Oscillations in the Spontaneous Fluorescence from Photosynthetic Reaction Centers

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The spontaneous fluorescence from the special pair primary electron donor in bacterial photosynthetic reaction centers has been measured at low temperature using fluorescence up-conversion following excitation of the special pair with 80 fs pulses from a mode-locked Ti:sapphire laser. Oscillations are observed during the first few picoseconds of the decay. The frequency of the oscillations and the detection wavelength dependence of their intensity are similar to results obtained by Vos, Martin, and co-workers using stimulated emission. This demonstrates that the oscillations are associated with the excited state of the special pair, not with stimulated Raman scattering. Vibrational coherence persists on the time scale of electron transfer. Possible relationships between these results and other observables are discussed.

The nature of the electronic excited state(s) which initiates photosynthesis is a key element for understanding the mechanism of ultrafast electron transfer. The excited species in photosynthetic reaction centers (RCs) from bacteria is a dimer or special pair of bacteriochlorophylls (BChls) denoted P. Although the electronic properties of BChl monomers are typical of any large aromatic molecule, the interactions between the BChls within the special pair and between the special pair and its protein environment lead to unique properties. Many static spectroscopies such as absorption, circular dichroism,[2] photochemical hole burning,[3,4] Stark,[5] and resonance Raman[6,7] spectroscopies have been used to characterize these unusual properties of P.

Recently, two new dynamical approaches have been added. Vos, Martin, and co-workers, in a series of *tour de force* experiments, demonstrated oscillations in the stimulated emission from [1]P when the special pair is impulsively excited with very short pulses.[1] Oscillations were initially observed in RCs which are incapable of electron transfer, where the [1]P lifetime is about 200 ps, and were subsequently demonstrated in functional RCs, at both cryogenic and room temperature. At the same time, two groups independently reported the direct measurement of spontaneous fluorescence from [1]P at room temperature using fluorescence up-conversion.[8,9] Both groups observed that the excited decay is biphasic, a result which had not previously been reported using transient absorption spectroscopy. The observation of a biphasic decay was also perplexing as photochemical hole-burning experiments at cryogenic temperatures exhibit zero-phonon line widths[3,4] which agree quite closely with those predicted from the single exponential kinetics measured by transient absorption spectroscopy. In order to investigate this further and to establish the experimental capability for electric-field-modulated measurements on the spontaneous fluorescence, we have developed the methodology for measuring spontaneous fluorescence with high signal-to-noise at low temperatures. In the course of these experiments, oscillations analogous to those reported by Vos et al.[1] were discovered, and this is the subject of this Letter. Vos et al.[1] have presented substantial evidence that the oscillations observed by stimulated emission are associated with the functionally important excited state of P; however, oscillations have been observed in the stimulated emission from other systems, notably studies of bacteriorhodopsin by Shank, Mathies,

and co-workers.[10] In that work, the oscillations were shown to be due to stimulated Raman scattering and thus reflect ground state properties rather than those of the reactive excited state.

Experimental Section

Fluorescence up-conversion has been used for many years to achieve better time resolution than is possible with electronic detection. (See e.g. ref 11 for an early application to biological samples.) The basic design considerations for the setup were taken from Shah.[12] Samples were excited using a mode-locked Ti:sapphire laser (Spectra Physics Tsunami) pumped by 6–10 W (all lines) from an argon ion laser (Spectra Physics Model 2080). The output was approximately 1 W at 82 MHz with pulses of ~70 fs in duration (sech[2] assumed) measured using autocorrelation (Clark Instruments AC-150). The measured spectral bandwidth of 15 nm gives a Fourier transform bandwidth 40% high than the theoretical limit. A glass flat was used to pick off ~40 mW of light for exciting the sample, the remainder being used for gating the fluorescence. Both the gate and excitation beams were vertically polarized. The excitation beam was attenuated to 10–20 mW with a 1 mm quartz neutral density filter and focused onto the sample using a 2.5 cm focal length, 2.5 cm diameter off-axis (60°) paraboloidal metal mirror (Janos, Inc.). To our knowledge this is the first up-conversion spectrometer utilizing off-axis paraboloidal mirrors. Off-axis parabolic mirrors are especially useful in the present application because they permit sufficient space around the sample to insert the equipment needed for maintaining low temperature (see below). The excitation beam passes through only ~ 2 mm of fused silica, CaF_2, and glass (see below) which introduces little dispersion (pulse broadening) before the sample is excited. If we assume a 75 fs pulse width at the sample, the resulting bandwidth for impulsive excitation is ~140 cm^{-1}.

