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HIGH-RESOLUTION OPTICAL ABSORPTION-DIFFERENCE SPECTRA OF THE TRIPLET STATE OF THE PRIMARY DONOR IN ISOLATED REACTION CENTERS OF THE PHOTOSYNTHETIC BACTERIA *RHODOPSEUDOMONAS SPHAEROIDES* R-26 AND *RHODOPSEUDOMONAS VIRIDIS* MEASURED WITH OPTICALLY DETECTED MAGNETIC RESONANCE AT 1.2 K

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We have recorded triplet optical absorption-difference spectra of the reaction center triplet state of isolated reaction centers from *Rhodopseudomonas sphaeroides* R-26 and *Rps. viridis* with optical absorption-detected electron spin resonance in zero magnetic field (ADMR) at 1.2 K. This technique is one to two orders of magnitude more sensitive than conventional flash absorption spectroscopy, and consequently allows a much higher spectral resolution. Besides the relatively broad bleachings and appearances found previously (see, e.g., Shuvalov V.A. and Parson W.W. (1981) Biochim. Biophys. Acta 638, 50–59) we have found strong, sharp oscillations in the wavelength regions 790–830 nm (*Rps. sphaeroides*) and 810–890 nm (*Rps. viridis*). For *Rps. viridis* these features are resolved into two band shifts (a blue shift at about 830 nm and a red shift at about 855 nm) and a strong, narrow absorption band at 838 nm. For *Rps. sphaeroides* R-26 the features are resolved into a red shift at about 810 nm and a strong absorption band at 807 nm. We conclude that the appearance of the absorption bands at 807 and 838 nm, respectively, is due to monomeric bacteriochlorophyll. Apparently, the exciton interaction between the pigments constituting the primary donor is much weaker in the triplet state than in the singlet state, and at low temperature the triplet is localized on one of the bacteriochlorophylls on an optical time scale. The fact that for *Rps. sphaeroides* the strong band shift and the monomeric band found at 1.2 K are absent at 293 K and very weak at 77 K indicates that these features are strongly temperature dependent. It seems, therefore, premature to ascribe the temperature dependence between 293 and 77 K of the intensity of the triplet absorption-difference spectrum at 810 nm (solely) to a delocalization of the triplet state on one of the accessory bacteriochlorophyll pigments.

Introduction

In purple bacteria, illumination with visible or near-infrared light generates two separated charges:

$$\mathrm{P\,BPh\,Q} \xrightarrow[\lesssim 10\ \mathrm{ps}]{h\nu} \mathrm{P^+\,BPh^-\,Q} \xrightarrow[200\ \mathrm{ps}]{} \mathrm{P^+\,BPh\,Q^-} \qquad (1)$$

where P represents the primary electron donor P-890 (*Rhodopseudomonas sphaeroides*) or P-990 (*Rps. viridis*) (the number refers to the maximum of the longest-wavelength absorption band in nanometers at cryogenic temperatures), a bacteriochlorophyll dimer [1]. BPh is the 'first' electron acceptor, a bacteriopheophytin monomer [2–4] and Q is the secondary or first stable acceptor, a ubiquinone- or menaquinone-iron complex [5,6]. The chemical free energy stored in the radicals is used for metabolic reactions in the cell on further electron transport. When Q is chemically

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; Mops, 4-morpholinepropanesulfonic acid; LDAO, lauryldimethylamine *N*-oxide; ADMR, optical absorption-detected electron spin resonance in zero magnetic field.

reduced before illumination electron transport from BPh^- to Q^- is severely hampered and the back-reaction

$$P^+ BPh^- Q^- \rightarrow P^T BPh Q^- \quad (2)$$

generates the triplet state of the primary donor, P^T, with a yield close to unity at cryogenic temperatures [2,7,8].

The assignment of the reactants in reaction 2 rests primarily on results of studies on isolated reaction centers of *Rps. sphaeroides* R-26 with flash spectroscopy [2,9–12]. A flash of light generates a state of the reaction center, called P^F, characterized by an optical absorption-difference spectrum that has been interpreted to be composed of the sum of an oxidized P-890 and reduced BPh [2,3]. This state decays with a half-time of 10–20 ns (depending on the temperature [2]) to another state, called P^R, with an optical difference spectrum resembling that of ^{1}BChl *a* $-$ ^{3}BChl *a* [2]. Hence, P^R was identified as the triplet state of P-890 resulting from the back-reaction [2]. This identification was strengthened by the earlier discovery of a photoinduced triplet state in reduced reaction centers by ESR [7], with a spin-polarization pattern [13] characteristic of a triplet state generated by radical recombination [14,15] and with similar decay kinetics at liquid helium temperatures to P^R [16,17], and by the discovery of a magnetic field effect on the yield of P^R [18,19].

