophores of *R. rubrum*.

Triplet states other than the reaction center triplet are produced when photochemistry in the reaction center is blocked by oxidation of the primary donor P, either chemically or by excitation with supersaturating light. The reaction center is then completely closed and no triplet of the primary donor can be formed, but instead a triplet can be generated in antenna pigments. If BChl is excited, a BChl triplet is formed, probably by intersystem crossing. If carotenoids are present, the triplet excitation is transferred to the carotenoids in about 20 ns [19]. We have studied the yield of antenna carotenoid triplets that are generated in *R. rubrum* upon excitation of the carotenoid itself at 530 nm and upon excitation of the BChl at 608 nm. The triplet yield in carotenoids by intersystem crossing is known to be very low [20]. The efficiency for transfer of the 530 nm excitation to BChl is about 0.3 [3,4] and it appeared interesting to find out whether light energy absorbed by carotenoids and not transferred to the reaction centers was rapidly degraded into heat as in vitro. To our surprise this was not the case. We observed an antenna carotenoid triplet quantum yield that was about one order of magnitude larger than the yield we found on BChl excitation in state P I X. A weak magnetic field strongly decreased the triplet quantum yield, indicating that the carotenoid triplet formed by 530 nm excitation is not formed by intramolecular intersystem crossing. The possible involvement of the radical pair mechanism or of triplet formation by fission of the excited singlet state into two triplet states will be discussed.

Materials and Methods

Rhodospirillum rubrum, strain S1, was grown as described elsewhere [21]. Deuterated cells were a generous gift from Dr. J.J. Katz. After centrifugation

the cells were diluted in fresh growth medium to an absorbance of 0.25 at 880 nm in a 0.5 cm cuvette. Chromatophores were prepared by sonication, followed by centrifugation and were resuspended in a buffer containing 50 mM morpholinopropane sulphonate and 50 mM KCl at pH 7. For deuterated material ^2H_2O (99.75%) was used instead of H_2O. Reaction center particles of *R. rubrum* were prepared as described elsewhere [21,22]. Prior to the experiments all solutions were bubbled with nitrogen for at least 10 min to remove oxygen. All experiments were done at room temperature.

Absorbance changes were measured in a single-beam spectrophotometer. In order to reduce actinic effects of measuring light, Schott interference filters were placed between the sample and the measuring light (a 250 W tungsten-iodine lamp). After passing the sample the measuring beam passed a Bausch and Lomb monochromator placed between the sample and the photomultiplier tube. The monochromator prevented scattered laser light and fluorescence from reaching the photomultiplier tube. For the same reason an additional Schott interference filter was placed between the sample and the monochromator. Actinic flashes were provided by a flash lamp-pumped dye laser (Lambda Physik FL3B, laser dye Rhodamine 6G, pulsewidth 400 ns), which was tuned to 608 nm with an interference filter, or by a frequency-doubled Nd-YAG laser (J.K. Lasers, pulsewidth 15 ns) at a wavelength of 530 nm. The laser energies were varied with neutral density filters and were monitored by deflecting a fraction of the laser beam onto a ground glass plate. The scattered light from this plate was measured by a silicon photodiode (Monsanto MD1), the photocurrent was electronically integrated and displayed on a Siemens Oscillomink recorder. The laser energy monitor was calibrated at 530 nm and at 608 nm by means of a calibrated photodiode (UDT PIN10) at the location of the cuvette.

The absorbance changes were measured by an EMI 9658 photomultiplier tube, which was connected via a current-to-voltage converter and a differential amplifier (Analog Devices 46K) to a Biomation 8100 transient recorder. The memory of the transient recorder was read out onto magnetic tape by a Digital Equipment Corp. PDP-9 computer, which was also used for further processing of the data. Measurements in a magnetic field were carried out with the set-up described in [15].

Fluorescence yields after the laser flash were measured with the apparatus described by Van Best [23]. The photomultiplier tube (Philips XP1005; S1 cathode) was protected from the scattered laser light by Schott KV550, Kodak Wratten 87C and Schott AL906 filters. The fluorescence was measured at 4.5 and 16 μs after the laser flash with a weak measuring flash (0.5 μs half-width; filter combination Schott KG 3/2, Calflex C, Schott AL433 and Schott BG 18/2; energy density about 20 nJ/cm^2). Luminescence induced in the apparatus itself prevented us from measuring the fluorescence at times less than 4 μs.

Results

The quantum yield of P^+ and carotenoid triplet formation

Fig. 1 shows a typical kinetic trace of the absorption of *R. rubrum* whole cells, measured at 580 nm, after a 530 nm Nd-YAG laser flash. Before the flash,

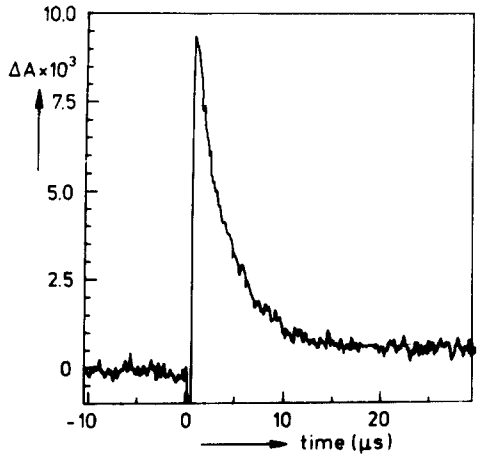

Fig. 1. Kinetics of the absorbance change at 580 nm in whole cells of *R. rubrum* after a 530 nm laser flash. Bacteriochlorophyll concentration 3.5 μM. No additions. Laser energy density 2 nE/cm^2 (about 5 photons absorbed per photosynthetic unit). Average of 16 flashes, spaced 5 s.