The fluorescence from the sample was collected with a 2.5 cm focal length, 2.5 cm diameter off-axis (90°) paraboloidal mirror which also collimates the fluorescence. The gate beam was aligned through an optical delay consisting of a retroreflector mounted on a galvanometer (Clark Instruments ODL-150). The ~1.5 mm diameter gate beam and 2.5 cm diameter collimated fluorescence were made parallel and projected onto a 7.5 cm diameter, 17.5 cm focal length off-axis (30°) paraboloidal mirror. With this mirror, the noncollinear gate and fluorescence beams were focused into a 1 mm BBO crystal (type I) which was used to up-convert the fluorescence by angle

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tuning. The up-converted light was recollimated using a CaF$_2$ lens. Because the up-conversion signal is small compared to the second harmonic of the gate pulse and the cross correlation of the gate beam and scattered excitation light from the sample, the signal was spatially filtered using an iris and a prism for frequency predispersion. A $^1/_4$ m single monochromator (SPEX 270M) with a band-pass of 2−4 nm was used to filter the up-conversion signal which was detected by a cooled photon counting PMT (Hamamatsu R928P) and counted using a 200 MHz photon counter (SRS-400, Stanford Research Instruments) using a 40 ms counting gate per time point.

Low temperature was achieved using a miniature Joule−Thompson refrigerator (MMR Technologies, Mountain View, CA) and a very thin sample geometry. The refrigerator is cooled by expansion of high-pressure argon or nitrogen through a series of ultrafine capillaries in a laminated glass cold finger, resulting in liquefaction of the coolant gas at the tip of the cold finger. The cooling power is ∼250 mW and is obtained without any detectable vibration. This latter aspect is crucial for maintaining good time resolution. The cold finger must be maintained under vacuum (< 10 mTorr). By cooling the entire refrigerator containing the glycerol/buffer liquid sample to ∼240 K under ambient pressure, the samples are sufficiently viscous that a vacuum can be applied without bubbling as the sample is cooled further by the refrigerator. This procedure and a new sample cell design described below were developed in collaboration with MMR Technologies. The sample cell consists of two pieces of 300 μm thick , 1 cm ^{2} glass (Dow Corning, glass code 0211) which were bonded using a low melting point solder glass (Electroscience Labs, Inc., King of Prussia, PA). A nominal thickness of 25 μm was achieved. It was found that for thicker sample cells the glycerol/buffer glass would shatter below 106 K. Operating at low temperature we routinely achieve an instrument response function (IRF) better than 130 fs (fwhm), as measured by up-converting scattered excitation light from the sample and fitting to a Gaussian function. For room temperature studies, a 1 mm path length quartz cuvette was used with rapid stirring by a small magnetic stir bar.

Wild type (WT), (M)Y210F, and (M)L214H (β mutant) *Rb. sphaeroides* were grown semiaerobically; WT was also grown photosynthetically (anaerobically). The mutant strains were generously provided by Professor C. Schenck, Colorado State University. RCs were isolated and quinone-depleted by the method of Okamura et al.[13] These RCs all contain carotenoid,[14] which is important for experiments using relatively high average power excitation. The ^{3}P quantum yield due to charge recombination in quinone-depleted RCs at 82 K is nearly 100%, and ^{3}P decays to the ground state in about 100 μs, causing a population bottleneck. In the presence of carotenoid, ^{3}P is quenched within hundreds of nanoseconds and returns to the ground state which minimizes, as much as possible, the potential for saturating the P absorption band (see below). All samples were dissolved in 1/1 (v/v) glycerol/buffer (10 mM Tris, pH 8.0) solution. The RCs were concentrated in order to achieve a sufficient OD in the 25 μm path length. With an OD$_{865}$ ∼ 0.05, we were able to obtain >5 kHz count rates with excitation at 865 nm at 82 K with 130 fs time resolution. Data were averaged until ≥40 000 counts were collected in the peak channel. To obtain a valid zero of time, cross correlations were obtained from scatter of the excitation beam by changing the wavelength of the monochromator, making no adjustment of the mirrors or crystal.

