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TRIPLET STATES OF MONOMERIC BACTERIOCHLOROPHYLL IN VITRO AND OF BACTERIOCHLOROPHYLL DIMERS IN ANTENNA AND REACTION CENTER COMPLEXES

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Triplet states of monomeric bacteriochlorophyll in solution, and of dimeric bacteriochlorophyll in photosynthetic reaction centers and antenna complexes isolated from *Rhodopseudomonas sphaeroides* and *Rps. viridis* were generated by excitation with short flashes. The triplet states were characterized by measurements of optical absorption spectra, decay kinetics as functions of temperature, quenching by O_2, and delayed fluorescence. The triplet state of the antenna dimer can be described as one molecule in the ground (singlet) state and one in the excited (triplet) state. Formation of the triplet disrupts exciton interactions between the two molecules, and the complex acquires a new absorption band due to the molecule in the ground state. In reaction centers, the formation of the triplet causes bleaching of the main absorption band of P, the reactive bacteriochlorophyll complex (870 nm in *Rps. sphaeroides*; 960 nm in *Rps. viridis*). Other absorption bands that have been considered to be exciton bands of P (810 nm in *Rps. sphaeroides*; 850 nm in *Rps. viridis*) appear to undergo shifts, but not to bleach. The latter bands probably are due to neighboring bacteriochlorophylls, not to P. The decay kinetics of the triplet state in reaction centers are strongly influenced by interactions with the anionic radical of the quinone that serves as an early electron acceptor. A new transient (20 ns) state of the reaction center was found when reaction centers were excited with supersaturating flashes.

Introduction

Reaction centers of photosynthetic bacteria contain four molecules of BChl and two of BPh [1]. Two of the BChls are thought to form a special dimer (P) [2–6]. When the reaction centers are excited with light, P undergoes photooxidation, probably by transferring an electron to another BChl (B). This forms a singlet charge-transfer state, $^1[P^+B^-]$ [7,8]. From B^-, an electron moves to one of the BPhs (H), and then on to a quinone (Q) [9–13]. If one blocks electron transfer between H and Q by extracting Q from the reaction centers or by reducing Q chemically before the excitation, the $[P^+H^-]$ radical pair decays in about 10 ns by several types of back-reactions. One of the back-reaction pathways leads to the formation of a triplet state, P^R [14,15]. Recent studies of reaction centers isolated from *Rhodopseudomonas sphaeroides* indicate that P^R is a thermal mixture of two different states [8]. The lower state appears to be largely a locally excited triplet state of P, ^3P, and the higher one largely a triplet charge-transfer state, $^3[P^+B^-]$. The present paper describes further studies on the triplet states of monomeric BChl in vitro, of dimeric BChl in pigment-protein complexes obtained from the antenna system of *Rps. sphaeroides*, and of P in reaction centers isolated from this species and from *Rps. viridis*.

In reaction centers from *Rps. sphaeroides* or other

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Abbreviations: SDS, sodium dodecyl sulfate; BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

species that contain BChl *a*, P has a major absorption band at 870 nm. This band bleaches when P is oxidized to P^+ or converted to ^3P. B and the fourth BChl absorb near 800 nm, and the formation of B^- is reflected by bleaching in this region. However, the absorption spectrum of reaction centers in the 800 nm region is not well understood. The oxidation of P causes an absorbance decrease at 810 nm and an increase at 790 nm, in addition to the bleaching at 870 nm, suggesting that P also has an absorption band near 810 nm. Excitonic interactions between the two BChls of P should result in a splitting of the long-wavelength (Q_y) absorption band of the BChls into two bands; Vermeglio and Clayton [16] and Rafferty and Clayton [17] have suggested that these are at 810 and 870 nm. The oxidation of the dimer to P^+ would disrupt the excitonic interactions, so that the absorption spectrum could become the sum of spectra for one BChl in the oxidized state and one in the unoxidized state. Vermeglio and Clayton [16] suggested that the unoxidized molecule in P^+ absorbs at 790 nm. These assignments were based on measurements of the linear dichroism of the light-induced absorbance changes in oriented films of reaction centers. Pearlstein [18] has pointed out, however, that the idea that P has a major absorption band at 810 nm is not easily reconciled with several other observations on the absorption spectrum of reaction centers. For example, the 870 nm band moves to 890 nm at 77 K, while the absorption in the 800 nm region sharpens, but does not move [19]. This is most easily explained if the 800 nm absorption is due to B and the fourth BChl, rather than P. An alternative interpretation of the light-induced absorbance changes in the 800 nm region is that the oxidation of P causes a blue shift in the absorption band of one or both of the other BChls. Observations on the linear dichroism of absorbance changes induced by excitation with polarized light (photodichroism) can be explained straightforwardly in this way [20].