all reaction centers are in the state P I X. The reduction time of P^+ is of the order of a millisecond [24]. Therefore the reaction centers will be in the state P I X of $P^+I X^{(-)}$ during the flash and no reaction center triplet is expected to be formed. The fast decaying part, with a lifetime of about 3.5 μs, has a carotenoid triplet minus singlet difference spectrum (see Fig. 4b) and is therefore ascribed to antenna carotenoid triplet. The slowly decaying part is ascribed to P^+ and an electrochromic contribution [25]. The light saturation characteristics of this slow component at 580 nm are the same as that of the absorbance decrease at 605 nm which is caused by P^+, and thus the slow phase of the absorbance change at 580 nm is proportional to the P^+ concentration. Fig. 2 displays the amplitude of the fast and of the slow component of the absorbance change at 580 nm as a function of excitation energy density at 608 nm (dye laser flash, Fig. 2a) or 530 nm (Nd-YAG laser flash, Fig. 2b). The quantum yields for P^+ formation can be estimated from the initial slopes of the curves. For 608 nm excitation the intersection of the tangent of the curve at zero energy with the maximal P^+ absorbance change occurs at an energy density of 0.062 nE/cm^2 absorbed. Since the BChl concentration is about 3.5 μM, the 5 mm cuvette contains about 1.75 nequivalents of BChl molecules per cm^2 of surface area. Thus the saturation energy corresponds to about 1 photon absorbed per 30 BChl molecules, i.e. about one per reaction center [24,26]. Hence, at 608 nm, at which wavelength the exciting light is mainly absorbed by BChl, the quantum yield for P photooxidation is close to unity. The initial quantum yield for antenna carotenoid triplet formation is very low and the rise of the saturation curve is sigmoidal. This, together with the high yield of P^+ formation, indicates that only after P^+ is formed by the first photon, triplet formation can take place. At an energy density of 0.12 nE/cm^2, which corresponds to about 2 photons absorbed per reaction center, the absorbance change of the carotenoid is $\Delta A_T \approx 6 \cdot 10^{-4}$. The maximal differential absorption coefficient for the spirilloxanthin triplet-minus-singlet spectrum in cyclohexane is

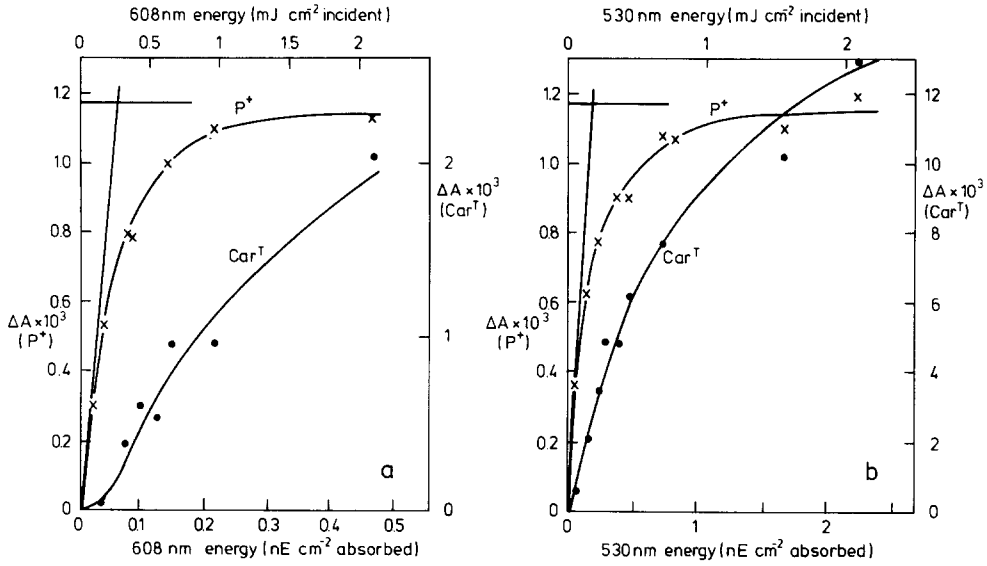

Fig. 2. Amplitude of the 3.5 μs (car^T) and slowly decaying (P^+) component of the absorbance change at 580 nm, as a function of laser energy for (a) 608 nm excitation, absorbed by bacteriochlorophyll, and (b) 530 nm excitation, mainly absorbed by carotenoids. Conditions as in Fig. 1.

about 43 $mM^{-1} \cdot cm^{-1}$ [20]. With this absorption coefficient a carotenoid triplet concentration is calculated of $2.8 \cdot 10^{-8}$ M, about 0.2 per reaction center. Since one quantum is used to produce P^+, the probability of the second quantum to make a carotenoid triplet i.e. the quantum yield of triplet formation under conditions where the reaction center is not trapping, is about 0.2. If the carotenoid triplet is formed via intersystem crossing in antenna BChl, we expect that the quantum yield of triplet formation is proportional to the fluorescence yield. The relative fluorescence yields in states P I X, $P^+I\,X^{(-)}$ and $P\,I\,X^-$ are 1, 4 and 2.4, respectively [44]. Hence the triplet yield in state P I X is about 0.05 and that in state $P\,I\,X^-$ about 0.12.

The saturation energy of P^+ formation for 530 nm excitation calculated from the initial slope of the saturation curve of Fig. 2b, is 0.19 nE/cm^2 absorbed. This corresponds to about 1 photon absorbed per 9 BChl molecules. Hence, for this wavelength, the quantum yield for P^+ formation is only about 0.3, which agrees well with the efficiency of energy transfer to the reaction center calculated from the relative fluorescence yield upon excitation in the carotenoid region and at 590 nm, absorbed by BChl [3]. Since about 10% of the light is absorbed by BChl, the efficiency of energy transfer from the carotenoid to BChl is about 0.22. However, the quantum yield of carotenoid triplet formation is much higher for 530 nm than that for 608 nm excitation (note that the scale of the cartenoid change in Fig. 2b is compressed by a factor of 5) and the rise of the saturation curve is no longer sigmoidal. The quantum yield calculated from the initial slope of Fig. 2b is about 0.3. About 30% of the energy is transferred to BChl or absorbed by it and will form triplets with a yield that depends on the state of the reaction center. In state P I X the triplet yield by these processes is $0.3 \times 0.05 = 0.015$ (see above). This results in a quantum yield of triplet formation on direct excitation of the carotenoid of 0.32.