Recently, a study of the optical difference spectrum of P^R as a function of temperature (300–77 K) revealed changes around 800 nm that were attributed to P^R being a mixture of two triplet states, one located on P-890 and one on an acceptor intermediate between P-890 and BPh [20]. This intermediate acceptor has been postulated on the basis of picosecond-flash spectroscopy to be one of the accessory B-800 BChl pigments in the reaction center [21]. At low temperatures, the triplet would be trapped on P-890, the activation energy of the temperature dependence of the temperature-induced change at 800 nm leading to an energy difference between P-890^T and P-800^T of 0.030 eV [20].

In view of the importance that the characteristic properties of P^R have for our understanding of the primary reactions in bacterial photosynthesis, a precise measurement of the absorption-difference spectrum P-890 (B-800) BPh Q^- $-$ P-860^T (B-800) BPh Q^- is clearly of much value. We have employed the newly introduced technique of magnetic resonance in zero magnetic field detected by the singlet ground-state absorption (ADMR) [22] to measure in isolated reaction centers of *Rps. sphaeroides* R-26 and of *Rps. viridis* this absorption difference spectrum at 1.2 K with a sensitivity that is more than an order of magnitude better than that obtainable with flash spectroscopy. The general aspects of the P^R spectra of both bacterial species investigated are similar to those obtained previously [2,20,23]. In reaction centers of *Rps. sphaeroides* R-26, bleachings at 890, 600 and 385 nm are observed together with the appearance of broad, rather unstructed bands between 410 and 580 nm and between 740 and 790 nm. The P^R spectrum of reaction centers of *Rps. viridis* shows bleachings at 400 and 1000 nm, while at 625 nm a band is present that may represent a bleaching partly masked by a triplet-triplet absorption band. Between 430 and 600 nm and between 710 and 790 nm broad bands appear. Surprisingly, in addition to those general features, we observe in both species new, strong and exceedingly sharp oscillations in the P^R spectrum between 790 and 830 nm (*Rps. sphaeroides*) and between 810 and 890 nm (*Rps. viridis*). To a first approximation, these features seem to be composed of two band shifts and the appearance of one sharp absorption band. The absence of these features in previously published spectra [20,23] measured between 300 and 77 K is probably at least partly due to the lower spectral resolution attainable by the inherently less sensitive flash technique. In addition, there may be temperature and site effects.

We ascribe the appearance of the strong narrow band at 807 and 838 nm for reaction centers of *Rps. sphaeroides* R-26 and *Rps. viridis*, respectively, to the absorption of a monomeric BChl of the dimeric primary donor in which the original singlet-singlet exciton interaction is broken by the formation of a triplet in which the triplet-triplet exciton interaction is much weaker.

Materials and Methods

Absorption-difference spectra

Singlet ground-state absorption-detected magnetic resonance in zero magnetic field (ADMR) was performed as described in Ref. 22. Briefly, a sample is continuously excited by broad banded light, resulting in a steady-state distribution of P-890, or P-990 over the ground-state S_0 and the triplet states T_x, T_y, T_z (Fig. 1). The equilibrium concentration of S_1 and other higher excited levels is negligible. When the triplet is irradiated with microwaves resonant between two of the three sublevels (which in general have different decay rates) at temperatures where spin-lattice relaxation is inhibited, the population of the levels is changed which indirectly results in a slight change in the entire equilibrium distribution. The perturbation in concentration of S_0 is detected via a small change in the transmittance. The state S_0 may be the single ground state of the molecule excited to the triplet state, but also the singlet ground state of molecules that in one way or another are perturbed by the presence of a triplet state on the primary donor. Hence, using microwaves of constant power at fixed frequency corresponding to one of the transitions between the levels T_x, T_y, T_z, and scanning the wavelength of the detecting light while keeping the photomultiplier current constant (see below), one records the optical absorption-difference spectrum of P^R.

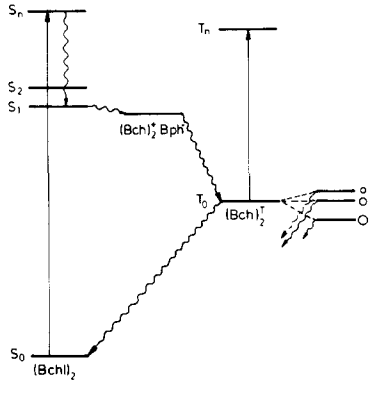

Fig. 1. Energy-level scheme of the special pair BChls. The triplet sublevels are shown on an enlarged scale. The relative populations, and the decay rates of the three triplet sublevels are indicated by open circles and arrows, respectively. By the application of microwaves resonant between two of the three triplet sublevels, the stationary triplet state concentration is decreased. With ADMR one monitors the changes in absorption brought about by converting part of the reaction centers from the triplet state to the singlet state. The triplet is assumed to be localized on one of the constituent pigments of the dimer.