Rps. viridis contains BChl *b* rather than BChl *a*, and the absorption bands of its reaction center lie at longer wavelengths than do those of *Rps. sphearoides*. At 77 K, the oxidation of P in *Rps. viridis* reaction centers causes absorbance increases near 808 and 832 nm, and bleaching near 835, 850 and 990 nm [21, 22]. Several investigators [12,22,23] have interpreted the 850 and 990 nm bands as the two exciton bands of P. Thornber et al. [22] suggest that the absorbance increase at 808 nm is due to the BChl that remains in the unoxidized state in P^+. They interpret the absorbance changes at 832 and 835 nm as a shift of a band due to a third BChl. The BChl that absorbs in this region is probably analogous to B in *Rps. sphaeroides*, because reduction of the electron-acceptor complex causes a bleaching at 832 nm [12,24,25]. Thornber et al. [22] suggest that the absorption band of the fourth BChl is at 812 nm. Shuvalov et al. [24,26] have offered a different set of assignments based on measurements of photodichroism. They suggest that the 850 nm band is due to the fourth BChl, rather than P, and that the oxidation of P causes a blue shift of this band, rather than a bleaching. Shuvalov and Asadov [25] viewed the 812 nm component as a contaminant, but Thornber et al. [22] have provided arguements against this.

The absorption spectrum of the triplet state can provide additional information on the interactions among the four BChls of the reaction center, and particularly on the interactions between the two BChls of P. Unless the exchange interaction between the two molecules is unusually strong, the triplet excitation will hop back and forth between the two relatively slowly. The absorption spectrum of P thus should be essentially the sum of the spectra for one BChl molecule in a triplet state and the other in the ground (singlet) state. If P has absorption bands at 810 and 870 nm in *Rps. sphaeroides* reaction centers, as Vermeglio and Clayton [16] suggest, the formation of ^3P should cause a bleaching of both bands, and the development of a new band due to the BChl that is in the ground state. Similarly, if the band assignments suggested by Holten et al. [12] and Thornber et al. [22] for *Rps. viridis* are correct, the formation of ^3P should cause bleaching at both 850 and 960 nm. The situation could become more complicated if the exchange interaction between the two BChls is exceptionally strong. This would cause the triplet excitation to hop back and forth rapidly between the molecules, and would cause a splitting or broadening of the absorption bands of the complex.

Materials and Methods

Reaction centers were obtained from *Rps. sphaeroides* strain R-26 by treatment of chromato-

phores with lauryldimethylamine oxide and chromatography on DEAE-Sephacel, as described elsewhere (Schenck, C.C., Blankenship, R.E. and Parson, W.W., unpublished data). Essentially the same procedure was used to obtain reaction centers from *Rps. viridis*. Concentrated solutions of reaction centers were dialyzed against 0.05% Triton X-100, 50 mM Tris-HCl (pH 8), and were diluted with the same buffer for measurements at room temperature, or with a mixture of this buffer and glycerol (1 : 2, w/w) for low temperatures. To reduce Q to Q^-, 1 mM sodium ascorbate and 1 mg/ml $Na_2S_2O_4$ were added in the dark. Further reduction of Q^- to Q^{2-} was obtained by illuminating the reaction centers for several minutes with continuous light in the presence of the same reductants plus 0.3 mM cytochrome *c*, followed by a dark period of 2–5 min to allow reoxidation of H^- [8]. To remove Q and Fe, reaction centers were treated with 0.2–0.3% SDS [8].

Large BChl-protein complexes from the antenna system were prepared by treating chromatophores of *Rps. spaeroides* R-26 with Triton X-100, followed by sucrose gradient centrifugation, as described by Sauer and Austin [27]. The particles were dissociated into small units by treatment with 0.15% SDS, just before they were used for measurements.

BChl was extracted from *Rps. sphaeroides* R-26 chromatophores with methanol, concentrated and transferred to pyridine for storage. For the measurements of delayed fluorescence, the BChl was chromatographed on powdered sugar [28].

Flash-induced absorbance changes were measured as described previously [8,12,14]. For measurements in the microsecond time range, a photomultiplier with an extended-red multialkali cathode (EMI 9658R) was used in the 500–900 nm region, a silicon photodiode (EG and G Co. DT-110) in the 900–1 000 nm region and a germanium photodiode (Judson Infrared J-16) in the 1 000–1 800 nm region. An ITT F4102 photomultiplier was used for nanosecond measurements. A Tektronix R7912 transient digitizer with a CP4165 controller was used for signal aquisition, signal averaging, baseline subtractions, and further analysis of the data. Excitation flashes were provided by a ruby laser (694 nm, 20–30 ns pulse width), and by a dye laser that was pumped by the ruby laser. 3,3-Diethylthiatricarbocyanine iodide was used in the dye laser to obtain 834 nm flashes, IR-144 (Eastman) for 857 nm, and DOTC (Exciton Corp.) for 740 nm.