Fluorescence decays were fit using a model function consisting of a sum of exponentials convoluted with the instrument response function. In the case of excitation into the accessory

monomeric BChls (B) at 800 nm, three exponentials were used with one having a negative amplitude to account for energy transfer yielding a reduced χ^2 < 1.2. When P excitation was used, no negative amplitude exponential was needed to obtain a good fit. However, with the presence of oscillations at low temperature (*vide infra*) reduced χ^2 values for two-exponential fits were somewhat large (χ^2 > 2), and it was found that a much better fit was obtained when three exponentials were used (χ^2 < 2). The reason for this is simply that a multiexponential model function is inadequate to take into account the presence of oscillations in the decay, so that the initial oscillation is fit (incorrectly) as a very fast, large amplitude decay component (see below).

Excitation fluence studies were performed to ensure that the up-conversion signal scaled linearly with the excitation power. These studies were performed by setting the optical delay to obtain the highest count rate. The count rate did indeed scale linearly with excitation fluence, but over time the count rate gradually decreased. At 40 mW excitation fluence the count rate decreased ∼30% over 5 min. The time dependence of the degradation of the up-conversion signal depended on the excitation fluence. For example, at 40 mW (82 K, 25 μm sample cell) the signal degrades quickly initially and then more slowly; whereas at excitation powers of 10−20 mW the degradation was linear. We attribute the degradation of the signal to a gradual bleaching of the RCs at the focus of the excitation beam. To compensate for the degradation even at the lower excitation power, the sample was translated randomly after each scan (a 50 ps range at 42 fs/point takes ∼1−3 min), and the optical delay was scanned in alternate directions. The ability to translate the sample precisely without loss of alignment is another very desirable feature of the miniature MMR refrigerator.