In the state $P I X^-$, the antenna triplet yield is expected to be slightly increased (by about $0.3 \times (0.12 - 0.05) = 0.02$), but in addition the reaction center triplet is formed by quanta that are absorbed by BChl or transferred to it. When dithionite is added to the sample, the slow phase in the absorbance change disappears and the yield of the fast component increased by about 20—30%. This corresponds to a reaction center triplet yield on carotenoid excitation of 0.04—0.06 and on BChl excitation of 0.13—0.2, in good agreement with the measured yield of triplet formation in reaction center preparations of *Rps. sphaeroides* R26 [12]. Chromatophores behave essentially the same as whole cells. However, in reaction center preparations the carotenoid triplet could only be observed under reducing conditions, both with 608 and 530 nm excitation.

The curve of the carotenoid triplet amplitude vs. laser energy for 530 nm excitation levels off at about 3 carotenoid triplets per reaction center and rises from that point with an almost constant slope of about 10% of the initial slope of the curve (not shown in Fig. 2). This indicates that the carotenoid triplet, generated by excitation of the carotenoid directly cannot be formed in all carotenoids, but only in about 20% [5].

In fully deuterated cells and chromatophores the yields of the antenna carotenoid triplet (in preparations under ambient redox conditions) and of the reaction center carotenoid triplet (reducing conditions) are about equal to those of protonated samples, both for 530 nm and 608 nm excitation, if we assume that the differential extinction coefficients are the same in normal and deuterated preparations. When X is reduced by adding dithionite the decay time of the reaction center carotenoid triplet in deuterated material is 6 μs, compared to 3.5 μs in protonated preparations. The lifetime of the antenna carotenoid triplet, formed in non-treated deuterated cells is 2—3 μs, both with 608 nm (BChl) and 530 nm (carotenoid) excitation. In protonated cells it is 3.3 μs at low triplet densities (about 1 per reaction center) and increases to about 3.6 μs at a density of 3 per reaction center.

Fluorescence quenching

In Fig. 3 the fluorescence yield at 4.5 and 16 μs after laser flashes is plotted as a function of the intensity of the excitation at 608 nm (Fig. 3a) and 530 nm (Fig. 3b) in *R. rubrum* at ambient redox potential. Since the lifetime of the carotenoid triplet is about 3.5 μs, at 4.5 μs about 28% of the carotenoid triplet is still present and at 16 μs only 1%. The increase of the fluorescence yield 16 μs after the laser flash (Figs. 3a and b) is caused by the presence of increasing concentrations of P^+ in the reaction center, formed in the laser flash. At 4.5 μs at higher intensities of the flashes the fluorescence is quenched by the carotenoid triplets [27]. The fluorescence changes due to P^+ upon 530 nm excitation show an apparently biphasic saturation behaviour because of the observed strong spatial inhomogeneities in the YAG-laser beam in this experiment. For this reason quantitative conclusions cannot be drawn from this experiment, but it is clear that, when the bacteria are excited at 530 nm the fluorescence at 4.5 μs is quenched for all intensities of the laser, while for 608 nm excitation a small quenching is observed only at high intensities. This is in agreement with the low triplet yield upon 608 nm excitation, and the high triplet yield on direct carotenoid excitation.

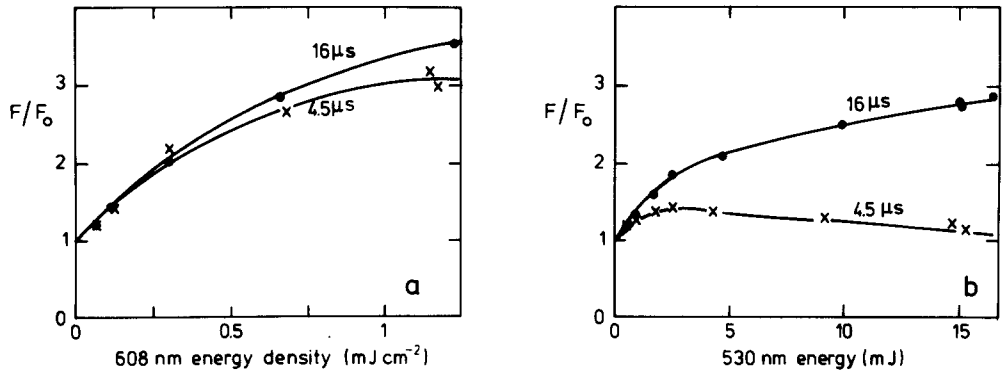

Fig. 3. Relative fluorescence yield of whole cells of *R. rubrum* wild type at 4.5 (X) and 16 (•) µs after a laser flash as a function of (a) incident laser energy density for 608 nm excitation and (b) of total laser energy for 530 nm excitation. Absorbance of the bacteria was 0.5 in a 5 mm cuvette. Due to inhomogeneity of the Nd-YAG laser, the energy density could not be calculated for 530 nm excitation and the fluorescence is plotted as a function of total energy.

