OBSERVATION OF A BACTERIOCHLOROPHYLL ANION RADICAL
DURING THE PRIMARY CHARGE SEPARATION IN A REACTION CENTER

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The primary light-induced charge separation in reaction centers of *Rhodobacter sphaeroides* was investigated with femtosecond time resolution. The absorption changes in the time range 100 fs to 1 ns observed after direct excitation of the primary donor P at 860 nm could only be explained by a kinetic model which uses three time constants. This finding supports the following reaction scheme: (i) the electronically excited primary donor P* decays with a time constant of 3.5 ps and populates a very short-lived intermediate involving a reduced accessory bacteriochlorophyll molecule; (ii) with a time constant of 0.9 ps the electron is transferred to the neighboring bacteriopheophytin molecule; and (iii) from there within 200 ps to the quinone.

1. Introduction

The primary photochemical event in the photosynthesis of bacteriochlorophyll-containing organisms is a light-induced charge separation within a transmembrane protein complex called the reaction center (RC). Recently, the crystal structures of the reaction centers of two purple bacteria were elucidated: *Rhodopseudomonas* (*Rps.*) *viridis* [1,2] and *Rhodobacter* (*Rb.*) *sphaeroides* [3,4]. The prosthetic groups in the transmembrane core of the reaction centers (4 bacteriochlorophylls, 2 bacteriopheophytins, 2 quinones, 1 iron) are arranged as two branches (A, B [*1]) in an approximate C_2 symmetry. Two bacteriochlorophyll molecules form the so-called special pair (P) located on the symmetry axis. Starting at the special pair, each branch consists of an accessory bacteriochlorophyll (B_A, B_B), a bacteriopheophytin (H_A, H_B), and a quinone (Q_A, Q_B).

Before the structure of the RC was known, information on the dynamics of light-induced charge sep-

aration had been obtained [7,8]: The special pair, P, functions as a primary electron donor. On the picosecond time scale the electron is transferred to a bacteriopheophytin H and from there to a quinone Q [9–17]. Additional experiments have shown that the electron transfer proceeds via the A branch [6,8,14].

Presently, there exist several theoretical models to describe the primary electron transfer in the reaction centers:

(i) Most obvious from the structure is a three-step model, where the electron is transferred in sequence from the special pair P to the neighboring bacteriochlorophyll B_A, then to the bacteriopheophytin H_A, and further on to the quinone Q_A [18–20]. In this model the energy of state P^+B^- must be below the energy of state P* and a finite occupation time of state P^+B^- is expected. The failure to demonstrate this state convincingly [12,16,17] has led to the proposal of two alternative mechanisms:

(ii) In the "super-exchange" model the energy of P* is lower than that of state P^+B^-. The latter is thus not populated. Instead, the electron immediately travels to the bacteriopheophytin H_A. Nevertheless,

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[*1] Indices A and B [5] correspond to branches L and M [6], respectively. Cation and anion radicals are indicated by their charges only (e.g. P^+, H^-).

bacteriochlorophyll B_A is functional as a virtual electron conductor [21,22].

(iii) Recently, a model was discussed where B* instead of P* acts as primary donor. Excitation energy transfer occurs from P* to B*, which is followed by a charge separation yielding B^+H^- and a subsequent charge transfer forming P^+BH^-Q [20].

A number of experiments on the primary reaction dynamics have been undertaken to test these models. Most recent data obtained for *Rhodobacter sphaeroides* (and also for *Rps. viridis*) were contradictory to model (i). Light absorption by the special pair band ($\lambda = 860$ nm) populates the lowest excited electronic state P* of P lasting for 2.8 ps. This information was directly deduced from the time dependence of the stimulated emission (i.e. gain) seen at the long-wavelength side of the P-absorption band [5,12]. Simultaneous reduction of bacteriopheophytin H to H^- was evaluated from data measured at 545 nm [12], where the bacteriopheophytin (H_A) absorbs. From the absorption decrease with the same time constant of 2.8 ps and from other data it was concluded that either no intermediate electron carrying state between P* and H^- exists, or, if it exists, its lifetime must be shorter than 100 fs [5,12]. It should be noted, however, that in earlier experiments – not performed under optimum conditions – evidence for such an intermediate was discussed based on the finding of a rapid transient component with decay constant ≈ 1 ps [13,15–17].

We have reinvestigated the kinetics of the primary processes of *Rb. sphaeroides* under optimized experimental conditions:

(i) The excitation wavelength was chosen to excite P in its 860 nm band.