Delayed fluorescence was measured with a gated S-1 photomultiplier (RCA 7102), followed by a high-gain preamplifier [8]. To gate the tube off, the cathode was biased at +65 V with respect to the third dynode, which was operated at about −1 kV. The first and second dynodes were held at −25 V with respect to the third. The tube was gated on for a short interval by switching the cathode bias to −150 V with respect to the third dynode. Trace 1 in Fig. 1 shows the switching artifact resulting from gating the tube on for 10 μs in the dark. For traces 2–5, a solution of the BChl-protein antenna complex from *Rps. sphaeroides* was excited with a 20 ns flash while the tube was gated off. At the time of the flash (upward arrow), the prompt fluorescence from the sample caused a small downward deflection of the trace. The tube then was gated on for 10 μs, starting at 12.5, 20, 30 or 40 μs after the flash. The gating pulse increased the gain of the tube by a factor of about 10^5. Because the response time of the preamplifier was relatively long (about 200 μs), the apparatus integrated the photocurrent due to delayed fluor-

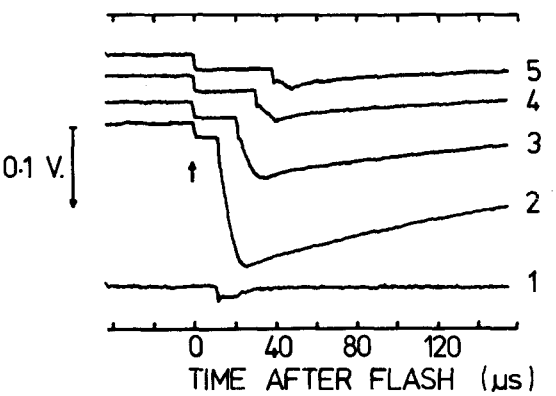

Fig. 1. Delayed fluorescence from an aerobic solution of the antenna complex isolated from *Rps. sphaeroides*. The sample had $A_{853} = 1.0$ and was in 0.15% SDS at 293 K. Fluorescence was detected through a Schott RG-1000 short-wavelength cut-off filter, which transmits beyond 1 μm. Curves 2, 3, 4 and 5: laser flash (857 nm, 20 ns, approx. 6 mJ/cm²) at time zero; photomultiplier gated on for 10 μs beginning 12.5, 20, 30 or 40 μs after the flash, respectively. Fluorescence gives downward deflections of the traces. Curve 1: excitation flash blocked; photomultiplier gated on for 10 μs beginning 12.5 μs after time zero. The downward deflection is an artifact.

escence during the 10 μs gate. We measured the signal at the end of the gate period, and subtracted the baseline due to the prompt fluorescence. The time resolution of the measurement was determined by the width of the gate, rather than by the speed of the amplifier.

Results and Discussion

Decay kinetics, delayed fluorescence, and quenching by O_2

Fig. 2 shows representative measurements of the absorbance changes that accompany the formation of the triplet states of BChl in several different systems. Measurements were made at many different wavelengths for each of these systems, with similar results. The triplet state of monomeric BChl in deoxygenated pyridine/glycerol solution decays with a halftime of about 40 μs at 293 K (curve A). Quenching by O_2 decreases the decay time to a few microseconds in aerobic solutions (not shown). The triplet state of the BChl dimer in the antenna complex isolated from *Rps. sphaeroides* decays with a halftime of about 100 μs in the absence of O_2 (curve B1). This triplet state also is quenched by O_2, but not as severely as the triplet state of free BChl; the halftime in aerobic solutions is about 35 μs (curve B2). In reaction centers of *Rps. viridis* that have Q reduced to Q^-, the triplet state P^R has a decay halftime of about 100 μs at 77 K (curve C). The halftime is about 10 μs at 293 K (not shown).

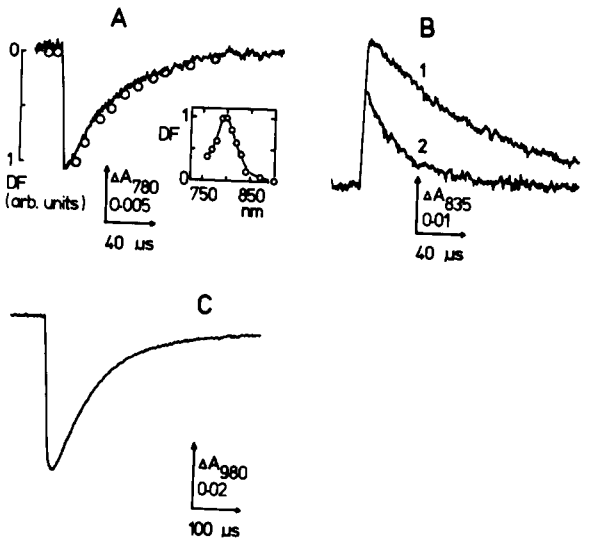

Fig. 2. Kinetics of absorbance changes reflecting formation of triplet states. (A) BChl (A_{780} = 0.32) in anaerobic pyridine/glycerol (1 : 1, w/w), 293 K; excitation at 694 nm (20 ns, 1 mJ/cm^2). The circles near the trace indicate delayed fluorescence (DF) from the sample, measured through an 830 nm interference filter. Inset: uncorrected emission spectrum of the delayed fluorescence, measured through a monochromator. (B) Antenna complex from *Rps. sphaeroides* (A_{853} = 1.6) in 0.15% SDS at 293 K, under anaerobic conditions (trace 1) and in the presence of atmospheric O_2 (trace 2); excitation as in Fig. 1. (C) *Rps. viridis* reaction centers at 77 K (A_{833} = 0.8 at 293 K) in the presence of ascorbate (1 mM) and $Na_2S_2O_4$ (1 mg/ml). Excitation at 740 nm (20 ns, 8 mJ/cm^2).

