Radical Pair Quantum Yield in Reaction Centers of Photosystem II of Green Plants and of the Bacterium *Rhodobacter sphaeroides*. Saturation Behavior with Sub-picosecond Pulses

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We have measured the radical pair quantum yield of reaction centers of photosystem II of green plants and of the purple bacterium *Rhodobacter sphaeroides* as a function of excitation density using sub-picosecond pulses at 77 K. The saturation behavior of both RCs can be described consistently by taking into account stimulated emission and the geometry of the polarized (magic angle) laser experiment. Our results show that the quantum yield of charge separation for the PS II RC is very high, about 1, and that no annihilation occurs under these conditions.

Introduction

The photochemical reaction center (RC) of photosystem II contains 6 Chl *a*, 2 Pheo *a*, and 2 β-carotene pigments[1−4] that are located on two polypeptides, D1 and D2. Excitation energy transfer from the antenna complexes to the RC and among the pigments in the RC is followed by the primary charge separation step: the transfer of an electron from the primary donor P680 to the photoactive Pheo *a*. Although the mechanism of electron transfer as well as the intrinsic charge separation time are still a matter of debate, the quantum yield of charge separation in the RC complex may be expected to be high, because the charge separation process is much faster than the excited state decay processes. However, since the secondary quinone acceptors are absent in the isolated RC, the radical pair state $P680^+Pheo^-$ is not stabilized by further downward electron transfer as is the case in the isolated bacterial RC, which contains quinones, and in the PS II in vivo. Therefore, despite the fast electron transfer from P680, the yield of the radical pair $P680^+Pheo^-$ after ∼100 ns is rather low at room temperature due to charge recombination to the excited state, triplet formation, and internal conversion occurring on a nanosecond time scale.

Several groups have studied the processes of energy transfer and charge separation in the PS II RC by (sub-) picosecond time-resolved measurements either at room temperature or low temperatures. Recently, Holzwarth and co-workers reported a study of the excitation intensity dependence of the kinetics.[5] They observed a saturation of the ΔOD signal of the radical pair state at high excitation densities, which was accompanied in the kinetics mainly by an acceleration of the fastest time constant, from ∼250 fs to ∼100 fs. They interpreted this as annihilation as a consequence of multiple photon excitation of RCs. A comparison of their induced ΔOD signals at a certain excitation intensity with those published by other groups[6−10] led them to conclude that they had performed their experiments on the PS II RC under annihilation-free conditions,[11] unlike the other groups.

Singlet−singlet or singlet−triplet annihilation has been studied in several photosynthetic antenna systems and is a useful method to determine, for example, the size of a complex; see for a review ref 12. It is, however, not very likely that in the case of sub-picosecond measurements on the PS II RC annihilation occurs to a significant extent. Most of the experiments employed "red" excitation, i.e. between 680 and 690 nm, in order to selectively excite the redmost absorbing states of the RC. Upon excitation by a photon, the red absorption bleaches and if a second photon arrives at this excited RC, it will induce stimulated emission by which the excited state is lost and no annihilation will occur.

Greenfield et al.[9] recently reported no dependence of the PS II RC kinetics on excitation density. They suggested that this could qualitatively be explained by the decreased absorption probability for a second photon if the first photon has bleached the absorption band, but they did not take into account the effect of stimulated emission. In this paper we report on measurements of the radical pair yield of the PS II RC as a function of excitation density at 77 K and compare the results to the saturation behavior of the bacterial RC of *Rhodobacter sphaeroides*. In our analysis we will explicitly take into account stimulated emission and the geometry of the polarized laser experiment. The results unambiguously show that the energy dependence can be adequately described this way and that no annihilation is observed up to excitation densities of 8 photons/RC.