Referring to Fig. 1, we can write for the intensity of the transmitted light I_{Tr} as a function of the wavelength λ:

$$I_{Tr}(\lambda) = I_{exc}\exp\left[-\left\{C_S(\lambda)[S_0] + C_T(\lambda)[T_0] + {}^TC_S(\lambda) \times \left[{}^TS_0\right] + D(\lambda)\right\}\right] \quad (1)$$

with $C_i(\lambda)$ constants depending on instrumental characteristics and reaction center concentration and $D(\lambda)$ the contribution of pigments unreactive to the presence of the triplet state; square brackets denote the fractional concentration; ${}^TS_0$ represents those singlet ground states that are correlated with the presence of the triplet state P-890^T (or P-990^T), such as new levels, shifted levels, etc., so that: that:

$$\left[{}^TS_0\right] = [T_0] \quad (2)$$

moreover:

$$[S_0] + [T_0] = 1 \quad (3)$$

Application of the resonant microwaves changes the (fractional) triplet concentration by $[\Delta T_0]$, hence, the singlet ground-state concentration by $-[\Delta T_0]$, and the ${}^TS_0$ concentration by $[\Delta T_0]$ (note the difference in sign). Thus, by a change of $[\Delta S_0]$ in the single ground-state concentration, we have for the change in the transmitted light:

$$\Delta I_{Tr}(\lambda) = I_{Tr}(\lambda) - I_{Tr}^{micr}(\lambda) = I_{exc}\exp\left[-\{A(\lambda) + D(\lambda)\}\right] \times \left(1 - \exp\left[-[\Delta S_0]\{C_S(\lambda) - C_T(\lambda) - {}^TC_S(\lambda)\}\right]\right) \quad (4)$$

with $A(\lambda) = C_S(\lambda)[S_0] + C_T(\lambda)[T_0] + {}^TC_S[{}^TS_0]$. The second exponent in Eqn. 4 is under our conditions of measurement always smaller than 0.01. Taking a Taylor expansion and retaining the first two terms we get:

$$\Delta I_{Tr}(\lambda) = I_{exc}\exp\left[-\{A(\lambda) + D(\lambda)\}\right] \times \left[[\Delta S_0]C_S(\lambda) - C_T(\lambda) - {}^TC_S(\lambda)\right] \quad (5)$$

and for the relative change in transmitted light:

$$\Delta I_{Tr}^{rel}(\lambda)=\frac{\Delta I_{Tr}(\lambda)}{I_{Tr}(\lambda)}=[\Delta S_0]\{C_S(\lambda)-C_T(\lambda)-{}^{T}C_S(\lambda)\} \quad (6)$$

The constants $C_i(\lambda)$ are proportional to the products of ϵ, the molar extinction coefficient, the optical path length and the reaction center concentration, so that we obtain:

$$\Delta I_{Tr}^{rel}(\lambda)\propto\{\epsilon_S(\lambda)-\epsilon_T(\lambda)-{}^{T}\epsilon_S(\lambda)\}[\Delta S_0]. \quad (7)$$

To enhance the signal-to-noise ratio, the microwaves are amplitude modulated and the signal is detected by lock-in amplification. This yields $\Delta I_{Tr}(\lambda)$; keeping the photomultiplier current output constant we correct for the wavelength dependence of the instrumental sensitivity and the number of quanta absorbed, so that by scanning the wavelength the true absorption-difference spectrum is obtained.

Instrumental Procedure

The 3-mm quartz tube containing the sample is placed in a four-window cryostat and cooled to 1.2 K by liquid helium under low pressure. It is enveloped by a helix containing the microwave field, and illuminated in front via a 10 cm water filter by a 24 V, 250 W tungsten-iodine lamp driven by a current-stabilized power supply (Oltronix B32-20R). Detection is via the back window, a Bausch and Lomb (f/4.4) 0.5 m monochromator (calibrated with a low-pressure mercury lamp) and an S1-type photomultiplier (EMI 9684B) cooled to −50°C or an extended red S20-type photomultiplier (EMI 9659B). ADMR spectra are recorded using amplitude modulation of the microwave power (frequency 665Hz; HP switch 33016 B) and lock-in amplification (PAR 5101) of the photomultiplier output. The microwaves are generated by an HP 8960 B sweep oscillator with an 8699 B insert and amplified by either a Varian solid-state amplifier VSP-7435-KL-496 to a level of 1 W or a Microwave Power Devices solid-state amplifier to a level of 10 W. The frequency is measured by an HP 5246 L counter with an HP 5245C frequency converter.

