

# An Alternative Pathway of Light-Induced Transmembrane Electron Transfer in Photosynthetic Reaction Centers of *Rhodobacter sphaeroides*

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**Abstract**—In the absorption spectrum of *Rhodobacter sphaeroides* reaction centers, a minor absorption band was found with a maximum at 1053 nm. The amplitude of this band is ~10,000 times less and its half-width is comparable to that of the long-wavelength absorption band of the primary electron donor P<sub>870</sub>. When the primary electron donor is excited by femtosecond light pulses at 870 nm, the absorption band at 1053 nm is increased manifold during the earliest stages of charge separation. The growth of this absorption band in difference absorption spectra precedes the appearance of stimulated emission at 935 nm and the appearance of the absorption band of anion-radical B<sub>A</sub><sup>-</sup> at 1020 nm, reported earlier by several researchers. When reaction centers are illuminated with 1064 nm light, the absorption spectrum undergoes changes indicating reduction of the primary electron acceptor Q<sub>A</sub>, with the primary electron donor P<sub>870</sub> remaining neutral. These photoinduced absorption changes reflect the formation of the long-lived radical state PB<sub>A</sub>H<sub>A</sub>Q<sub>A</sub><sup>-</sup>.

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Reaction centers (RC) of purple photosynthetic bacteria are transmembrane pigment–protein complexes that carry out primary conversion of light energy into electrochemical energy of separated charges during photosynthesis. The spatial structure of RCs was determined with high resolution [1, 2]. *Rhodobacter sphaeroides* RCs consists of three protein subunits (L, M, and H) and 10 cofactors of electron transfer: four bacteriochlorophyll (BChl) molecules, two of which form a dimer of the electron donor special pair P, and two others are accessory BChl (B<sub>A</sub> and B<sub>B</sub>); two bacteriopheophytin (BPheo) molecules (H<sub>A</sub> and H<sub>B</sub>); two ubiquinone molecules (Q<sub>A</sub> and Q<sub>B</sub>); an ion of non-heme iron, and a carotenoid molecule. A key element of reaction centers is the primary electron donor P, which, in its excited state P\*, can transfer an electron to the primary acceptor in a few picoseconds, inducing all the following photosynthetic reactions.

In many laboratories it has been shown that within 3 ps under illumination, a charge-separated state P<sup>+</sup>H<sub>A</sub><sup>-</sup> is formed in reaction centers [3-7]. Electron transfer from the excited donor P\* to the H<sub>A</sub> molecule occurs in two stages with an intermediate electron acceptor, the B<sub>A</sub> molecule [8-13]. The kinetics of rise and decay of the absorption band of the anion-radical B<sub>A</sub><sup>-</sup> at 1020 nm at room temperature is consistent with a kinetic scheme in which an electron is transferred from the excited donor P\* to a B<sub>A</sub> molecule with a time constant of 3.5 ps, and then from B<sub>A</sub><sup>-</sup> to H<sub>A</sub> with a time constant of 0.9 ps [4]. With a time delay of 200 ps, an electron crosses the membrane and is localized at the primary quinone acceptor Q<sub>A</sub> [14-16].

Much less is known about the processes taking place in reaction centers during the earliest, femtosecond delay times. This is because with time delays from 0 to ~200 fs, changes in light absorption are largely masked by a coherent action of ultrashort excitation and probing light pulses. The interaction of pulses causes perturbations that are expressed in difference absorption spectra as waves that distort or obscure minor changes in light absorption.

It was shown earlier that during excitation of primary electron donor P with ultrashort light pulses (~20 fs), irre-

*Abbreviations:* B<sub>A</sub> and B<sub>B</sub>, accessory bacteriochlorophylls; BChl, bacteriochlorophyll; BPheo, bacteriopheophytin; ET, electron transfer; H<sub>A</sub> and H<sub>B</sub>, bacteriopheophytins serving as electron acceptors; P, BChl special pair (P<sub>A</sub> and P<sub>B</sub>) that is a primary electron donor; RC, reaction center.