(ii) Photon exposure was kept low to excite only a small fraction of the reaction centers during each pulse.

(iii) The time resolution was better than 100 fs.

(iv) The wavelengths of the probing pulses were varied from 500 to 1000 nm.

(v) Data were collected for delay times (t_D) up to 1 ns, allowing a quantitative comparison with kinetic models.

(vi) Data were obtained at highest precision at those wavelengths and delay times which were most indicative for a short-lived intermediate.

For evaluation of the experimental data we used two different kinetic models (depicted in fig. 3). Model (a) is based on three states connected by two kinetic constants and does not account for a short-lived intermediate. Model (b) connects four states by three time constants and allows for such an intermediate. The experimental results reported here clearly favor model (b) and suggest that the additional intermediate is a P^+B^-HQ state.

2. Experimental

Reaction centers of *Rb. sphaeroides* ATCC 17023 were isolated from phototrophically grown cells essentially as described in ref. [23], except for the following modifications [24]. Crude chromatophores were solubilized in 20 mM Tris (pH 8.0), 0.25% lauryldimethylaminoxide (LDAO), 125 mM NaCl, 0.5 mM phenylmethylsulfonylfluoride (PMSF) for 45 min at room temperature in the dark and subsequently centrifuged at 144000g, 4°C for 120 min. The resulting pellet was subjected to a second solubilization step (same concentration and same buffer without PMSF). The combined reaction center containing supernatant were diluted with an equal volume of 20 mM Tris (pH 8.0) containing 0.25 LDAO and directly applied to a DEAE 52 anion-exchange cellulose column. The column was then washed with increasing concentrations of NaCl (80, 110, 130, 155 mM) in 20 mM Tris (pH 8.0) containing 0.08% LDAO. Pure reaction centers were eluted at 185 mM NaCl. The reaction center solution was dialyzed against 20 mM Tris (pH 8.0) containing 0.08% LDAO and afterwards concentrated to 78 μM by ultrafiltration. Reaction centers from the carotenoid-less mutant *Rb. sphaeroides* R26.1 were prepared with LDAO and then transferred to tris-buffer (10 mM, pH 8) containing Triton X-100 (1.9 mM) as previously described [25]. The samples were used directly in the spectroscopic experiments or after appropriate dilution. Measurements were done at 24°C in cuvettes with 1 mm path length and under stirring.

A synchronously pumped unidirectional ring dye laser (UDR) [26] generated pulses of duration 60 fs at a wavelength of 860 nm. A three-stage dye amplifier increased the energy of single pulses to 20 μJ (repetition rate 10 Hz). For excitation and probing each of these pulses was split into two parts. The ex-

citation pulse (0.3 μJ) was focused to a spot diameter of 0.5 mm, providing an energy density of 150 μJ/cm^2 in the sample. This photon exposure excited approximately 13% of the reaction centers. The exciting pulse had a band width of 10 nm. At wavelengths below 840 nm no spectral components of the exciting pulses were detected. Consequently, there was no direct excitation of B (or H) via their absorption bands at $\lambda < 840$ nm. The probing pulse passed an adjustable delay line and was then focused onto a thin jet of ethylene glycol to generate a femtosecond light continuum. A 10–18 nm wide portion of this continuum was selected by means of a special dispersion compensating spectrometer [27]. The energy density of the probing pulse was less than 7 μJ/cm^2. Exciting and probing pulses were polarized parallel and for each probing wavelength λ_{pr} the experimental response function was recorded. Typical values of 130 fs were obtained, allowing the determination of time constants somewhat below 100 fs.

The weak photon exposure necessarily caused only small changes of transmission ($<2\%$) in the samples. These small absorption changes could be accurately measured by a sensitive difference detection system [28]. For each delay time t_D the average of at least 1000 absorption measurements was calculated.

3. Results

The inset of fig. 1 depicts the absorption spectrum of the reaction centers of *Rb. sphaeroides* R26.1. The broad absorption band at 860 nm is due to the electronic transition of lowest energy in the special pair P (Q_y transition) and irradiation in that range ascertains selective excitation. The Q_y transitions of the other pigments occur at shorter wavelengths, at 805 (mainly due to the two bacteriochlorophylls B) and 760 nm (bacteriopheophytins H); the corresponding Q_x transitions occur at 600 (P and B) and 540 nm (H).