Sample preparation

Reaction centers of *Rps. sphaeroides* R-26 were prepared following the procedure of Feher and Okamura [24]. Reaction centers of *Rps. viridis* were prepared by the following procedure. Chromatophores were suspended in 0.01 M Tris-HCl buffer, pH 8.0, to an absorbance at 1015 nm of 25 in 10 mm and incubated for 5–7.5 min with 5% LDAO at room temperature. Then the suspension was layered on top of a linear 0.1–1.0 M sucrose gradient in 0.01 Tris-HCl buffer, pH 8.0, containing 1 mM EDTA and 0.1% LDAO and centrifuged for 17 h at 175000 × *g*.

The reaction centers of *Rps. sphaeroïdes* R-26 and *Rps. viridis* were passed once or twice over a DEAE column by elution with an NaCl gradient; their optical absorbance spectrum confirmed to that in the literature [24,25], with an A_{280}/A_{800} ratio (*Rps. sphaeroides* R-26) of 1.28 ± 0.02 for one and 1.18 ± 0.02 for two DEAE passages and with an A_{280}/A_{800} ratio (*Rps. viridis*) of 2.35 ± 0.1 for one and 2.03 ± 0.03 for two DEAE passages. The reactions centers of *Rps. viridis* contained only small amounts of carotenoids (see also Ref. 24). After chromatography the samples were concentrated in 10 mM Mops buffer, pH 8, to the required absorbance. The samples were diluted to 65% (v/v) with ethylene glycol to obtain optically clear samples upon freezing slowly to 77 K (absorbance 0.3 in 3 mm at the longest-wavelength absorption band). Reduction of the primary acceptor of reaction centers of *Rps. sphaeroides* R-26 was carried out by addition of 10 mM sodium dithonite under anaerobic conditions. The reaction centers of *Rps. viridis* were frozen under continuous light excitation after the addition of 10 mM sodium ascorbate.

Results

In Figs. 2 and 3 the P^R spectrum of isolated reaction centers of *Rps. sphaeroides* R-26 is shown; figs. 4 and 5 show the P^R spectrum of isolated reaction centers of *Rps. viridis*. We measured the P^R spectrum with the ADMR technique [22] point by point with a time constant of the lock-in amplifier of 3 s for a period of 20 s per point. The sign of the microwave transitions at the longest-wavelength absorption band of both bacterial species corresponds to a decrease in transmittance, i.e., an increase in the ground-state population of

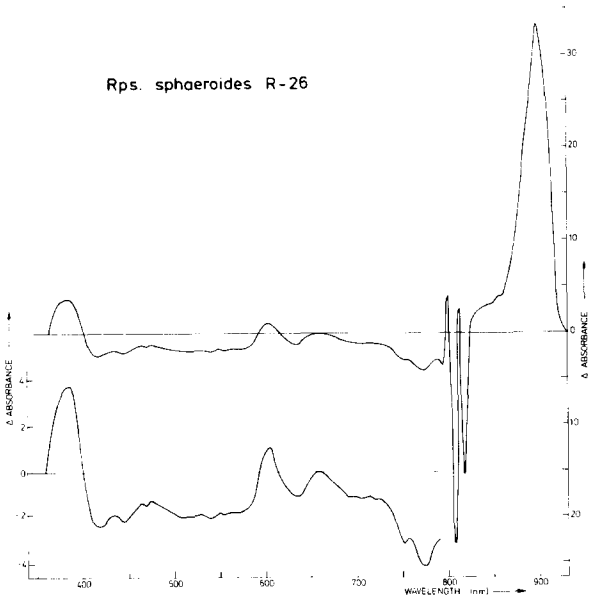

Fig. 2. The absorption-difference spectrum of isolated reaction centers of *Rps. sphaeroides* R-26 recorded by ADMR at 1.2 K with resonant microwaves at 656 MHz. The figure represents a P-890^T – P-890 spectrum. The optical resolution is 3.2 nm in the 420–930 nm range and 6.4 nm in the 360–420 nm range. For further experimental details see text.