Material and Methods

PS II RC (D1-D2-cytochrome *b*-559) complexes were isolated from spinach by means of a short Triton X-100 treatment of CP47-RC complexes as described earlier.[4,13] The samples were characterized by a ratio of the room-temperature absorption at 416 and 435 nm of 1.20. They contained Chl *a*, Pheo *a*, and β-carotene in a ratio of 6.3/2.0/2.0 according to the method reported in ref 4. The samples were diluted in a buffer containing 20 mM BisTris (pH 6.5), 20 mM NaCl, 0.03% *n*-dodecyl-β,D-maltoside, and 80% (v/v) glycerol in a cuvette with 1.5 mm path length. The *Rb. sphaeroides* bacterial RCs are membrane-bound RC-only complexes, prepared as described in refs 14 and 15. The samples were placed in an Oxford DN 1704 nitrogen bath cryostat. Pump−probe measurements were performed using a 30 Hz laser system described in detail elsewhere.[10,13] The system had an instrument response (cross correlation of pump and probe pulse) fwhm of 250−280 fs, from which we estimate the pump pulse to be ∼180 fs. Excitation was at 684 nm with a narrow-band (5 nm fwhm) interference filter for the PS II RC experiments and at 883 nm with a broad-band filter (fwhm 27 nm) for the *Rb. sphaeroides* RC experiments. The excitation energy of the pump beam was varied with optical density filters between 30 nJ/pulse and 3.3 μJ/pulse. The pump beam was focused with a 20 cm lens to a spot size in the sample of ∼275 μm in diameter for the

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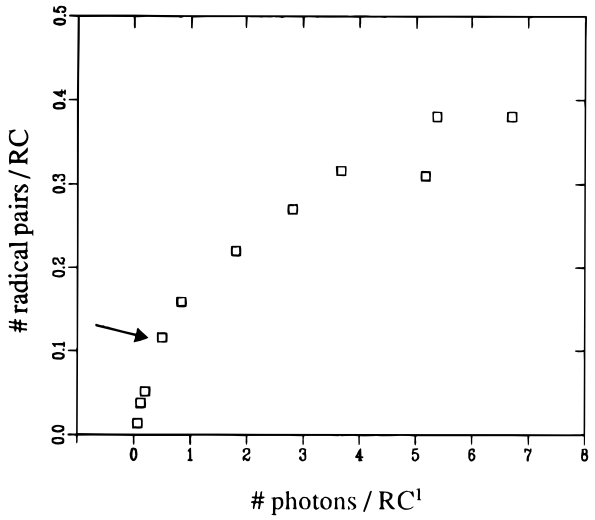

Figure 1. Number of radical pairs per RC as a function of the number of photons per RC of PS II at 77 K upon 684 nm excitation. (Actually no. photons/RC corresponds to $n_{photons}(1 - T)/n_{RC}$ in the illuminated volume; see text.) Calibration of the *y*- and *x*-axes is described in the first two sections of the Results.

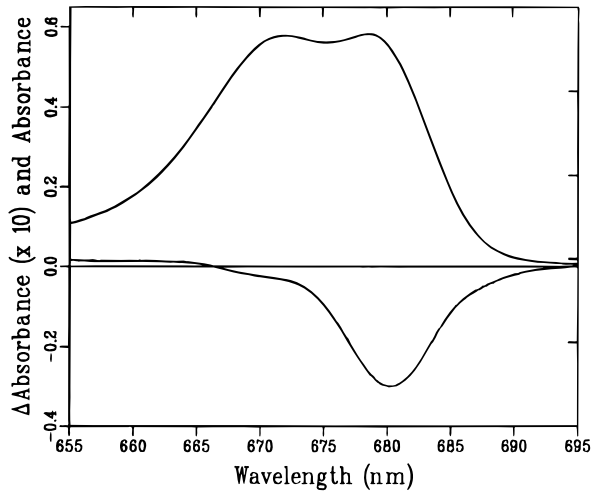

Figure 2. Absorption spectrum of the PS II RC at 77 K (top), and the ΔOD spectrum multiplied by 10 (bottom), at $t = 150$ ps, $P = 240$ nJ/pulse or 0.5 photons/RC.

experiments at 684 nm and to 300 μm for the experiments at 883 nm; the focus of the probe beam was slightly smaller. The probe beam was polarized under magic angle (54.7°) with respect to the pump beam; for anisotropy measurements also the signal with the probe beam polarized parallel with respect to the pump beam was recorded.

