# The absence of a spectroscopically resolved intermediate state $P^+B^-$ in bacterial photosynthesis

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Reaction centers from the photosynthetic bacterium *Rhodopseudomonas sphaeroides* have been excited either in the bacteriopheophytin band at 760 nm or in the accessory bacteriochlorophyll (B) band around 800 nm with laser pulses of 150 fs duration. Upon monitoring in the absorption band of the primary donor (P) at 860 nm, ultrafast energy transfer is observed which leads to the excited state of P in less than 100 fs. A transient bleaching recovering in 400 ±100 fs is specifically detected upon excitation and observation in the 800 nm absorption band of B. However, upon direct excitation of P in the near infrared and using either normal or borohydride-treated reaction centers, we have found no spectral or kinetic evidence indicating the presence of a transient intermediate state such as $P^+B^-$.

*Bacterial photosynthesis* *Energy transfer* *Charge separation* *Femtosecond spectroscopy*

## 1. INTRODUCTION

The remarkably efficient separation and stabilization of electric charges, which constitute the key processes of photosynthesis, occur in a transmembrane chlorophyll-protein complex named the reaction center. Reaction centers isolated from purple photosynthetic bacteria contain several polypeptides, four bacteriochlorophylls, two bacteriopheophytins and at least one quinone ($Q_A$). In the case of the reaction center from *Rhodopseudomonas sphaeroides* R26, the main absorption bands of the pigments are located at 865, 800, 760, 600 and 540 nm (fig.1a). The 865 nm band, which bleaches upon (photo)oxidation of the reaction center, is ascribed to the primary electron donor (P), a dimer of bacteriochlorophyll. The 800 nm band is assigned to the $Q_Y$ transition of the two other 'accessory' bacteriochlorophylls (B) while the 600 nm band corresponds to the $Q_X$ transition of all four bacteriochlorophylls. The bands at 760 nm and around 540 nm are attributed to the $Q_Y$ and $Q_X$ transitions of the two bacteriopheophytins (H), respectively. The X-ray structure of the reaction center from a related bacterium (*Rps. viridis*) shows that the two bacteriochlorophylls constituting P, the two B and the two H molecules, are organized with C-2 symmetry, thus defining two 'branches' of pigments extending from P, with only one of them directed towards $Q_A$ [1]. The P, B and H pigments are non-covalently bound to two polypeptides (named L and M) forming a scaffold of mainly transmembrane $\alpha$-helices which are also symmetrically organized with respect to the C-2 axis. We will denote $B_L$ ($B_M$) and $H_L$ ($H_M$) the B and H molecules associated with the L(M) polypeptide. $B_L$ ($B_M$) is located in close proximity to both P and $H_L$ ($H_M$), with center-to-center distances of about 11 Å. $H_L$, which can be spectrally resolved from $H_M$ in the 540 nm region, is the chromophore located at the closest distance from $Q_A$ [1].

Picosecond spectroscopy on *Rps. sphaeroides*

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reaction centers has revealed the presence of short-lived intermediates in the electron transfer process [2]. Previous studies using 530 or 610 nm excitation have demonstrated the appearance of $P^+$ and $H_L^-$ within 5–10 ps after the excitation pulse. This step is followed by the electron transfer to $Q_A$ in about 200 ps. However, the occurrence of earlier electron transfer step(s) and the involvement of a B molecule in these processes are still strongly debated [3–9]. Using relatively strong 25–50-ps pulses at 880 nm to excite reaction centers from *Rps. rubrum* directly in the P band, Shuvalov et al. [3] and Kryukov et al. [4] were the first to ascribe to the state $P^+B^-$ a transient bleaching observed around 800 nm. They reported a lifetime of 35 ± 5 ps for the recovery of this bleaching and the concomitant appearance of $H_L^-$. Several groups have reported a similar component of bleaching when reaction centers are excited with an excessive density of photons either in the visible or near infrared [2,5,6]. Later Shuvalov and Klevanik [7], using pulses comparable in duration and wavelength to those used in their previous study [3], reported that in *Rps. sphaeroides* reaction centers, the state $P^+B^-$ appeared in less than 1 ps and disappeared in 7 ± 2 ps to generate the state $P^+H_L^-$. At the same time Borisov et al. [8], using 4-ps, 870-nm pulses of rather low energy and probing the putative $B^-$ state around 800 nm, found no evidence for it. Subsequently, Kirmaier et al. [9], presenting a thorough review of the literature, a critical analysis of the calculation procedures used in [7] to estimate the contribution of $B^-$ at early time, and some new measurements, came to the conclusion that there was no convincing evidence that $P^+B^-$ is a kinetically or spectrally resolved intermediary state in the charge separation process. Very recently, Shuvalov and Duysens [10], using 33-ps pulses at 880 nm, have investigated reaction centers from *Rps. sphaeroides* R26 in which the $B_M$ molecule had been specifically removed or altered by borohydride treatment [11–13]. They analyzed a transient bleaching around 800 nm in terms of an early intermediate containing only 35% of the state $P^+B_L^-$ (the remaining 65% being contributed by P*) decaying to the state $P^+H_L^-$ in 4 ± 1 ps.