Time-resolved absorption changes are plotted in figs. 1 and 2 as a function of delay times between -1 and $+1$ ps on a linear scale and from 1 ps to 1 ns on a logarithmic scale. The experimental data points are shown as full circles. The curves are calculated according to the kinetic model (a) (three components,
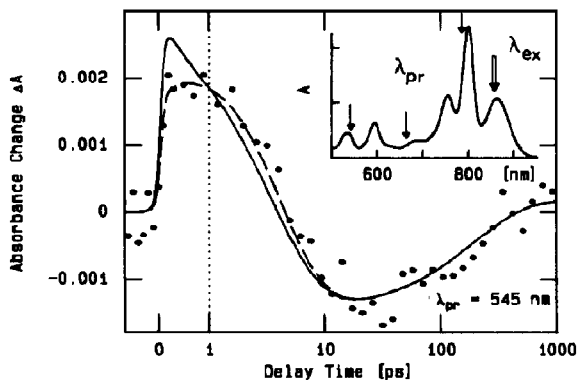

Fig. 1. Time-resolved absorption data (full circles) taken at a probing wavelength of 454 nm for reaction centers of *Rb. sphaeroides* R26.1 (excitation wavelength 860 nm). The delay time scale is linear between -1 and $+1$ ps and logarithmic for $t_D > 1$ ps. The curves are calculated according to two models presented in fig. 3 and discussed in the text. The inset depicts the absorption spectrum of the sample.

solid line) and (b) (four components, dashed line).

The results in fig. 1 were obtained for a probing wavelength of $\lambda_{pr} = 545$ nm. At this wavelength the bacteriopheophytin H_A absorbs (see inset) and absorption changes include those due to changes of the redox state of H_A. First, the absorption increases instantaneously with the excitation pulse due to formation of P*. In the time domain of 1 to 10 ps an absorption decrease is observed, which is reversed with a time constant of 200 ps. The three-component model (a) (on the basis of the time constants 3.5 and 200 ps) yields the time course shown by the solid line. It correctly describes the experimental data at late delay times $t_D > 10$ ps. At shorter times, however, a systematic difference between experimental data points and the predicted curve is seen which cannot be eliminated for any choice of time constants. Only the introduction of a fourth kinetic component with a time constant of 0.9 ps gives satisfactory agreement between data points and calculated curve (dashed line).

Strong evidence for the existence of an additional intermediate is also obtained from data taken at other probing wavelengths. In figs. 2a and 2b data are shown for probing wavelengths of 785 and 665 nm. At 785 nm, where the bacteriochlorophylls and the bacteriopheophytins absorb (fig. 2a), positive ab-
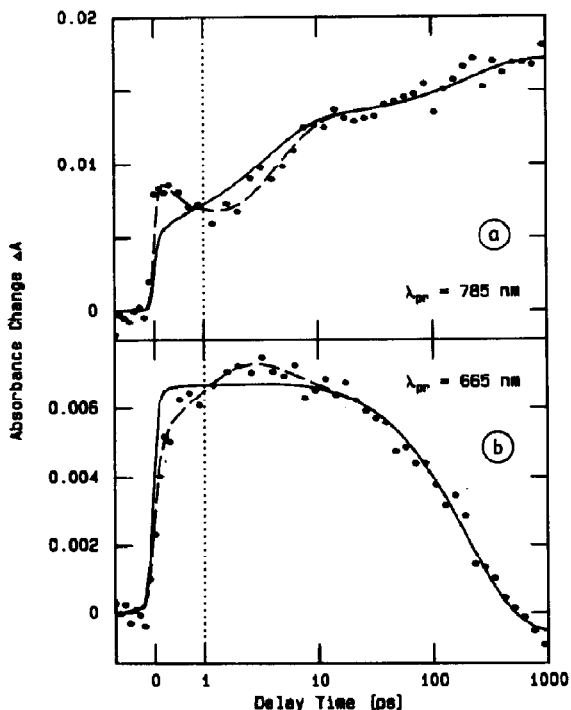
Fig. 2. Time-resolved absorption data (full circles) for probing wavelengths of 785 nm (a) and 665 nm (b) for reaction centers of wild-type *Rb. sphaeroides* ATCC 17023 (excitation 860 nm). The curves are calculated for the two models of fig. 3.

sorption changes are found throughout the time range analyzed: The first rapid rise of absorption follows the excitation pulse to a first distinct maximum at 300 fs. Then absorption decreases until $t_D = 1.2$ ps. A subsequent absorption increase proceeds in the 3 ps time domain, followed by a second slow increase in the 200 ps range. The solid curve represents the absorption change predicted by the three-component model (a) and clearly deviates from the experimental values in the time range of zero to 10 ps. The four-component model (b) on the other hand, fits the experimental data quite well, in particular during the first picosecond (dashed line).