Results

The spontaneous fluorescence from wild-type RCs at 298 and 82 K are compared in Figure 1. As found by previous investigators,[8,9] the fluorescence at room temperature is not well described by a single exponential. It can be better fit by two exponentials, with time constants of 2.11 and 7.63 ps (relative amplitudes of 80.6% and 19.4%, respectively), in agreement with the data in the literature.[8,9] The signal-to-noise of our data is sufficiently good that the limitations of using a multiexponential model are apparent, even for the room temperature data. First, the early parts of the decays are poorly fit, due to the incipient onset of oscillations. Although this can be fit by including another decay component, such a fit is deceptive. The amplitude of the oscillatory component at a given temperature depends on the excitation bandwidth;[1] thus, if the analysis of decay components is to be pushed to the limit, it is essential that transform-limited excitation pulses be used. Second, although several exponentials (at least two) can be used to fit the decays, equally good or superior fits can be obtained using a distribution of lifetimes around a mean value, as was demonstrated using maximum entropy analysis (results not shown). This is consistent with a distribution of electron transfer rates and is compatible with the physical model in ref 15 in which the heterogeneous kinetics are postulated to result from a distribution of driving forces for the initial electron transfer reaction. Of course, it is equally compatible with a distribution in any other factor(s) which affects the electron transfer rate. Third, we investigated whether the heterogeneity in the carotenoid for RCs grown semiaerobically[14] might contribute to the heterogeneity of the decay kinetics; within the signal-to-noise, no dependence was observed (data not shown).
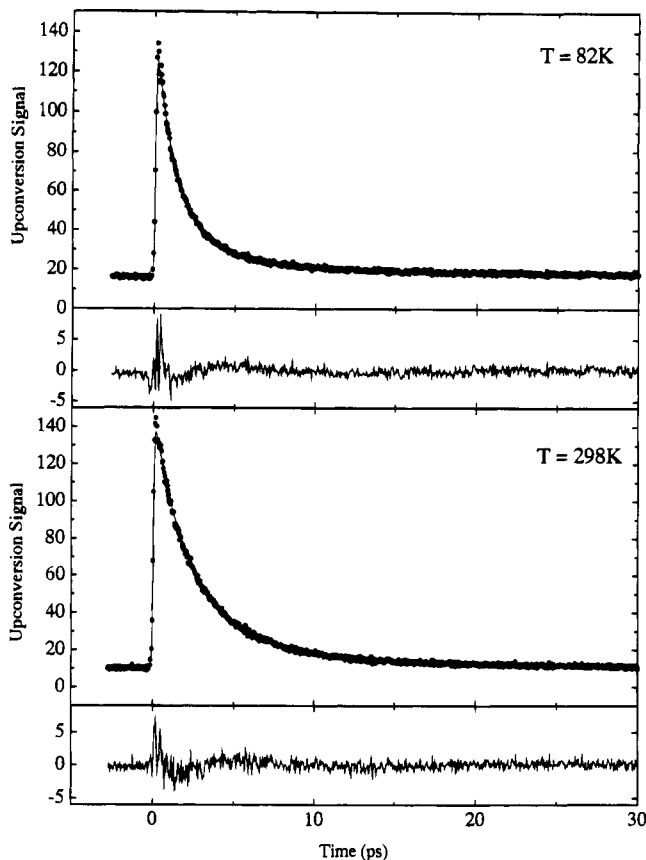
Figure 1. Comparison of fluorescence decays for quninone-depleted WT RCs at 82 and 298 K. The decays have been fit to a two-exponential model function convolved with the IRF. The residuals from the fit are unweighted. The IRF for the room temperature scan was 170 fs, and the sample OD (850 nm) was 0.3 in a 1 mm path length quartz cuvette. The IRF for low-temperature scans was 126 fs with a sample OD (800 nm) of ~0.08 in a 25 μm path length glass cell. Excitation was at 867 nm with emission detected at 950 nm.

Ultrafast spontaneous fluorescence decay data have not previously been reported at low temperature. The major decay component is considerably faster than at room temperature, but the overall decay is still found to be roughly biphasic. Using a biexponential fit, one obtains best-fit values of 1.43 and 10.89 ps (relative amplitudes of 90.9% and 9.1%, respectively) at 82 K, a result which is consistent with recent reports of low temperature transient absorption and stimulated emission,[16] but not the older literature.[17-19] As noted above, the biexponential model function is inadequate when oscillations are present. A better, albeit meaningless, fit was obtained with the inclusion of a third exponential function. The lifetimes and amplitudes (in parentheses) obtained from this procedure are 0.92 ± 0.17 ps (67 ± 7%), 3.1 ± 0.4 ps (28 ± 6%), and 20 ± 10 ps (5 ± 1%), for excitation at 867 nm and emission at 950 nm.

