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Electron Transfer in the Reaction Center of the Photosynthetic Bacterium *Rb. sphaeroides* R-26 Measured by Transient Absorption in the Blue Spectral Range

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Electron transfer in the reaction center of the purple photosynthetic bacterium *Rb. sphaeroides* R-26 has been studied at room temperature by transient absorption spectroscopy with a time resolution of 120 fs. Measurements of absorption changes were performed in the range from 400 nm up to 680 nm, after excitation with a laser pulse of 80 fs duration within the absorption band of the bacteriochlorophyll at 800 nm. The excited state of the primary donor, characterized by the absorbance changes extending over the whole spectral range investigated, appeared within 120 fs and gave rise to the bleaching of the Q_x absorption band of bacteriochlorophyll at 600 nm, increased further by electron transfer to bacteriopheophytin in ~3 ps. Photoreduction of the bacteriopheophytin acceptor detected at 546 nm and 670 nm proceeded with the same time constant. Multiphase absorbance changes were relatively the largest in the blue spectral range between 415 nm and 450 nm. Apart from the immediate absorbance increase due to excitation of the primary donor, another fast increasing phase was detected characterized by a wavelength dependent time constant—from ~5.5 ps at 415 nm to ~1.9 ps at 450 nm. Both the photooxidised primary donor and photoreduced bacteriopheophytin contributed to the amplitude of this phase. The electron transfer from the reduced bacteriopheophytin to a quinone acceptor was observed as a decrease in the intensity of the transient absorption bands at about 422 nm and at 670 nm, with disappearance of the bleaching at 546 nm and increase of the bleaching at 600 nm, all in ~200 ps.

KEY WORDS: Purple bacteria; reaction center; primary radical pair; transient absorption spectroscopy.

INTRODUCTION

Photosynthetic reaction centers (RC) are mainly hydrophobic pigment-protein complexes in which trans-membrane charge separation and stabilization processes occur driving all the subsequent chemistry of photosynthesis. After isolation and purification of RC from purple bacteria [1], they were the object of many studies using different spectroscopic techniques [2–5]. The X-ray structural study of the reaction centers from *Rhodobacter sphaeroides* [6,7] has shown the detailed organization of the prosthetic groups (4 bacteriochlorophylls *a*, 2 bacteriopheophytins *a*, 1 or 2 ubiquinones, and 1 non-heme iron) anchored to the 2 poplypeptides L and M composed of 10 α-helices. Both subunits as well as the prosthetic groups are organized with approximately C-2 symmetry

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about the axis starting from the iron atom to the center of the primary electron donor (P) which is a dimer of bacteriochlorophyll *a*. Two bacteriopheophytins *a* ($BPhe_L$ and $BPhe_M$) are located on either side of P, and two additional bacteriochlorophylls *a* ($BChl_L$ and $BChl_M$) are positioned in between $BPhe_L$ or $BPhe_M$ and P.

After light excitation of the reaction center, the reactions start from the singlet excited state $^1P^*$ of a primary electron donor P which decays in some picoseconds by ultrafast electron transfer to a primary electron acceptor $BPhe_L$, creating the primary radical pair $P^+BPhe_L^-$ (see [5] for a review). After primary charge separation the electron is transferred to a secondary electron acceptor, the ubiquinone Q_A, in about 200 ps [8–10]. When Q_A is prereduced or removed, the forward electron transfer is blocked at the level of $BPhe_L^-$ and the primary radical pair decays by charge recombination. This reaction has been studied extensively for isolated reaction centers of *Rb. sphaeroides* (see [11] for a review).