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versible electron transfer from the excited primary electron donor to the nearest acceptor, the  $B_A$  molecule, is accompanied by a reversible, oscillating electron transfer [12, 13, 17, 18]. This oscillating process of electron transfer is caused by nuclear wave packet motion along the potential surfaces of the excited primary donor  $P^*$  and the radical pair  $P^+B_A^-$ . Due to this oscillating process, an electron localizes on a  $B_A$  molecule already within 100 fs after the act of light absorption.

On the other hand, within the same early time delays of ~100–200 fs, absorption changes were observed in *Rba. sphaeroides* reaction centers that reflect charge separation within the dimer of the special pair and the formation of a short-lived state with charge transfer,  $P^*$  ( $P_A^{\delta+}P_B^{\delta-}$ ) [19, 20].

The same conclusion was reached by other authors [21] who found a spectral component that may reflect charge separation within the excited primary electron donor in the infrared range (1000–1600  $\text{cm}^{-1}$ ) of the absorption spectrum of *Rba. sphaeroides* reaction centers within 200 fs after light absorption.

Recently, Australian researchers discovered a minor band with a maximum at 705 nm within the long-wavelength absorption spectrum region of photosystem 2 complexes [22]. Excitation of complexes in this absorption band caused photochemical charge separation.

Zhu et al. [23] detected reduction of active bacteriopheophytin  $H_A$  at unexpectedly short delay times of  $\leq 500$  fs, which does not correspond to the generally accepted simple scheme of two-stage electron transfer with rate constants of 3 and 1 ps.

Thus, the processes taking place in reaction centers of *Rba. sphaeroides* during the earliest femtosecond delay times remain unclear.

## MATERIALS AND METHODS

Wild-type reaction centers were isolated from the corresponding strain of purple bacteria *Rba. sphaeroides* as described earlier [24], diluted in 20 mM Tris-HCl buffer (pH 8.0) with 0.1% Triton X-100, and concentrated to optical density 0.5 at 760 nm (with 1-mm optical pathlength). Absorption spectra for the reaction centers were measured at room temperature with a UV1600PC spectrophotometer (Shimadzu, Japan). The long-wavelength range of the absorption spectrum, 950–1100 nm, was measured on samples with optical density of 5 at 870 nm (with 10-mm optical pathlength) and accumulated to improve signal-to-noise ratio. The spectra were averaged over ten measurements and normalized by the optical pathlength.

To carry out femtosecond measurements, 5 mM sodium dithionite was added to the reaction centers and they were illuminated with low intensity white light for 5 min to reduce the primary electron acceptor  $Q_A$ .

Femto-second difference absorption spectra were obtained using a pump–probe spectrometer. A MaiTai SP titanium-sapphire laser (Spectra-Physics, USA) was used to obtain pulses of ~25 fs duration with wavelength 800 nm and repetition rate 84 MHz. Generator pulses were routed into a Spitfire Ace optical regenerative amplifier (Spectra-Physics), and ~35 fs pulses were obtained at the output with energy ~4 mJ and repetition rate 50 Hz. The light beam coming out of the amplifier was split into two beams. The first beam was attenuated to the energy of ~0.3  $\mu\text{J}$ /pulse and focused in a water-filled cuvette with 5-mm thickness to generate a continuum, which was used for probing pulses. The second beam with the energy of ~17  $\mu\text{J}$ /pulse was used to generate a continuum on a sapphire with ~2-mm thickness. From the resulting continuum, light with wavelength  $>850$  nm was cut-off by an RG850 light filter (Newport, USA) and used for sample excitation. Excitation and probing pulses were focused in the plane of a rotating quartz cuvette with 1-mm thickness containing the sample and overlapped at the same point within the sample volume. Relative polarization of excitation pulse was parallel to measuring pulse. A time delay between pumping and probing pulses was varied with a PI-M531.DD computer-controlled optical delay (Physik Instrumente, Germany). After the sample cuvette, the probing pulse was directed to the entrance slit of a Spectra Pro 2300i spectrograph (Acton Research Corporation, USA). The spectra were registered with a Pixis 400BR CCD camera (Princeton Instruments, USA). To obtain a difference absorption spectrum, the excitation pulse was blocked by a 3501 chopper (NewFocus, USA) during each time delay. Approximately 10% of primary electron donors in reaction centers were excited by the pulse. Difference absorption spectra for each time delay were obtained by averaging 1000–5000 measurements.