Figure 2 shows the first few picoseconds of the spontaneous fluorescence decay from WT, (M)L214H, and (M)Y210F RCs at 82 K following excitation in the special pair Q_y band at 866 nm. Especially for the mutants, whose excited decay is longer than WT, it is evident that there is structure in the decay. No oscillations in the spontaneous emission from ^{1}P were evident within the signal-to-noise when the monomeric BChls in the RC were excited at 803 nm and transfer their energy to P. Figure 3 shows measurements of the decay of WT RCs at 82 K as a function of the detection wavelength. It is observed that the intensity of the oscillations is largest when the emission is detected at the red edge of the band and is smallest near the maximum of the emission.
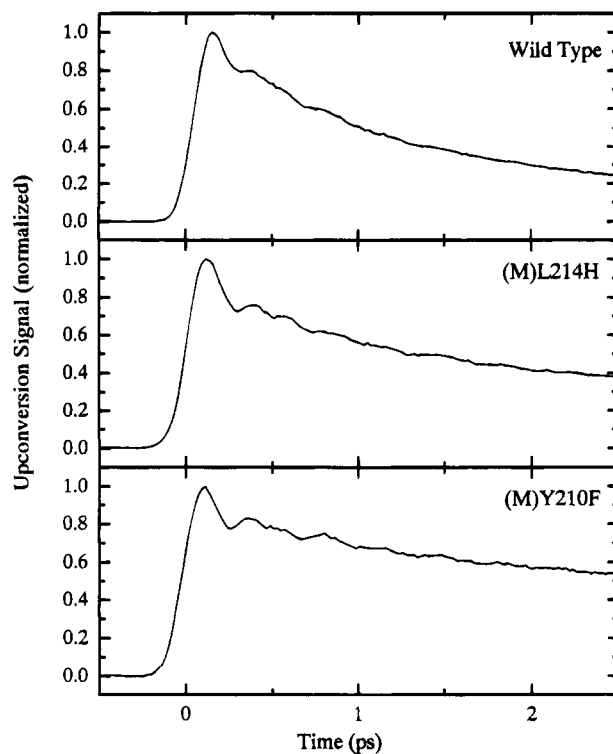

Figure 2. Comparison of low-temperature (82 K) spontaneous fluorescence from quinone-depleted WT, (M)L214H, and (M)Y210F RCs in the first few picoseconds, emphasizing the oscillations. Excitation is at 866 nm with emission at 960 nm.
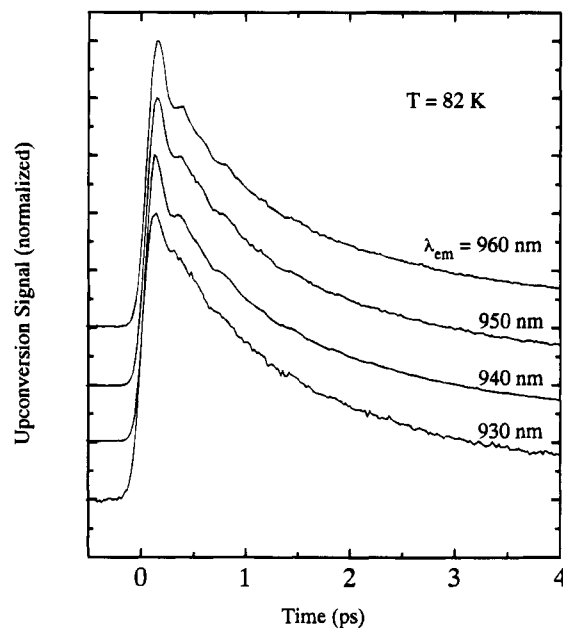

Figure 3. Detection wavelength dependence of the amplitude of the oscillations for WT RCs (82 K, excitation at 866 nm, the emission maximum at this temperature is 920 nm). Each scan took 1.5–2 h.