Absorption spectra of the carotenoid triplets

In Fig. 4a the absorption difference spectrum is displayed of the carotenoid triplet in intact cells generated by 608 nm excitation. X has been reduced by adding dithionite. The spectrum agrees well with the one measured by Monger et al. [19] in chromatophores at low redox potential, which were excited by 694 nm ruby laser flashes. It shows the bleaching of the absorption bands at 480 and 510 nm, and the appearance of bands at 580 and 430 nm. This spectrum is similar to a spirilloxanthin triplet-minus-singlet spectrum, shifted to the red by 20 nm [20]. Fig. 4b shows the difference spectrum of the antenna carotenoid, generated by the 530 nm Nd-YAG laser flash and by the 608 nm dye laser flash in chromatophores under ambient redox potential. For wavelengths

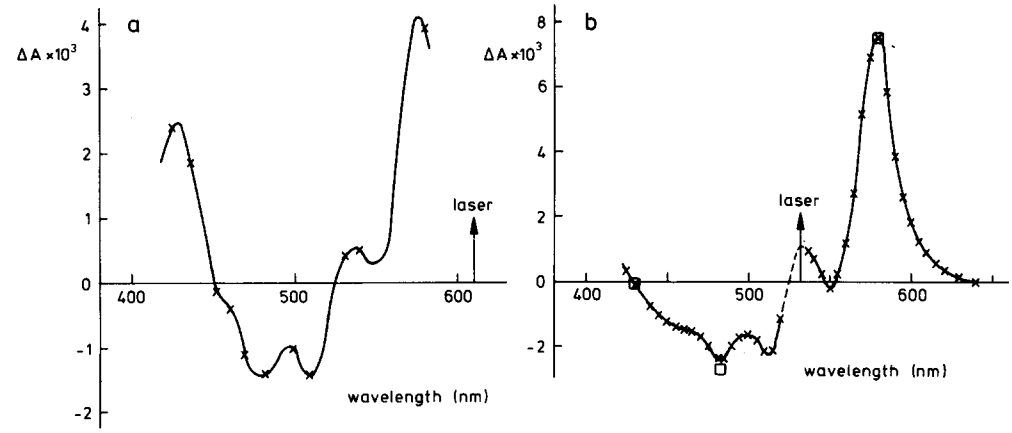

Fig. 4. (a) Absorption difference spectrum of the carotenoid triplet induced by 608 nm laser flashes in whole cells at low redox potential. 100 mM solid sodium dithionite added. (b) Absorption difference spectrum of the carotenoid triplet induced by (X) 530 nm excitation, conditions as in Fig. 1, and (□) 608 nm excitation in cells in which P is oxidized by continuous white background illumination. The latter spectrum is normalized at 580 nm to the change for 530 nm excitation.

above 470 nm the spectrum is almost identical to the spectrum of the reaction center triplet. However, the peak at 430 nm, which is present in the reaction center carotenoid triplet spectrum, is absent.

The magnetic field effect

In Fig. 5 we show the influence of a magnetic field on the yield of the carotenoid triplet in chromatophores of normal (Fig. 5a) and deuterated (Fig. 5b) *R. rubrum* chromatophores in state $P\,I\,X^-$ upon 608 nm (BChl) excitation. For both types of preparations the decrease in high field is about 10% and the magnetic field at which half the effect is reached, $B_{1/2}$, is about 70 mT. Furthermore, a lag of about 40 mT is observed, which may indicate that there is appreciable interaction between the spins on P^+ and I^-, or between one of these spins and a third spin, for instance on the reduced primary acceptor X^- [17]. At low fields dephasing of the spins is inhibited by this interaction. The extent of the magnetic field effect is comparable to that found in chromatophores and whole cells of *Rps. sphaeroides* [9,15]. The absence of an effect of deuteration is remarkable, since it has generally been assumed that the spin dephasing responsible for the generation of the triplet state of the radical pair P^+I^- is caused by hyperfine interactions, which for the deuteron are lower by a factor of 6.5.

If the antenna carotenoid triplet which is created by 530 nm excitation is formed by intramolecular intersystem crossing, then one would not expect to observe an effect of a magnetic field. However, as shown in Fig. 6, a magnetic field does have a large effect on the yield of the antenna carotenoid triplet

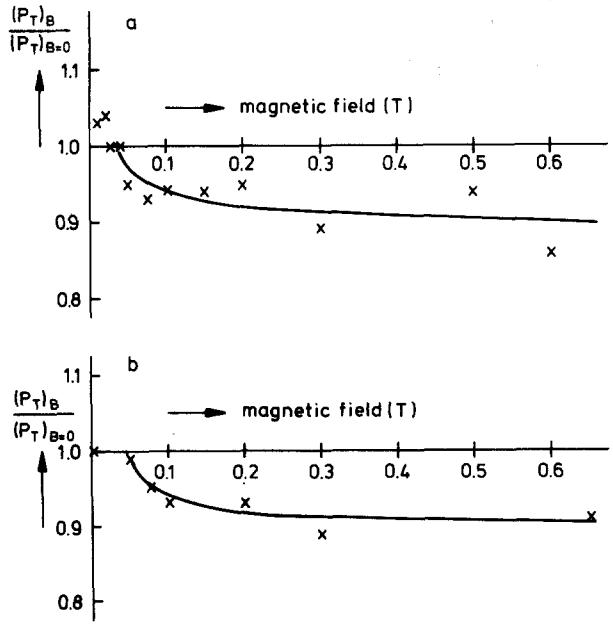

Fig. 5. Influence of a magnetic field on the yield of the reaction center carotenoid triplet for (a) normal and (b) deuterated chromatophores of *R. rubrum*. 100 mM dithionite added, excitation wavelength 608 nm.