These discrepancies appear to be due to a combination of unsatisfactory experimental conditions such as pulses of a duration longer than the phenomena under investigation, ill-suited excitation wavelengths in which the excitation is not directly created on P but rather on H or B, and/or excessive excitation energies which can lead to nonlinear processes. It is quite clear that the answer to such contradictory conclusions has to be sought using near-infrared subpicosecond pulses of low energy. However, the first studies using shorter pulses were done upon excitation around 600 nm. Using 0.7-ps pulses of quite high energy to excite reaction centers from *Rps. sphaeroides*, Holten et al. [2] found that the state $P^+H_L^-$ rises with a time constant of about 4 ps. An initial transient state was detected but it could not be determined whether this initial transient was P*, $P^+B^-$ or possibly some other state resulting from multiple excitation of the reaction center. In a more recent study, Parson et al. [14], using low energy, 2-ps pulses at 610 nm, observed the same kinetics of about 4 ps for the disappearance of P* (monitored by the stimulated emission around 900 nm) and for the appearance of $H_L^-$ and proposed the reaction scheme $P^* \longrightarrow P^+H_L^-$. In the first study combining very short pulses (150 fs) and near-infrared (850 nm) excitation, Martin et al. [15] could conclusively demonstrate (i) the ultrafast (< 100 fs) appearance of the broad absorption of P* and (ii) the absence of a transient bleaching around 800 nm. They also observed stimulated emission from P* and, upon monitoring at a variety of wavelengths, confirmed the reaction scheme proposed in [14] but reported a time constant of 2.8 ± 0.2 ps for the reaction. Woodbury et al. [16], using 0.8-ps pulses at 610 nm, extended the initial investigation [14] and reported a transient bleaching at 800 nm which they assigned to energy transfer from B* to P rather than to a transient reduction of B.

Here, we have extended the work in [15] on *Rps. sphaeroides* to investigate (i) the excitation energy transfer from H and B to P, (ii) the transient absorbance changes upon excitation and detection in the 800 nm band and (iii) the electron transfer kinetics in the borohydride-modified reaction centers. The results from these experiments, together with comparable studies done on the reaction center from *Rps. viridis* [17], allow us to conclude that the state $P^+B^-$ cannot be spectrally or kinetically resolved in the reaction center from purple photosynthetic bacteria.

## 2. MATERIALS AND METHODS

The femtosecond laser pump-probe setup, using the continuum amplification techniques with styril 9 or LDS 867 (Exciton, Dayton, OH) dyes, has been described [15,17]. The energy of the pump pulse was adjusted so that about 20% of the reaction centers were excited on each laser pulse. The preparation and conditions for handling the samples of *Rps. sphaeroides* R26 reaction centers were as described [15].

## 3. RESULTS AND DISCUSSION

### 3.1. *Excitation energy transfer from H or B to P*

Upon excitation in H at 760 nm or in B at 790 and 810 nm, the bleaching of the ground state of P monitored at 860 nm occurs in less than 100 fs, i.e. as fast as upon direct excitation of P at 850 nm (fig.1b). Previous measurements on *Rps. sphaeroides* reaction centers have been interpreted in terms of finite energy-transfer steps amongst the chromophores: Moskowitz and Malley [18] have estimated a time constant of about 10 ps for the energy transfer from H to P, while Akhmanov et al. [6] have calculated values that are one order of magnitude shorter. More recently, Woodbury et al. [16] have proposed an energy-transfer step occurring within about 1.5 ps between the B molecules and P in order to rationalize a fast transient bleaching of the 800 nm band of B observed upon excitation at 610 nm. The ultrafast (< 100 fs) transfer between H or B and P demonstrated in fig.1b is thus significantly shorter than these previous estimates and implies a very close proximity of the chromophores, as is indeed observed in the molecular model of the reaction center of *Rps. viridis* [1]. Furthermore, upon excitation in H or in B the lifetime of P*, as measured by the decay of the stimulated emission, is still 2.8 ps [19] as it is upon direct excitation of P [15].

