Biochimica et Biophysica Acta, 592 (1980) 461—477

BBA 47924

SUBPICOSECOND AND PICOSECOND STUDIES OF ELECTRON TRANSFER INTERMEDIATES IN *RHODOPSEUDOMONAS SPHAEROIDES* REACTION CENTERS

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(Received January 22nd, 1980)

Key words: Electron transfer; Reaction center; Picosecond spectroscopy; Bacteriochlorophyll; Bacteriopheophytin; (Rps. sphaeroides)

Summary

The primary electron transfer processes in isolated reaction centers of *Rhodopseudomonas sphaeroides* have been investigated with subpicosecond and picosecond spectroscopic techniques. Spectra and kinetics of the absorbance changes following excitation with 0.7-ps 610-nm pulses, absorbed predominantly by bacteriochlorophyll (BChl), indicate that the radical pair state P^+BPh^-, in which an electron has been transferred from the BChl dimer (P) to a bacteriopheophytin (BPh), is formed with a time constant no greater than 4 ps. The initial absorbance changes also reveal an earlier state, which could be an excited singlet state, or a P^+BChl^- radical pair.

The bleaching at 870 nm produced by 7 ps excitation pulses at 530 nm (absorbed by BPh) or at 600 nm (absorbed predominantly by BChl) shows no resolvable delay with respect to standard compounds in solution, suggesting that the time for energy transfer from BPh to P is less than 7 ps. However, the bleaching in the BPh band at 545 nm following 7-ps 600-nm excitation, exhibits an 8- to 10-ps lag with respect to standard compounds. This finding is qualitatively similar to the 35-ps delay previously observed at 760 nm by Shuvalov at al. (Shuvalov, V.A., Klevanik, A.V., Sharkov, A.V., Matveetz, Y.A. and Kryukov, P.G. (1978) FEBS Lett. 91, 135—139) when 25-ps 880-nm excitation flashes were used. A delay in the bleaching approximately equal to the width of the excitation flash can be explained in terms of the opposing effects of bleaching due to the reduction of BPh, and absorbance increases

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin.

due to short-lived excited states (probably of BChl) that turn over rapidly during the flash.

The decay of the initial bleaching at 800 nm produced by 7-ps 530- or 600-nm excitation flashes shows a fast component with a 30-ps time constant, in addition to a slower component having the 200-ps kinetics expected for the decay of P^+BPh^-. The dependence on excitation intensity of the absorbance changes due to the 30-ps component indicate that the quantum yield of the state responsible for this step is lower than that observed for the primary electron transfer reactions. This suggests that at least part of the transient bleaching at 800 nm is due to a secondary process, possibly caused by excitation with an excessive number of photons. If the 800-nm absorbing BChl (B) acts as an intermediate electron carrier in the primary photochemical reaction, electron transfer between B and the BPh must have a time constant no greater than 4 ps.

Introduction

Spectroscopic investigations of light-induced charge separation in reaction centers from photosynthetic bacteria have revealed a sequence of several extremely rapid electron transfer steps. However, there is still some question as to the number of intermediary states involved and to the rates of the reactions that connect them. To explore the earliest of these steps, we have studied the events that result from excitation of isolated reaction centers with 7-ps flashes at 530 and 600 nm and with 0.7-ps flashes at 610 nm.

Isolated reaction centers from photosynthetic bacteria contain four molecules of bacteriochlorophyll (BChl), two of bacteriopheophytin (BPh), one or two quinones, a nonheme Fe atom, and three polypeptides [1—3]. Measurements of optical absorbance changes and ESR signals have shown that the primary photochemical process is the oxidation of a BChl complex involving two of the four BChls [4—6]. The complex is referred to as '*P*-870', '*P*-960' or 'P'. P has a strong absorption band near 870 nm in reaction centers from bacterial species that contain BChl-a, and near 960 nm in those that contain BChl-b. This band bleaches when the dimer is oxidized to the radical cation (P^+), or is raised to an excited singlet state (P^*) or triplet state [7,8].

If purified reaction centers are excited by a short flash of light, a transient radical pair state is generated within a few picoseconds. The radical pair evidently includes P^+ and a radical anion (BPh^-) involving one of the two BPhs [7,9—14]. In a second step that takes about 200 ps, an electron moves from BPh^- to one of the quinones (Q). This step can be blocked by extracting Q or reducing it chemically before the excitation, and the P^+BPh^- radical pair then lives for about 10 ns [7,8,15,16].

Exactly how an electron gets from P to BPh is not clear. Several groups of investigators have reported that, when reaction centers were excited with 530- or 626-nm flashes lasting about 7 ps, absorbance changes reflecting the formation of P^+BPh^- were complete within the instrumental resolution time of about 7 ps [7,9—14]. These absorbance changes include the bleaching of P's absorption band at 870 or 960 nm, the bleaching of bands at 545 and 760 nm due to

the BPh, and the formation of a new band near 670 nm due to BPh^-. Most picosecond studies have used 530-nm excitation flashes, which are absorbed mainly by the two BPhs, rather than by P itself. It has been presumed that the excited BPh transfers energy rapidly to P, and that the excited BPh immediately extracts an electron from P, or from one of the other BChls. In addition, absorbance changes due to the excited singlet state of BPh (BPh^*) could have been confused with those reflecting the formation of BPh^- and could have given the impression that the electron transfer reaction is faster than it really is [17].

Shuvalov et al. [17] have suggested that electron transfer does not occur directly between P^* and the BPh, but rather proceeds by way of one of the other BChls (*P*-800, or B):

$$P^*B\ BPh \rightarrow P^+B^-\ BPh \rightarrow P^+B\ BPh^- \qquad \text{(Model 1)}$$

In support of this scheme, Shuvalov et al. [17] and Kryukov et al. [18] have reported that, when *Rhodospirillum rubrum* reaction centers were excited with 880-nm flashes lasting about 25 ps, which presumably excited P directly, the BPh appeared not to become reduced immediately. They measured the rate of BPh^- formation from the bleaching of the absorption bands at 545 and 760 nm. The bleaching at 760 nm lagged by about 30 ps after the center of the flash. At times preceeding the absorbance decreases at 545 and 760 nm, they observed a transient bleaching at 800 nm, which they ascribed to the reduction of B. A similar transient bleaching has been described by Rockley et al. [9] and by Holten et al. [7], both of whom used 530-nm excitation. From the kinetics of the absorbance changes, Shuvalov et al. [17] put an upper limit of 15 ps on the first step on Model 1, and a time constant of about 35 ps on the second step.