The lifetime of P^R depends on whether Q^- is present. With *Rps. sphaeroides* reaction centers at 293 K, P^R decays with a halftime of about 12 μs in the presence of Q^-. and 90 μs if Q is reduced to Q^{2-} or removed (along with Fe) by treatment of the reaction centers with SDS (Fig. 3 and Ref. 8). The decay slows down with decreasing temperature, but becomes independent of both temperature and the state of Q about 160 K (Fig. 3). In the high-temperature region, the apparent activation energy for the decay rate constant is 0.095 eV in reaction centers with Q^- and 0.015 eV in those with Q^{2-}. The temperature dependence of the decay in reaction centers containing Q^- has been described previously [14]. A similar activation energy (0.085 eV) was measured in the high-temperature region (Fig. 5 of Ref. 14) but a lower number was reported because of a computational error.

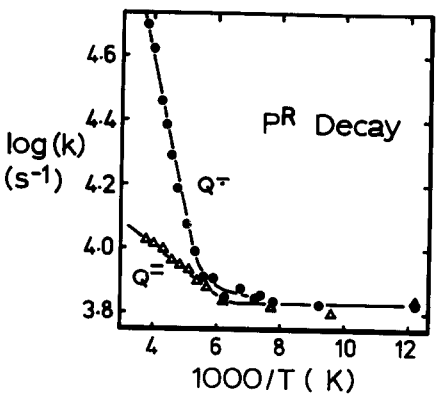

Fig. 3. Temperature dependence of rate constant for the decay of P^R in *Rps. sphaeroides* R-26 reaction centers (A_{802} = 1.0) containing Q^- or Q^{2-}. Excitation at 857 nm (20 ns, 6 mJ/cm^2).

Exchange interactions involving the unpaired electron on the semiquinone could explain the relatively fast decay of P^R in reaction centers containing Q^-. Part of the activation energy could reflect the thermal equilibrium between the two components of P^R, ^3P and $^3[P^+B^-]$. $^3[P^+B^-]$, which lies about 0.03 eV above ^3P [8], could interact more strongly with Q^- than ^3P does, if B is closer to Q than P is. (The exchange interaction between Q^- and B^- also could be temperature dependent). The decay kinetics of P^R at low temperatures put an upper limit of about $8 \cdot 10^3$ s^{-1} on the effective rate constant for spin exchange between ^3P and Q^-. Using an empirical expression described by Likhtenstein et al. [29], this corresponds to a minimal edge-to-edge distance of about 13 Å between the two species.

In reaction centers of *Rps. sphaeroides*, P^R is not quenched by O_2. This point was investigated with reaction centers that were treated with SDS to remove Q. Reaction centers of *Rps. viridis* differed in that O_2 did accelerate the decay of P^R slightly in these.

Anaerobic solutions of BChl or of *Rps. sphaeroides* reaction centers emit a weak delayed fluorescence with a lifetime comparable to that of the excited triplet states [8]. The open circles in Fig. 2A show the decay kinetics of the delayed fluorescence from a deoxygenated BChl solution. The inset in this figure shows the emission spectrum, which is essentially the same as that of the prompt fluorescence from the excited singlet state (not shown).

The intensity and lifetime of the delayed fluorescence from reaction centers depend on the redox state of Q (Fig. 4). The measurements summarized in this figure were made at 920 nm, in the time interval from 12.5 to 22.5 μs after excitation with laser flashes at 857 nm. As expected, no delayed fluorescence is observed when Q is in the oxidized state. Under these conditions, the flash results in electron transfer to the quinone and P^R is not formed [14]. When Q is reduced to Q^- by the addition of ascorbate and $Na_2S_2O_4$, one sees delayed fluorescence. We could not measure the lifetime of this luminescence accurately, but is similar to the 12 μs lifetime of P^R. When Q^- is reduced further to Q^{2-} by illumination in the presence of reduced cytochrome *c*, the decay halftimes of P^R and the delayed fluorescence increase to about 90 μs and are the same within experimental