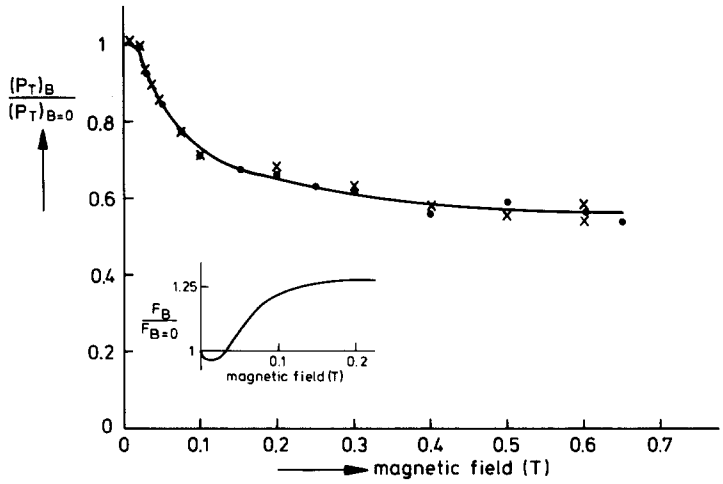

Fig. 6. Effect of a magnetic field on the carotenoid triplet formed by 530 nm excitation in (•) normal and (X) deuterated chromatophores of *R. rubrum*. Conditions as in Fig. 1. Insert: magnetic field effect on prompt fluorescence of tetracene as a function of magnetic field (from Ref. 42).

generated by the YAG laser in non-treated chromatophores. The effect is much more pronounced than the effect on the reaction center triplet and is present in both normal and deuterated material. At 0.6 T the triplet yield is 45% lower than the yield in zero field; $B_{1/2}$ and the initial lags are of the same order of magnitude as the corresponding values of the reaction center carotenoid triplet, generated by 608 nm excitation, as measured both in normal and deuterated cells. The total triplet yield decreases by 45%. If we assume that the antenna triplet yield by intersystem crossing is not affected by a magnetic field, the quantum yield of formation of the carotenoid triplet directly from excited carotenoid decreased from 0.32 to 0.17. The extent of the magnetic field effect does not depend on the laser energy. Within experimental accuracy (about 3%) no effect of a magnetic field on the quantum yield of the oxidation of the primary donor was observed. The unusually high carotenoid triplet yield and the pronounced effect of a magnetic field on this yield indicate that the carotenoid triplet, generated by 530 nm excitation, is not formed by intramolecular intersystem crossing, but that another mechanism is responsible for the formation of the triplet.

Discussion

Pathways of carotenoid triplet formation

The various ways of carotenoid triplet formation are summarized in the scheme in Fig. 7 and are discussed in the following sections. Antenna carotenoid triplet can be formed by intersystem crossing in the antenna BChl that is excited either directly, or via excitation transfer from singlet excited carotenoid, and by transfer of the BChl triplet excitation to the carotenoid. The yield of this process is about 0.2 if the reaction centers are in the state $P^+I\,X^-$ and about 0.05 if they are in the state P I X. Furthermore the carotenoid

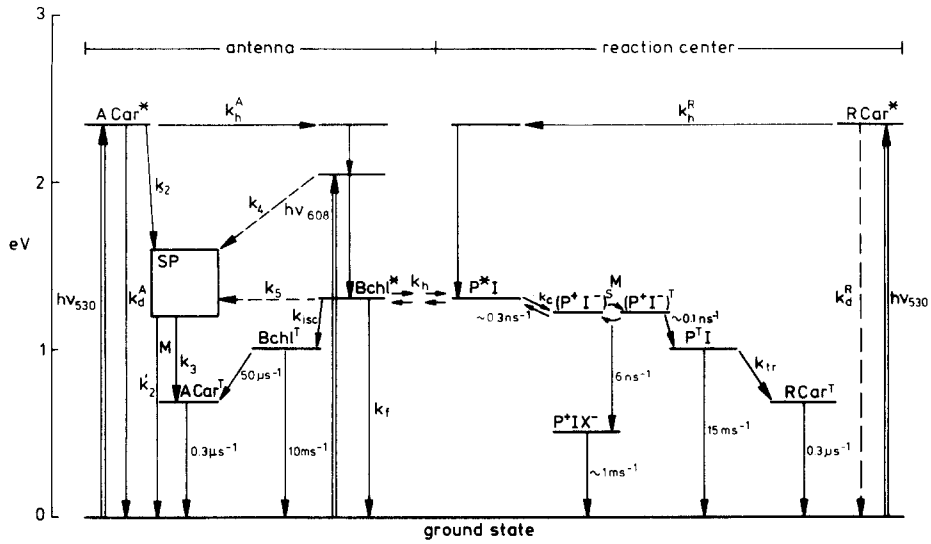

Fig. 7. Scheme of triplet formation, energy transfer and electron transfer reactions in the antenna and reaction center of *R. rubrum*. ACar are antenna carotenoids, RCar is reaction center carotenoid. A superscript T denotes a triplet state. SP is the spin-pair intermediate in antenna carotenoid triplet formation. Double arrows are light reactions at the wavelengths indicated. k_h, k_h^A and k_h^R are the rate constants for energy transfer between BChl molecules, from carotenoid to BChl in the antenna and in the reaction center respectively. k_h^A is averaged over all antenna carotenoids. k_f and k_{isc} are the fluorescence and intersystem crossing rate constants of antenna BChl. k_c is the charge separation rate, k_{tr} is the rate of transfer of triplet excitation from P to RCar. k_{tr} must be large since P^T is not observed in the presence of carotenoids. The reactions marked M are magnetic field dependent. The decay rates of $BChl^T$ and P^T to the ground state are measured in the carotenoidless mutant G9 [6,19]. The values of the recombination rates of $P^+ I^-$ are estimated in the Appendix. The ratio of the decay rate of excited RCar to the ground state, k_d^R, to the transfer rate k_h^R is not known for intact reaction centers. The other rate constants are described in the text.

triplet can be formed from the singlet excited carotenoid via an intermediate spin-pair state SP. The pair of spins may recombine to the singlet ground state or to the carotenoid triplet state. The proportion of the products is magnetic field-dependent.