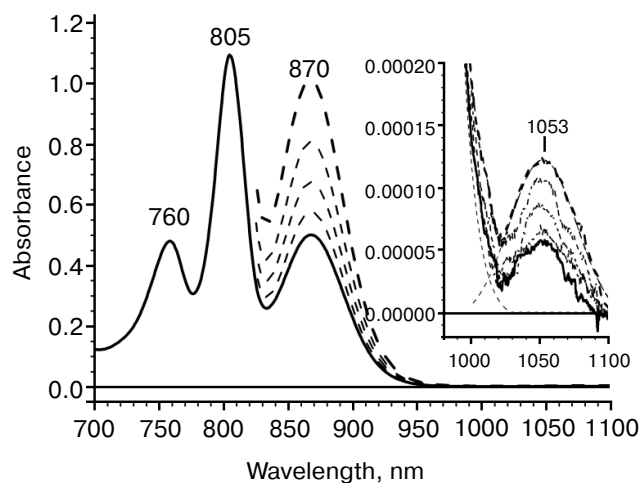
Stationary photoinduced difference absorption spectra for  $P^+Q_A^-/PQ_A$  were recorded on a UV1800 spectrophotometer (Shimadzu, Japan) during illumination of the sample with excitation light in the direction perpendicular to that of the measuring beam. The sample was illuminated during spectrum registration, the duration of which was ~80 s. A series of pulses from a Nd:YAG LS-2130 laser was used as the excitation light source (pulse duration 10 ns; repetition rate 20 Hz; wavelength 532 or 1064 nm).

## RESULTS AND DISCUSSION

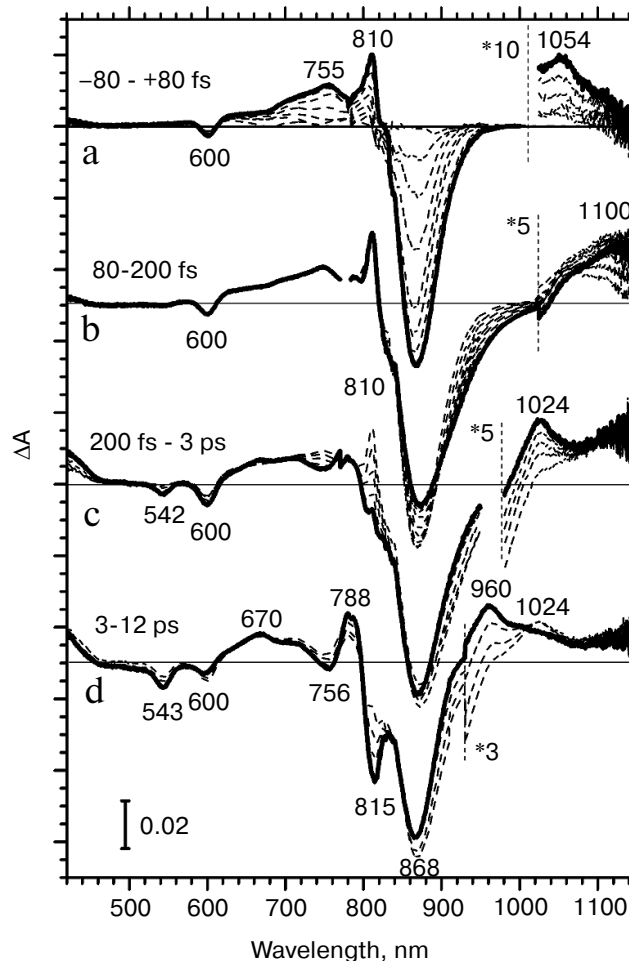
Figure 1 presents the room temperature absorption spectrum of reaction centers of the purple bacteria *Rba. sphaeroides*. The absorption band with maximum at 870 nm reflects a low-energy transition within the dimer of the special pair P; the absorption maxima at 805 and 760 nm correspond to absorption of accessory bacterio-