Results

Figure 1 shows the saturation curve of the radical pair (RP) yield of the PS II RC at 77 K. In the following sections it will be explained by which methods this curve has been obtained, in particular how we have calibrated the *x*- and *y*-axes. Note that this curve shows a distinct saturation behavior that already sets in at less than 0.1 RP/RC. From this experiment we can estimate the quantum yield of radical pair formation, from which the magnitude of the bleached oscillator strength of the excited state can then be determined.

Number of Radical Pairs per RC. The radical pair spectrum shown in Figure 2 was measured at a delay time of $t = 150$ ps. From the calculation of the ratio of the integrals of the induced bleached absorption and of the absorbance (from 660 to 700 nm for the OD spectrum and 670 to 700 nm for ΔOD, see Figure 2) the fraction of bleached pigments is obtained, which for a laser energy of $P = 240$ nJ/pulse is 2.1%. However, one has to take into account the contribution of the vibrational bands to the absorption spectrum (about 13%[16]) as well as the absorption of the radical pair state. From the absorption increase at 660 nm, one can in first approximation assume that the induced excited state absorption is ∼10% of the bleached signal (i.e. taking a structureless absorption in the 660−700 nm region). With these corrections, the fraction of bleached pigments is $2.1 \times 1.25 = 2.6\%$. To convert this number to the fraction of RCs in a radical pair state, an assumption has to be made concerning the magnitude of the oscillator strength of the radical pair. Suppose that the radical pair consists of 1 Pheo and 1 Chl; this leads to a relative oscillator strength of 1.6/7.2 (the oscillator strength of Chl *a* relative to that of Pheo *a* is 1/0.6 and there are 6 Chls and 2 Pheos present in the PS II RC). With these numbers we conclude that $2.6\% \times 7.2/1.6 = 12\%$ of the RCs are in the radical pair state in this particular experiment, which is indicated in Figure 1 by the arrow. The corresponding number of photons is calculated in the next section.

Number of Photons per RC. For the calibration of the *x*-axis of Figure 1 we have calculated the number of absorbed photons per RC using the Lambert−Beers law. Whether the photon is actually absorbed or stimulates emission is merely determined by the ground and excited state populations. Therefore the photons that may stimulate emission are included, since the Einstein coefficients of absorption and stimulated emission are equal.

To calculate the number of photons per RC, we need the absorption cross section of a RC, i.e. the effective extinction coefficient of the RC. On the basis of the extinction coefficients of Chl *a* and Pheo *a* in 80% acetone, Eijckelhoff and Dekker obtained a good fit of the acetone extract spectrum of the RC with a pigment ratio of 6.3 Chls and 2 Pheos.[4] A first estimate of the RC extinction coefficient therefore would be $6.3 \times 8.6 \times 10^4 + 2 \times 5.2 \times 10^4 = 645.8$ mM^{-1} cm^{-1}. However, a simulation of the (intact) RC absorption spectrum with the absorption spectra of Chl *a* and Pheo *a* in acetone given above led to a ratio of the Q_y/Q_x band that was a factor 0.89 too low.[4] This indicates that the RC extinction coefficient is indeed smaller, $\epsilon_{max} = 575$ mM^{-1} cm^{-1}, due to the fact that the RC absorption band is broader (20 nm) than the Chl and Pheo bands in acetone (18 nm).

At 77 K, the Q_y absorption is both stronger and narrower than at room temperature (RT). A comparison of absorption spectra taken at these two temperatures shows that $\epsilon_{678\ nm} = 676$ mM^{-1} cm^{-1} at 77 K. With this value one can calculate the effective absorption cross section at 77 K, at 678 nm, to be 18.8 Å^2/RC; the effective absorption cross section σ of the RC in a *nondilute* sample can be calculated by averaging over the path length. In this case σ corresponds to the area each RC occupies in a sample of OD 0.3 cm^{-1}, in a sample volume of (1 cm × 1 cm × 1 cm) such that the transmission of the sample is 50%:

$$C = \frac{\mathrm{OD}N_A}{\epsilon} = \frac{0.3(6 \times 10^{23})}{676 \times 10^3} = 2.66 \times 10^{14}\mathrm{RC/mL} \quad (1)$$

$$\text{area covered by RCs} = \frac{1}{2}\,\mathrm{cm}^2 = \frac{1}{2}(10^8 \times 10^8)\ \text{Å}^2 \quad (2)$$

$$\sigma = \frac{5 \times 10^{15}}{2.66 \times 10^{14}} = 18.8\ \text{Å}^2/\mathrm{RC} \quad (3)$$

with OD the optical density in cm^{-1}, N_A the Avogadro number, and ϵ, the extinction coefficient.

