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Phosphorescence from the primary electron donor in *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* reaction centers

Larry Takiff and Steven G. Boxer

Department of Chemistry, Stanford University, Stanford, CA (U.S.A.)

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Phosphorescence has been detected from the dimeric primary electron donor in *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis* reaction centers. For *Rb. sphaeroides* the emission maximum occurs at 1318 ± 2 nm at 20 K and is nearly independent of temperature. The emission linewidth (full width at half maximum) is 240 ± 20 cm^{-1} at 20 K and increases to 580 ± 40 cm^{-1} at 280 K. The phosphorescence lifetime is identical to that of the triplet state of the primary electron donor measured by ground state recovery or triplet–triplet absorption kinetics; likewise, the same magnetic-field effect is observed for the triplet state quantum yield by phosphorescence and absorption measurements. A comparison of the difference between the fluorescence and phosphorescence maxima of the special pair (3424 cm^{-1}) and of pure monomeric bacteriochlorophyll *a* (4600 cm^{-1}) and the observation that the phosphorescence linewidth for the special pair in the reaction center is smaller than the fluorescence linewidth suggest that the triplet state has weak charge-transfer character. For *Rps. viridis* the emission maximum occurs at 1497 ± 2 nm and the energy difference between the maxima of the fluorescence and phosphorescence is 3130 cm^{-1}, compared to 4210 cm^{-1} for pure monomeric bacteriochlorophyll *b*.

Introduction

The triplet state of the primary electron donor in reaction centers from photosynthetic bacteria and green plants is formed by charge recombination when secondary electron transfer is blocked by prior reduction or removal of quinone (Fig. 1) [1]. In bacterial reaction centers the excited singlet state of the primary electron donor or special pair, ^1P, transfers an electron in 3–5 ps to the initial electron acceptor, I, to form the radical-ion pair, P^+I^- [2,3], which recombines in blocked reaction centers to form the ground or excited triplet state of P. In quinone-depleted reaction centers from *Rhodobacter sphaeroides*, R-26 mutant, the quantum yield of ^3P formation is about 0.3 at room temperature and about 0.9 at 20 K [4–6]; the ^3P lifetime is about 30 μs at room temperature and 150 μs at 20 K. Until now, the triplet state has been detected optically by triplet–triplet absorption ($T_n \leftarrow T_1$) or by recovery of the special pair ground state absorption; transitions among the magnetic sublevels of ^3P have been extensively studied by EPR [7] and optically detected magnetic resonance [8] spectroscopies. Much less is known about the triplet state of *Rps. viridis*, but it

Abbreviations: BChl, bacteriochlorophyll; EDTA, ethylenediaminetetracetic acid; EPR, electron paramagnetic resonance; FWHM, full width at half maximum; Q, quinone; Q_y, absorption band for lowest energy singlet electronic state; PVA, poly(vinyl alcohol); P, primary electron donor; ^1P, singlet-excited state of P; ^3P, triplet-excited state of P; I, primary electron acceptor; LDAO, lauryldimethylamine *N*-oxide.

Correspondence: S.G. Boxer, Department of Chemistry, Stanford University, Stanford, CA 94305, U.S.A.

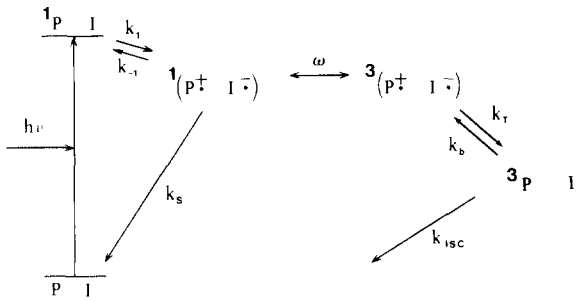

Fig. 1. Reaction scheme describing the intermediates, kinetics and energetics for the initial photochemistry in Q-depleted reaction centers from *Rb. sphaeroides*. P is the primary electron donor, consisting of two bacteriochlorophylls; I is the initial electron acceptor which is a bacteriopheophytin.

appears to be similar to that of *Rb. sphaeroides*. Direct absorption from the ground state ($T_1 \leftarrow S_0$) or phosphorescent emission ($T_1 \rightarrow S_0$) have not been observed either from reaction centers or pure bacteriochlorophylls, presumably because of the very low cross-section for excitation, the very low emission quantum yield, and the difficulty of working in a wavelength region further into the infrared than can be detected with conventional photomultiplier tubes. Recent advances in near infra-red detectors, notably ultra-high sensitivity cooled germanium detectors, now make it possible to make measurements in the 1000–1700 nm region with high sensitivity. Using such a device, we have been able to detect phosphorescence from the primary electron donor in *Rb. sphaeroides* and *Rps. viridis* reaction centers and from pure bacteriochlorophylls *a* and *b* (Takiff, L. and Boxer, S.G., unpublished data). A preliminary account of this work was presented earlier [9].