chlorophyll ( $B_A$  and  $B_B$ ) and bacteriopheophytin ( $H_A$  and  $H_B$ ), respectively. In the long-wavelength spectral range, positive absorption is observed; it is caused by light scattering of reaction center (RC) pigment–protein complexes. Against the background of RC complex light scattering, a minor absorption band can be observed with maximum at 1053 nm (Fig. 1, inset). This absorption band remains in the absorption spectra of reaction centers that were adapted to the dark during a long time and corresponds specifically to the reaction center complex, since its amplitude changes proportionally to concentration of complexes, just as the amplitudes of other absorption bands of reaction centers do (Fig. 1, dashed line). The amplitude of the absorption band at 1053 nm is 9000 times less than that of the primary electron donor absorption band at 870 nm. At the same time, half-widths of these bands are practically the same, which may indicate an association of the band at 1053 nm to the low-energy  $Q_y$  transition of P. Therefore, our data suggest the presence of an electron transition not described earlier in *Rba. sphaeroides* RC that is lower by 0.2 eV compared to the  $Q_y$  transition of P.

The extremely small amplitude of the absorption band at 1053 nm indicates that it corresponds to a low-intensity optical transition. In particular, it can be proposed that the absorption band at 1053 nm reflects a “hot” transition from one vibrational level of the ground state  $S_0$  of the primary electron donor P to its lowest singlet excited level  $S_1$ . The proposed vibrational level is located  $2050\text{ cm}^{-1}$  higher than  $S_0$ . According to Boltzmann temperature distribution, the population of such a vibrational level at room temperature would be



**Fig. 1.** Absorption spectrum for reaction centers (RC) of wild-type *Rhodospirillum rubrum* measured at room temperature with 1-mm optical pathlength. The dashed lines show absorption spectra measured with increased RC concentration. Inset: absorption spectra in 980–1100 nm range obtained by accumulating 10 measurements.



**Fig. 2.** Difference light-minus-dark absorption spectra for reaction centers of *Rhodospirillum rubrum* measured at room temperature: a) from  $-80$  to  $80$  fs; b) from  $80$  to  $200$  fs; c) from  $200$  fs to  $3$  ps; d) from  $3$  to  $12$  ps. The spectra were obtained by accumulating 5000 measurements.

$\sim 10^{-4}$ , which corresponds to the experimentally measured ratio of amplitudes of absorption bands at 1053 and 870 nm. With this assumption, the fact that half-widths of these absorption bands match would also be explained.

Another and perhaps more probable proposition is attributing the absorption band at 1053 nm to a spin-forbidden singlet–triplet ( $S_0 \rightarrow T_1$ ) P transition. It was shown earlier that the maximum of P phosphorescence spectrum in *Rba. sphaeroides* RCs is located at 1318 nm [25, 26]. Given these data, the model considered here assumes the existence of a Stokes shift of  $\sim 260$  nm. Even though this value appears to be unusually high, such a possibility should not be excluded, since a  $S_0 \rightarrow T_1$  transition was not detected experimentally due to it being spin-forbidden. It should be noted that in the triplet state with the lifetime of  $\sim 1\ \mu\text{s}$  the probability for relaxation of vibronic levels is significantly higher than that for a singlet state.

Excitation light induces transmembrane electron transfer from the excited primary electron donor  $P^*$  to the primary quinone acceptor  $Q_A$  in reaction centers. Various stages of this process can be directly observed by changes in the absorption of the reaction centers occurring at different time delays after a femtosecond excitation of the primary electron donor. Figure 2 presents difference absorption spectra of *Rba. sphaeroides* RCs reflecting evolution of light energy conversion into the energy of charge separated states. For instance, in reaction centers with pre-reduced quinone, the  $P^+H_A^-$  state is formed within 12–15 ps (Fig. 2d).

With a time delay of 200 fs, an excited electron leaves the special pair dimer and is transferred to the nearest acceptor, the  $B_A$  molecule. This is indicated by an increase in the anion-radical  $B_A^-$  absorption band in difference spectra at 1024 nm, which occurs in the ion-radical pair  $P^+B_A^-$  within 2.5 ps (Fig. 2c). Absorption changes with time delays of less than 200 fs apparently occur in the excited primary electron donor  $P_{870}$ .

At time delays from 80 to 200 fs after light absorption, stimulated emission increases at 935 nm, and absorption increases at 1100 nm (Fig. 2b). Earlier, we associated these changes in absorption to the formation of a charge transfer complex  $P^*$  ( $P_A^{\delta+}P_B^{\delta-}$ ), since the absorption band at 1100 nm is absent in the dark absorption spectrum, and it appears simultaneously with the increase in stimulated emission at 935 nm in femtosecond difference absorption spectra [20, 21].