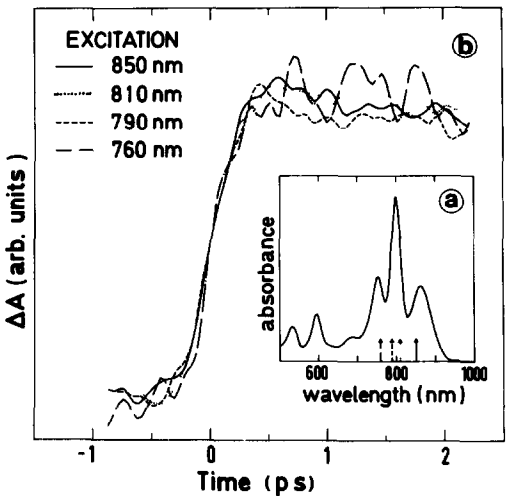


Fig.1. (a) Absorption spectrum of reaction centers from *Rps. sphaeroides* R26. (b) Kinetics of the rise of the bleaching observed at 860 nm upon excitation with 150-fs pulses at the indicated wavelengths demonstrating ultrafast (< 100 fs) energy transfer from H and B to P (see text for details).

### 3.2. *Excitation and detection in the 800 nm absorption band of B*

Upon excitation and observation in the absorption band of B around 800 nm a fast transient bleaching is observed. The recovery of this bleaching can be fitted with a 400 ± 100 fs time constant (fig.2). Such a fast transient bleaching of B can be expected before the excited species B*

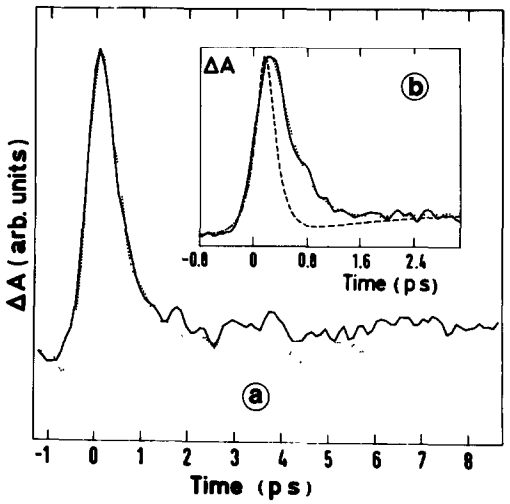


Fig.2. Kinetics of absorbance changes at 800 nm following excitation at 807 nm of reaction centers from *Rps. sphaeroides*. (a) Prior to the measurement the reaction centers were poised in the state $PH_LQ_A$ (no addition; ——) or in the state $P^+H_LQ_A$ (10 mM potassium ferricyanide; ···). (b) The fits assume a 400 fs (···) or a 100 fs (– – –) relaxation for the fast transient bleaching.

transfers its excitation energy to P. However, fig.1b clearly demonstrates that the energy transfer from B* to P takes place in less than 100 fs. Although the signal-to-noise ratio in fig.1b does not allow us to exclude the possibility that about 10% of P* is generated in 500 fs, we note that a quantum yield of $P^+$ formation of 0.93 has been reported for *Rps. sphaeroides* reaction centers excited at 800 nm, compared to a yield of essentially 1.0 when P is excited directly [20]. This decreased yield compared to that observed upon direct excitation of P could be rationalized by assuming two populations (or two conformational states) of the reaction center with different decays of B*. The fast transient bleaching would then include the contributions (i) of a small fraction of the B* population relaxing to the B ground state in about 500 fs and (ii) of most of the B* states transferring to P in about 50 fs. Due to the fact that the kinetics are measured with pulses longer than this characteristic time, the maximum amplitude of the about 50 fs contribution is attenuated by a factor of roughly 6 while the about 500 fs component is almost unaffected. In addition, it seems likely that the transient signal also includes a contribution of stimulated emission from B* [17]. An alternative to the scheme discussed above would be a situation where a small fraction (around 10%) of photooxidized reaction centers is responsible for the fast transient bleaching. However, this interpretation can be ruled out in view of the observation of this transient relaxing in about 400 fs even after chemical oxidation of the reaction centers (state $P^+H_LQ_A$, fig.2a). This striking observation can be rationalized with our model which primarily involves excited states of B and not of P or H. The characteristics of the transient bleaching described here upon excitation and detection in the 800 nm band of *Rps. sphaeroides* thus parallel closely the transient bleaching detected in the 830 nm band of the B molecules in *Rps. viridis* upon selective excitation in the H or B bands [17].