One of the attractive features of Model 1 is that it provides a role for one of the two 'extra' BChls (B). Similar schemes based on direct reactions between P^* and BPh leave this role unspecified. Even Model 1, however, is silent with regard to the roles of the fourth BChl and the second BPh.

In spite of the attractiveness of Model 1, the evidence supporting it is ambiguous. The transient bleaching near 800 nm could be due to a secondary process, rather than to a step in the normal electron transfer sequence. The interpretation of absorbance changes in this region is particularly complicated, because P and the two other BChls all appear to contribute to the absorption here [19—25]. Adding to the problem picosecond absorbance measurements generally involve high excitation intensities, so that each reaction center may absorb more than one photon. In this paper, we shall show that the quantum yield of the transient bleaching at 800 nm is lower than that of the primary electron transfer reaction, at least under some conditions. Further, the transient bleaching can occur even if P is already oxidized. These observations suggest that the bleaching is due, at least in part, to a secondary process. Excitation flashes at 880 nm probably are less likely to cause multiple excitations than are flashes at 530 or 600 nm, because oxidation of P by the first photon would decrease the absorption cross-section at 880 nm by 85—90% [1]. However, the residual absorption at 880 nm could be far from negligible if the flash intensity is high.

Another objection to Model 1 is that, if the P^+B^- radical pair is an intermediate in the electron transfer sequence, one might expect to trap this state by excitation after chemical reduction of the BPh. This would be analogous to the trapping of P^+BPh^- after reduction or extraction of Q. Netzel et al. [26] and Holten et al. [7] both have reported that excitation of *Rhodopseudomonas viridis* reaction centers after reduction of the BPh does not result in the formation of any detectable radical pair states. This favors the view that there are no intermediate carriers between P^* and BPh. Holten et al. [7] did see the formation of what appeared to be P^*. The excited state decayed in about 20 ps, without detectable reduction of another BChl or BPh. Similar results have been obtained recently in *Rps. sphaeroides* but with a somewhat longer lifetime (350 ps) for the excited state (Schenk, C.C., Parson, W.W., Holten, D. and Windsor, M.W., unpublished data). A reservation concerning these experiments is that the reduction of BPh could have adverse effects on electron transfer between P^* and B.

Finally, the 880-nm excitation flashes that Shuvalov et al. [17] and Kryukov et al. [18] used were relatively long (25 ps). As we shall discuss below, measurements made during the time of the excitation flash may be complicated by absorbance changes due to very short-lived excited states. Because the electron transfer reactions are so rapid, studies with shorter pulses are needed. Unfortunately, shorter flashes at 880 nm are difficult to generate. In the present work, we have explored the initial electron transfer steps using 7-ps flashes at 600 and 530 nm and 0.7-ps flashes at 610 nm. The 600 and 610 nm flashes are absorbed predominantly by the BChls, so that interference from absorbance changes due to BPh^* is unlikely to be a major problem. The results indicate that, if B does act as an intermediate electron carrier, electron transfer between B^- and the BPh must take no longer than 4 ps.

Methods and Materials

The picosecond apparatus is basically the same as that described previously [27], but with several modifications. A single 1060-nm pulse of 7 ± 1.5 ps duration (determined by two-photon fluorescence in rhodamine-6G) is selected from the pulse train of a modelocked neodymium-glass laser, amplified twice and frequency doubled with about 15% efficiency. After passing through a series of optical components the 530-nm excitation pulses have an energy of 1.5 mJ in a 1.4 ± 0.2 mm diameter spot at the sample. Excitation pulses at 600 nm are generated via the stimulated Raman process by focusing the 530-nm radiation into a 5 cm cell of perdeutero-cyclohexane. Residual 530-nm light is removed with colored glass filters. The 600-nm pulses have an energy of 200 μJ in a 1.4 ± 0.2 mm spot at the sample.

The energy of the pump pulse on each shot was measured by diverting about 4% of the 530 or 600 nm pulse to a photodiode, whose output was displayed on a storage oscilloscope. These readings were calibrated with a ballistic thermophile placed at the sample position. Measurements of absorbance changes as a function of excitation energy were made by attenuating the pump pulse energy with neutral density filters of known transmittances at 530 and 600 nm. Unattenuated flashes were used for all other measurements. The 7-ps

probe pulses are generated by focusing the 1060-nm radiation remaining after frequency doubling into a 10 cm cell of CCl_4. Absorbance changes are measured as previously described [27]. Picosecond measurements were performed in 1, 2 or 5 mm path cells.

The subpicosecond apparatus has been described previously [28,29]. The initial pulse train consists of subpicosecond optical pulses at 610 nm from a passively modelocked continuous wave dye laser. Pulses are obtained by acousto-optic dumping from the laser cavity at a repetition rate of 10 per second and then passed through three dye laser amplifiers pumped by a frequency-doubled neodymium-yttrium aluminum garnet laser, also operated at 10 pulses per second. The amplified 610 nm pulse train is split into two parts. The first beam traverses an optical delay line based on a digitally-controlled, stepping motor-driven translation stage. It then is focused to a 0.5 mm spot on a 1 cm path sample cuvette. The pulses in this pump beam are of 0.7-ps duration and have energies of 100 μJ. The second beam is focused into a 1-cm cell of CCl_4 to form a broad-band probe pulse. Residual 610-nm light is removed with a colored glass filter. Additional filters are used to isolate the desired spectral region of the probe beam, which is split into two beams vertically. One beam is focused onto the same region of the sample cuvette as the pump beam, while the second probe beam passes through an unexcited region of the sample and provides the reference (ground state) absorbance measurement. The probe pulses also have 0.7 ps duration, but have energies of less than 1 μJ.

The probe beams are dispersed by a 1/4 Jarrell-Ash monochromator (with exit slit removed) onto two tracks of a vidicon detector coupled to a Princeton Applied Research model 1215 optical multichannel analyzer and dedicated computer. In one mode of operation, the probe wavelength is fixed, and the translation state is used to obtain an essentially continuous measurement of ΔA as a function of time. Alternatively, difference spectra covering an 80-nm region are taken with a fixed delay between the pump and probe pulses. The coincidence (zero) time of the pump and probe pulses was obtained as follows: The CCl_4 cell was left in the probe light path but was moved out of focus so that the continuum was not generated. The glass filters were removed from the probe light path, and a $K_2H_2PO_4$ crystal was placed in the sample position. Second harmonic (305 nm) light generated when the 610-nm pulses in the pump and probe beams coincided was measured. Corrections were applied for delays introduced by the glass filters during absorbance measurements. The error in determining the zero time is ±1 ps.