Although the triplet state is not formed in appreciable yield in functioning photosynthetic reaction centers, its properties in blocked reaction centers have proven to be to very useful for understanding the mechanism of the initial charge separation steps. Until now only the absolute energy of the singlet state was known. The energy of the ^{3}P state places a lower limit on the energy of the initial charge-separated product state, P^+I^-; the energy of P^+I^- provides the fundamental information which is needed to analyze the energetics of photoconversion in photosynthesis. For *Rb. sphaeroides* the free energy of the ^{3}P state has been estimated by measuring the temperature dependence of the amplitude of the delayed fluorescence component whose lifetime is comparable with that of the triplet state [10]. Delayed fluorescence measurements are very valuable for estimating the energies of a variety of reactive intermediates [11,12], but there has been no independent check of their reliability. Furthermore, this method is based on several assumptions, making an independent measurement of the P^+I^- energy desirable. We have shown that the energy of P^+I^- can also be obtained by measuring activated reformation of P^+I^- from ^{3}P (Ref. 13; see also Goldstein, R.A., Takiff, L. and Boxer, S.G., unpublished data). Although the energy difference between P^+I^- and ^{3}P can be estimated quite accurately from such measurements, the absolute energy of P^+I^- obtained by this approach is only as good as the value for energy of ^{3}P. Finally, the nature of the ^{3}P state itself has been the subject of much discussion, especially in comparison with the properties of the first singlet-excited state and radical cation of P. In particular, the relative contributions of charge-transfer and exciton interactions in the excited states of dimeric P have been the subject of several theoretical analyses [14–17]. As shown in the following sections, the phosphorescence spectrum of ^{3}P provides information which bears on each of these issues.

Experimental

Q_A-containing reaction centers of the R-26 mutant of *Rb. sphaeroides* were prepared by standard methods [18]; quinone depletion was performed on an ion-exchange column [19]. Reaction centers in 10 mM Tris-HCl (pH 8.0) 0.1% lauryldimethylamine *N*-oxide, 0.1 mM EDTA were embedded in poly(vinyl alcohol) films (PVA, Aldrich, average M_r 125 000). Films used for phosphorescence experiments each had an absorbance of about 2 at 803 nm and two or three were stacked (reabsorption is not an issue in these measurements); those used for fluorescence or transient absorption were about one-tenth as concentrated. Q-depleted reaction centers in films were determined to be more than 99% Q-depleted by transient absorption kinetics; Q-containing reaction centers in films were more than 90% Q_A-containing. Aqueous samples were prepared using

0.1% Triton X-100 or sodium cholate as the detergent, 67% (v/v) glycerol, and were deoxygenated by repeated cycles of partial evacuation followed by addition of Ar. About 1 mg solid sodium dithionite was then added and the samples were transferred anaerobically to a quartz cuvette which was sealed under vacuum.

Rps. viridis reaction centers were prepared by a procedure similar to that for *Rb. sphaeroides* except that a sucrose density gradient centrifugation replaced the second extraction with LDAO (Middendorf, D., personal communication). The reaction centers were exchanged into deuterium oxide with 10 mM Tris-HCl (pH 8.0), 0.1% LDAO, 0.1 mM EDTA, diluted to 67% glycerol-d_3, and deoxygenated by partial evacuation followed by backfilling with argon. The quinone was pre-reduced by cooling the sample to 77 K in the presence of 20 mM sodium ascorbate (pH 8.0), while under cw illumination from a xenon lamp (approx. 200 mW/cm^2, absorbance at 830 nm about 3 in a 3 mm quartz cuvette held in an optical dewar). Quantitative quinone reduction and triplet formation under these conditions was verified by transient absorption spectroscopy.