Development of emission of very short laser pulses (down to 100 fs duration and tens of microjoules in each pulse) covering a wide range of wavelengths, has made it possible to probe the kinetics of charge separation and stabilization with a high accuracy. Knowing the size of the core antenna system (about 40 bacteriochlorophylls), the quantum yield of charge separation (better than 98% [12]), the lifetime of antenna fluorescence in the *Rb. sphaeroides* RC-less mutant PM8 (1.1 ns [13]), and assuming full equilibration of the excitation over the antennas and the RC dimer, it has been calculated that the characteristic time of charge separation must be a few ps or less. Martin *et al.* [14] for the first time demonstrated that in RC of *Rb. sphaeroides* R26 one-step charge separation takes place in 2.8 $\pm$ 0.3 ps and that no evidence is found for a second, faster kinetics attributable to the reduction of $BChl_L$, the intermediate accessory molecule. The opposite conclusion was drawn from the experimental study by Zinth *et al.* [15,16]. At selected wavelengths within the 800 nm absorption band the biphasic kinetics were found, with a time constant of 3.5 $\pm$ 0.4 ps attributed to creation of $BChl_L^-$ and 0.9 $\pm$ 0.3 ps connected with reduction of $BPhe_L$. From different types of measurements it was estimated that the forward energy transfer from the reduced $BPhe_L$ to Q_A occurs in 200 $\pm$ 50 ps [10,17].

In this paper we report a study of the absorbance changes in the blue range of the spectrum associated with the electron transfer in reaction center from *Rb. sphaeroides* R-26 with 120 fs time resolution. Due to direct excitation of the Q_y absorption band of $BChl_L$ and $BChl_M$ at 800 nm and its instantaneous transfer to P [18], we could study absorption bands far from this wavelength.

MATERIALS AND METHODS

Chromatophores were prepared with a French press (3 times, 14000 psi) in the presence of DNase. Short centrifugation was applied to remove unbroken cells and large fragments, and ultracentrifugation to obtain a pellet of chromatophores. The pellet was resuspended in Tris pH 8 (10 mM) and solubilized with 1% LDAO (lauryldimethylamine-N-oxide; non-ionic detergent) for about 10 min in the dark. Solution was ultracentrifuged and treated with ammonium sulfate (saturated). Dialysis was applied overnight against Tris pH8 10 mM, 0.1% LDAO. The solution obtained was passed over DEAE column, eluted with a gradient of NaCl (+0.1% LDAO. Tris pH 8 10 mM; NaCl concentration 20 mM–180 mM. The RC were released at 180 mM. If needed concentration with centriprep 100 was performed. All procedures were performed on ice.

Isolated reaction centers prepared as described above and in [19,20] were resuspended at 10 μM RCs in 10 ml of 10 mM Tris, pH 8.0, 0.1% LDAO, 0.1 mM Na-ascorbate. RCs suspension was continuously flowing through the excited volume of the sample cell at a rate up to 10 ml/min, which enabled the total exchange of the excited volume within 50 ms. The sample cell (laboratory made) with the windows made of fused silica plates, has the absorption layer of 1 mm in thickness.

The method of transient absorption measurements with a time resolution of 120 fs has been described in detail [21]. Excitation flashes of 80 fs duration at 800 nm were provided by a laser (Spectra Physics) at a repetition rate of 100 Hz. A CCD camera was used for absorption measurements in the range from 400–680 nm. Analysis of the traces was based on the three exponential fits.

RESULTS AND DISCUSSION

Figure 1 shows a ground state absorption spectrum of photosynthetic purple bacteria *Rb. sphaeroides* reaction centers. The excitation and transient absorption measured here wavelengths are indicated. The figure shows the main absorption bands and their attribution to the components of the photosynthetic membrane.

Figure 2 shows the absorbance changes measured in the spectral region studied from 400 nm to 680 nm. The exemplary short delay times between the probe and