Reaction centers were isolated from *Rps. sphaeroides* strains R-26 and 2.4.1 as described previously [16,30,31]. Their concentrations were determined by using an extinction coefficient of 288 $mM^{-1} \cdot cm^{-1}$ at 802 nm [1]. The samples were at room temperature for all measurements, and were at moderate redox potentials (near +250 mV) except where specified. BChl and BPh were obtained from *Rps. sphaeroides* as described previously [32]. Micellar solutions were prepared by injecting concentrated ethanolic solutions of BChl into 0.1 M aqueous cetyltrimethylammonium bromide.

Results and Discussion

Fig. 1 shows spectra of the absorbance changes that occur in the 560—950 nm region, upon excitation of reaction centers from *Rps. sphaeroides* with 7-ps flashes at 530 nm. The measurements in part A of the figure were made at moderate redox potentials, as were most of the other measurements described below. The open circles show the absorbance changes 20 ps after the excitation, when the reaction centers are predominantly in the state P^+BPh^-; the filled circles show measurements at 2 ns, when the reaction centers are in the state $P^+BPh\ Q^-$. The spectra are similar to spectra that have been obtained previously with *Rps. sphaeroides* strain R-26 and from other species of bacteria [7,9,10,17,18]. Their major features are a bleaching at 870 nm due to the oxidation of P, and bleaching at 760 nm and the formation of a broad absorption band from 620 to 720 nm due to the reduction of BPh. The oxidation of P also results in an absorption decrease near 805 nm and an increase near 790 nm. An additional absorbance decrease centered near 800 nm is superimposed on these absorbance changes in the spectrum measured at 20 ps. Part of the additional bleaching appears to be due to one or more BChl molecules that interact with the BPh that undergoes reduction [7,20—26,33—36], and part reflects the 30-ps transient state that was described in the Introduction. Part B of Fig. 1 contains measurements at high redox potential; we shall return to these later.

Measurements of the kinetics of the initial absorbance changes at several

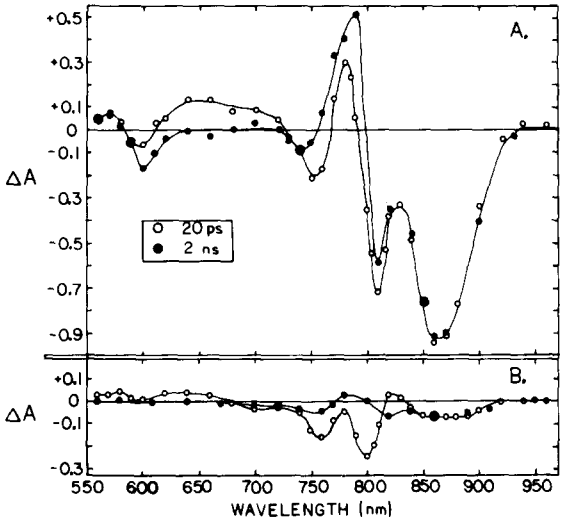

Fig. 1. Absorbance changes resulting from excitation of *Rps. sphaeroides* strain 2.4.1. reaction centers (suspended in 0.1 M phosphate, 0.3% lauryl dimethylamine oxide, pH 7.5) with 530-nm pulses lasting 7 ps; (A) moderate potential and (B) high potential. The data presented in (A) were normalized to that for 16 μM reaction centers in a 5 mm path, but were actually taken in 31 μM reaction centers in a 2 mm path. The high potential sample (B) was prepared by adding to the sample of (A) aliquots of 10 mM potassium ferricyanide in the same buffer as the reaction centers, followed by the addition of solid potassium ferrycyanide until the observed bleaching at 870 nm had reached a level unchanged by the addition of more oxidant. The resulting sample (B) contained 16 μM reaction centers RCs in a 5 mm pathlength cell. The indicated delay times apply to 870 nm; the actual delay times are greater at shorter wavelengths, by up to 17 ps at 550 nm according to the curve in Fig. 3.