Transient absorption kinetics were measured by monitoring the change in absorption at 868 nm for *Rb. sphaeroides* or 752.5 nm for *Rps. viridis* after subsaturating 8 ns excitation flashes (532 nm for *Rb. sphaeroides*, 608 nm for *Rps. viridis,* 10 Hz) with a time resolution of about 1 μs. Triplet lifetimes and relative triplet quantum yields were obtained by fitting the decay data to a single exponential plus a baseline.

A time-resolved luminescence spectrometer was built around an ultra-sensitive liquid nitrogen cooled germanium photodiode detector (Model 403L, ADC Corp., Fresno, CA). Film samples were squeezed against a gold-coated copper sample holder with a quartz flat. The copper sample holders were attached to the cold finger of a closed-cycle helium refrigerator whose temperature could be varied between 20 and 280 K. Aqueous samples in quartz cuvettes were held at 77 K in a liquid nitrogen optical dewar. Phosphorescence was observed by exciting samples with about 80% saturating 8 ns light pulses at 532 nm for *Rb. sphaeroides* or 608 nm for *Rps. viridis* from a Q-switched Nd : YAG or YAG-pumped dye laser operating at 10 Hz. For some experiments, the dye laser output was Raman shifted with high pressure H_2 gas to 850–900 nm. Bandpass filters (RT-830, Hoya Corp.) allowed only the first Stokes–Raman lines to excite the sample. A spherical mirror focussed luminescence from the sample onto the entrance slit of a monochromator equipped with a 1 μm blazed, 600 groove/mm grating (bandwidth 19 nm, linewidth measurements were corrected for this bandwidth). The output of the monochromator was focussed through an 850 nm long-pass filter onto the detector. The optics were aligned using the prompt fluorescence of reaction centers. The output of the detector (time constant, approx. 1 ms) was processed with a boxcar averager (gate duration, 1 ms) and digitized in a minicomputer.

In order to discriminate the red edge of the relatively intense fluorescence (completely decayed by about 30 ns [11]) from the more slowly decaying and very weak phosphorescence (lifetime on the order of 100 μs, vide infra), a light chopper was mounted in front of the monochromator entrance slit. The synchronous output signal from the chopper was divided in frequency to about 10 Hz, delayed by an adjustable time, and used to trigger the pulsed laser and the boxcar. By adjusting the delay, the time between the laser pulse and when the chopper wheel unblocked the detection system could be varied. To record phosphorescence spectra, this delay was set so that the laser pulse (and the prompt fluorescence) occurred just prior to the unblocking of the slit by the chopper wheel. To measure luminescence kinetics, the delay was increased further in set amounts, and the luminescence signal was recorded at each delay time value *.

The method used to measure the luminescence decay kinetics can be modeled as follows. The edge of the chopper wheel opening is taken to reach the monochromator slit in the variable delay time, τ_d, relative to the laser flash, and uncovers it linearly in time τ_u (the slit remains uncovered during the entire subsequent decay). The detector puts out a signal whose height is proportional to the time integral of the intensity of the lumines-

* While Ge detectors with a faster time response are available, they are not sensitive enough to detect the very weak luminescence described in this paper.

cence that strikes it; the boxcar output signal, S, is proportional to the height of the detector signal. Then:

$$S = A\left(\int_{\tau_d}^{\tau_d+\tau_u} \frac{t-\tau_d}{\tau_u} e^{-kt}\,dt + \int_{\tau_d+\tau_u}^{\infty} e^{-kt}\,dt\right) + B$$

$$= \frac{A}{k^2\tau_u}(1-e^{-k\tau_u})\,e^{-k\tau_d} + B$$

where A and B are constants, and k is the luminescence decay rate constant. The signal measured in this manner should be a single exponential to a baseline when plotted against the variable delay time τ_d, and the decay rate of this signal is the decay rate of the luminescence. An important requirement of this method is that the chopper speed be sufficiently high that the wheel moves from completely occluding to completely exposing the detection system before the luminescence has decayed away. The chopper used for these experiments (PAR Model 192) operated at 100 rps with an effective radius of 10 cm and could be swept past a 2.5 mm slit in about 40 μs, which was thus the system's time response for this size slit.