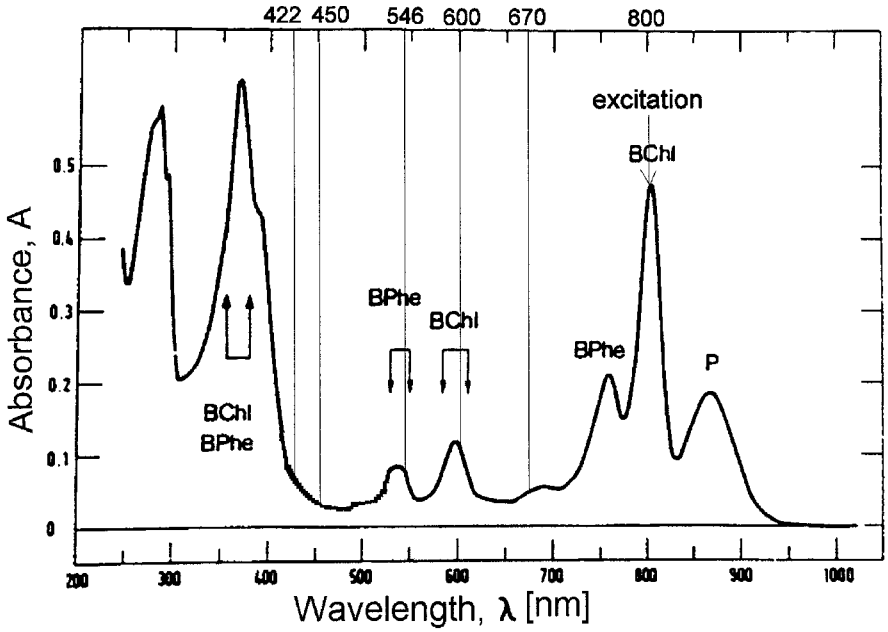

Fig. 1. Absorption spectrum of photosynthetic purple bacteria *Rhodobacter sphaeroides* reaction centers. The excitation and transient absorption wavelengths are indicated.

the excitation pulse at 800 nm have been chosen as (a) 1 ps, 2 ps, 5 ps and 10 ps, and the exemplary long delay times as (b) 20 ps, 100 ps, 200 ps, 600 ps. In both cases, the traces marked with −3 ps correspond to the signal obtained at 0 delay time. The differences spectra at these time intervals can be assigned to P*, P^+, $BPhe_L^-$, and Q_A^-. The time evolution of the spectra reflects the excitation of the primary electron donor, P → P*, and the electron transfer reactions P* → $P^+BPhe_L^-$ and $P^+BPhe_L^-$ → $P^+Q_A^-$. To analyze the rates of the two latter reactions the absorption changes 1 ps to 1 ns after excitation were averaged over 5 nm intervals and fitted with a constant plus three exponentials:

$$\Delta A_{fit} = A_0 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2) + A_3\exp(-t/\tau_3) \quad (1)$$

In this relation the time constant τ_1 reflecting the energy transfer reaction, P → P* (exactly PBChl → PBChl* → P*BChl) was fixed to 120 fs due to the time resolution of our measuring system, τ_2 is the time constant for P* → $P^+BPhe_L^-$ electron transfer, τ_3 is the time constant for electron transfer from $BPhe_L^-$ to Q_A and t is the time after excitation. Using these time dependent absorbance changes, the kinetics discussed below are shown in Figs. 3–6 for chosen wavelengths. They can be assigned to excited, oxidized and/or reduced cofactors participating in the electron transfer.

Figure 3 shows the absorbance changes observed at 422 nm ascribed to four bacteriochlorophylls and to two bacteriopheophytins. After an initial instantaneous increase of absorption due to the reaction of excited states of the bacteriochlorophylls in 120 fs, further absorbance changes are due to (a) creation of P^+ and $BPhe_L^-$ in τ_2 = 4.8 ± 1.7 ps and (b) subsequent transfer of electron from bacteriopheophytin to quinone molecule in τ_3 = 250 ± 90 ps. The non-disappearing part of the signal is due to P^+.

The creation of the reduced bacteriopheophytin in the state P^+BPhe^- is accompanied by a bleaching of the Q_X band of BPhe at 546 nm. Figure 4 presents the changes of absorption at 546 nm. The can be fitted by an initial instantaneous increase of absorption with 120 fs followed by two other phases. (a) The fast phase leads to an induced bleaching with a time constant τ_2 = 3 ps, and (b) the slow phase represents reoxidation of bacteriopheophytin with a time constant of τ_3 = 230 ± 160 ps. In this case the non-disappearing part of the signal is also due to P^+.