P (Ref. 22, and unpublished results). The broad features of the P^R spectra of both bacterial species investigated are quite similar to those obtained previously [2,20,23]. In reaction centers of *Rps. sphaeroides* R-26 bleachings at 900, 600 and 385 nm are observed, together with the appearance of broad, rather unstructured bands between 410 and 580 nm and between 740 and 790 nm. The P^R spectrum of reaction centers of *Rps. viridis* shows bleachings at 400 and 1000 nm, while at 625 nm a band is present that may represent a bleaching partly masked by a triplet absorption band. Between 430 and 600 nm and between 710 and 790 nm broad, rather unstructured bands appear. The width and maximum of the bleaching at 900 nm (*Rps. sphaeroides* R-26) and 1000 nm (*Rps. viridis*) in the P^R spectrum differ from the longest-wavelength absorption band, because they depend somewhat on the frequency of the resonant microwave field (unpublished results). We explain these small differences by so-called site-dependent properties of the reaction centers. A site is defined as an environment of the pigments which gives rise to a particular set of zero-field splitting parameters $|D|$ and $|E|$, hence a particular set of microwave frequencies. The broad bands of the P^R spectrum of reaction centers of *Rps. sphaeroides* R-26 and *Rps. viridis* are similar to those previously measured [20,23], albeit that our technique allows the determination of a much more detailed structure.

Surprisingly, in addition to the broad features, we observe strong and exceedingly sharp oscilla-

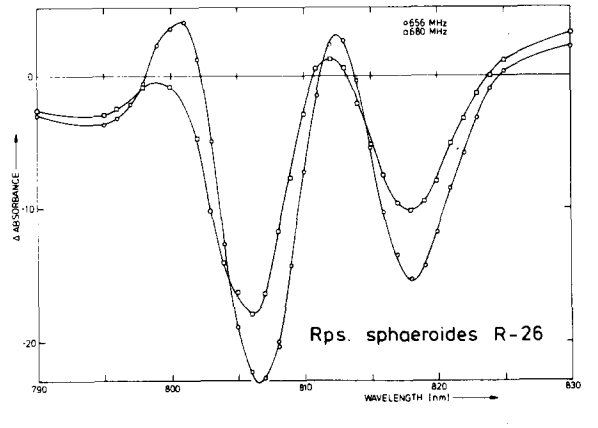

Fig. 3. The absorption-difference spectrum of isolated reaction centers of *Rps. sphaeroides* R-26 recorded by ADMR at 1.2 K with resonant microwaves at 656 MHz (○) or 680 MHz (□). The optical resolution is 3.2 nm.

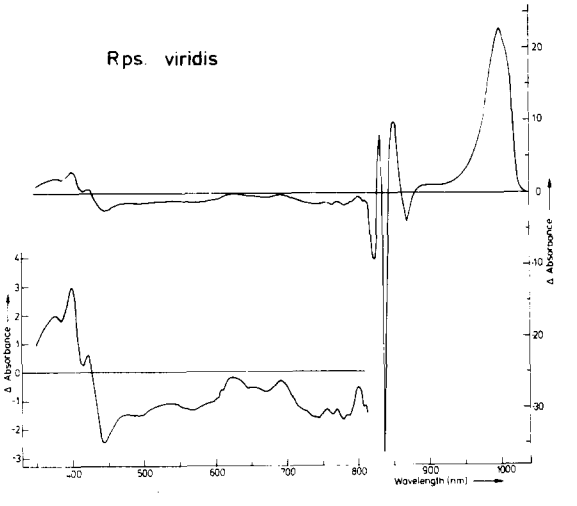

Fig. 4. The absorption-difference spectrum of isolated reaction centers of *Rps. viridis* recorded by ADMR at 1.2 K with resonant microwaves at 356 MHz. The figure represents a P990^T – P-990 spectrum. The optical resolution is 2.4 nm in the 450–790 and 900–1050 nm range, 1.6 nm in the 790–900 nm range and 6.4 nm in the 350–450 nm range.

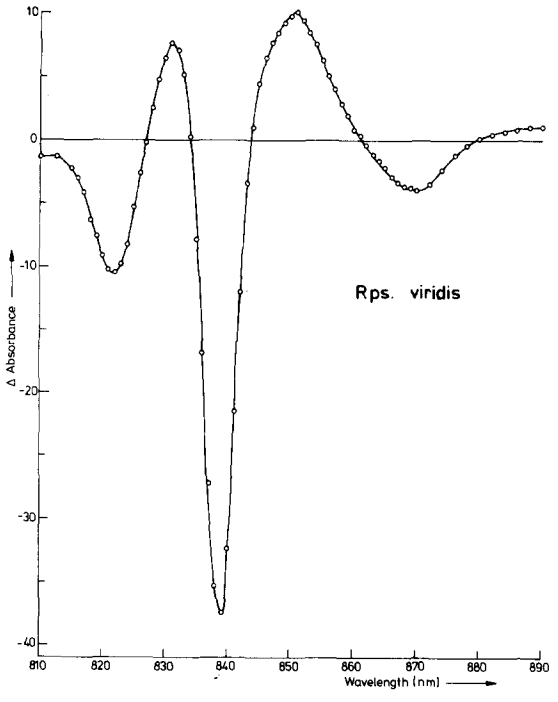

Fig. 5. The absorption-difference spectrum of isolated reaction centers of *Rps. viridis* recorded by ADMR at 1.2 K with microwaves at 356 MHz. The optical resolution is 1.6 nm.