Reaction center carotenoid triplets are formed by excitation of the antenna pigments or of the reaction center directly and are observed only when the primary acceptor X is reduced, i.e. when the reaction $P^+I^-X \rightarrow P^+I\,X^-$ is inhibited. In reaction center preparations the carotenoid triplet is only observed in state $P\,I\,X^-$, both with BChl and carotenoid excitation. Thus the process of carotenoid triplet formation directly from singlet excited carotenoid takes place only in antenna carotenoids.

Absorption spectra

The antenna carotenoid triplet spectrum, both excited by 608 and 530 nm flashes, does not show the peak at 430 nm present in the reaction center carotenoid triplet spectrum. In the carotenoid triplet difference spectrum of Cogdell et al. [6] in reaction center preparations of *R. rubrum*, the absorption change at 430 nm is about 1.5 times as large as the change at 580 nm, while in our spectrum it is only about 0.7 of the 580 nm change. This is in agreement

with our observation that, under reducing conditions, the antenna triplet yield and the reaction center triplet yield are about equal, and that our difference spectrum is composed of about equal contributions of antenna carotenoid, which lacks the peak at 430 nm and reaction center carotenoid, in which the 430 nm peak is present. An explanation for the difference between antenna and reaction center carotenoid spectra may be that the interaction of the carotenoid with the surroundings differs for antenna and reaction center, and that the peak arises from an interaction with the primary donor. Another explanation may be that in the reaction center the carotenoid is a mono*cis* configuration, while in the antenna the carotenoid is all-*trans* [28,29]. In the in vitro absorption spectrum *cis*-isomers show a peak about 140 nm below the longest wavelength maximum [30]. The wavelength difference between the 430 nm peak and the peak in the in vivo absorption difference spectrum (580 nm) is about 150 nm, so the 430 nm peak may be caused by a *cis*-peak in the triplet-triplet absorption spectrum.

The reaction center triplet

It is generally accepted that in the reaction center the carotenoid triplet state is generated via triplet excitation transfer from the donor triplet P^T, that is formed through electron-hole recombination in the radical pair P^+I^-. Direct evidence for this mechanism comes from measurements of the rise and decay kinetics of P^+, I^- and of the triplet state [7]. Indirect evidence is furnished by the spin polarization of the reaction center BChl triplet at low temperatures [31,32] and by the magnetic field effects on the triplet and emission yield [9,14—16]. Up to now, the singlet-to-triplet conversion due to a dephasing of the spins on P^+ and I^- was believed to be governed predominantly by hyperfine interactions within the radicals. However, we do not observe an effect of substituting all hydrogen atoms by deuterium, which has a much lower magnetic moment, corroborating a recent report by Blankenship and Parson [33]. This indicates that the hyperfine interaction is not the factor that determines the conversion frequency. The central conversion frequency is governed by the difference in electronic *g*-value ($\Delta g \approx 9 \cdot 10^{-4}$ for the BChl-Bph pair [34]) and by the interactions between the spins of the radical pair and between one (or both) of these spins and a third species, while the width of the frequency distribution is determined primarily by the hyperfine interactions. It is shown in the Appendix that accepting the hyperfine interactions to be the sole responsible agent for spin dephasing (the Δg term can be neglected for fields less than about 1 tesla) leads to a 45% decrease in triplet yield and an isotope effect of 60% on the magnetic field effect, when no spin-spin interaction is present. Introducing an interaction of —5 G gives figures of 55 and 50%, respectively. These values are at variance with the experimental results, which show no isotope effect on the triplet yield and on the magnetic field effect. We conclude that, at least in *R. rubrum*, the dephasing of the spins is primarily governed by external influences (such as from the reduced secondary acceptor X^-) and that the hyperfine interactions play only a minor role in the dephasing.

The decrease of the decay rate of the reaction center carotenoid triplet by a factor of two upon deuterium substitution agrees with the isotope effect on the triplet decay rate that is generally observed in conjugated systems [35]. The

increase of the decay rate of the antenna carotenoid triplet upon deuteration remains to be explained.

In reaction center preparations the carotenoid triplet is observed only when the primary acceptor X is reduced, also when the reaction center carotenoid itself is directly excited at 530 nm. Hence in reaction centers direct triplet formation upon carotenoid excitation does not compete with the excitation transfer. This suggests that in the *R. rubrum* wild type reaction center the efficiency of energy transfer $car^*P \rightarrow car\ P^*$ is much higher than in the antenna of intact cells. However, Boucher et al. [28] have found a transfer efficiency of 0.20 in reaction preparations of the carotenoidless mutant *R. rubrum* G9, which were reconstituted with spirilloxanthin.

The antenna carotenoid triplet

Antenna carotenoid triplets are generated by two processes. The first process is intersystem crossing in BChl, which is excited either directly or via singlet excitation transfer from carotenoids, followed by transfer of the BChl triplet excitation to a carotenoid molecule through short-range exchange interaction. The quantum yield of this process depends on the state of the reaction centers and is about 0.2 for photons absorbed by BChl when the reaction centers are non-trapping (state $P^+I\ X^{(-)}$). This yield is much higher than the carotenoid triplet yield of 0.02 observed by Monger et al. [19] upon 834 nm excitation of *Rps. sphaeroides* PM8 dpl, a mutant that lacks reaction centers. This might be explained by assuming that, when BChl is excited to a higher electronic state, a triplet can be formed from highly excited BChl by the same or a similar mechanism as for triplet formation upon carotenoid excitation at 530 nm (rate constant k_4 in Fig. 7) and that, when BChl is excited at 834 nm, only intersystem crossing is possible. Thus the rate k_5 would be negligible. In this case the magnetic field dependence of the triplet yield in cells in state $P\ I\ X^-$ upon 608 nm excitation is not only caused by the reaction center, but, at least in part, by antenna carotenoid. This might also be an explanation for the similarity of the curves of Figs. 5 and 6. However, if an appreciable part of the antenna carotenoid triplet is formed by this mechanism, the yield would not depend on the state of the reaction center and the saturation curve would not show a sigmoidal behaviour. Furthermore the quantum yield of photochemistry would be decreased appreciably by this loss process. The triplet quantum yield we calculate agrees with the triplet quantum yield found in algae [36] and with the quantum yield that can be calculated from the data of Connolly et al. [37], who measured a triplet yield of 0.75—0.8 in a 30 ns laser flash with an energy of 10 photons per molecule in BChl in solution. Assuming that the decay of excited BChl to the ground state is caused only by fluorescence, the rate constant for this decay is $5.6 \cdot 10^7\ s^{-1}$ [38]. The observed triplet yield is obtained with an intersystem crossing rate constant of $(6.5—7.5) \cdot 10^7\ s^{-1}$. Thus, in BChl in vitro the quantum yield of intersystem crossing is about 20—40% larger than that of fluorescence.

