The question of the intermediate state P^+BChl^- in bacterial photosynthesis

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In bacterial photosynthesis it has been proposed that an electron is transferred from the excited singlet state (P*) of the primary electron donor first to a bacteriochlorophyll (BChl) molecule, generating a P^+BChl charge-transfer state, and that $BChl^-$ subsequently (4–7 ps) transfers an electron to a bacteriopheophytin molecule. Here we review the evidence for and against the intermediacy of P^+BChl^- and present picosecond transient absorption and kinetic data that bear on this point. We conclude that there is no convincing evidence that P^+BChl is a kinetically or spectrally resolved intermediary state in the charge separation process.

Bacterial photosynthesis *Reaction center* *Early intermediate* *Bacteriochlorophyll* *Bacteriopheophytin* *Quinone*

1. INTRODUCTION

Photoexcitation of bacterial reaction centers prepares the excited singlet state (P*) of the primary electron donor (P), a complex involving two of the 4 bacteriochlorophyll (BChl) molecules [1]. It is generally accepted that within 10 ps an electron from P* causes reduction of an intermediary electron acceptor (I), thought to be a complex that involves a bacteriopheophytin (BPh) and one of the remaining BChls. The odd electron on I^- appears to be largely, if not completely, localized on the BPh at or below room temperature [2–5]. (It has been suggested that P^+BChl^- and P^+BPh^- are in thermal equilibrium and that ~60% of unpaired electron density on the acceptor is on the BPh at 295 K, and more at lower temperatures [6].) Reduction of the BPh causes a bleaching of the BPh's Q_X and Q_Y absorption bands at 545 and 765 nm, and the development of a broad, new band at 665 nm. Absorption changes that are observed in regions of the spectrum normally associated with the BChls (600 and 800 nm in *Rhodopseudomonas sphaeroides*) have been interpreted as arising from electrochromic effects of BPh^- on a BChl, disruption of excitonic interactions between the BChl and P or BPh, or the partial reduction of the BChl [1–9]. The electron is subsequently transferred from BPh^- to a quinone (Q) in ~200 ps at room temperature [5,9–22].

Most picosecond studies on the primary events have employed flashes that lasted either 5–10 or 25–50 ps. Such studies have placed an upper limit of 5–10 ps on the time constants for both the oxidation of P and the reduction of I (BPh) [5,10–14, 16–19,22–24]. Holten et al. [19], using 0.7 ps excitation flashes at 610 nm, reported a rise time of ~4 ps for appearance of the bleaching at 545 nm and the absorption increase near 670 nm. The authors attributed these kinetics to the reduction of BPh to BPh^- (I to I^-). Prior to the 4-ps step (near $t = 0$), a different spectrum was observed. It could not be determined whether this initial transient was P*, P^+BChl^-, or possibly some other state resulting from multiple excitations of the reaction center. It was concluded that if the transient was P^+BChl^-, then it must decay to P^+BPh^- in about 4 ps. However, these results should not be

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taken as supporting the identification of P^+BChl^- as an early intermediate state.

The reaction center can be trapped in state BPh^- by continuous illumination at low potential in the presence of reduced cytochrome-c [2,3,7,8]. Picosecond studies on *Rps. viridis* [14] and *Rps. sphaeroides* [25] reaction centers in this state revealed a transient that exhibited bleaching in the 870-nm band in *Rps. sphaeroides* (960-nm in *Rps. viridis*), but not the strong bleaching at 800 nm (830 nm) expected for $P^+BChl^-BPh^-$. Picosecond studies on *Rps. viridis* and *Chromatium vinosum* reaction centers containing BPh^- at low temperature showed also that neither P^+ nor the triplet state, P^R, was formed after light activation [23]. However, the authors could not exclude the possibility that reduction of the BPh pushes the energy of $P^+BChl^-BPh^-$ above that of P*, preventing its formation in these experiments [14,23,25].

Shuvalov et al. [17] and Kryukov et al. [26] were the first to report evidence from picosecond studies for P^+BChl^-. These studies employed relatively strong 25–50 ps, 880-nm excitation flashes that pumped the long-wavelength transition of P directly. A 30-ps transient was observed that exhibited strong bleaching at 800 nm. This transient was assigned as P^+BChl^-. However, several groups using 5–10 or 30–50 ps flashes have observed a 30–50 ps 'extra' component to the bleaching near 800 nm [18,19,27] (830 nm in *Rps. viridis* [14]) when reaction centers are excited with an excessive number of photons, either in the visible or near-infrared.