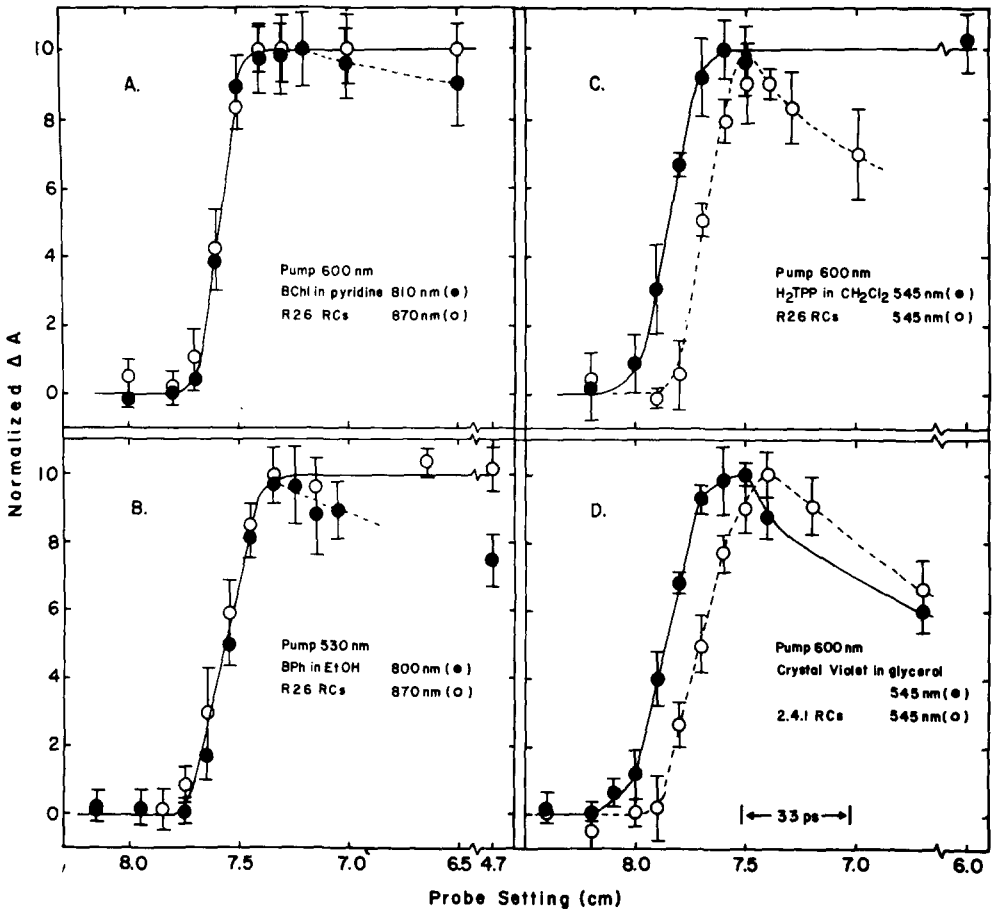

Fig. 2. Kinetics of absorbance changes upon excitation of reaction centers with 7-ps flashes. Normalized ΔA is plotted versus probe delay line setting for reaction centers and standard compounds in solution with pump and probe pulses of various wavelengths. The absolute value of the probe setting is unimportant, but a 1 mm change in the setting gives a change of 2 mm in optical path, corresponding to a time delay of 6.7 ps between arrival of pump and probe pulses at the sample. The delay time increases from left to right. For each frame, data for reaction centers and standard compounds were taken in the same pathlength cells: 2 mm for (A) and (B), 5 mm for (C) and (D). Each point represents the average of between 5 and 8 measurements with the standard deviation indicated. The concentrations and maximum absorbance changes for each sample are as follows, with the maximum absorbance changes in parenthesis: (A) 53 μM BChl (—0.53) and 8 μM reaction centers (—0.19); (B) 92 μM BPh (—0.31) and 12 μM reaction centers (—0.23); (C) 100 μM H_2TPP (+0.15) and 32 μM reaction centers (—0.11); (D) 50 μM crystal violet (—0.39) and 25 μM reaction centers (—0.09). Reaction centers were suspended in 50 mM Tris, pH 8, 0.05% Triton X-100 for (A) and (B) and in 10 mM Tris, pH 8, 0.1% lauryl dimethylamine oxide, 10 μM EDTA for (C) and (D). Similar results were obtained for (C) and (D) when BChl in pyridine or zinc tetraphenylchlorin were used for the standard compounds. Measurements like those shown in (A) and (B) were repeated with several reaction center preparations at each pump wavelength and yielded the same results within experimental error.

wavelengths are shown in Fig. 2. The open circles in Fig. 2A and B show the bleaching kinetics at 870 nm, upon excitation at 600 nm (Fig. 2A) or 530 nm (Fig. 2B). To establish the timing of the measuring pulses relative to the excitation flashes, we measured the 'instantaneous' absorbance changes accompanying the excitation of BChl to its excited singlet state in pyridine solution,

or of BPh in ethanol (filled circles). These measurements had to be made at somewhat shorter wavelengths (810 and 800 nm). To determine the delay by which measuring light at these wavelengths lagged behind light at 870 nm, we made similar measurements with BChl in pyridine at 820, 810, 770, 650, 545, and 450 nm and with BChl in aqueous micellar solutions at 830, 780 and 530 nm. Extrapolation of the measurements to 870 nm indicates that 800 and 810 nm light is delayed by less than 2 ps relative to 870 nm. (The difference in arrival times at the sample of the various wavelengths in the probe pulse is called 'chirping'. It is severe in the visible region of the spectrum but becomes progressively less important at longer wavelengths. The chirping results from the different velocities with which light of various wavelenghts travels through the CCl_4 cell where the continuum is generated, and through other components of the apparatus.)

The data in Figs. 2A and 2B show that there is no significant delay in the bleaching of P at 870 nm, after excitation at either 600 or 530 nm. These measurements do not provide any information of the rate of electron transfer, because absorbance changes due to the formation of P^+ and P^* would be indistinguishable at 870 nm. The lack of a delay in the bleaching after excitation at 600 nm is not surprising, because light of this wavelength should generate P^* directly. From the results obtained with 530-nm pulses, on the other hand, the transfer of energy from BPh^* to P would appear to be complete in less than 7 ps. This is somewhat shorter than the time of 10 ± 3 ps that Moskowitz and Malley [37] have estimated for energy transfer. Moskowitz and Malley's estimate was based partly on an apparent lag of 12 ps in absorbance changes measured at 1242 nm, and partly on the observation that saturating 530-nm flashes caused only about 60% of the P to undergo photo-oxidation. They interpreted the latter observation to mean that quenching of BPh^* by stimulated emission competes with energy transfer to P. We found some evidence for this effect, but it was not as pronounced as that reported by Moskowitz and Malley [37]. In three determinations with different preparations, the bleaching at 870 nm caused by saturating 530 nm flashes was 87 ± 5% of that expected [1] for complete photo-oxidation. The bleaching caused by saturating 600 nm flashes was 100 ± 5% of the expected amount.

Measurements of the bleaching at 803 nm after excitation at either 600 or 530 nm also showed no detectable lag relative to the absorbance changes obtained with BChl or BPh in solution (data not presented). In this case, the reference measurement could be made at the same wavelength, so that chirping was not a problem.

Figs. 2C and D show similar measurements of the absorbance changes at 545 nm, upon excitation at 600 nm. Absorbance changes obtained by exciting tetraphenylporphin in CH_2Cl_2 and crystal violet in glycerol were used to determine the time of the flash (filled circles). The absorbance changes in the standards were measured at 545 nm, so no corrections for chirping were needed. The absorbance decrease at 545 nm in the reaction centers is clearly delayed by 8 to 10 ps with respect to the absorbance changes seen with the standards.

The lag in the bleaching at 545 nm agrees qualitatively with the results that Shuvalov et al. [17] and Kruykov et al. [18] have obtained at 760 nm using 25-ps 880-nm flashes, but the delay that we observe is only about one-third as

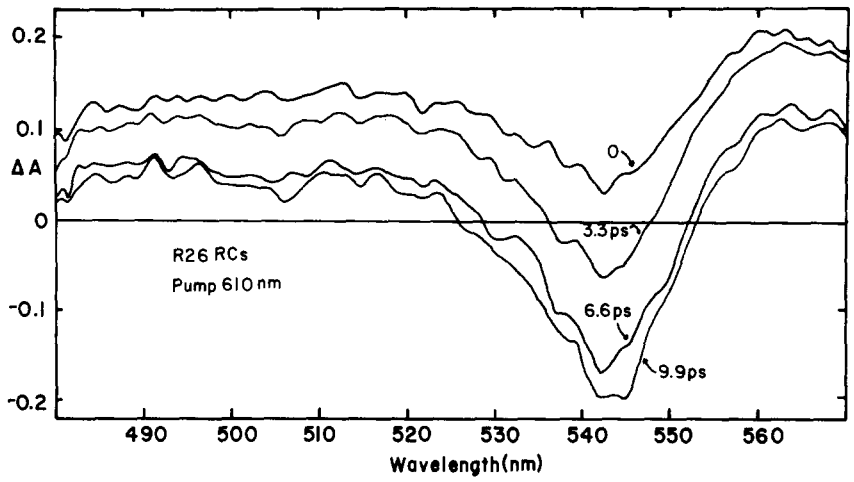

Fig. 3. Spectra of transient absorption changes observed at various delay times following excitation of 12 μM R-26 reaction centers (in 50 mM Tris, pH 8, 0.05% lauryl dimethylamine oxide) with 0.7-ps 610-nm pulses. Spectra taken at delays of 13.2 and 16.5 ps (not shown) were the same within error as that shown for a 9.9-ps delay. Each spectrum is the average of approx. 400 measurements. The zero time was measured at 610 nm and has an uncertainty of ±1 ps across the spectrum.