The experimental results of fig. 2b obtained with $\lambda_{pr} = 665$ nm present the spectral range where the reduced bacteriochlorophylls and the reduced bacteriopheophytins absorb [30]. Once more, the results confirm the existence of the 0.9 ps component. At this wavelength the instantaneous rise of absorption

is followed by a second and slower increase. At later times absorption decreases with time constants of 3.5 and 200 ps. Again a least-squares fit of the three-component model (a) (solid line) does not satisfactorily explain the data between 0.2 and 10 ps, while the four-component model (b) is in full agreement (dashed line).

Additional results – not documented in this Letter – should be briefly reported:

(a) Transient changes of absorption at probing wavelengths of 690 nm, and between 760 and 800 nm, confirm the existence of an intermediate with a time constant of 0.9 ps. This component is not seen in the Q_y absorption and gain range of the special pair between 860 and 970 nm. Here the absorption changes decay monoexponentially.

(b) Experiments ($\lambda_{pr} = 860$ nm) have been performed at a photon exposure reduced by a factor of three. Under these conditions only 4% of the RCs are excited by the laser pulse. The same absorption characteristics including the 0.9 ps component were found.

(c) Three different samples, RC from wild-type *Rb. sphaeroides* ATCC 17023, wild-type RC with sodium ascorbate added (0.2 mM), and RC from the carotenoid-deficient strain R26, were compared. For all samples the 0.9 ps component is required to explain the observed absorption changes.

4. Discussion

A theoretical description of the time-resolved absorption data consists of rate equations for the population of molecular states I_j. The first state I_1 is populated by optical excitation. Sequential relaxation with time constants τ_j leads to daughter states (see fig. 3) [*2]. It can be shown that the absorption change $\Delta A(t_D)$ found under these conditions is a sum of single-sided exponentials with time constants τ_j convolved with the experimental response function [31]. Comparison of the calculated curve and the experimental data allows the determination of the amplitudes of the various components, i.e. the difference of absorption cross-sections as well as the decay time constants of the individual states. At some probing

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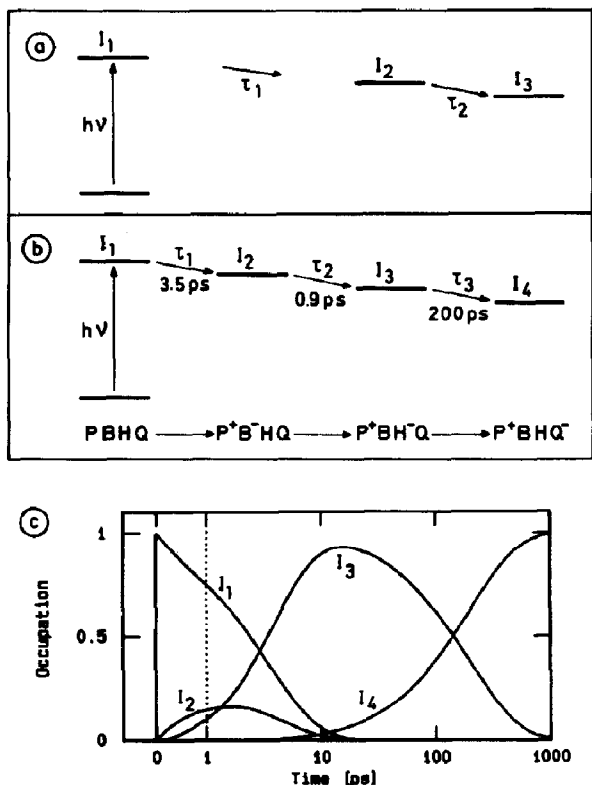[*2] An unbranched reaction scheme is assumed here.

Fig. 3. (a, b) Schemes of the sequence of states considered as alternatives for electron transfer in reaction centers. Models in (a) and (b) refer to the solid and dashed lines of the absorbance changes in figs. 1 and 2, respectively. (c) Population densities of the states I_j as functions of time calculated according to model (b). Note that the population of I_2 (P^+B^-HQ) remains smaller than 16% due to slow population (3.5 ps) and fast depopulation (0.9 ps) of this state.

wavelengths individual kinetic components may not be apparent. We note that the shortest time constant is always seen at the beginning of the time-resolved measurements, irrespective of its location in the reaction sequence.