In the experiment described in the previous section the power incident on the cryostat was 240 nJ/pulse. This corresponds to 8.2×10^{11} photons, of which 10% is lost on each of the three cryostat windows, leaving 6.0×10^{11} photons. Multiplication of the spectral distribution of the excitation pulse by $(1 - 10^{-OD})$ at every wavelength yielded a spectral distribution of the excited subset of pigments centered at 683 nm and fwhm 5 nm. It showed that 42% of the photons, i.e. 2.5×10^{11}, are absorbed (at very low excitation densities, otherwise stimulated emission becomes important, as demonstrated below). The diameter of the pump spot in the sample was about 275 mm, or 6×10^{12} Å^2, so the excitation density was 0.043 photons/Å^2.

Since we need for our calculation the cross section around the wavelength of excitation, we multiplied the cross section spectrum of the RC with the spectral distribution of the excited subset of pigments, centered at 683 nm. This yields for this laser power an excitation density of 0.50 photons/RC. The point measured at this excitation density is marked in Figure 1 with an arrow.

Radical Pair Yield of RCs of *Rb. sphaeroides.* The curve of Figure 1 saturates only very slowly; even at 7 photons/RC the radical pair yield is less than 0.4. To compare it with a system that has a known quantum yield, we measured the saturation curve of the radical pair $P870^+BPheo^-$ in bacterial reaction centers of *Rb. sphaeroides* for which the quantum yield of charge separation has been precisely established to be 1.[17] The number of radical pairs and absorbed photons per RC were calculated according to the methods described in the previous sections for the PS II RC. The number of radical pairs per RC was obtained from the ratio of the integral from 850 to 940 nm of the ΔOD spectrum, measured at a time delay of 30 ps, i.e. 10 times longer than the charge separation time, and of the OD spectrum. Underlying the bleached P870 band is an unstructered band due to $BPheo^-$ absorption with an extinction coefficient of 17 mM^{-1} cm^{-1}.[18] With an extinction coefficient of the RC of 288 mM^{-1} cm^{-1} at 800 nm, at RT,[19] the extinction coefficient at 870 nm is 110 $mM^{-1}cm^{-1}$.[19] The ΔOD signal was therefore corrected with a factor 1.18. At 890 nm, at 77 K, we determined the extinction coefficient to be 195 mM^{-1} cm^{-1}. With this value we calculated an effective cross section at 890 nm of 5.42 Å^2/RC.

Figure 3 shows that the saturation of the radical pair of the bacterial RC is very similar to that of the PS II RC up to the highest measured excitation density of 2.5 photons/RC for the bacterial RC.

Saturation of the Radical Pair Yield in a Polarized Experiment. In this section we will calculate how the saturation curve in Figure1 can be calculated from a simple model including stimulated emission and the specific geometry of a polarized experiment.

Taking the Einstein coefficients for absorption and stimulated emission equal and neglecting excited state decay processes during the time scale of the excitation pulse, the fraction of RCs in the excited state is given by

$$N_2 = (1/2)(1 - e^{-2n(\theta)}) \tag{4}$$

where $n(\theta)$ is the number of absorbed photons per RC. The probability that an RC is excited is proportional to $(\mu \cdot \mathbf{E})^2$, where m is the absorption transition dipole moment of a RC pigment and $\mathbf{E}$ the electric field vector. Since $\mathbf{E}^2 \sim I \sim n$, $n(\theta) = n \cos^2 \theta$, where θ is the angle between the (vertically polarized) exciting laser pulse and the transition dipole of the pigment; see Figure 4. Consequently, the pigments with a dipole moment