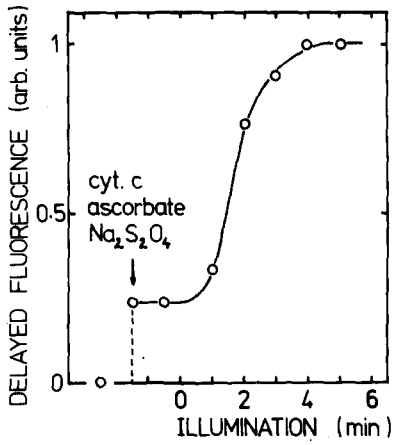

Fig. 4. Delayed fluorescence measured through a 920 nm interference filter in the 12.5–22.5 μs interval after excitation of *Rps. sphaeroides* R-26 reaction centers (A_{860} = 1.1) with Q in different redox states (see text for details). Excitation at 847 nm (20 ns, 6 mJ/cm^2). cyt. c, cytochrome *c*.

error [8]. The delayed fluorescence measured in the 12.5–22.5 μs time interval increases by a factor of about 4. Delayed fluorescence with a similar lifetime and intensity is seen in reaction centers that are depleted of Q and Fe by treatment with SDS [8]. The intensity and lifetime of the delayed fluorescence from the depleted reaction centers are not affected by the presence of O_2. This agrees with the observation that O_2 does not quench P^R in reaction centers from *Rps. sphaeroides*.

The quenching of the triplet state of free BChl by O_2 results in the promotion of O_2 to its excited singlet ($^1\Delta_g$) state, which luminesces at 1.25 μm [30, 31]. Aerobic solutions of free BChl or of the BChl-protein antenna complex from *Rps. sphaeroides* gave a short-lived, delayed emission in this wavelength region (Fig. 1), but aerobic, Q-depleted reaction centers from *Rps. sphaeroides* did not. In addition, aerobic solutions of BChl or of the antenna complex gave a short-lived, but relatively strong delayed fluorescence that had the same emission spectrum as the prompt fluorescence from these samples. This delayed fluorescence may result from collisions between triplet BChl and singlet O_2, which could generate excited singlet BChl and ground state (triplet) O_2.

The delayed fluorescence seen in the absence of O_2 probably results from thermal repopulation of the

excited singlet state [8]. With both free BChl and reaction centers of *Rps. sphaeroides*, the delayed fluorescence has an activation energy of 0.40 ± 0.02 eV [8]. This means that the triplet state of free BChl lies about 1.15 eV above the ground state, and ^3P about 0.98 eV above the ground state of P. Singlet O_2 lies about 1.00 eV above the ground state [30, 31]. This evidently is far enough below 1.15 eV to allow O_2 to quench the triplet state of free BChl effectively, but not low enough to allow effective quenching in *Rps. sphaeroides* reaction centers.

Absorption spectra

Fig. 5A and B shows spectra of the absorbance changes that accompany the formation of the triplet states of free BChl and of the BChl-protein antenna complex. The formation of the triplet state of BChl in pyridine/glycerol solution causes a bleaching of the Q_y absorption band at 780 nm (Fig. 5A and Refs. 13 and 32). The Q_x band at 600 nm also bleaches [32], but there are only very small absorbance changes at

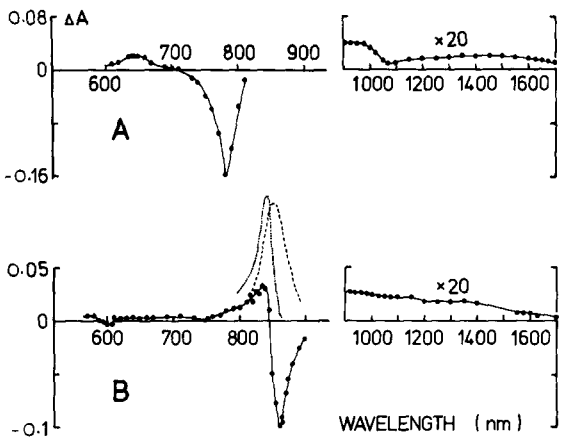

Fig. 5. (A) Difference spectrum of absorbance changes accompanying formation of the triplet state of BChl *a* (A_{780} = 0.7) in anaerobic pyridine at 293 K. Excitation at 740 nm (20 ns, 8 mJ/cm^2) for measurements at wavelengths between 600 and 730 nm, and at 694 nm (20 ns, 10 mJ/cm^2) for longer wavelengths; normalized at 730 nm. (B) Absorbance changes accompanying formation of the triplet state of the BChl dimer in the antenna complex from *Rps. sphaeroides* (A_{853} = 1.3) under anaerobic conditions at 293 K. Excitation at 834 nm for the 850–865 nm region, and at 857 nm (20 ns, 6 mJ/cm^2) for other wavelengths. (– – –) Absorption of the sample before excitation. (· · · · · ·) Calculated absorption spectrum of the complex in the triplet state (see text).

wavelengths between the Q_x and Q_y bands, and in the 1.0–1.7 μm region. The absorbance changes seen with the BChl dimer in the antenna complex are significantly different. The main absorption band of the complex is at 853 nm (dashed curve in Fig. 5B). Formation of the triplet state causes a bleaching on the long-wavelength side of this band, but on the short-wavelength side one sees an absorbance increase peaking at 835 nm (filled circles in Fig. 5B).