The transient bleaching around 800 nm previously reported upon excitation of *Rps. sphaeroides* reaction centers at 610 nm with 0.7–0.8-ps pulses [2,16] can be ascribed to the same effect as discussed above. The 600 nm region corresponds to the $Q_X$ transition of the four bacteriochlorophylls. A bleaching of the $Q_Y$ transition of the B molecules should thus be accompanied by a corresponding bleaching of their $Q_X$ transition. In view of the identical absorption changes of *Rps. viridis* and *Rps. sphaeroides* reaction centers upon excitation and detection in their respective $Q_Y$ transition of B [17], this effect constitutes in our view the simplest interpretation of the fast transient bleaching at 620 nm reported for *Rps. viridis* and which has been assigned to a transient reduction of $B_L$ occurring prior to the reduction of $H_L$ [21].

### 3.3. *Initial electron transfer*

We have recently demonstrated that in the reaction center of *Rps. viridis* no transient bleaching at 830 nm lasting more than 100 fs can be observed upon direct excitation within the $Q_Y$ absorption band of P with 150-fs pulses and we have given conclusive evidence that in this system, like in reaction centers from *Rps. sphaeroides*, the state $P^+H_L^-$ was generated directly from P* with a time constant of 2.8 ± 0.2 ps ([17], see also [22,23]). A comparison of the main experimental results regarding the initial electron transfer in reaction centers from *Rps. sphaeroides* [15] and *Rps. viridis* [17] is presented in fig.3 to demonstrate the striking similarities in the kinetics of the transient absorbance changes for these two species of purple bacteria.

By treating reaction centers with sodium borohydride, it is possible to remove essentially the accessory $B_M$ molecule [11,12], although it has also been proposed, on the basis of circular dichroism data, that in some of the centers $B_M$ was replaced by a bacteriopheophytin [13]. Such modified reaction centers have recently been reported [10] to exhibit a fast transient bleaching in the 800 nm band upon excitation at 880 nm with pulses of 33 ps duration (but with only partial temporal overlap between the pump and probe pulses). This fast transient bleaching around 800 nm has been taken as a proof of the existence of the $P^+B_L^-$ state [10]. We have investigated the kinetics of such modified reaction centers (kindly provided by V. Shuvalov) upon excitation at 870 nm with pulses of 150 fs duration, using a low absorbance at 870 nm (in order to match the sample conditions used in [10]) and less than 20% of the reaction centers were excited. Under these conditions the kinetics at the investigated wavelengths of 930 and 805 nm are identical to those observed with unmodified reac-