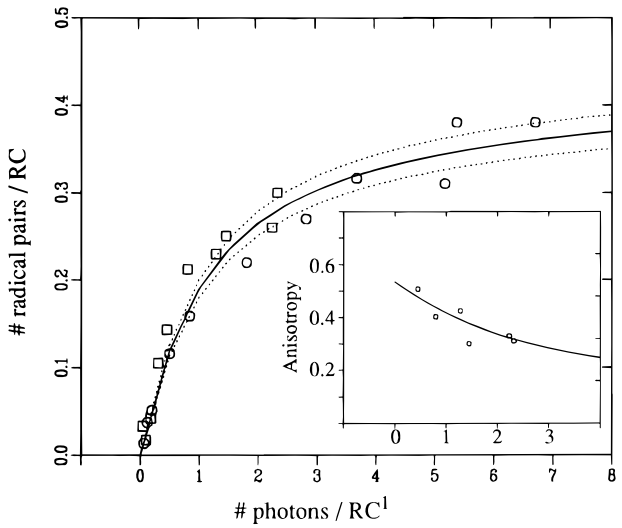

Figure 3. Radical pair saturation curve of the PS II RC (circles) and of the RC of *Rb. sphaeroides* (squares) at 77 K. The solid line represents the radical pair yield according to eq 7, with $Q = 0.95$, whereas the dotted lines represent $Q = 1.0$ and 0.9, respectively (see text). The inset shows the anisotropy of the ΔOD spectrum of *Rb. sphaeroides* and the calculated anisotropy (see text).

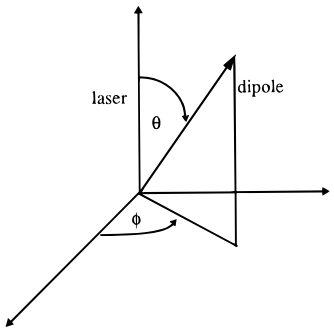

Figure 4. Geometry of the polarized experiment.

that makes a small angle with the polarization of the excitation pulse have a much larger probability to be excited than those which have their dipole moment perpendicular to the laser beam. The expressions for the vertically and horizontally polarized delta absorption are obtained by multiplying the probability of detection in the parallel or perpendicular direction with the fraction of excited RCs and averaged over all directions (see also ref 20):

$$\Delta OD_V = 3\int_0^{2\pi}\int_0^{\pi} \frac{d\phi \sin\theta \, d\theta}{4\pi} \frac{\cos^2\theta}{2}(1 - e^{-2n\cos^2\theta})$$

$$= \frac{1}{2} + \frac{3}{8n}\left(e^{-2n} - \sqrt{\frac{\pi}{8n}}\,\mathrm{erf}(\sqrt{2n})\right) \tag{5}$$

For the perpendicular delta absorption

$$\Delta OD_H = 3\int_0^{2\pi}\int_0^{\pi} \frac{d\phi \sin\theta \, d\theta}{4\pi} \frac{\cos^2\phi \sin^2\theta}{2}(1 - e^{-2n\cos^2\theta})$$

$$= \frac{1}{2} - \frac{3}{16n}\left(e^{-2n} - \frac{1-4n}{4}\sqrt{\frac{\pi}{2n}}\,\mathrm{erf}(\sqrt{2n})\right) \tag{6}$$

The factor 3 enters in (5) and (6) since the absorption of a perfectly aligned sample measured with light polarized along the axis of alignment would be 3 times higher than that of an isotropic sample.

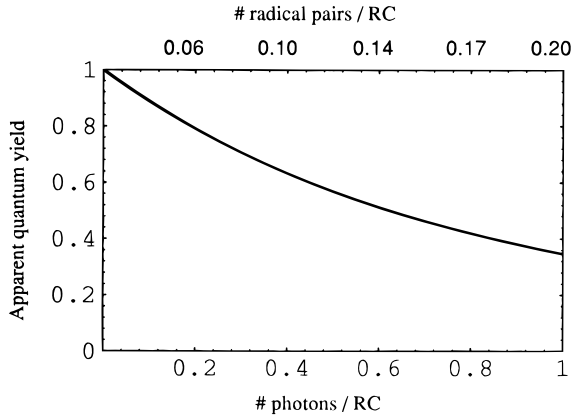

Figure 5. Relative number of radical pairs formed per photon as a function of excitation density. For comparison, the corresponding number of radical pairs per RC are also shown (upper axis).