The response of the detection system varied with wavelength. This response was corrected by measuring the spectrum of a standard lamp which was treated as a blackbody radiation source, and by dividing all spectra by the system response curve. The correction was small in the region in which the phosphorescence was observed. The germanium detector has a high cross section for absorption of gamma rays; these appear as random spikes in the spectrum that decay with the time constant of the boxcar. Since they are easily identified, they were removed from the spectra manually. The magnetic field effect on the luminescence intensity was measured by setting the monochromator (the bandwidth was 38 nm for these measurements) to the signal peak and averaging the luminescence signal for 128 s. The field was then set to zero and the measurement was repeated; dividing the signal measured at a given field by that measured at zero field yielded a relative luminescence intensity at that field. Averages and standard deviations of four repetitions at each field were calculated. A small correction was made for the baseline signal recorded far to the red of the phosphorescence peak. Magnetic fields of up to 300 G were applied with a Helmholtz coil mounted outside the refrigerator.

Results

Rhodobacter sphaeroides

A luminescence signal was detected to the red of the fluorescence for quinone-depleted *Rb. sphaeroides* reaction centers. The observed signal at 20 K is shown in Fig. 2; it has an emission maximum of 1318 ± 2 nm (7589 ± 10 cm^{-1}, 0.941 eV) and full width at half maximum (FWHM) of 240 ± 20 cm^{-1}. Also shown in Fig. 2 is the absence of luminescence in this wavelength region from a quinone-containing sample under identical conditions. For the latter sample ^{3}P is not formed because electron transfer from I^- to Q_A is much faster than charge recombination between P^+ and I^-. The luminescence signal for Q-depleted reaction centers was measured as a function of temperature; spectra at 20 and 280 K are compared in Fig. 3B. The luminescence signal is much smaller at higher temperatures because the quantum yield of formation of ^{3}P is lower, the triplet lifetime is considerably shorter, and the band broadens. As seen in Fig. 3B, the peak maximum of the luminescence does not change appreciably with temperature (1307 ± 4 nm at 280 K), while the luminescence linewidth (FWHM) increases from 240 ± 20 cm^{-1} at 20 K to 580 ± 40 cm^{-1} at 280 K. It is also seen that the line broadening is not symmetric. This result is compared with the tem-

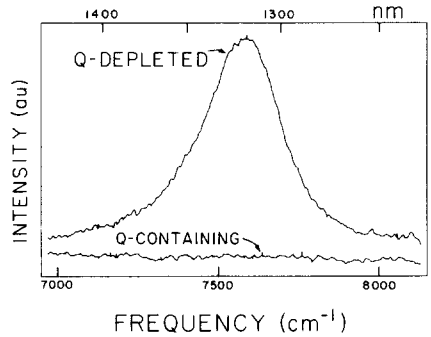

Fig. 2. Luminescence spectrum of quinone-depleted *Rb. sphaeroides* reaction centers in a PVA film at 20 K compared with that of a comparably concentrated quinone-containing reaction center film taken under identical conditions.

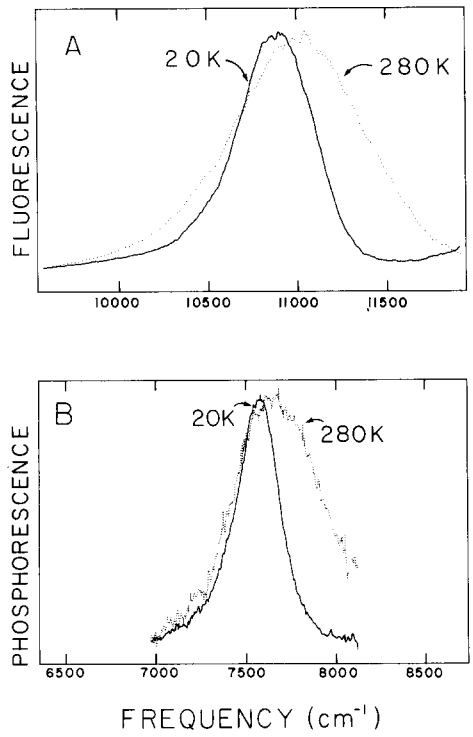

Fig. 3. (A) Fluorescence spectra of Q-depleted *Rb. sphaeroides* reaction centers in a PVA film at 20 and 280 K; (B) phosphorescence spectra of quinone-depleted reaction centers in a PVA film at 20 K and 280 K. The data for the two temperatures is scaled so that the red-most points are the same and the peak amplitudes are the same to facilitate comparison of the peak maxima and lineshapes.