During the earliest time delays, from –80 to 80 fs, changes in difference absorption spectra are due to the transition of  $P_{870}$  to the excited state (Fig. 2a). A bleaching of P absorption bands at 870 and 600 nm occurs due to the primary donor transitioning to the lowest exciton excited level  $S_1$  and to an increase in stimulated emission of the excited state at 870 nm. Positive absorption bands appear at 755, 810, and 1053 nm, which reflect the properties of absorption of the excited primary electron donor. The absorption band at 1053 nm appears in difference spectra during delay times that are as small as if it were one of the absorption bands of the excited primary electron donor  $P^*$ .

From the fact that the minor absorption band at 1053 nm is observed in a steady-state absorption spectrum (Fig. 1), it follows that this band reflects the  $S_0 \rightarrow S_x^*$  transition. If this absorption band corresponds to the primary electron donor, then an absorption decrease might be expected in this range on difference absorption spectra (Fig. 2a). However, during primary electron donor excitation by 870 nm light, the absorption band at 1053 nm is increased multifold within the earliest time delays. Such a time delay is so small that it is comparable to the magnitude of light dispersion in the probing pulse.

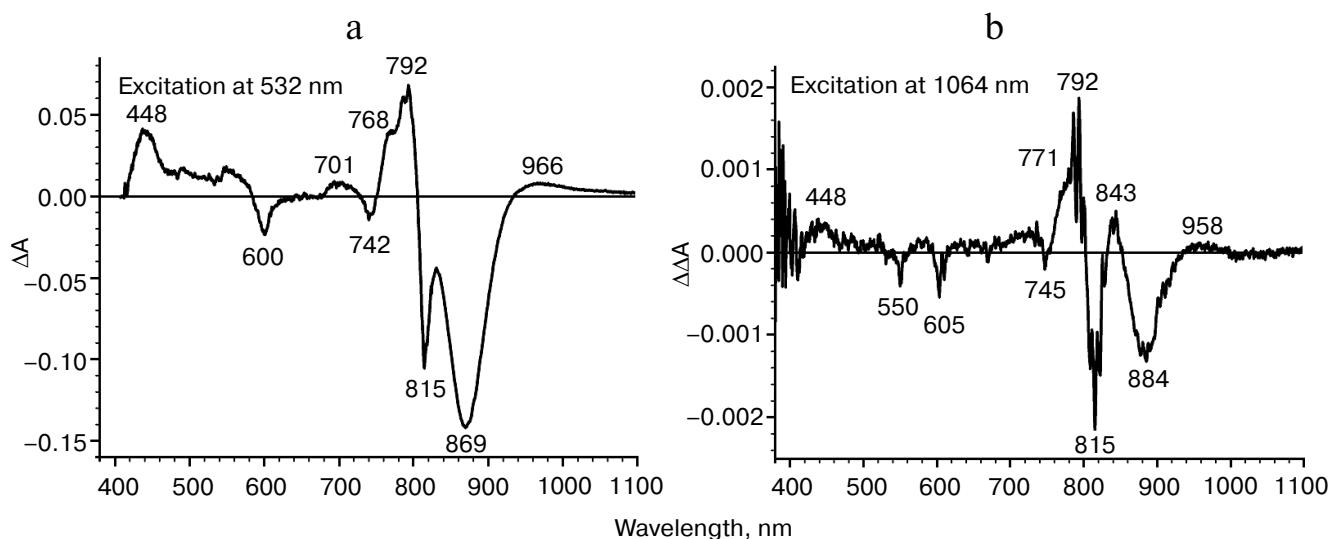
We can evaluate the degree of increase in absorption band at 1053 nm. Optical density of the absorption band at 1053 nm is  $\sim 0.00006$  OD in the dark absorption spec-

trum. It increases up to  $\sim 0.004$  OD in femtosecond difference spectra that is by  $\sim 100$ -fold. Because a primary electron donor is excited only in  $\sim 10\%$  of RCs during femtosecond measurements, this number should be increased by an order of magnitude. Therefore, during the excitation of a primary electron donor, absorption at 1053 nm increases by three orders of magnitude.