The isotropic ΔOD signal, detected under magic angle, and the anisotropy are given by

$$\Delta OD_{iso} = (1/3)(\Delta OD_V + 2\Delta OD_H) \quad (7)$$

$$r = \frac{\Delta OD_V - \Delta OD_H}{\Delta OD_V + 2\Delta OD_H} \quad (8)$$

Figure 3 shows the curve given by eq 7 multiplied with a fit parameter Q, which in principle reflects the quantum yield of radical pair formation. With $Q = 0.956 \pm 0.05$ the curve reproduces the saturation of the radical pair for both the PS II RC and the bacterial RC quite well. The inset of Figure 3 shows the decrease of the anisotropy calculated with eq 8 due to saturation (solid line), together with the measured anisotropy of the radical pair state of *Rb. sphaeroides*. These results demonstrate that stimulated emission and the geometry of the polarized light experiment are sufficient to describe the observed saturation behavior.

The measured anisotropy of the bacterial RC is quite high and extrapolates at low excitation density to a value of 0.55. To explain such high anisotropy, one has to assume an induced absorption polarized perpendicular to the bleached absorption with an amplitude ratio of 0.25/1.25, respectively. This is in reasonable agreement with the induced $BPheo^-$ absorption, of which at RT the ratio of extinction coefficients with the P870 absorption is 0.18/1.18 (refs 18, 19; see above) and which is almost perpendicularly polarized.

In Figure 5 we have plotted the slope of the curve of Figure 3, i.e. the derivative of eq 7, and normalized it to the slope at very low excitation density (no. photons/RC $\rightarrow$ 0). It shows that saturation starts to play a role already at very low excitation density. For example, at ~0.1 radical pairs/RC, which corresponds to 0.4 photons/RC, the apparent quantum yield is 63% mainly due to saturating photoselection.

Net Oscillator Strength of the Excited State. Since the curves of the radical pair yields of the PS II RC and the bacterial RC *Rb. sphaeroides* coincide, and the latter has been shown to have a near unity quantum yield,[17] we conclude that the PS II radical pair quantum yield is about 1. We can now calculate the net bleached oscillator strength of the excited PS II RC state by comparing it to that of the radical pair state. For this we need the fraction of bleached, excited RCs obtained under the same conditions as the fraction of RCs in the radical pair state calculated in the first section.

To get the ΔOD spectrum at $t = 0$, the instrument response has to be deconvoluted. We used for that the sum of the decay-associated spectra that result from a global analysis of the data (not shown). We calculate the ratio of the integrals of the $\Delta OD_{t=0}$ and OD spectra (from 660 to 700 nm for the OD and 674 to 700 nm for ΔOD), which yields a bleaching of 4.5%. Assuming that half of this signal is due to stimulated emission, and with the correction factor of 1.25 (for the contribution of vibrational bands, as previously used for the calculation of the relative amount of radical pairs), this gives $4.5 \times 0.5 \times 1.25 =$ 2.8% bleached oscillator strength. By comparing this value to the value found for the radical pair spectrum, we can estimate the relative oscillator strength of the initially excited state. If we would assume that one photon bleaches one Chl oscillator strength, this would correspond to $2.8\% \times 7.2 = 20\%$ excited RCs. The corresponding fraction of radical pairs formed is 12%, and since the radical pair quantum yield is close to unity, this indicates that the net bleached oscillator strength upon excitation actually is about 1.7 times higher than that of a single chlorophyll.

Discussion and Conclusions

As discussed above, the saturation curves for the PS II RC and for the *Rb. sphaeroides* RC coincide, from which we conclude that the yield of radical pair formation in the PS II RC at 77 K is close to 1. For large excitation densities, the observed ΔOD signal never exceeds a bleaching corresponding to 40% of the RCs in the radical pair state (see Figure 1). This is a natural consequence of the occurrence of stimulated emission. By taking into account the saturation effects of stimulated emission and the specific geometry of a polarized, magic angle experiment, a satisfactory agreement with the experimental data of both type of RCs is obtained. The radical pair yields for the PS II RC are best fitted by a curve that describes the saturation of the excited state with $Q = 0.956 \pm 0.05$. This number is obtained by assuming that the radical pair conists of one Chl and one Pheo pigment and therefore a relative oscillator strength of 1.6 per 6 Chls and 2 Pheos. The pigment content of the PS II however has been reported to be nonstoichiometric, i.e. 6.3 Chl *a* per 2 Pheo *a*.[4] Using these numbers the radical pair yield curve would be even better fitted taking $Q = 1.06 \pm 0.05$.