Figure 5 presents the absorbance changes at 600 nm attributed mainly to the bleaching of the Q_X band of P. After immediate bleaching of the absorption with an instrumental limited rise time of 120 fs, further bleaching with a τ_2 = 3 ps time constant is connected with photooxidation of P to P^+ and is accompanied by a relatively smaller absorbance increase due to reduction of $BPhe_L^-$

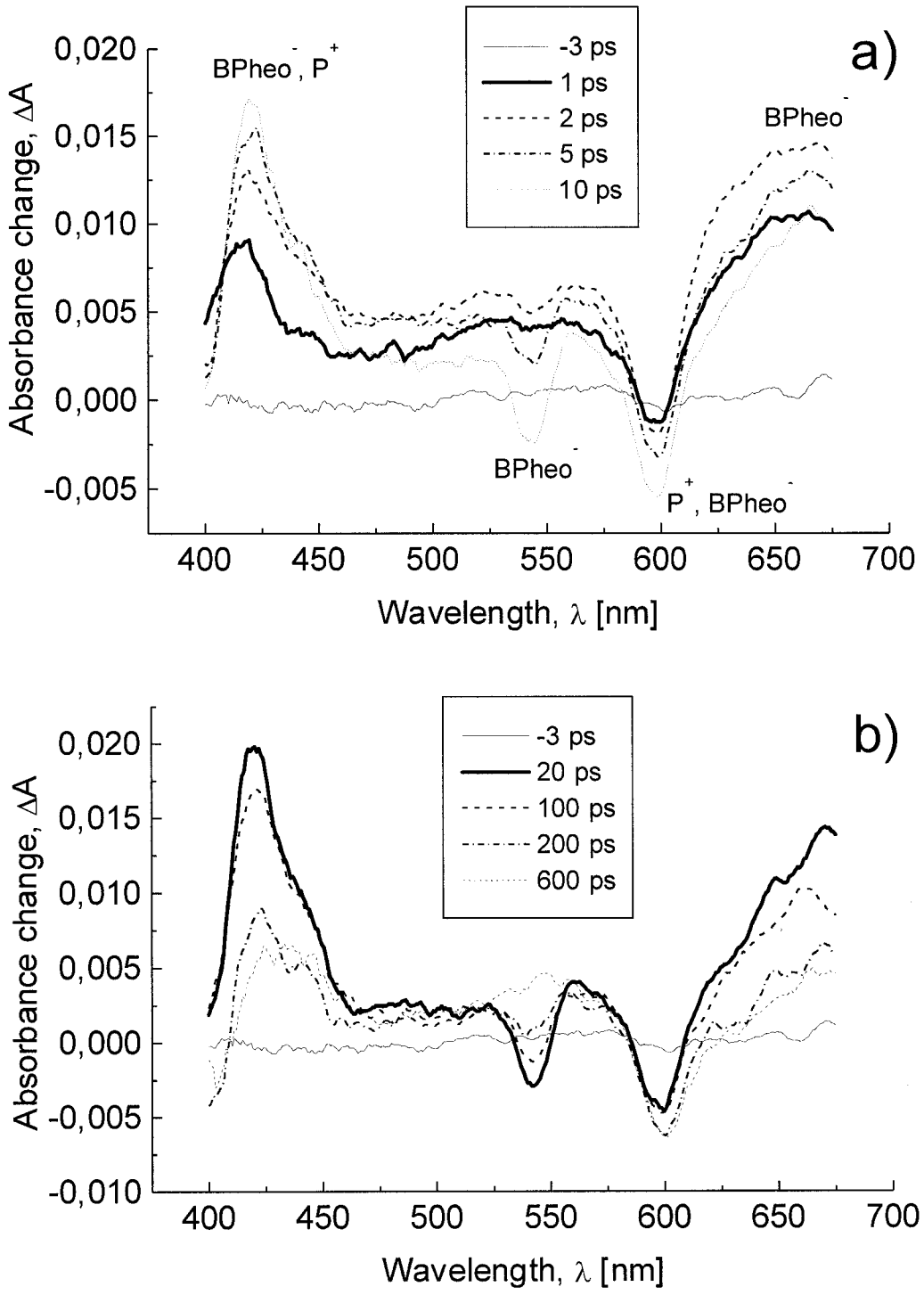

Fig. 2. Absorbance changes observed in *Rb. sphaeroides* R-26 reaction centers at various delay times for (a) small range of delay time and (b) large range of delay time.

(Fig. 5a). Reoxidation of $BPhe_L^-$ by electron transfer to Q_A leads to increase of the bleaching in $\tau_3 = 180 \pm 70$ ps (Fig. 5b).