We treated two different models for comparison with experimental data. The first simpler model (a) (from which solid curves in figs. 1 and 2 were derived) describes electron transfer as involving three states I_1 to I_3 which are connected by two time constants, $\tau_1 = 3.5$ and $\tau_2 = 200$ ps (fig. 3a). The second model (b) uses four states, I_1 to I_4, and three relax-

ational processes with time constants $\tau_1 = 3.5$, $\tau_2 = 0.9$, $\tau_3 = 200$ ps (see fig. 3b).

The first state I_1 to be considered is the excited electronic state P^*. It is observed in the wavelength range 860 to 970 nm, where only a monoexponential absorption change with a time constant of 3.5 ± 0.4 ps is found. As a consequence, this time constant must be the first one (τ_1) in the sequence of model (a) and model (b). Our value of the decay time of P^* is somewhat larger than the 2.8 ± 0.2 ps published previously [12].

The intermediates of the electron transfer process are seen at shorter wavelengths, for instance at 545, 665, and 785 nm. At these wavelengths the experimental data at early delay times are not well accounted for by the prediction of the three-component model (a) (solid lines) but strongly suggest a fourth component. The corresponding time constant is short, with a value of 0.9 ± 0.3 ps. The analysis of the time-resolved absorption data – the time dependence and the absorption amplitudes at various probing wavelengths – indicate that the molecular intermediate related to the additional component must occur after the 3.5 ps relaxation of P^* and prior to the 200 ps process leading to a reduced quinone. In the framework of a sequential model this result leads to the conclusion that an additional intermediate I_2 is required. The most obvious candidate is the configuration P^+B^-HQ containing a bacteriochlorophyll anion radical. This interpretation is supported by the following observations:

(i) I_2 shows strongly reduced absorption at the wavelength 797 nm, where the accessory bacteriochlorophyll B absorbs (Q_y band) and where B^- has a strongly reduced absorption [30].

(ii) During the rapid decay of I_2 the absorption recovers.

(iii) The relatively slow population (3.5 ps) and faster depopulation (0.9 ps) of the state B^- gives rise to a small transient population of 16% only (see fig. 3c). This small concentration of B^- makes the experimental observation a non-trivial task.

(iv) I_2 does not show absorption decrease in the wavelength range where bacteriopheophytin absorbs (e.g. at 454 nm).

State I_3 represents the next intermediate where the electron has reached the bacteriopheophytin H forming the configuration P^+BH^-Q. The related de-

crease of the H absorption at 545 nm is clearly seen after the decay of state I_2 and prior to the formation of I_4 (see fig. 1). Finally, in state I_4 the electron arrives at the quinone Q_A. The remaining absorption changes are due to the configuration P^+BHQ^-.

At this point, previously reported data should be mentioned. Several authors have reported the observation of a 1 ps component [12,13,15,17] after femtosecond excitation of RC at wavelengths around 610 nm. Since this excitation wavelength does not start the charge separation process at the lowest electronic level of the primary donor P, the data were disregarded as evidence for an additional intermediate in the electron-transfer process.

Experiments performed at the favorable excitation wavelength around 860 nm but with pulse durations of 30 ps reported a short-lived component preceding the bacteriopheophytin [16,17]. Unfortunately, the long pulses and limited time resolution did not permit firm conclusions.

Detailed investigations with femtosecond pulses at the optimum excitation wavelength of 860 nm were reported on RC from *Rb. sphaeroides* [12]. Some of these experiments done with probing wavelengths of 860 to 900 nm clearly showed the monoexponential decay of P^*. But the data on the formation of the first ground-state species contained substantial "statistical noise". These experiments were not able to rule out a weak (15–20%) contribution of an additional short-lived intermediate [18].

We have performed a detailed experimental study of the primary charge transfer processes in the reaction centers of *Rb. sphaeroides*. The analysis of the kinetic data with a linear model strongly suggests that the primary charge transfer to the bacteriopheophytin H proceeds via the accessory bacteriochlorophyll B as a true intermediate. The following reaction scheme results: After excitation of the special pair P an electron is transferred with a time constant of 3.5 ps to the accessory bacteriochlorophyll B. Here the electron resides for the short time of 0.9 ps before it reduces the bacteriopheophytin H.

Acknowledgement

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