The PS II RC has two near-degenerate red states[21−25] which in principle could both be excited and these excitations eventually annihilate. We have not taken this into account in the calculations presented here because we expect that this will introduce only a minor modification to the fitted curve due to the particular nature of the two near-degenerate red states: First of all the inhomogeneity of the two electronic transitions makes one of the two more resonant with the excitation pulse than the other, and therefore the probability of exciting both is reduced. Furthermore, by measuring the anisotropy on a sub-picosecond time scale,[26] these two red states have been shown to have approximately orthogonal transition dipoles by which the probability to excite both in a polarized laser experiment is small. As can be deduced from the RT decay time of the anisotropy of 0.4 ps,[26] energy transfer from one state to the other takes about 0.8 ps. Therefore even in the PS II RC with two near-degenerate red states, the causes for saturation as presented in this article describe the major part of the observations.

The net bleached oscillator strength of the excited state of 1.7 corresponds to the oscillator strength of one Chl and one Pheo and agrees with the oscillator strength of the red states according to the multimer model,[27] which yields red states with an oscillator strength of 1−2 Chls each.[26] It suggests, however, that there is no significant amount of excited state absorption or strong two-exciton transition in the multimer, which may be surprising.

As we have shown, the saturation of the radical pair can be fully explained without taking into account annihilation. We

can only speculate about a possible explanation for the variation in the kinetics observed when the excitation density is increased.[5] The saturation of the excited state due to stimulated emission induced by the excitation pulse will lead to a distortion of the kinetics. At moderately high excitation densities the pigments that are excited by photons from the center of the spectrum of the excitation pulse will saturate faster than those on the edges. This leads to an apparent shorter excitation pulse at the center of the band. Therefore, at excitation densities larger than ~0.1 photons/RC, there will be a kinetic component due to the time-dependent broadening (as a result of saturation effects) of the ΔOD spectrum on a time scale shorter than the excitation pulse. Such a component has to be added to the fit to account for this process. If not, it must be expected that in the fit the fastest component accelerates with increasing excitation density, which may be from ~250 fs to ~100 fs as indeed observed by Müller et al.[5] for their fastest component. In a complex data analysis which involves four components, the slower ones will also be affected in order to compensate for the acceleration of the fastest component. This was, for instance, observed for the second component in the study by Müller et al. which accelerates from 2.7 ps to 1.1 ps. In the study of Greenfield et al.[9] the fit to the data starts only after 300−400 fs, due to some interfering oscillations during the first 100 fs, and indeed in that study no dependence of the kinetics on the excitation intensity was observed. This explanation may however not be sufficient to explain kinetic changes occurring on a 50 ps time scale.[5] At RT, the description of the PS II RC as an effective one-state system may break down due to the larger homogeneous bandwidths and the thermal energy that lead to a decrease of the photoselective effect of the excitation pulse. A certain amount of annihilation may therefore occur in these types of measurements, in addition to the saturation effects described here.

We conclude that even at rather low excitation densities saturation occurs. At low excitation densities the major cause for this is photoselection, whereas when a significant amount of RCs are excited, stimulated emission becomes important. The only observable effect this has on the kinetics is an apparent (not due to a real kinetic process) broadening of the ΔOD spectra during the excitation pulse. This effect has been observed, and accounted for, in our previous sub-picosecond measurements,[10,13] in which we found a component that followed the excitation pulse instantaneously and had a decay-associated spectrum that described the broadening of the ΔOD spectra on a time scale shorther than 100 fs. Although we previously attributed this component to a coherent coupling artifact, it may very easily arise from the type of saturation effects occurring at the center of the ΔOD spectrum that we have discussed here. A similar observation was reported for bacterial reaction centers,[28] and also in this case part of the pulse-limited broadening could be due to saturation effects.

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