tions between 790 and 830 nm (*Rps. sphaeroides* R-26) and between 810 and 890 nm (*Rps. viridis*). For those bands also, site effects are observed (Fig. 3), although to a lesser extent than for the 890 nm (*Rps. sphaeroides* R-26) and the 990 nm (*Rps. viridis*) band. For reaction centers of *Rps. viridis*, Shuvalov and Parson [23] observed with the flash technique at 77 K three bands between 810 and 890 nm. However, the structure of the oscillations is different from ours. For reaction centers of *Rps. sphaeroides* R-26 at 77 K these authors did not observe the rapidly oscillating features we find between 790 and 830 nm [20,23]. This absence, and the different structure for *Rps. viridis*, seem to be due at least partly to the lower resolution of the inherently less sensitive flash technique. This is especially clear for reaction centers of *Rps. viridis*. In addition, site effects and temperature may play a role, especially for reaction centers of *Rps. sphaeroides* R-26.

Discussion

In a monomer pigment the formation of a triplet causes the bleaching of the singlet-singlet absorption spectrum and the appearance of the triplet-triplet absorption spectrum. We ascribe the bleachings at 385, 600 and 900 nm for *Rps. sphaeroides* R-26 and those at 400, 625 and 1000 nm for *Rps. viridis* to the bleaching of the singlet absorption spectrum of P-890, or P-990. It is interesting to compare the relative amplitudes of the bleachings in the P^R spectra at these wavelengths, corrected for the broad triplet-triplet absorption, with the relative amplitudes of the corresponding bands in the absorption spectrum. If we take six pigments absorbing at 385 nm (400 nm), four pigments at 600 nm and two pigments at 890 nm (990 nm), and if we assume a total bleaching of the 890 nm (990 nm) band by triplet formation, we then find a bleaching of about 0.7 ± 0.3 pigment molecules per reaction center at 600 and 385 nm (400 nm). The fact that less than two pigments bleach at the high-wavelength absorption bands cannot be explained by a delocalization of the triplet on a BPh, as there are no bleachings of sufficient intensity in the 530–550 nm region. It is unlikely that an appreciable part of the missing intensity of the bleaching at 600 nm is due to a more or less narrow triplet-triplet absorption band, as in the in vitro difference spectrum of ^{3}BChl-^{1}BChl no such band is found. Thus, we are led to the conclusion that the triplet in P-890^T is localized on one of the constituent pigments of P-890, in the sense that only (incoherent) hopping is allowed. The broad, rather unstructured bands that for *Rps. sphaeroides* R-26 appear between 410 and 580 nm and between 740 and 790 nm, and for *Rps. viridis* between 430 and 600 nm and between 710 and 790 nm, are probably mainly due to the triplet-triplet absorption spectrum of P^T.

The strong, sharp oscillations between 790 and 830 nm and between 810 and 890 nm for reaction centers of *Rps. sphaeroides* R-26 and *Rps. viridis*, respectively, are new and exciting features of P^R. They cannot be caused solely by spectral shifts in the 790–830 nm region (*Rps. sphaeroides* R-26) and the 810–890 nm region (*Rps. viridis*), because the integrated bleachings in those regions (positive in Figs. 3 and 5) are much smaller than the in-

tegrated appearances (negative in Figs. 3 and 5). In BChl *a* in vitro no strong and sharp triplet-triplet absorption bands are found [23]. This makes it highly unlikely that the large difference between the integrated bleachings and appearances is caused by strong triplet-triplet absorption. This is especially clear for reaction centers of *Rps. viridis* for which the absorption bands of the various pigments and pigment complexes are better resolved than in the BChl *a*-containing species. By inspection of Fig. 5 we ascribe the peaks at 822 and 831 nm to a blue shift of the absorption band of a pigment absorbing at about 827–831 nm and the peaks at 850 and 869 nm to a red shift of the absorption band of another pigment absorbing at about 850–860 nm. The very strong peak at 838 nm then represents the appearance of a new band, which is correlated with the formation of P^T. Although the situation is less clearcut for reaction centers of *Rps. sphaeroides* R-26, the features between 790 and 830 nm are by analogy to *Rps. viridis* well explained by a shift to longer wavelength of the absorption band of a pigment absorbing at about 808–813 n and the appearance of a new band at 807 nm, which partly masks the shift at 808–813 nm. The intensity of the band at 801 nm is dependent on microwave frequency (Fig. 3); at present, we cannot tell whether it corresponds to the band shift at 827–831 nm in reaction centers of *Rps. viridis*.