Figure 6 represents changes in absorbance at 670 nm assigned to the appearance of the excited state of P in 120 fs followed by (a) a positive fast phase well-fitted

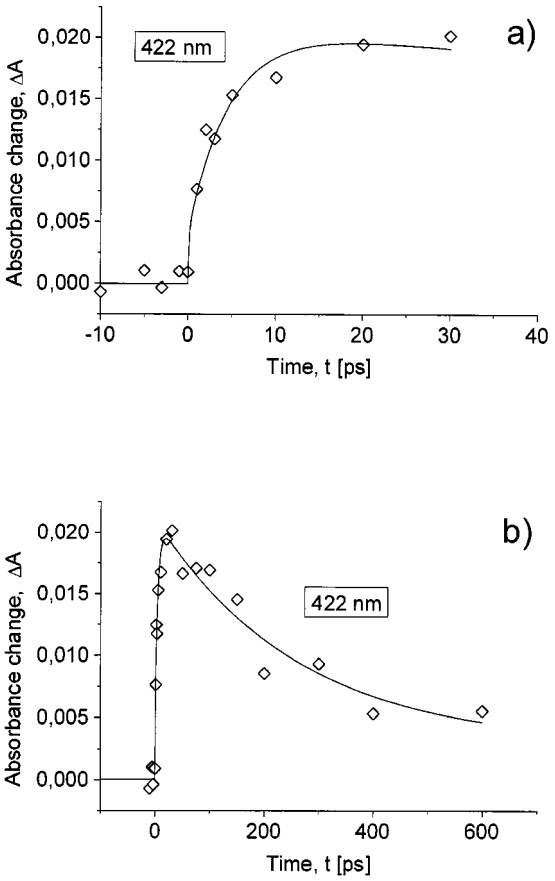

Fig. 3. Kinetics of absorbance changes at 422 nm measured at room temperature for the reaction centers from *Rb. sphaeroides* R26 upon the excitation at 800 nm: (a) in 40 ps time window; (b) in 600 ps time window. The smooth curve represents the best fit to the observed kinetics. It includes the convolution of the pump and probe white pulse, with $\tau_1 = 120$ fs ($A_1 = -0.004 \pm 0.002$) response time of the apparatus, creation of P^+ and $BPhe_L^-$ in $\tau_2 = (4.8 \pm 1.7)$ ps ($A_2 = -0.016 \pm 0.002$) and subsequent transfer of electron from $BPhe_L^-$ to Q_A molecule in $\tau_3 = (250 \pm 90)$ ps ($A_3 = 0.018 \pm 0.003$); ($A_0 = 0.003 \pm 0.003$).

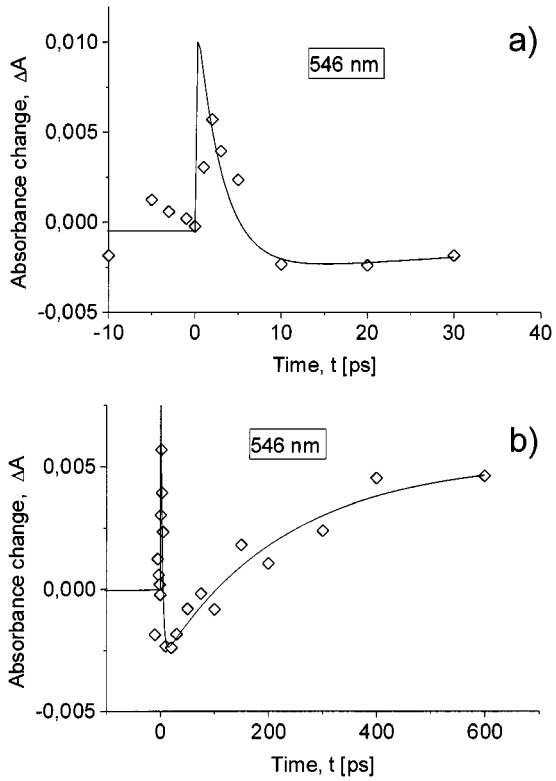

Fig. 4. Kinetics of absorbance changes at 546 nm measured at room temperature for the reaction centers from *Rb. sphaeroides* R26 upon excitation at 800 nm (a) in 40 ps time window and (b) in 600 ps time window. The smooth curve represents the best fit to the observed kinetics. It includes the convolution of the pump and probe white pulse with 120 fs ($A_1 = -0.014$) response time of the apparatus, the creation of reduced bacteriopheophytin BPh^- accompanied by a bleaching of the Q_x band of $BPhe_L$ with a time constant $\tau_2 = 3$ ps ($A_2 = 0.015$) and reoxidation of bacteriopheophytin with a time constant of $\tau_3 = (230 \pm 160)$ ps ($A_3 = -0.008 \pm 0.002$); ($A_0 = 0.005 \pm 0.003$).