perature dependence of the fluorescence in Fig. 3B (emission maxima, 913 and 900 nm at 20 and 280 K, respectively; linewidths, 460 and 790 cm^{-1} at 20 and 280 K, respectively). The ratio of the integrated intensity of the 1318 nm luminescence to that of the fluorescence is about $5 \cdot 10^{-5}$. Excitation of the Q_y band of P at 853, 872 and 890 nm all gave luminescence bands centered at 1320 ± 2 nm at 20 K (data not shown). The FWHM of this luminescence was about 20% less than that due to excitation at 532 nm.

We were concerned that the luminescence signal might be due to emission from singlet oxygen, which is known to luminescence at 1270 nm in aqueous solution (4 μs lifetime) [20–22]. Singlet oxygen may be generated by energy transfer from ^3P to triplet oxygen [23,24]. An aqueous Q-depleted sample was prepared containing sodium dithionite so that no oxygen would be present. The same signal was seen as in the film sample in Fig. 2; a dithionite-reduced Q-containing sample likewise showed the same luminescence signal at 20 K. Under these conditions Q_A is reduced to Q_A^-, and ^3P is formed in appreciable yield.

The decay kinetics of the luminescence signal are compared with those measured by transient absorption at 20 K in Fig. 4 (different samples from the same batch of reaction centers were used for these experiments because a much more concentrated sample is required to quantitate the emission). In both cases the data fit very well to a single exponential with $1/k_{phos} = 152 \pm 4$ μs and $1/k_{abs} = 148 \pm 2$ μs. The magnetic-field effect on the relative triplet yield was measured both in absorption and emission at 80 K as shown in Fig. 5. Although the signal-to-noise ratio is much poorer for the emission measurement, the magnetic-field effects measured by these two different methods agree to within the experimental error.

Rhodopseudomonas viridis

A luminescence signal from photoreduced *Rps. viridis* reaction centers was detected at 1497 ± 2 nm (6680 ± 10 cm^{-1}, 0.828 eV) at 77 K. The FWHM of this band is 190 ± 40 cm^{-1}. The band is not symmetric: it appears to have a long-wavelength shoulder, as does the fluorescence. The

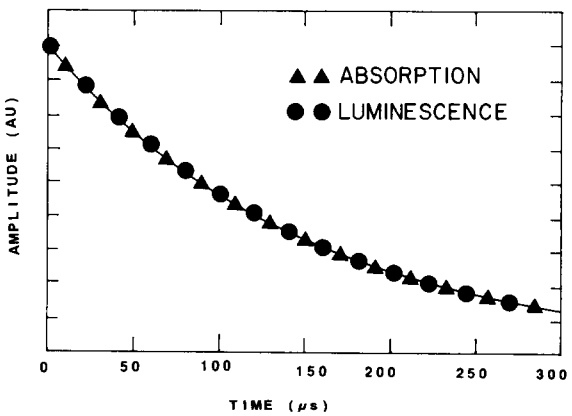

Fig. 4. Decay kinetics of the emission signal centered at 1318 nm (circles) and recovery kinetics of the ground state absorption at 868 nm (triangles) for quinone-depleted *Rb. sphaeroides* reaction centers embedded in a PVA film at 20 K. The solid line is a fit of the emission data to a single exponential plus a baseline. $1/k_{phos} = 152 \pm 4$ μs; $1/k_{abs} = 148 \pm 2$ μs.

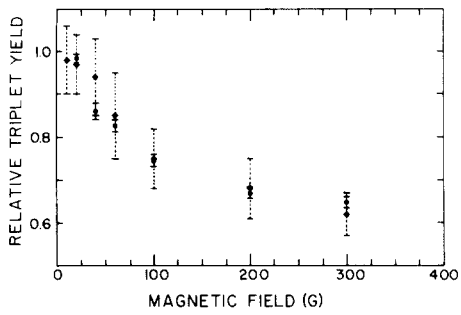

Fig. 5. Magnetic-field effect on the intensity of the 1318 nm delayed luminescence (diamonds with dashed, larger error bars) compared to that on the triplet yield measured by transient absorption (circles with solid, smaller error bars). Both measurements made on Q-depleted *Rb. sphaeroides* reaction centers in PVA films at 80 K and are relative to the zero-field values.