We interpret the appearance of the new bands at 807 nm (*Rps. sphaeroides*) and at 838 nm (*Rps. viridis*) as the absorptions due to a BChl monomer of the P-890 and P-990 dimers, respectively, in which the singlet-singlet exciton interaction is broken by the formation of a triplet state with much weaker triplet-triplet exciton interaction. Apparently, the triplet excitation is on an optical time scale localized on monomeric BChl. It may, however, hop between the two monomers with a frequency exceeding 100 MHz. This would explain the lower values of the zero-field splitting parameters $|D|$ and $|E|$ of P-890^T and P-990^T compared to those of monomeric BChl *a* and BChl *b*, respectively [7,26–30]. The interpretation of the 807 nm (*Rps. sphaeroides* R-26) and 838 nm (*Rps. viridis*) bands by the emergence of monomeric BChl absorption explains the small amplitude of the bleachings in the Soret region and at 600 nm (*Rps. sphaeroides* R-26) of the singlet spectrum of P compared to the amplitude of the bleaching of the longest-wavelength absorption bands. We note that our interpretation of the triplet absorption-difference spectrum of *Rps. viridis* contrasts with that of Shuvalov and Parson [23] in three respects. These authors assign the 830 nm band to an emerging monomer BChl *b* absorption band, whereas features at 842 and 852 nm are attributed to a blue shift of an absorption band at 850 nm. Our spectrum (Fig. 5) clearly shows that the 850 nm band is subjected to a red shift, that the 830 nm band forms part of a blue shift and that the monomer absorbs at 838 nm.

If the above interpretation of the 807 nm (*Rps. sphaeroides* R-26) and 838 nm (*Rps. viridis*) band is correct, then there is a large difference in width of the emerging monomer absorption band and the absorption band of the special pair dimer. The narrow monomer absorption bands resemble the narrow absorption bands found at low temperature in matrix-isolated Chl *a* in vitro [31]. This would indicate that the interaction between the two monomers in P^T, or between one of the monomers and the protein matrix, that must be invoked to explain the red shift with respect to the in vitro absorption, is uniform and specific. This conclusion has been reached earlier on the basis of the narrow FDMR bands observed in photosynthetic bacteria [32]. The emergence of the monomer absorption band at 807 nm (*Rps. sphaeroides* R-26) and 838 nm (*Rps. viridis*), i.e., at wavelengths close to the infrared absorption in vitro, suggests that the red shift of P-890 (P-990) with respect to in vitro BChl is mainly due to strong exciton interaction between the two BChls of the special pair, and not to BChl-protein interactions. The large width of the special pair might be caused by the short lifetime of $^1P^*$ in open reaction centers, or by a random distribution of sites with a slightly different singlet-singlet exciton interaction (red shift). Alternatively, mixing of the excited-singlet exciton states and the nuclear vibrational states of the special pair might contribute to the width of the 890 nm (990 nm) band.

It is instructive to compare our singlet-minus-triplet absorption difference spectra with the absorption difference spectra (reduced-minus-oxidized) obtained by Thornber et al. [25], Paillo-

tin et al. [33] and Shuvalov et al. [34,35] for reaction centers of *Rps. viridis* and by Clayton et al. [36,38] for reaction centers of *Rps. sphaeroides* R-26. First, we note that for both types of spectrum, the bleachings due to the disappearance of the singlet absorption spectrum of the special pair dimer, should be similar (if measured at the same temperature even identical). Secondly, the appearance of new (monomer) bands need not be similar. This is because the triplet absorption spectrum is different from the oxidized absorption spectrum, whereas also the coupling strength in ($BChl^+$/BChl):($BChl^+$/BChl*) and the ($BChl^T$/BChl):($BChl^T$/BChl*) systems need not be the same so that the position of the first excited state of the (weakly coupled) monomer might be slightly different for both reaction center states. Note that if the pigments in P^T or P^+ are strongly coupled, no monomer band is expected to appear.