by the time constant $\tau_2 = 3$ ps and (b) a negative slow phase, fitted by a $\tau_3 = 210 \pm 60$ ps time constant. The 3 ps phase is assigned to creation of $BPhe_L^-$ in 3 ps and a subsequent decrease of this absorbance change in 210 ± 60 ps reflects the transfer of an electron to the ubiquinone acceptor Q_A.

In Figure 7 the traces of the normalized absorbance changes measured in the blue range of the spectrum in 30 ps time window are presented for a few chosen wavelengths. One can see that the fits of all traces in this small time window can be obtained with two exponentials. The first exponential term of the fitting function corresponds to the instrumental rise time of $\tau_1 = 120$ fs, whereas the second one depends on the wavelength. The shorter the wavelength, the longer the τ_2 time constant, changing from 6.3 ps at 422 nm to 1.9 ps at 450 nm. The monotonic wavelength dependence of τ_2 time constant is shown in Fig. 8. A similar dependence, not only of τ_2, but also of τ_3 time constants, was observed in the red region of the spectrum between 787 nm and 815 nm [22]. It was found that at room temperature the time constant for the initial charge separation and subsequent electron transfer to Q_A encompasses a range of values $\tau_2 = \sim 1.3$–4 ps, and $\tau_3 = \sim 100$–320 ps, depending on the wavelength at which the kinetics is followed. It was suggested that this reflects a distribution of the reaction centers (or a few conformers), differing in distances or orientations between the cofactors, hydrogen bonding, or other pigment-protein interactions. Consequently, different subpopulations of RCs are characterized both by slightly different spectral properties and primary charge separation kinetics. Such an explanation is consistent with a potential energy sur-

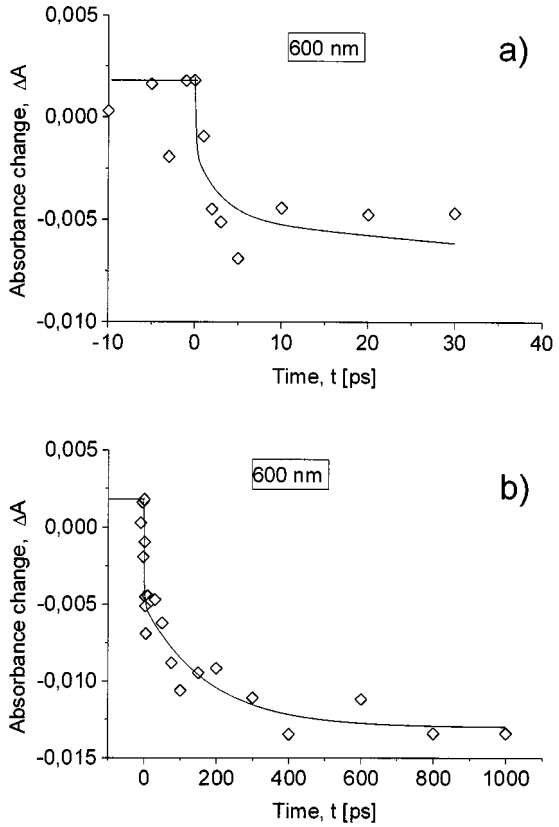

Fig. 5. Kinetics of absorbance changes at 600 nm measured at room temperature for the reaction centers from *Rb. sphaeroides* R26 upon excitation at 800 nm (a) in 40 ps time window and (b) 600 ps time window. The smooth curve represents the best fit to the observed kinetics. It includes the convolution of the pump and probe white pulse, with 120 fs ($A_1 = 0.004 \pm 0.002$) response time of the apparatus, the bleaching with $\tau_2 = 3$ ps ($A_2 = 0.003 \pm 0.002$) time constant connected with photooxidation of P and creation of reduced bacteriopheophytin, followed by reoxidation of BPh_L^- with a time constant of $\tau_3 = (180 \pm 70)$ ps ($A_3 = -0.008 \pm 0.001$); ($A_0 = 0.013 \pm 0.001$).