signal is not present in un-photoreduced samples, where the triplet state is not formed. When a photoreduced sample is warmed, allowing reoxidation of the quinone, then cooled again without photoreduction, the 1497 nm luminescence decreases by approximately the same amount as the transient absorbance signal associated with the triplet state. The ratio of the integrated intensity of the 1497 nm luminescence to that of the fluorescence from a reduced sample is about $1 \cdot 10^{-4}$. The luminescence decay kinetics fit well to a single exponential of lifetime 91 ± 5 μs, compared to the triplet lifetime of 95 ± 1 μs measured on the same sample by transient absorption. Although oxygen has been reported to increase the decay rate of the triplet state slightly [23], the triplet energy which we infer is incompatible with sensitization of singlet oxygen formation by the special pair triplet state in this species (vide infra).

Discussion

The near-infrared luminescence is phosphorescence from ^{3}P

We believe that the luminescence signal shown in Fig. 2 for Q-depleted *Rb. sphaeroides* reaction centers is due to phosphorescence from ^{3}P. The signal is exceedingly weak and for this reason we have carried out a rather extensive series of control experiments. The luminescence signal is only present in reaction centers which are known to form the ^{3}P state. The decay kinetics and magnetic-field effects are identical to those measured for ^{3}P by transient absorption. In principle the ^{3}P state could be sensitizing the emission of singlet oxygen, but the same signal was present in samples where oxygen was removed both by outgassing and chemical reduction with dithionite. The signal is not observed in the absence of reaction centers. Based on the known fluorescence quantum yield of about $4 \cdot 10^{-4}$ [25], the quantum yield of ^{3}P phosphorescence is about $2 \cdot 10^{-8}$, assuming that our reaction centers have no more or less fluorescence due to degraded reaction centers than those used in Ref. 25.

Similarly, we believe that the 1497 nm luminescence from *Rps. viridis* reaction centers is due to phosphorescence of the ^{3}P state in this species. The appearance and disappearance of the signal matches that of the triplet state, and the lifetime of the signal is identical to that of the triplet state. Based on the fluorescence quantum yield of about $8 \cdot 10^{-4}$ [26], the quantum yield of ^{3}P phosphorescence is about $1 \cdot 10^{-7}$, assuming that our reaction centers have no more or less fluorescence due to degraded reaction centers than those used in Ref. 26.

Charge transfer character in the ^{3}P state

It is interesting to compare the properties of the singlet and triplet excited states of P. The Q_y absorption band of P in both species is shifted considerably to the red of the Q_y absorption band of the monomeric BChls in the reaction center complex or the pure BChls in organic solvents. This is attributed to the combination of an exciton interaction and mixing with charge transfer states [14–16]; the relative importance of these interactions is not yet certain. For the particular geometry in the special pair [27–29], the exciton interaction is expected to be large and the lower energy exciton component carries considerably more oscillator strength than the upper exciton component, so that this interaction leads to a red shift. We have shown that there is a very large change in dipole moment associated with excitation of P [30,31]. The linewidth of the Q_y absorption band of the special pair (approx. 500 cm^{-1} at 77 K) is substantially greater than that of the monomeric BChl band in the reaction center (approx. 300 cm^{-1}) or pure monomeric BChl *a* in a solid

matrix (approx. 300 cm^{-1} in 2-methyltetrahydrofuran containing 12.5% pyridine at 77 K). We have also shown that it is not possible to burn a narrow hole in the special pair Q_y absorption band [32,33], a result which may be associated with strong electron-phonon coupling [32–34]. Both the absorption and fluorescence bands of P narrow considerably as the temperature is lowered; in *Rb. sphaeroides* both are nearly twice as wide at 20 and 280 K as the phosphorescence from ^3P at the same temperatures (Fig. 3); in *Rps. viridis* the absorption band is about twice as wide, and the fluorescence about three times as wide, as the phosphorescence at 77 K.