long as theirs. Although the difference between the delays could be due to our use of a different species of bacteria, another explanation suggests itself. In both cases, the delay is approximately equal to the width of the excitation flash. Such a delay could be explained by the assumption that the excitation flash causes two different types of absorbance changes at this wavelength. An absorbance increase with a very short decay time could be superimposed on a longer-lived absorbance decrease due to the reduction of BPh. The net absorbance change during the flash then could be positive, negative or zero, depending on the intensity and length of the flash and on the kinetics and quantum yields of the two opposing effects. After the flash, the absorbance increase would decay, and the longer-lived absorbance decrease would remain. A rapidly decaying absorbance increase could be due to P^*, and electron carrier between P^* and BPh, or to short-lived excited states produced by excitation of the reaction centers with an excessive number of photons.

If this interpretation is correct, the use of even shorter excitation flashes might decrease the lag in the bleaching at 545 nm still further. Fig. 3 shows spectra of the absorbance changes in the region around 545 nm, following excitation with 610-nm flashes lasting approx. 0.7 ps. Chirping of the probe beam in the subpicosecond apparatus is much less severe than that in the picosecond apparatus because a shorter cell is used for generating the continuum and mirrors are used instead of prisms for steering the beam to the sample position. The change in zero time across the spectrum in Fig. 3 is less than 1 ps. (The same is true for the other subpicosecond measurements presented below.) The spectrum measured during or within a few ps after the flash shows an absorbance increase over the entire wavlength range from 480 to 570 nm. Following the flash, the absorbance increase rapidly decays to a smaller amplitude (generally not zero), and is replaced by an absorbance decrease near 545 nm.

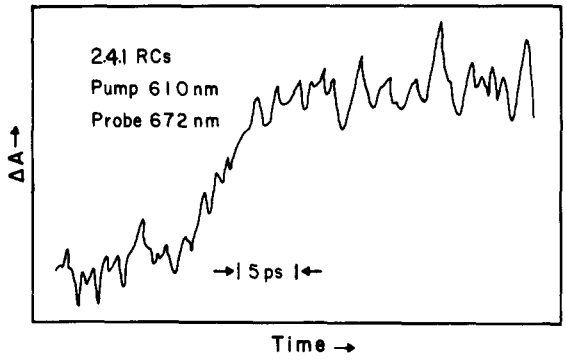

Fig. 4. Kinetics of the absorbance increase at 672 nm produced by excitation of 40 μM 2.4.1. reaction centers (in 10 mM Tris, pH 8, 0.1% lauryl dimethylamine oxide, 10 μM EDTA) with 0.7-ps pulses at 610 nm.

Judging from the difference between the absorbance change at 544 nm and the weighted mean of the changes at 526 and 554 nm, a trough around 545 nm appears to have developed to about 1/2 of its maximum depth even in the earliest measurement. The quatitative significance of this is not clear, because of the ±1 ps uncertainty in the timing of the excitation. The trough continues to grow in depth in the next few ps after the flash, and is fully developed by about 7 ps.

The bleaching at 545 nm shown in figs. 2C, 2D and 3 almost certainly reflects the formation of BPh^-, rather than BPh*, because the 600 and 610 nm excitation flashes fall within one of the absorption bands of the BChl in the reaction center and are well removed from bands due to the BPh. If P^+B^- is an intermediate in the electron transfer sequence, it must decay to P^+BPh^- with a time constant of no more than 4 ps.

The formation of BPh^- also can be detected from its broad absorption band in the 620—720 nm region (Fig. 1). Fig. 4 shows the kinetics of the absorption increase at 672 nm, following excitation with the 0.7 ps 610 nm flashes. The

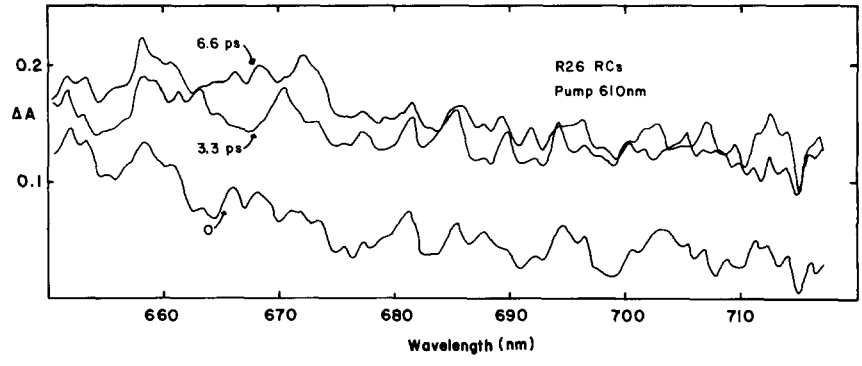

Fig. 5. Spectra of transient absorption changes following excitation of R26 reaction centers with 0.7 ps 610 nm pulses. Spectra at 9.9 ps and longer times were the same within error as that taken at 6.6 ps. Other conditions were as in Fig. 4.