To calculate the absorption spectrum of the antenna complex in the triplet state, we added the normalized absorbance changes to the original spectrum. For the normalization, we assumed that complete conversion into the triplet state would cause complete bleaching on the long-wavelength side of the Q_y band. The triplet-state absorption spectrum calculated in this way has a well defined band at 840 nm (dotted curve in Fig. 5B). The area under the new band is about 70% of that under the original band, but the uncertainty in the normalization procedure limits the significance of this comparison.

A reasonable interpretation of these observations is that the triplet state of the antenna dimer consists essentially of one molecule in a triplet state, and one in the ground state, as discussed in the Introduction. The ground-state molecule evidently has an absorption band centered at a slightly shorter wavelength than the original band of the dimer. This interpretation agrees with the recent finding by Rafferty et al. [33] that, when one of the molecules is destroyed by prolonged illumination in the presence of O_2, the remaining molecule absorbs at wavelengths about 7 nm shorter than those of the original band.

Fig, 6A shows spectra of the absorbance changes that result from the formation of triplet states in reaction centers from *Rps. viridis*. At 293 K, the absorbance changes include a bleaching of P's main absorption band at 960 nm and the formation of a new band near 830 nm (Fig. 6A). The absorbance changes in the 830–850 nm region are more complex at low temperatures (Fig. 6B). At 77 K, the formation of P^R causes absorbance increases at 830 and 842 nm, and decreases at 852 and 980 nm.

In line with the discussion above, we suggest that the absorbance increase at 830 nm is due predominantly to an absorption band of a ground-state monomeric BChl in ^3P. As mentioned in the Introduction, a new absorption band at 808 nm is formed

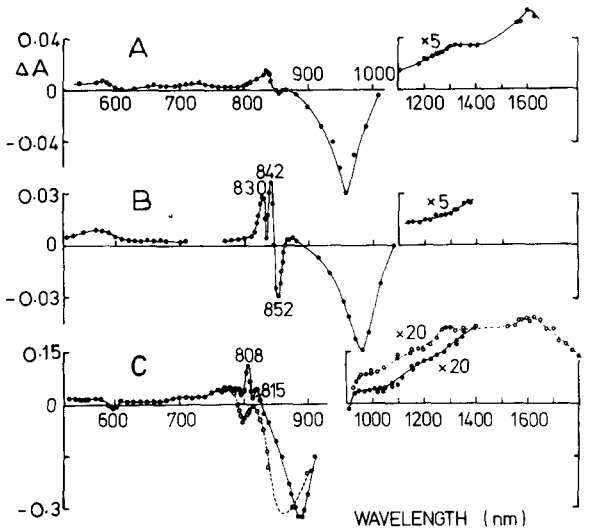

Fig. 6. (A) Difference spectrum of absorbance changes accompanying the formation of P^R in *Rps. viridis* reaction centers (A_{833} = 0.8) in the presence of 0.15% SDS at 293 K. Excitation at 857 nm (20 ns, 6 mJ/cm^2) for the 710–840 nm region and at 834 nm (20 ns, 6 mJ/cm^2) for other wavelengths. (B) Same as A except at 77 K, in the absence of SDS and the presence of ascorbate and $Na_2S_2O_4$. Excitation at 857 nm (20 ns, 2 mJ/cm^2) for the 1 140–1 380 nm region, and at 740 nm (20 ns, 1.5 mJ/cm^2) for other wavelengths. (C) Difference spectrum of absorbance changes accompanying the formation of P^R in *Rps. sphaeroides* R-26 reaction centers (A_{802} = 1.1 at 293 K) in the presence of ascorbate and $Na_2S_2O_4$, at 77 K (•———•) and at 293 K (○– – –○). Excitation at 740 nm (20 ns, 2 mJ/cm^2) for the 840–1 400 nm region and at 857 nm (20 ns, 1 mJ/cm^2) for other wavelengths. Some of the data shown with open circles are taken from Ref. 8.

when P undergoes oxidation to P^+. That band has been attributed to monomeric BChl in the unoxidized state. Interactions with the other (oxidized) molecule could shift the absorption band in P^+ relative to its position in ^3P.

The separation between the 830 nm band in ^3P and the 960 nm absorption band of P is much greater than the separation between the bands at 840 and 853 nm in the antenna complex. If our interpretation of the 830 nm band is correct, this implies that the exciton interaction between the two BChls in P is considerably stronger than that in the antenna complex. If the exciton splitting is symmetric on either side of 830 nm, the higher exciton band would be at 730 nm. The reaction centers do not have an absorption band in this region [21,22], possibly because the geometry of P is such that almost all of the dipole strength goes to the 960 nm band.