The interactions which determine the energy of the lowest triplet state of P relative to that of a monomeric BChl are expected to be quite different from those affecting the singlet state energy. The exciton interaction will be very much smaller in the triplet state because the $T_1 \leftarrow S_0$ transition is forbidden. Thus, charge-transfer interactions may be relatively more important. There have been many studies of the triplet states of electron donor-acceptor complexes, beginning with the early work of Iwata and Nagakura [34,35]. For a donor-acceptor complex, charge transfer leads to a reduction of the zero-field splitting parameters, D and E, relative to those in the parent monomers [36]. This reduction is a result of the increase in average distance between the unpaired electrons which decreases the magnetic dipolar interaction. Less information is available for symmetric molecular dimers. In most covalently connected dimers the zero-field splitting parameters are very similar to that of the monomer [37–39].

The zero-field splitting parameter *D* is observed to be about 20% smaller for ^3P than for monomeric BChl in both species at cryogenic temperatures [40–42]. Several physical models have been used to describe the nature of the ^3P state, ranging from strictly localized excitation on one of the two macrocycles of the special pair [43], predominant localization with some charge transfer to the other macrocycle [44], delocalization over the two macrocycles [45,46], and temperature-dependent delocalization onto a monomer BChl [10]. The descriptions vary partly because different types of measurement sample different aspects of the electronic structure of ^3P and different time scales. Thus, the zero-field splitting parameters sample a time-scale on the order of the inverse of the zero-field splitting energy (fraction of a ns time-scale), electron nuclear double resonance and spin echo spectroscopy sample a time-scale on the order of the inverse of the hyperfine energies (typically tens of ns), and the singlet-minus-triplet [43] and triplet-triplet [10] absorption spectra sample the optical time-scale (as does our phosphorescence measurement). Recent measurements of the principal axes of the zero-field tensor in reaction center single crystals [44] demonstrate that none of the principal axes coincides with the pseudo-C_2 symmetry axis seen by X-ray crystallography in *Rps. viridis* reaction centers [27], whereas preliminary reports indicate that one of these axes does coincide in *Rb. sphaeroides* reaction centers [48]. This is taken to indicate that the triplet state is much more symmetric in *Rb. sphaeroides* than in *Rps. viridis* [48] *. A quantitative analysis of the data for *Rps. viridis* requires about 23% charge-transfer character in the ^3P state to account for the observed reduction in zero-field splitting parameters [44]. Scherer and Fischer [17] recently attempted to calculate the orientation of the principal magnetic axes using the model of Kooyman and Schaafsma [49]. The degree of delocalization of the triplet state was used as a parameter in the fit to the experimental data, however, the fit proved to be insensitive to this parameter.

The difference in energy between the first excited singlet and triplet states (the singlet-triplet splitting) and the spectral lineshapes should also provide insight into the nature of the ^3P state. The electron–electron exchange interaction will be reduced in a charge-transfer complex, leading to a smaller singlet-triplet splitting in the complex compared with the monomers comprising the complex [36]. For example, the singlet-triplet splitting of the fluorene-tetracyanobenzene donor-acceptor complex is only approx. 1100 cm^{-1} compared to a fluorene monomer energy difference of

* We note that the difference in dipole moment between the ground and singlet excited state of P is identical in *Rb. sphaeroides* and *Rps. viridis* reaction centers [30,31], suggesting that whatever asymmetry is responsible for the large value of the difference dipole moment is comparable in the two species.

9300 cm^{-1}; the reduction in zero-field splitting parameters also indicates substantial charge-transfer character, estimated to be about 70% [50]. It has also been found that the phosphorescence lines of complexes possessing a substantial degree of charge-transfer character are considerably broader than those whose excited states are relatively more neutral [36]. This is rationalized by the much larger interaction with the solvent for a polar excited state, a result which is well known for polar singlet excited states (e.g., the ^1P state).

We find that the energy difference between the maxima of the fluorescence and phosphorescence of P is 3290 cm^{-1} for *Rb. sphaeroides* at 20 K and 3130 cm^{-1} for *Rps. viridis* at 77 K. These values are not identical to the singlet-triplet splitting because they are not corrected for possible differences in the Stokes shift between absorbance and fluorescence vs. $T_1 \leftarrow S_0$ absorption and phosphorescence. However, we expect such differences to be small (up to 200 cm^{-1}). These energy differences are each about 30% smaller than those measured for the pure monomers which comprise P: 4580 cm^{-1} for bacteriochlorophyll *a*, 4210 cm^{-1} for bacteriochlorophyll *b* (six-coordinate complexes) (Takiff, L. and Boxer, S.G., unpublished data), or chlorophyll *a* (approx. 4700 cm^{-1}) [51,52]. This reduction in singlet-triplet splitting is consistent with some charge-transfer character in the ^3P state. The reduction is very similar for the two species, suggesting a similar degree of charge-transfer in each. This observation contradicts the hypothesis mentioned earlier that the triplet is much more localized in *Rps. viridis* than in *Rb. sphaeroides* reaction centers [44].