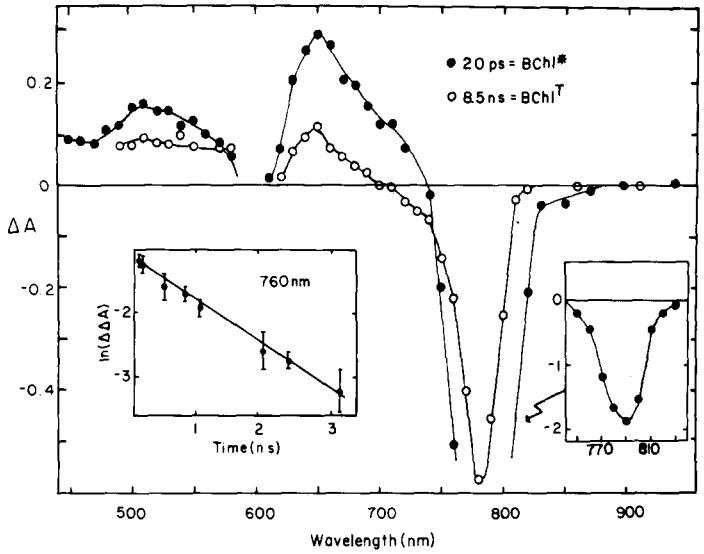

Fig. 6. Spectra of absorbance changes accompanying the formation of the lowest excited singlet ($BChl^*$) and excited triplet ($BChl^T$) states for 53 μM BChl in undegassed pyridine solution. The excitation flashes were 7-ps pulses at 600 nm. The delay time of 20 ps for the $BChl^*$ spectrum was determined at 800 nm; the delay increases at shorter wavelengths according to the curve shown in Fig. 3. At 450 nm the delay time is approx. 60 ps. However this causes little distortion of the spectrum, since the time constant for the decay of $BChl^*$ is 1.6 ± 0.1 ns (inset at lower left). The data for ground state bleaching in the region about 790 nm in the 20-ps spectrum (inset at lower right) were obtained with a sample diluted by a factor of 5 and then normalized to the same scale as the rest of the figure. The quantity $\Delta\Delta A$ on the ordinate of the decay time measurement shown in the left-hand inset is equal to $\Delta A(t) - \Delta A(\infty)$, where $\Delta A(t)$ is the absorbance change at time t, and ΔA (∞) is the absorbance change at the asymptote of the decay curve. $\Delta A(\infty)$ generally was obtained from measurements made between 7 and 9 ns after the excitation. The standard errors for the time constants reported include the error introduced by the uncertainty in determination of $\Delta A(\infty)$. This uncertainty was typically $\Delta A \simeq 0.025$.

absorbance increase at 672 nm appears to develop with a time constant of about 4 ps.

Spectra of the early absorbance changes across the entire 650—715 nm region are given in Fig. 5. During or within a few picoseconds of the flash, one sees a new absorption band that appears to peak near 650 nm. (There is a similar peak near 650 nm in a spectrum reported by Shuvalov et al. [17].) The absorbance on the red side of this band continues to grow in the 3—7 ps period after the flash, so that by 7 ps the spectrum has the featureless form associated with P^+BPh^- (Fig. 1). These measurements and those of Fig. 4 support the conclusion drawn from Fig. 3 that electron transfer to BPh occurs with a time constant no greater than 4 ps. However the measurements made at 545 nm are more compelling than those made at 672 nm because, as Shuvalov et al. [17] have pointed out, B^- could resemble BPh^- in having an absorption band in the latter region. In addition, the short-lived states that give the broad absorbance increases in the 480—570 nm region and the peak near 650 nm probably contribute to the initial stages of the absorbance increase at 672 nm.

What accounts for the absorbance increases that occur throughout the visible region of the spectrum during the flash? One possibility would be the putative P^+B^- radical pair. Another is excited singlet states of the BChls. Fig. 6 shows spectra of the absorbance changes that accompany the excitation of BChl to its

excited singlet and triplet states in pyridine. The spectra include broad absorption bands due to the excited states, broken by troughs in regions where the ground state absorbs. Note that the excited states have absorption peaks near 650 nm. The absorption spectrum of BChl* has not been reported previously, but similar spectra have been obtained for BPh* in solution [32], for P* in *Rps. viridis* [7], and for the triplet states of BChl and BPh in a variety of solvents [32,38]. (The inset in Fig. 6 shows the decay kinetics of BChl* in a pyridine solution similar to that used for the main part of the figure; the decay time is 1.7 ± 0.2 ns. This solution was not depleted of O_2. Similar measurements with deoxygenated solutions in pyridine or pyridine/ethanol (1 : 3, v/v) gave a decay time of 2.7 ± 0.2 ns.) Connolly, et al. [40] reported fluorescence lifetimes for BChl ranging from 2 ns in methanol to 3.1 ns in pyridine. Their measurements were made from single photon counting. Goedheer [39] reported a fluorescence lifetime of 1.6 ns calculated from measurements of fluorescence depolarization in various solvents.

One excited state that should be present at times preceeding the reduction of BPh is P*. The observation that the early absorbance increase decays approximately in parallel with the reduction of BPh (Fig. 3) thus suggests that the initial absorbance changes are due to P* and that electron transfer occurs directly from P* to BPh. However, excited states of the two other BChls also could contribute to the absorbance increases observed during the flash. These probably would be indistinguishable from P* in the visible region of the spectrum. The 610-nm excitation flashes presumably would be absorbed by the other BChls, as well as by P. The importance of excited states of the other BChls is likely to depend on the intensity and duration of the flash. Strong flashes will cause multiple excitations of each reaction center, and nonlinear processes such as exciton annihilation and stimulated emission could shorten the lifetimes of the excited states. The excited states thus could turn over very rapidly during the flash, making it extremely difficult to interpret the absorbance changes during and shortly after the flash. For this reason, we consider the value of 4 ps derived from the measurements of Figs. 3 and 4 to be only an upper-limit for the time constant for the reduction of BPh.

In the hope of distinguishing between P^+B^-, P*, and excited states of the other BChls, we studied the absorbance changes that occur in the 750—815 nm region of the spectrum. Fig. 7 shows measurements made with 610-nm flashes lasting 0.7 ps. At 1.8 ps after the flash, one sees a large absorbance decrease centered near 803 nm. The initial bleaching decays with multiphasic kinetics and is replaced by an absorbance decrease centered near 810 nm and an absorbance increase near 790 nm (cf. Fig. 1). This relaxation is only partially completed in the 15 ps time interval covered in Fig. 7.