The observation that the formation of P^R at 293 K causes only small absorbance changes in the 850 nm region (Fig. 6A) suggests that P does not have a strong absorption band near 850 nm. The sharp positive and negative absorbance changes seen at 842 and 852 nm at 77 K could be explained by a small blue shift of an absorption band near 850 nm, if this band is due to one of the BChls other than P, as suggested by Shuvalov et al. [24,26]. A shift of the band could result from changing interactions between this molecule and P. This would agree with the view that the oxidation of P to P^+ causes a blue shift of the 850 nm absorption band [24,26]. A small band shift would cause much more pronounced absorbance changes at 77 K than at 293 K, because the absorption band at 850 nm is considerably sharper at the lower temperature [21,22]. The absorbance changes seen at 293 K also could include a contribution from a band shift at about 850 nm, but they may be complicated by absorbance changes due to $^3[P^+B^-]$.

The apparent shift of the 850 nm band in P^R means that the BChl that absorbs here interacts closely with P. This is significant, because the BChl (B) that has been implicated in electron transfer between P and H does not absorb at 850 nm, but rather at 830 nm [12,24,25]. The triplet spectrum (Fig. 6B) may also include contributions from a band shift near 830 nm; this could account for part of the absorbance increase at 830 nm and the trough at 835 nm. But the shift of the 850 nm band appears to be particularly pronounced. This suggests that the 850 nm BChl is at least as close to P as B is, and that it could participate in electron transfer from P to B.

Fig. 6C shows measurements made with *Rps. sphaeroides* reaction centers at 77 K (filled circles) and 293 K (open circles). At 77 K, the formation of P^R causes a bleaching of P's absorption bands at 880 and 600 nm, and small absorbance increases in the 620–820 nm region. The spectrum is similar to one obtained previously at this temperature [8], except that it shows additional fine structure in the 795–820 nm region. There are small troughs at 798 and 815 nm, and peaks at 808 and 820 nm. These features were less pronounced in the data reported previously, probably because the measurements were

made with lower spectral resolution.

As in *Rps. viridis*, the formation of ^3P in *Rps. sphaeroides* at 77 K does not cause a bleaching in the 810–815 nm region, where the higher energy exciton band of P has been suggested [16,17] to lie. There actually is a small absorbance increase here. Part of the fine structure in the 795–820 nm region could be due to the formation of $^3[P^+B^-]$. The reduction of B causes a bleaching at 798 nm that is quite pronounced at higher temperatures (Ref. 8 and open circles in Fig. 6C), and this would still be significant at 77 K. The peak at 808 nm and the trough at 815 nm could be explained by a blue shift of an absorption band near 810 nm. This could be analogous to the shift of the 850 nm band in *Rps. viridis* (Fig. 6A). As in *Rps. viridis*, the BChl of which the spectrum appears to be most strongly perturbed by the formation of ^3P is not the BChl that has been implicated previously in electron transfer. Again, this suggests that the fourth BChl could participate in electron transfer from P to B.

The absorbance changes seen in *Rps. sphaeroides* reaction centers differ qualitatively from those seen in *Rps. viridis* reaction centers or in the antenna complex, in that there is no well defined new absorption band. Instead, a very broad band appears to underlie the small peaks and thoughs discussed above, extending from about 740 to 825 nm. The reason for this difference is unclear. Perhaps the triplet excitation hops between the two BChls of P so rapidly that ^3P cannot be described adequately as a simple combination of one excited molecule and one molecule in the ground state. The difference between the optical spectra of ^3P in *Rps. sphaeroides* and *Rps. viridis* then would be consistent with previous studies of the EPR and ENDOR spectra of P^+ which have suggested that electron delocalization between the two molecules may be stronger in *Rps. sphaeroides* than it is in *Rps. viridis* [34]. However, Davis et al. [35] recently have pointed out that the electron delocalization could be similar in the two species, because the differences in the EPR and ENDOR spectra could reflect different degrees of twisting in the bacteriochlorophylls.

In both *Rps. viridis* and *Rps. sphaeroides* reaction centers, the formation of P^R causes absorbance increases in the 1.0–1.7 μm region (Fig. 6). These are considerably larger than the absorbance increases seen in this region with free BChl or with the antenna complex (Fig. 5). At 293 K, the absorbance changes include a shoulder at about 1 260 nm in *Rps. sphaeroides* (Fig. 6C, open circles) and 1 300 nm in *Rps. viridis* (Fig. 6A). This can be explained by the presence of $^3[P^+B^-]$, because P^+ has absorption bands at these wavelengths [8]. The shoulders are not seen at 77 K, when P^R probably is predominantly in the form ^3P [8] (Fig. 6B and closed circles in Fig. 6C). The broad absorption band that remains at 77 K could represent excitation to the second locally excited triplet state. This transition has little dipole strength in monomeric BChl [36], but it could become more strongly allowed as a result of interactions between the two BChls of P, or between P and its environment. Another possibility is that the absorption band represents a charge-transfer transition, in which an electron moves from one molecule of BChl in ^3P to the other, or from ^3P to one of the other BChls. A similar suggestion has been offered to explain the 1 260 nm band of P^+ [8].