The absorption-difference spectrum of P^+ for *Rps. viridis* shows at 77–100 K strong bleachings at 990 and 848 nm, a shoulder at about 840 nm and two emerging bands at 832 and 808 nm [25,33,34]. The two different interpretations of the absorption-difference spectrum of P^+ and the absorption spectrum by Thornber et al. [25] and Paillotin et al. [33] as compared to those of Shuvalov et al. [34,35] differ mainly in the assignment of the 813 and 850 nm absorption bands. Thornber et al. [25] and Paillotin et al. [33] base their interpretation on the work of Clayton et al. [36] for *Rps. sphaeroides* R-26. These authors interprete the 832/840 nm feature in the absorption difference spectrum of P^+ as an electronic shift, the 848 nm as an exciton partner of the 990 nm band and the 808 nm as an emerging P^+ band. In the absorption spectrum the 787–790 nm band is ascribed to BPh *b*, the 812–813 and 834–835 nm bands to two accessory BChl *b* pigments and the 850 nm band to the exciton partner of P-990. Shuvalov et al. [34,35] ascribe the 833 and 850 nm bands in the absorption spectrum to the two accessory BChl *b* pigments, the 790 nm band to BPh *b* and the 813 nm band to an impurity. The absorption difference spectrum of P^+ is ascribed to a decrease and a shift of the band at 833 to 829 nm, a decrease and blue shift of the band at 850 nm and the appearance of a band at 807 nm. In the interpretation of Shuvalov et al. [34,35], a bleaching of an exciton partner of P-990 is not observed. Our results confirm the interpretation of the absorption spectrum by Shuvalov et al. [34,35], i.e., we see shifts of two pigments absorbing around 827–831 and 850–860 nm and we do not observe a bleaching of an exciton partner of P-990 in the range 350–1050 nm.

The assignment of the 813 nm band to an impurity [35] is in our opinion not well founded. The absorption spectrum of our preparation at 1.2 K shows clearly defined peaks at 787 and 812 nm, a strong peak at 830 nm and a weak shoulder at 850 nm (data not shown). The fact that the 812 nm band is observed i n different laboratories using different preparative techniques (Refs. 22 and 35, and this work) suggests that this band is an intrinsic part of the reaction center spectrum. Quite clearly, it does not react to the presence of a triplet state (Figs. 4 and 5), a behavior which is analogous to that of the 787 nm band that is ascribed to one [35] or two [25] BPh. If one adheres to the idea that analogous to reaction centers of *Rps. sphaeroides* R-26, in *Rps. viridis* there are only six pigments per reaction center (there is to our knowledge no proof for this assertion), then it seems likely that both the 787 and the 812 nm bands are due to BPh *b* monomers. Alternatively, one may abandon the constriction of six pigments per reaction center.

Turning to reaction centers of *Rps. sphaeroides* R-26, our interpretation of the absorption-difference spectrum of P^R contrasts with the interpretation of the absorption-difference spectrum of $P\text{-}890^+$ given by Clayton et al. [36–38]. Specifically, we see no bleaching of an excition partner of P-890 which Clayton et al. postulated to occur at 812 nm (at room temperature). In our opinion, the feature at 812 nm forms part of a band shift.

If the 817 nm feature represents a red shift of one of the accesory BChl *a* pigments, then this pigment should have close interaction with the primary donor. As such it could function as an intermediate acceptor as suggested by Shuvalov and Parson [20]. Note, however, that the interpretation of the 817 nm band as a red shift and the 807 nm as a monomeric absorption band rests on the analogy with *Rps. viridis* and on the fact that the 807 nm band is the strongest band between 790 and 830 nm. An alternative interpretation in

which the 807 nm feature is part of a blue shift and the 817 nm band represents the monomer absorption band is not excluded. In *Rps. viridis* this ambiguity does not arise and both accessory pigments seem to have practically equally strong interaction with the primary donor.

The presence of sharp oscillations between 790 and 830 nm in the 1.5 K absorption-difference spectrum of P^R of reaction centers of *Rps. sphaeroides* R-26 casts some doubt on the interpretation of Shuvalov and Parson [20] of the temperature dependence (300–77 K) of P^R in this wavelength region. These authors ascribe the bleaching at 797 nm observed at room temperature to delocalization of the triplet state on one of the accessory BChl pigments, that according to those authors serves as an intermediary electron acceptor before BPh. The decrease in this bleaching with lower temperature is in their opinion due to increased localization of the triplet on P-890. At 77 K the spectrum of P^R used for the interpretation did not show any structure between 780 and 830 nm, but later work revealed small peaks and troughs in this region [23]. The absence of these features in the earlier work was ascribed to the lower spectral resolution used at that time. The strong features we observe at 1.2 K are quite probably analogous to the small peaks and troughs seen in Ref. 23 at 77 K, the difference in aspect primarily being due to our higher spectral resolution. In addition, temperature effects may be partly responsible for the difference between our work and that of Shuvalov and Parson [23].

Whatever the exact cause of the difference between the 1.2 and 77 K P^R spectrum, it seems clear that the original interpretation of the temperature effect on the 797 nm bleaching should be reevaluated, taking into account temperature-dependent band shifts and the appearance of a monomer absorption band.

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