Discussion

Low Temperature Spontaneous Fluorescence Decay. Until recently, most measurements of kinetics for the primary charge separation reaction in RCs were analyzed using a single exponential function. Thus, it was surprising that at least two decay components were required to fit the spontaneous fluorescence data in the 2–10 ps time range at room temperature.[8,9] Recent transient absorption data with high signal-to-noise are consistent with this finding.[16] When the low-temperature decay is fit with two exponentials, the shorter component dominates.
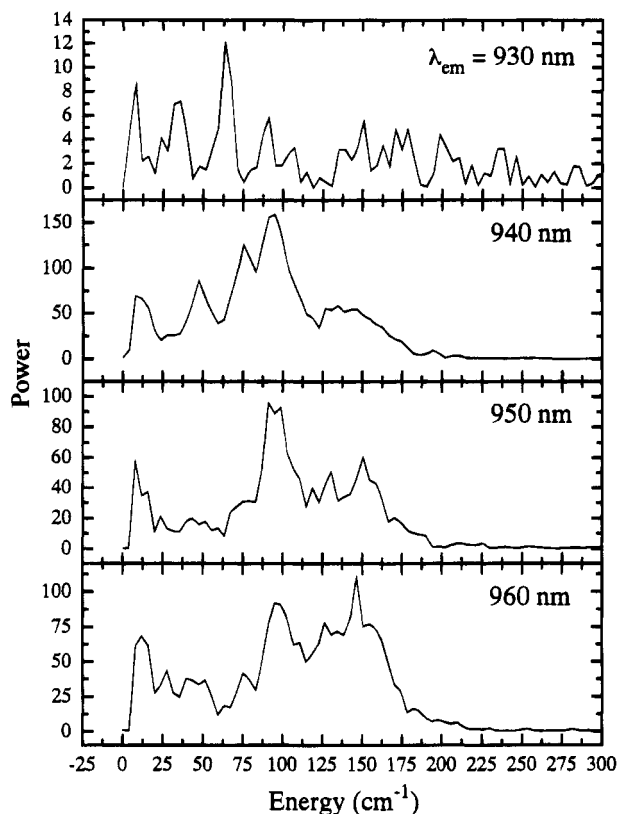
Figure 4. Frequency components in the oscillatory part of the spontaneous fluorescence decay from WT RCs at 82 K obtained by Fourier transformation. The spectral resolution depends on the number of points in the data set which is transformed, in this case ~4 cm^{-1}/point.

The relatively small amplitude of the long-lived component at low temperature may explain why it was not observed in photochemical hole-burning experiments on RCs.[3,4,20]

Oscillations in Spontaneous Fluorescence. It is evident from the decays in Figure 2 that oscillations are superimposed on the decay of the spontaneous fluorescence from RCs which are undergoing electron transfer. There are many approaches to extracting further information on the oscillatory contribution to the decay from the underlying nonexponential population decay. The oscillations were isolated by fitting the population decays to three exponentials whose lifetimes were fixed at the values cited above and allowing the amplitudes to float, leaving residual oscillations shown. A two-exponential fit, where all components are free to vary, or a high-order (5−7) polynomial fit gave similar results except in the low-frequency region (0−30 cm^{-1}). It is useful to Fourier transform the oscillatory component; however, we have found that important details such as peak intensities and widths in the transformed spectra depend sensitively on the method used to subtract off the underlying ^{1}P population decay. With this caveat, Fourier transform spectra are shown in Figure 4. The dominant frequency components are similar to those observed by Vos et al.[1] and by resonance Raman spectroscopy of P.[6]

These data lead to a number of conclusions. (1) Because we are observing the oscillations on the spontaneous fluorescence, there is no question that the oscillations are associated with the excited state of P. (2) There is a reasonably close correspondence between the oscillation frequencies we have observed and the low frequency modes observed in the resonance Raman spectrum of P both at room[6] and low[21] temperatures. Resonance Raman spectroscopy probes ground state modes which are coupled to excitation of P, and the data presented here and in ref 1 demonstrate that similar frequencies

are coupled in the excited state. This makes it unlikely that there are large structural rearrangements within the special pair upon photoexcitation, which could alter low-frequency modes. There could, of course, be significant structural rearrangements whose vibrational signatures might lie at higher frequencies than the bandwidth we can excite impulsively or which are not strongly coupled to electronic excitation of P. (3) The emission wavelength dependence of the amplitude of the oscillations closely parallels what has been observed and studied in detail by Vos et al.,[1] consistent with coherent wave packet propagation on the excited state potential surface. (4) The decay of vibrational coherence is on the order of 1 ps. Qualitatively, this implies that vibrational population relaxation (T_1) is occurring on this or a longer time scale. Although a quantitative analysis of the decay time for each coupled mode would be most desirable, such an analysis, especially for low-frequency modes, depends sensitively on the methods used to subtract off the underlying decay as described above for the analysis of frequencies. (5) It is evident from the comparison in Figure 2 that oscillations in the (M)Y210F and (M)L214H mutants are similar to that of WT. The modes which are impulsively excited at 850 nm are believed to be associated exclusively with P. Residue (M)210 is in close proximity to B_L; however, it is also close to P and to H_L, and mutations such as (M)Y210F lead to changes in the oxidation potential of P.[15] Although there is no evidence that changes in the vicinity of B_L or H_L lead to major changes in the frequency of oscillations following impulsive excitation of P, such effects, if present, might affect very low frequency modes,[1] which are difficult to obtain reliably from data like that in Figure 2. Experiments on mutants which affect P are in progress.