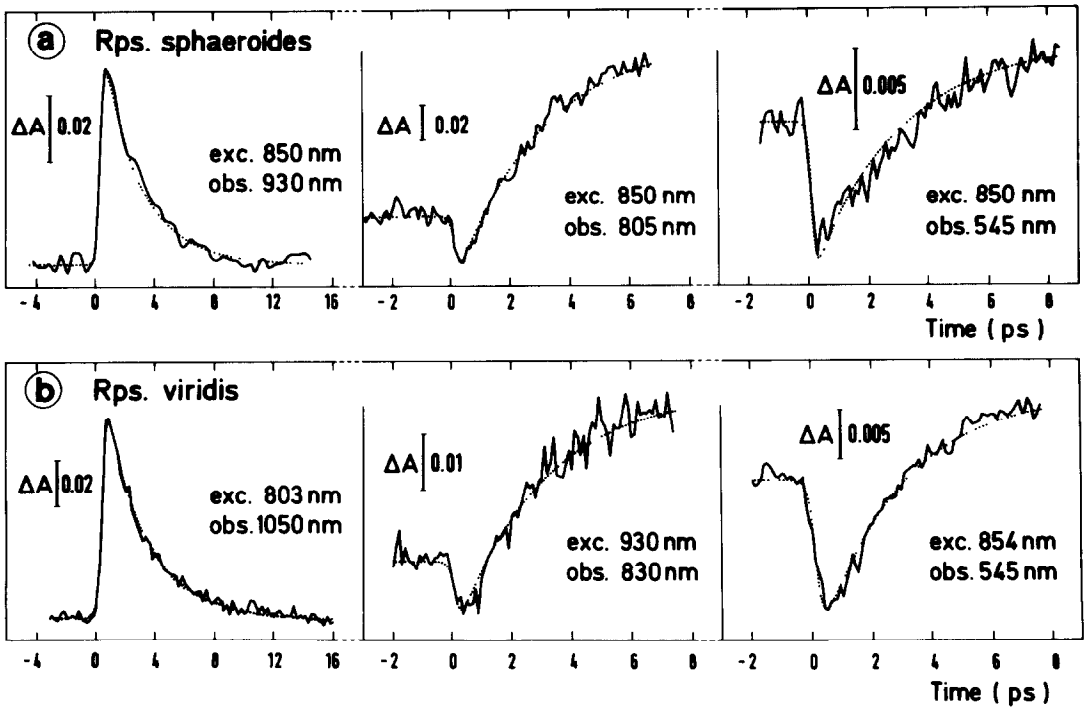


Fig.3. Transient absorbance changes in reaction centers from *Rps. sphaeroides* (a [15]) and *Rps. viridis* (b [17]) showing the decay of the stimulated emission (left) and the instantaneous absorbance increase followed by the development of a bleaching assigned to the bandshift of the accessory bacteriochlorophylls (middle) or to the reduction of the bacteriopheophytin $H_L$ (right). The best fits (···) assume a combination of an 'instantaneous' absorbance change and of exponential kinetics with a 2.8 ps time constant.

tion centers (fig.3a). More specifically no fast transient bleaching could be detected at 805 nm and the kinetics are well fitted with a component of about 3 ps time constant [19]. It thus appears that the observations reported in [10] using partial temporal overlap of long duration (33 ps) pulses could not be confirmed upon excitation with ultrafast (150 fs) pulses at the same wavelength. In this respect it is important to note that when a fast transient bleaching is present around 800 nm, such as upon excitation in the B absorption band, we can unambiguously resolve such a bleaching (fig.2). Thus, the difference in the results of the two experiments has to be sought either in the differences in the excitation and detection conditions or in the analysis of the data.

## 4. CONCLUSIONS

Upon excitation of reaction centers from *Rps. sphaeroides* in H (at 760 nm) or in B (around 800 nm), ultrafast (< 100 fs) energy transfer is observed leading to the formation of P*. This state decays with a time constant of 2.8 ± 0.2 ps to generate the radical pair $P^+H_L^-$ with no spectral or kinetic evidence for a transient bleaching around 800 nm which could be assigned to the state $P^+B^-$ [15]. However, when both the excitation and detection are localized in the 800 nm band, a fast transient bleaching recovering with a 400 ± 100 fs time constant is observed and could be mistakenly interpreted as a transient state such as $P^+B^-$.

When comparing the reaction centers from *Rps. sphaeroides* ([15,19], this study) and from *Rps. viridis* [17], the excitation energy transfer among H, B, and P as well as the characteristics of the initial charge separation appear identical in terms of both the kinetics of the transient absorbance changes and the nature of the ionized species. Taken together with other spectral evidence [24,25], these conclusions thus further strengthen the proposal that the geometrical organization of

the chromophores is essentially the same in the reaction center of these two organisms and that the structure of the reaction center from *Rps. sphaeroides* can be reasonably well approximated by the model derived for *Rps. viridis* [1].

One of the most unexpected features of the organization of the chromophores in the reaction center of *Rps. viridis* is the presence of the two almost perfectly symmetrical L and M branches of pigments extending from P. This has led to some speculations on the possible role (vestigial or functional) of the M branch [26,27]. Our observation that the electron transfer process and kinetics remain unchanged upon removal (and/or modifications) of the accessory $B_M$ molecule extends to the femtosecond time scale the results on the photoreactions of these reaction centers in the millisecond and picosecond regimes [12,13] and leads us to the conclusion that the $B_M$ molecule plays apparently no role in the $P^* \longrightarrow P^+H_L^-$ charge separation.

Although our studies [15,17,19] demonstrate that an intermediate state $P^+B^-$ lasting more than 100 fs cannot be spectrally or kinetically resolved, this should not be taken as an indication that the $B_L$ molecule plays no role in the initial electron transfer. As discussed in [15,16] it could serve to lower the energy barrier between P* and $P^+H_L^-$. It could also participate more directly in the electron transfer process: for example, the P* state may contain, in addition to an internal charge transfer state $P^{\pm}$, a small amount of $P^+B_L^-$ transferring very rapidly to $P^+H_L^-$ so that no detectable transient concentration of the state $P^+B_L^-$ could be measured.

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