The decay kinetics of the bleaching at 803 nm after excitation with 7 ps pulses at 530 and 600 nm are shown in Fig. 8 (open circles and squares). In both cases, the decay has a slow component with a time constant of about 200 ps. This probably reflects the absorbance changes associated with BPh^- (or more accurately, with a BChl that interacts with the BPh that undergoes reduction). The time constant for the slow component is essentially the same as that for electron transfer from BPh^- to Q, as measured at 545 or 670 nm (Fig. 8 closed circles and squares, and Refs. 7, 9—14, 17). Preceeding the slow phase of

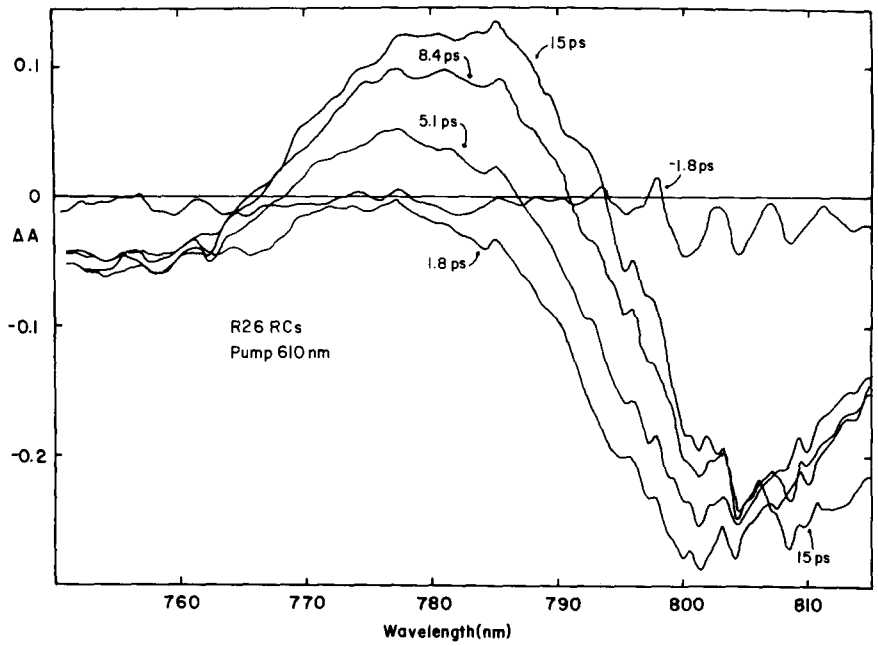

Fig. 7. Spectra of absorbance changes measured at various delay times following excitation of 2 μM R-26 reaction centers (in 50 mM Tris, pH 8, 0.05% lauryl dimethylamine oxide) with 0.7-ps flashes at 610 nm. As shown by the measurement at —1.8 ps, the data at wavelengths beyond 800 nm have a poorer signal to noise ratio than those at shorter wavelengths because of falling vidicon detector response in this region of the spectrum.

the relaxation at 803 nm, there is a fast phase that can be seen most clearly after excitation with the stronger 530-nm flashes (Fig. 8 open circles). The time constant of the faster phase is about 30 ps.

The 30 ps component of the bleaching at 803 nm seems unlikely to be due to P^+B^-, because its decay kinetics do not match the kinetics of electron transfer to BPh (Figs. 2—5). It could be due to a relaxation of the interactions among the components of the raction center (as Rockley et al. [9] have suggested), or to other excited states of the BChls. To distinguish between these possibilities, we investigated its dependence on the flash intensity. Fig. 9A shows the absorbance changes measured at 803 nm at 13 ps and at 73 ps after excitation with 530-nm flashes. The measurements at 73 ps reflect mainly the component with the slower decay kinetics; those at 13 ps reflect the sum of the two components. (Note that the absorbance changes measured at the two times are plotted with the different vertical scales, so as to normalize them at the highest flash intensities.) The saturation curve for the slow component (filled circles) is essentially the same as that for the absorbance changes due to BPh^- measured at 680 nm 13 ps after excitation (Fig. 9B). Both reach half their maximum amplitudes when the excitation pulses have an incident intensity of approx. 1.5 mJ · cm^{-2} (6.9×10^{15} quanta per cm^2). This is close to what one expects if the formation of P^+BPh^- occurs with a quantum yield near 1.0. (The absorption cross-section of R-26 reaction centers at 530 nm is approx. 1.7×10^{-16} cm^2; 6.9×10^{15} quanta per cm^2 gives 1.2 excitations per reaction center

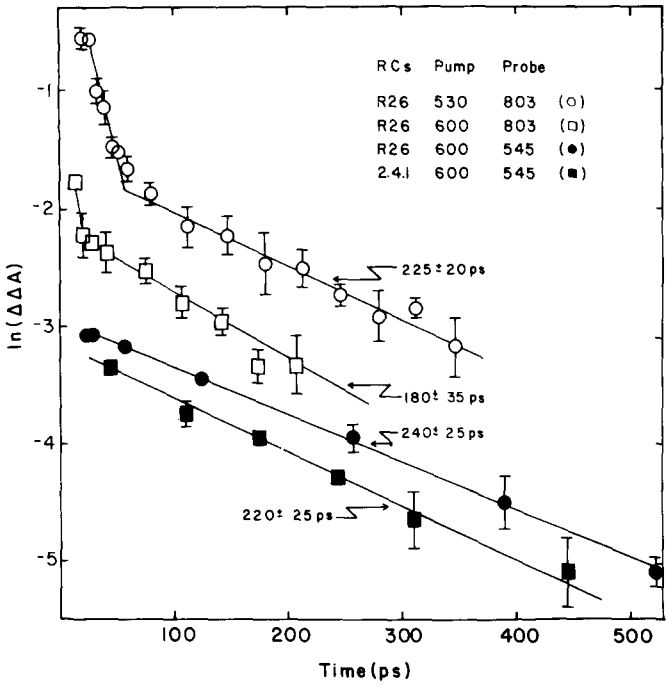

Fig. 8. Decay kinetics of the initial bleaching at 545 and 803 nm in R-26 and 2.4.1 reaction centers, following excitation with 7-ps flashes at 530 or 600 nm. Each point is the average of between 5 and 8 measurements. The curves have been displaced vertically in units of $\Delta\Delta A$ as follows: ○, 0; □, —1.1; ●, —1.5; ■, —1.7. Reaction center concentrations were 32 μM and 25 μM for strains R-26 and 2.4.1, respectively. Pathlengths of 2 mm were used for measurements at 803 nm, and 5 mm for those at 545 nm. The average of nine measurements for (○) gives lifetimes of 30 ± 15 and 200 ± 25 ps at full or 25% flash strength and three measurements for (□) gives 190 ± 30 ps.

at the front surface of the sample cell and less in deeper regions of the cell.) The fast component of the bleaching has quite a different dependence on the flash intensity: it does not reach saturation even with the strongest flashes that we can obtain (Fig. 9A, crosses). This result disagrees with a less extensive series of measurements reported previously by Rockley et al. [9].