The second way of antenna carotenoid triplet formation is directly from singlet excited carotenoids. The quantum yield of this process is about 0.32 and about half of that in a magnetic field of 0.6 T. The decrease in a magnetic field is a strong indication that between the excited singlet and the triplet

carotenoid an intermediate state is present, which contains two spins (state SP in Fig. 7). The recombination product of these spins can be either the singlet or the triplet state (the quintet state is energetically inaccessible), the proportion of which is magnetic field-dependent. Such an intermediate state is for example a radical pair. A charge separation may take place between the excited carotenoid and a BChl or another cellular constituent, and the resulting charges may recombine to form either the singlet ground or excited state or the BChl or carotenoid triplet state. When the BChl triplet is formed, it is rapidly transferred to the carotenoid.

The calculated efficiencies of energy transfer (0.22) and of triplet formation (0.32) for carotenoid that is excited by 530 nm excitation are only average values. The triplet yield seems to saturate at about 3 carotenoid triplets per reaction center, i.e. a triplet state in about 20% of the carotenoids [5]. This, and the fact that the lifetime of the triplet increases with increasing laser energy, indicate that the yield of the triplet formation is not the same for all carotenoids. In order to explain the average triplet quantum yield of 0.32 part of the carotenoids must have a triplet quantum yield close to one and a low transfer efficiency. The remaining carotenoids have a lower triplet quantum yield and a higher transfer efficiency. However, both types of carotenoids cannot be distinguished spectrally and the carotenoid triplet generated by direct excitation quenches the BChl fluorescence as efficiently as the triplet generated by intersystem crossing in BChl.

Singlet fission

An alternative for the radical pair mechanism as a pathway for antenna triplet formation is provided by the singlet fission, also called triplet pair mechanism, as observed in crystals of aromatic molecules (for an introduction and review, see Ref. 39). The energy of the lowest excited singlet state of spirilloxanthin is about 2.2 eV, while the triplet energy is estimated to be 0.65 eV [20] and the triplet energy of BChl about 1.0 eV [40,41]. Therefore the fission of one singlet excitation into a pair of triplets $S_1 + S_0 \rightarrow$ [TT], either into two carotenoid triplets (homofission) or into one carotenoid triplet and one BChl triplet (heterofission) is energetically possible. In organic crystals this process can be very efficient [39]. The main features of the triplet pair mechanism are summarized in Fig. 8. The two triplets generated by fission may diffuse apart (rate constant k_{-1}) or they may recombine and form a singlet (rate constants k_2 and k_2') or a triplet (rate constant k_3) state, depending on the spin state of the complex [TT]. This complex has nine spin-states, designated by $[TT]_l$. In general each of these states will have singlet, triplet and quintet character. The spin amplitudes of the wave functions are given by C_S^l, C_T^l and C_Q^l for singlet, triplet and quintet, respectively, and satisfy the relationships $|C_S^l|^2 + |C_T^l|^2 + |C_Q^l|^2 = 1$ for $l = 1, \ldots, 9$,

$$\sum_{l=1}^{9} |C_S^l|^2 = 1 , \qquad \sum_{l=1}^{9} |C_T^l|^2 = 3 \qquad \text{and} \sum_{l=1}^{9} |C_Q^l|^2 = 5 .$$

As fission and fusion processes are spin-conserving, fission takes place to a pair state which has some singlet character. Migration of the triplet excitation is not very likely, because at the highest triplet densities we generate with our

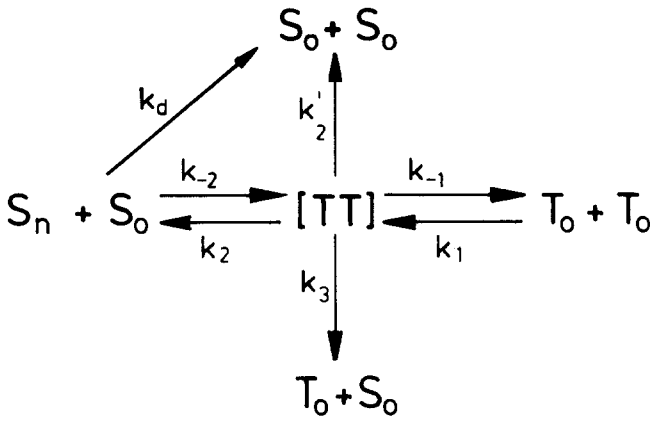

Fig. 8. Scheme for singlet fission and triplet-triplet annihilation. S_0 is the ground state; S_n is an excited carotenoid singlet state; [TT] is the intermediate triplet pair state, consisting of nine substates and T_0 is the lowest carotenoid triplet state.