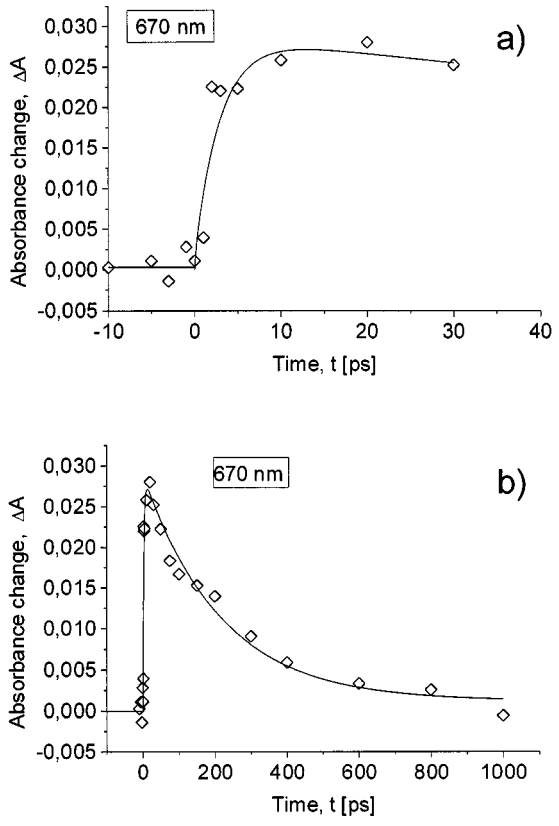

Fig. 6. Kinetics of absorbance changes at 670 nm (a) measured at room temperature for the reaction centers from *Rb. sphaeroides* R26 upon excitation at 800 nm (a) in 40 ps time window and (b) in 600 ps time window. The smooth curve represents the best fit to the observed kinetics. It includes the convolution of the pump and probe white pulse, with 120 fs ($A_1 = -0.002 \pm 0.005$) response time of the apparatus, the bleaching assigned to creation of $BPhe_L^-$ in $\tau_2 = 3$ ps ($A_2 = -0.026 \pm 0.005$) and subsequent decrease of this absorbance in $\tau_3 = (210 \pm 60)$ ps ($A_3 = 0.028 \pm 0.002$) reflecting the transfer of electron to quinon acceptor Q_A; ($A_0 = 0.001 \pm 0.002$).

face containing barriers between different substrates of the distribution, making their interconversion slower (>1ns) than the primary electron transfer reactions. These barriers may reflect restricted pigment/protein motions involving hydrogen bonds, porphyrin ring puckering/flattening, or torsional motions of various types. Our findings can be interpreted in the same way as in [22], i.e., that a distribution (or a few conformers) of RCs exists at room temperature and that the possible conformers do not interconvert in the time scale of charge separation. Each member of this population is able to perform primary photochemistry, but with different rates of electron transfer. We have not found a monotonic dependence of the τ_3 time constant on the wavelength in the blue range of the spectrum. This fact could be explained by additional participation of the ubiquinone Q_A^- absorbance band at 445 nm [23–25]. In spite of simplification made in the present analysis, that electron is transferred directly from P* to $BPhe_L$. The observed dispersion in the rates τ_2 is also consistent with two theoretical models which take into account an intermediate states, P^+BChl^-: the "super-exchange" model and the three-step model. In the "super-exchange" model the energy of P* is lower than that of P^+BChl^- state and the latter is not populated. The electron immediately travels to the bacteriopheophytin $BPhe_L$ and bacteriochlorophyll $BChl_L$ functions as a virtual electron conductor [26,27]. The most obvious from the structural data is the three-step model, when the electron is transferred in sequence from the excited special pair P* to bacteriochlorophyll $BChl_L$, then to the bacteriopheophytin $BPhe_L$, and further on to the quinone Q_A [15,16,28–30]. It was not justified to apply the three-step