The saturation curves shown in Fig. 9 indicate that the component of the bleaching at 803 nm that relaxes with 30-ps kinetics probably is not involved in the primary electron transfer reaction. Instead, it seems likely to reflect a state that is generated when the reaction centers are excited with an excessive number of photons. In agreement with this conclusion, transient bleaching with similar decay kinetics also can be seen when P has been oxidized with $K_3Fe(CN)_6$. Fig. 1B shows spectra of the absorbance changes that result from excitation of reaction centers with 7-ps 530-nm flashes under these conditions. The amplitude of the bleaching at 870 nm is greatly diminished compared to that seen at moderate redox potentials (Fig. 1A), indicating that about 90% of the P was in the oxidized state. Transient bleaching near 800 nm dominates the spectrum measured at 20 ps. Most of this bleaching decays with a time constant of approximately 30 ps (data not shown). In the oxidized reaction centers one also sees bleaching of the BPh absorption band at 760 nm. Part of this decays

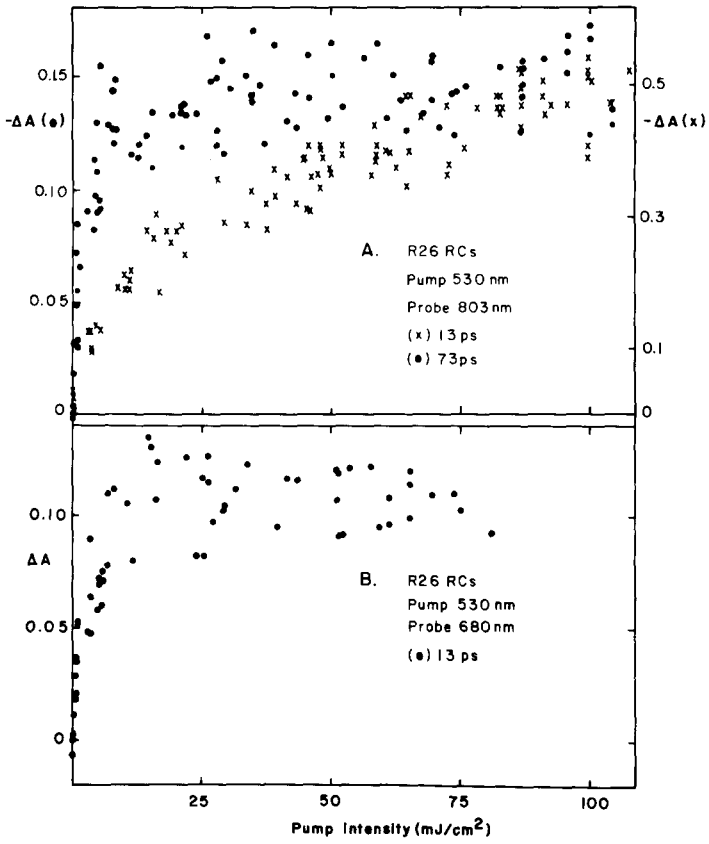

Fig. 9. Dependence of bleaching at 803 nm (A) and absorbance increase at 680 nm (B) on the intensity of 7-ps 530-nm excitation pulses. The energy of each pulse was measured with a calibrated photodiode as described in Methods and Materials. Each point gives the result of a single laser shot. The delay times indicated were determined at the measuring wavelengths with both reaction centers and standard compounds. For (A) the absorbance scale for the 13-ps data is on the right, while that for results at the longer time is the left; the scales are selected to normalize the data at the highest flash intensities. The reaction center concentrations and pathlengths are 18 μM in 2 mm for (A) and 25 μM in 5 mm for (B). The intensity dependence at each set of pump and probe wavelengths was repeated at least three times with the same results within experimental error. Results similar to those in (A) were found also when 600-nm excitation flashes were used.

in the 30-ps time range, but most decays more slowly, on the time scale of 0.4 to 2 ns.

Akhamanov et al. [41] have studied the transient bleaching near 800 nm in *Rps. rubrum* after excitation with 50-ps 870-nm flashes. They too have concluded that at least part of the bleaching is not linked to the primary electron transfer reaction. A likely identification of the transient state is an excited singlet state of the 'extra' BChl. On this interpretation, one would expect that strong 7-ps flashes would cause absorbance increases with similar decay kinetics throughout the visible region of the spectrum. Fig. 1B shows transient absorbance increases in the expected places, but the absorbance changes are relatively small and we did not attempt to measure their decay kinetics accurately. Another possibility is that the short-lived component is not an absorbance

decrease at all but rather stimulated emission from an excited state. Reaction centers have a fluorescence maximum near 800 nm [42]. Emission stimulated by the probe light would be spatially and spectrally coherent with the probe pulse and would appear as an increased transmission by the sample.

It is perhaps worth emphasizing that the interpretation of the 30 ps component in terms of an excited singlet state is not inconsistent with the suggestion that similar excited states turn over more rapidly than this during the excitation flash. As mentioned above, excited states could be quenched by stimulated emission or singlet-singlet annihilation during and at short times after the flash. The 30-ps lifetime itself is already much shorter than the 2-ns lifetime of BChl* in solution (Fig. 6), indicating that special quenching processes can occur in the reaction center. The observation of an unexpectedly short (20 ps) lifetime for P* in reduced reaction centers has been discussed previously [7].

As mentioned above, the 600- and 610-nm excitation flashes that we have used could be absorbed by the other BChls as well as by P. Energy transfer from the other BChls to P presumably occurs within a few picoseconds, as long as P has not been oxidized. It is conceivable, however, that the excitation of B results in direct electron transfer from B* to BPh, followed by electron transfer from P to B^+. While we cannot rule out this sequence of steps there is evidence that it is not a particularly favorable pathway: the quantum yield of P^+ is somewhat lower with 800 nm excitation than it is when P is excited directly at longer wavelengths [43].

In conclusion, electron transfer from P* to BPh appears to occur with a time constant of no more than 4 ps. The 30-ps bleaching at 803 nm appears to be largely, if not entirely, due to excited states that are not involved in the electron transfer reaction. The correlation that Shuvalov et al. [17] and Kryukov et al. [18] observed between the 35-ps relaxation near 800 nm and a 35-ps delay in the bleaching at 760 nm can be plausible attributed to their use of an excitation flash lasting 25 ps. Absorbance changes measured during and shortly after the flash can be complicated by the presence of very short-lived excited states. For the same reason, however, it is clear that we cannot rule out the possibility that P^+B^- is an intermediate in the electron transfer sequence, provided that it decays to P^+BPh^- within 4 ps.

Acknowledgements

We are indebted to Dr. R.E. Blankenship for stimulating discussion and for help with the preparation of the reaction centers. Mona Hauser assisted with some of the picosecond measurements. This work was supported by National Science Foundation grants PCM-77-13290-AO2 and PCM79-02911.

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