laser flash (about 3 per reaction center, that is about 1 per 5 carotenoid molecules) the lifetime of the triplets should be considerably shortened, due to triplet-triplet annihilation. This we do not observe. In contrast, we observe an increase of the lifetime of about 10%, in going from 1 triplet per reaction center ($\tau = 3.3$ μs) to 3 triplets per reaction center ($\tau = 3.6$ μs). If we neglect migration of the triplets and spin relaxation within the pair, the yields of (single) triplets will be given by

$$P_F \sum_{l=1}^{9} \frac{k_3 |C_T^l|^2 |C_S^l|^2}{k_2' |C_S^l|^2 + k_3 |C_T^l|^2}$$

in which $P_F |C_S^l|^2$ is the probability of fission to state $[TT]_l$, $P_F = k_{-2}/(k_{-2} + k_d)$ is the total fission probability, k_d is the rate constant for the decay of the excited carotenoid to the ground state (see also Fig. 7), $k_3 |C_T^l|^2$ is the recombination rate of state $[TT]_l$ to the triplet state and $k_2' |C_S^l|^2$ that to the ground state. $k_2 |C_S^l|^2$, the recombination rate to the singlet excited state is neglected because of the large energy difference between this state and the triplet pair state (at least 0.5 eV). A quintet state on a single molecule is energetically inaccessible since it requires the excitation of two electrons simultaneously. If homofission (the triplets are generated on equal molecules) takes place the nine states are either pure triplet or mixed singlet-quintet states. Consequently no triplet can be formed by recombination of a triplet pair that is formed in a singlet state. For heterofission, all nine states are a mixture of singlet, triplet and quintet wave functions, and a triplet can be formed. Additionally, the spin states of the pair will be mixed by application of a magnetic field for which the Zeeman energy of the pair states is about equal to the zero field splitting energy and hence the triplet yield from recombination will increase. In a magnetic field high compared to the zero-field splitting energy the individual spins are quantized along the magnetic field and only two states will have singlet character. Consequently the triplet yield by triplet recombination will be lower in a high magnetic field than in zero magnetic field. If the triplet pair can annihilate to the excited singlet state, as in tetracene, the magnetic field has an effect on the fluorescence complementary to that described here

for the effect on the triplet yield, i.e. a decrease for fields in the order of the zero field splitting energy and an increase at high field. It is seen that the shape of the curve depicted in the insert of Fig. 6 is complementary to the curve for the triplet yield.

The high triplet yield, the low rate of excitation transfer from carotenoid to BChl, the magnetic field effect and the absence of a deuteration effect are well explained by the triplet pair mechanism. But this does not constitute definite proof. In principle, the above observations would also be accomodated by the radical pair mechanism. Triplet production by singlet fission is quasiinstantaneous, whereas triplet formation via the back reaction of two geminate radicals necessitates a time lag of the order of the lifetime of the radical pair. This lifetime must be several ns in order to dephase the initial singlet state of the combined radicals enough to produce the large triplet amplitude corresponding to the high yield of the antenna carotenoid triplet. Obviously, fast optical spectroscopy could decide between the two mechanisms.

Appendix

The effect of deuteration on the reaction center triplet yield and on the magnetic field effect can be estimated using the one-nuclear spin approximation of the hyperfine contribution to the radical pair mechanism given by Haberkorn and Michel-Beyerle [18]. The triplet yield in reaction centers in zero magnetic field is given by

$$\phi_T(0) = \frac{3A^2 k_t (k_s + k_t)}{(3A^2 + 4k_s k_t)(k_s + k_t)^2 + 16 k_s k_t (J - (A/2))^2} \tag{1}$$

and that in high magnetic field (neglecting dephasing of the spins by difference in g-values of P^+ and I^-) is

$$\phi_T(\infty) = \frac{A^2 k_t (k_s + k_t)}{(A^2 + 4k_s k_t)(k_s + k_t)^2 + 16 k_s k_t J^2} \tag{2}$$

in which k_s and k_t are the recombination rates of singlet and triplet $P^+ I^-$ respectively, A is the hyperfine interaction, concentrated on a single nucleus and J is the exchange interaction of the spins on P^+ and I^- (A and J in s^{-1}). We substitute the value for the hyperfine splitting constant of P^+ and I^- for protonated material $A_H = 0.233$ ns^{-1} [18] and choose k_s and k_t such that the measured and calculated values of the reaction center triplet yield [12] and the magnetic field effect [14,15] in protonated bacterial reaction centers agree. For $J = 0$, $k_s = 0.34$ ns^{-1} and $k_t = 0.079$ ns^{-1} we obtain from Eqns. 1 and 2 $\phi_T^H(0) = 0.10$ and $\Delta_H = |\phi_T^H(\infty) - \phi_T^H(0)|/\phi_T^H(0) = 0.37$. Replacing now in the calculation of A, all protons by deuterons, we obtain [18] $A_{2_H} = 0.122$ ns^{-1}. With the same values of J, k_s and k_t this results in $\phi_T^{2H}(0) = 0.055$ and $\Delta_{2_H} = 0.59$. Recently evidence was obtained that $|J|$ is 2—10 G [43] *. For $J = -5$ G (0.09 ns^{-1}) we obtain the measured values for $\phi_T^H(0)$ and Δ_H for $k_s = 0.32$ and $k_t = 0.14$ ns^{-1}. Replacing in Eqns. 1 and 2 A_H by A_{2_H} we obtain $\phi_T^{2H}(0) = 0.044$ and $\Delta_{2_H} = 0.55$. Thus, if it is assumed that hyperfine interactions are solely respon-

sible for triplet formation, the triplet yield is expected to decrease appreciably and the magnetic field effect to increase significantly when the bacteria are deuterated. When an exchange interaction J between P^+ and I^- is present the triplet yield in reaction centers will be decreased more by deuteration than when this interaction is absent. The magnetic field effect Δ_{2_H} is not much affected by J.

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* The definitions of J in Refs. 18 and 43 differ by a factor 2.

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