It is known that by illuminating RC with continuous light, absorption changes can be observed that are caused by the formation of the charge-separated state,  $P^+Q_A^-$ , whose lifetime is approximately 100 ms at room temperature. For instance, during RC illumination by 532 nm light, the difference absorption spectrum is observed that is shown in Fig. 3a. The spectrum includes: (i) bleaching of primary electron donor absorption bands at 870 and 600 nm and the appearance of a positive band in the absorption range of quinone  $Q_A^-$  anion-radical at 448 nm; (ii) positive absorption changes in the long-wavelength spectrum range, corresponding to  $P^+$ ; (iii) electrochromic shifts of accessory bacteriochlorophyll absorption bands at 800 nm and of bacteriopheophytin at 760 nm. All these changes indicate formation of a charge-separated state  $P^+Q_A^-$ . The same difference absorption spectrum is observed during excitation of reaction centers with light of any other wavelength.

If the absorption band at 1053 nm corresponds to the primary electron donor or to the complex with charge transfer,  $P_A^{\delta+}P_B^{\delta-}$ , then during excitation of reaction centers in this absorption band, oxidation of the primary electron donor might be expected if the quantum energy were not so low.

During excitation by 1064 nm light, at least two processes take place in reaction centers: (i) long-lived absorption changes, including short-wavelength shifts of primary electron absorption bands at 870 nm and of accessory bacteriochlorophyll at 800 nm, similar to electrochromic shifts. It is however significant that no positive absorption bands of known ion-radical RC pigment forms are observed in the spectrum (data not shown). These changes in absorption are apparently not connected to electron transfer, but may rather be due to a local variation in sample temperature. Alongside these absorption changes in RCs, a second process occurs – accumulation of a state in which a positive absorption band is appeared at 450 nm on the minute time scale. To minimize the contribution of the first process, we measured a double-difference absorption spectrum after illuminating RCs by 1064 nm light, as shown in Fig. 3b. In this spectrum, positive changes in absorption at 450 nm and a long-wavelength H shift at 760 nm seem to indicate quinone reduction. In the long-wavelength absorption range, there is no significant bleaching of primary electron donor absorption band associated to a transition of P into the cation-radical form, but rather a short-wavelength shift is observed, which is apparently caused by the contribution from the first process. The lifetimes of the absorption changes



**Fig. 3.** Difference light-minus-dark absorption spectra for reaction centers of *Rhodobacter sphaeroides* measured at room temperature under constant illumination by nanosecond light pulses with frequency 20 Hz: a) excitation light with wavelength 532 nm; b) double-difference spectrum, excitation light with wavelength 1064 nm. The spectra were obtained by accumulating 10 measurements.

shown in the figure increase up to tens of minutes. These results suggest that photoinduced quinone reduction takes place; however, there are no facts indicating whether the reducing electron belongs to the dimer of the special pair or some other component of the RC complex.

Thus, during the excitation of reaction centers with 1064 nm light, changes occur in the absorption spectrum indicating reduction of the primary electron acceptor  $Q_A$ , while the primary electron donor  $P_{870}$  apparently remains neutral. Absence of charge recombination suggests that these photoinduced changes in absorption reflect the formation of the long-lived state  $PQ_A^-$ .

Summarizing these data, we note the following. Earlier we discovered an absorption band at 1100 nm, which we interpreted as a mixed state of the excited primary electron donor and the complex with charge transfer. However, according to the results represented in the current study, the nature of evolution of the primary electron donor excited state is apparently a more complex process. We found difference spectra of excited state  $P^*$  to contain also a shorter wavelength band at 1053 nm registered at ultrashort time delays ( $\sim 100$  fs). It is significant that a low-intensity optical transition in this spectrum range is also observed on the long-wavelength  $Q_Y$  slope of the absorption band of RC primary electron donor. Selective RC excitation within this absorption band at 1064 nm causes photoinduced formation of a primary quinone acceptor anion-radical. Although the nature of the electron donor and the mechanism of  $Q_A^-$  formation remain unknown in this case, further investigations in this field may facilitate a more detailed understanding of characteristics of the primary charge separation mechanism in bacterial RCs.

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