Speculation. The observation of oscillations is the rule rather than the exception when fluorescence is measured following excitation with very short pulses [see e.g. ref 22]. Thus, although an interesting physical phenomenon, it remains to be demonstrated that the oscillations are important for understanding the early events in photosynthesis. As with the resonance Raman results, it is certainly significant that low-frequency modes are strongly coupled to electronic excitation of P, in contrast to monomeric B, and that excess vibrational energy in these modes relaxes on the same time scale as the initial electron transfer reaction in WT. As we learn more about the properties of ^{1}P and attempt to integrate all of the available data into a unified picture, it is becoming evident that some unusual photophysical or even photochemical processes may be occurring. We conclude with some brief speculations on this subject.

One interpretation of the near absence of structure in photochemical hole-burning experiments on P is that there is a very fast vibrational relaxation process which effectively broadens the vibrational levels in ^{1}P.[4] A mechanism of this sort is required if only two modes are assumed to be coupled to the electronic excitation; however, we have shown that this is less significant if several low-frequency modes are strongly coupled,[3] as is seen in the resonance Raman spectrum.[6] At a more quantitative level, however, this does not solve the problem.[23] Absolute resonance Raman cross sections have been measured for P and B.[6] Both at room temperature[6] and low temperature,[23] the cross section for P appears to be considerably smaller than for B. All other things being equal, the resonance Raman cross section will be attenuated by excited (virtual) state dynamics, specifically electronic dephasing. For example, the very rapid transfer of energy from ^{1}B to P (150 fs[24]) is expected to attenuate the resonance Raman cross section of B relative to a pure, isolated monomeric BChl. The observation that the cross section for P is even smaller than for B can be interpreted as

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suggesting that excited state dynamics even faster than 150 fs are occurring. The long-lived (picoseconds) oscillations seen in [1]P suggest that such a rapid excited state process is not vibrational relaxation; however, there could be a very fast electronic dephasing process.

It is usually assumed that the state which is initially excited (the resonant transition in resonance Raman, hole-burning, or Stark effect spectroscopy) and the state probed by spontaneous fluorescence are the same state, but this may not be the case. A variety of experimental data[5,25] and theoretical conjectures[26] suggest that other electronic states, notably charge transfer states, may be nearly resonant with and strongly coupled to the exciton states of P. These CT states are usually considered to be dark states; however, it is conceivable that there might be sufficient intensity borrowing so that both relatively strong absorption into the initial state and relatively strong spontaneous fluorescence from the second state are possible, and both states could possess considerable CT character. This suggests a highly speculative scenario in which an extremely rapid electronic conversion process is postulated to occur from the initially excited state to a nearby state(s), and the state observed by spontaneous emission is a coherent photoproduct. This proposal is compatible with measurements of the steady-state fluorescence line shape in an applied electric field[27] and may provide a decay pathway from the initially excited state to the ground state in an applied electric field.[28] Furthermore, it could explain the emergence of two absorption bands in the Stark spectrum of mutants with an unsymmetrical special pair.[29,30] We have presented evidence that the excited state of P has a very large polarizability, consistent with strong mixing with other electronic states, analogous, surprisingly, to the properties of polyenes.[5] It would be remarkable if this analogy extends even further, and the excited state dynamics of [1]P actually resembles that of the visual pigments. This speculative hypothesis is currently being tested.

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