We also observe, however, that the ^3P phosphorescence linewidth is substantially narrower in both species than the singlet absorption or fluorescence (Fig. 3) which suggests a substantially weaker interaction of the triplet state than the singlet state with the environment. Interestingly, the *Rb. sphaeroides* phosphorescence linewidth is quite similar to that for pure BChl *a*, whose singlet absorption, fluorescence and phosphorescence linewidths are all about 300 cm^{-1} (measured in 2-methyltetrahydrofuran/ pyridine at 77 K) (Takiff, L. and Boxer, S.G., unpublished data). The phosphorescence linewidth of *Rps. viridis* is only about half of that measured for pure BChl *b*.

Temperature and excitation wavelength dependence in Rb. sphaeroides

In contrast to the absorption and fluorescence of P, we find that the ^3P phosphorescence maximum does not change appreciably with temperature in *Rb. sphaeroides* (Fig. 3). The energy difference between the maxima of fluorescence and phosphorescence increases by only 160 cm^{-1} between 20 and 280 K. Thus, it is very unlikely that the degree of delocalization of this state is strongly temperature dependent [10,47]. The origin of the change of the absorption and fluorescence with temperature is not fully understood; it may reflect strong electron-phonon coupling in ^1P [53], a result which we and others have argued is consistent with photochemical holeburning data [32–34]. The much smaller shift in the phosphorescence with temperature thus argues for much weaker electron-phonon coupling in the triplet state than in the singlet state. Finally, we note that a small (20%) phosphorescence line narrowing was observed when P was excited directly in its Q_y absorption band compared with higher energy excitation. Phosphorescence line narrowing has been reported following direct excitation of the triplet state of simple aromatic molecules [54], but is quite rare following excitation of the singlet state [55]. The existence of some degree of correlation between the site energies of the singlet and triplet states was suggested by Den Blanken and Hoff [43] in absorbance-detected magnetic resonance studies. They concluded that there is a small inhomogeneous broadening of both the singlet state and the zero-field splitting values in the triplet state, that these were at least partly due to the same mechanism and, thus, might be correlated.

Charge-separation energetics in Rb. sphaeroides

Based on the discussion above concerning the degree of charge-transfer character in the ^3P state, it is likely that the Stokes shift for the triplet state is at most as large as that for the singlet state and is probably considerably smaller *. Adding one half of the approx. 570 cm^{-1} Stokes shift in the

* In principle it should be possible to measure the phosphorescence excitation spectrum; however, the absorption cross section is expected to be incredibly small, and it is difficult to obtain tunable high intensity excitation light in the 1000–1400 nm range.

singlet state (quinone-depleted reaction centers in PVA at 77 K) to the energy of the phosphorescence maximum (7589 cm^{-1}) gives a triplet state energy of 7874 cm^{-1} or 0.98 eV; if the Stokes shift were 150 cm^{-1} (as it is for the singlet excited state of pure BChl *a*), the value is 7664 cm^{-1} (0.95 eV). This energy agrees reasonably well with that estimated by Parson and Shuvalov from delayed fluorescence measurements (approx. 8300 cm^{-1} [10]).

We have recently determined the standard free energy difference between the ^3P state and the radical pair states in *Rb. sphaeroides* reaction centers (Ref. 13; see also Goldstein, R.A., Takiff, L. and Boxer, S.G., unpublished data). Based on data taken at a magnetic field of 135 kG, we obtained a standard free-energy difference at room temperature of 1360 ± 40 cm^{-1}. In combination with the ^3P free energy of 7500 cm^{-1} (the energy of about 7730 cm^{-1} minus kT ln 3 = 230 cm^{-1} to account for the spin multiplicity of the triplet state) determined here and the ^1P energy of 11220 cm^{-1} (from absorption and fluorescence measurements), we conclude that the free energy of the P^+I^- state is 9090 cm^{-1} and the standard free energy change for the initial charge separation step in *Rb. sphaeroides* is 2130 cm^{-1} (0.264 eV).

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