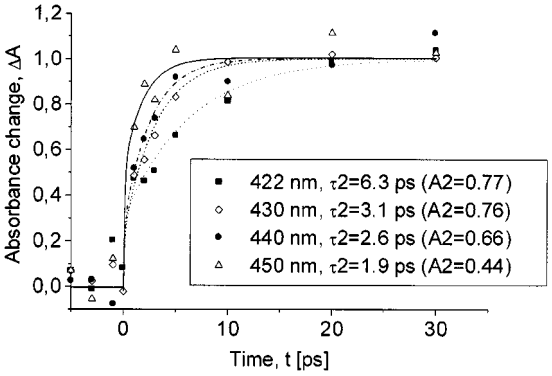

Fig. 7. Kinetics of normalized absorbance changes in blue range of spectrum measured at room temperature for reaction centers from *Rb. sphaeroides* R26 upon excitation at 800 nm in 30 ps time window. The traces were fitted with the two exponential function: $\Delta A_{fit} = 1 + A_1\exp(-t/\tau_1) + A_2\exp(-t/\tau_2)$, where τ_1 was fixed to 0.12 ps and $A_1 = 1 - A_2$.

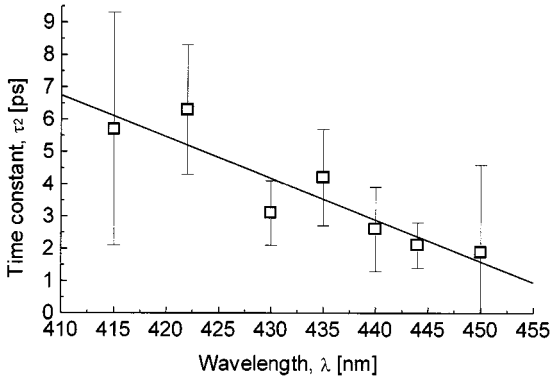

Fig. 8. Wavelength dependence of τ_2 time constant in the blue range of the transient absorption spectrum.

model for the interpretation of our results because of small contribution of $BChl^-$ in the absorbance change and because of insufficient accuracy of the measurement.

Recently another model of primary charge separation was proposed in which the excited bacteriochlorphyll $BChl_L^*$, instead of the excited special pair P*, acts as the primary electron donor [29]. An electron is transferred from $BChl_L$* to $BPhe_L$, giving $BChl_L^+BPhe_L^-$, and a subsequent charge transfer forms $P^+BChl_LBPhe_L^-Q_A$. Brederode and van Grondelle have experimentally supported this new ultrafast pathway of charge separation [31]. Thus, one may assume that there exist two populations of RCs with competing pathways of charge separation having different appearance rates, the first giving $BChl_L^+BPhe_L^-$ from $BChl_L$*, and the second creating P* and next $P^+BPhe_L^-$ from $BChl_L$*. If, in addition, absorbance changes around 420 nm from both populations are different on the time scale of charge separation due to formation of two different charge separated states, then dependence of $\tau_2(\lambda)$ the observed by us could be explained. The source of different absorbance changes of the states $BChl_L^+BPhe_L^-$ and $P^+BPhe_L^-$ in the blue region could be different primary donors and/or electrochromic shift of $BPhe^-$ due to interaction with positively charged $BChl_L^+$.

Also, it has been recently shown, that structural rearrangements involving dissociation and movements of LHCII have been induced by heat treatment of thylakoid membranes [32]. It has been hypothesized that the light-induced rearrangements in these membranes are driven by a thermooptic effect, i.e. thermal fluctuations due to the dissipation of excess excitation energies [33]. It can be shown that dissipation of the energy of one red photon over a small part of the photosynthetic membrane results in a heat jump of ~65°C [34]. This is a transient effect in the ps time scale and it can induce the structural rearrangement of the membranes. Such light induced reversible and irreversible changes in the photosynthetic membranes would influence of course the absorption features of the cofactors in the reaction center.

The absorption spectrum of *Rb. sphaeroides* R-26 in the blue range is composed of many overlapping bands and further studies are required to provide a better explanation of the phenomena described.

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