After this work had been completed, we learned that Dr. A. Vermeglio had made very similar absorbance measurements on the triplet states of the *Rps. sphaeroides* antenna complex and *Rps. viridis* reaction centers.

A new state generated by supersaturating flashes

The studies on reaction center described above were performed with electron transfer from H^- to Q blocked by the prior reduction or extraction of Q. The radical-pair state (P^F) that is generated by the flash under these conditions appears to be an equilibrium mixture of $^1[P^+B^-]$ and $^1[P^+H^-]$ [8]. If the flash intensity is subsaturating, P^F decays with a lifetime of 10–20 ns, depending on the temperature and on the state of Q [8,14]. One of its decay paths leads to P^R; one probably leads from $^1[P^+B^-]$ directly to the ground state; and there may also be decay paths involving triplet states other than P^R (Schenck, C.C., Blankenship, R.E. and Parson, W.W., unpublished data). If the flash intensity is increased so that the reaction centers are excited with a second photon while they are still in state P^F, P is converted transiently into another state that has not been described previously. The inset in Fig. 7 shows representative measurements of the formation and decay of this state. The upper trace is a profile of the excitation flash. The middle and lower traces show the flash-

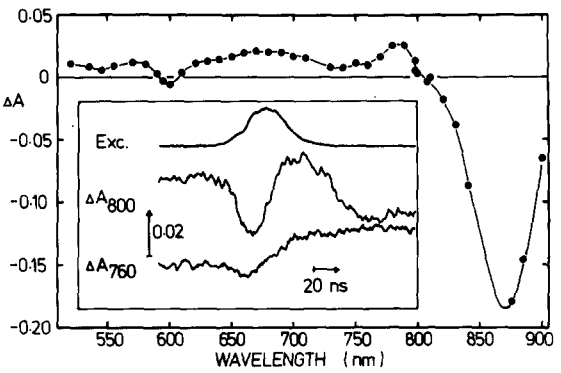

Fig. 7. Inset: Kinetics of absorbance changes at 760 and 800 nm during excitation of *Rps. sphaeroides* R-26 reaction centers ($A_{802} = 0.8$) in the presence of ascorbate and $Na_2S_2O_4$ at 293 K. The upper trace shows the excitation (Exc.) profile (857 nm, 6 mJ/cm^2). Spectrum: absorbance changes measured 24 ns after the centers of the excitation flashes, as in the inset. The baseline for the absorbance change is taken before the flash, when the reaction centers are still in the ground state.

induced absorbance changes at 800 and 760 nm, respectively. These measurements were made at 293 K, with *Rps. sphaeroides* reaction centers containing Q^-. At 800 nm, the flash initially causes an absorbance decrease that is probably due to the formation of $^1[P^+B^-]$ [8]. The new state forms later during the flash, and results in an absorbance increase. After the flash, the new state decays with a halftime of about 20 ns. The final absorbance change is negative, as expected for the formation of P^R (cf. open circles in Fig. 6C). At 760 nm, the flash first causes a small absorbance decrease that probably reflects the formation of $^1[P^+H^-]$ [8]. The new state and P^R both give absorbance increases at this wavelength.

The filled circles in Fig. 7 show the spectrum of the absorbance changes that accompany the formation of the new state. The absorbance changes include a bleaching of P's absorption bands at 600 and 870 nm, and the development of broad absorption bands from 620 to 795 nm. The spectrum differs in many ways from that of state P^F (cf. Fig. 2 in Ref. 8). It is closer to the spectrum that we ascribe to ^3P, differing from it mainly in having a larger absorbance increase in the 670 nm region. The spectrum also is similar to one that was obtained recently by picosecond excitation of reaction centers that had H in the reduced form [37]. The transient state seen under those conditions had a much shorter lifetime (340 ps), and it could be generated by excitation with subsaturating flashes.

The identification of the new state is not clear. The absorption band at 670 nm suggests the formation of an anion radical of BChl or BPh [11], but the lack of a bleaching at 545, 760 or 800 nm indicates that no BChl or BPh other than P is involved. From the bleaching at 870 nm, the state clearly does involve P. One possibility, which was suggested previously for the 340 ps state seen in strongly reduced reaction centers [37], is a charge-transfer state in which one BChl molecule of P is oxidized and the other reduced. Such a state might form upon the absorption of a second photon by the return of an electron from B^- or H^- to P^+; it could be either a singlet or a triplet state, and its lifetime could depend on the conditions of the other components of the reaction center.

The major relevance of the new state to the other observations presented above is that the formation of the state causes little or no bleaching in the 810 nm region. This agrees with the view that P does not have a major absorption band here. In addition, may of the measurements on reaction centers described above were made with relatively strong excitation flashes, so that part of the P^R evidently was formed by way of the new state. The absorption spectrum and decay kinetics of the P^R that is formed in this way appear to be identical to those of the P^R that is formed with subsaturating flashes.

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