Akhamanov et al. [18] made measurements at 295 K on *Rps. sphaeroides* reaction centers having Q reduced, using low-intensity 30-ps, 880 nm excitation flashes (1 photon/reaction center). They also showed a difference spectrum that was dominated by a strong bleaching at 800 nm. However, the spectrum was calculated by subtracting from the transient difference spectrum at $t = 0$, a spectrum for P^+Q^- obtained by CW illumination (after normalization at 870 nm). It was concluded from the calculated spectrum that P^+BChl^- made a substantial contribution to the absorption changes at $t = 0$. There are difficulties in the interpretation of such calculated spectra, as discussed below.

Shuvalov and Klevanik [22] recently reported a rise time of 7 ± 2 ps for reduction of the BPh in *Rps. sphaeroides*, using 28-ps excitation flashes at 870 nm. They also showed a difference spectrum (110 K) calculated by first multiplying a spectrum taken at an 85-ps delay by a fraction (based on measurements at 785 nm) and then subtracting the resultant spectrum from one acquired at the center of the excitation flash ($t = 0$). The rationale was to correct the early transient spectrum for the contribution of P^+I^-. The authors justified the correction procedure on the view that the 110 K difference spectra for P^+I^- and P^+Q^- do not differ from one another in the 800-nm region (but see below). The calculated spectrum showed a sharp trough at 800 nm, a smaller bleaching at 870 nm, and a weak absorption increase near 765 nm. The calculated spectrum was attributed to P^+BChl^-.

We argue here that these calculated spectra necessarily show a large negative feature near 800 nm (815 nm in *Chloroflexus* [21,28]; 830 nm in *Rps. viridis* [9,14]), but not conclusively because of the presence of P^+BChl^-. The 'correction' spectrum used by Akhamanov et al. [18] is that of P^+Q^-. The 85-ps spectrum used for the correction procedure by Shuvalov and Klevanik [27] contains a significant (~50%) contribution from P^+Q^- (see below). The spectra for P^+Q^- and P^+I^- differ significantly in the 800-nm region at room temperature [9–11,17,21,25] and at all temperatures over the ranges 5–295 K [5]. These differences are particularly clear in transient difference spectra acquired using broad-band (2D) detection [5,9,21]. Subtraction of a P^+Q^- spectrum from a P^+I^- difference spectrum necessarily gives a sharp trough near 800 nm, as reported previously [9,21,25]. Here we show low-temperature transient difference spectra that further demonstrate this point (see fig.1). Photoselection must also be taken into account when exciting in the long-wavelength band of P [9,28,29].

We also present data that agree with previous evidence that the growth of bleaching in the Q_X ground-state band of BPh lags behind the bleaching in the 870-nm band of P. This lag, and similar effects observed in the near-infrared absorption changes associated with BChl, are observed using either 600- or 867 nm excitation flashes. The lags are consistent with the view expressed by Holten et al. [19], Shuvalov et al. [17], Kirmaier et al. [9], and Shuvalov and Klevanik [22] that reduction of I (BPh) takes 4–7 ps. However, we conclude that

there is presently no time-resolved spectral evidence to support the prior formation of P^+BChl^-.

2. MATERIALS AND METHODS

The picosecond transient absorption spectrometer [9] and facility for low-temperature measurements [5] are described elsewhere. The energy of the 867 -nm excitation flashes are ≤ 1 μJ, giving < 1 photon per reaction center at the sample. The energy and focussing of the 600-nm flashes gives 1–5 photons per reaction center. Under these conditions, we observe neither the spectral distortions nor the 30-ps transient found previously when more intense 532- or 880-nm flashes were employed [14,18,19,27]. (We also observe these extra features when intense 532-nm flashes are used.) Except for polarization effects (see below) and the overall amplitudes of the absorption changes, excitation flashes at 600 or 867 nm gave identical spectral and kinetic results, where comparisons could be made. Standard deviations in ΔA are typically $\pm$ 0.006. *Rps. sphaeroides* R26 reaction centers were prepared as described [30], and suspended in Tris (pH 8.0)/Triton X-100. Measurements at 76 K were made on samples in 65% glycerol/buffer. Kodak IR-132 dye was used as received and dissolved in spectral grade dimethyl sulfoxide for the rise time measurements.

3. RESULTS AND DISCUSSION

Fig.1A shows 76 K near-infrared absorption difference spectra for *Rps. sphaeroides* reaction centers in 65% glycerol at two time delays with respect to the peak of a 30-ps, 600-nm excitation flash. The 1.6-ns (dashed) difference spectrum can be assigned to P^+Q^-. The 30-ps (solid) spectrum is due mainly to P^+I^-, but contains a 25% contribution from P^+Q^-, considering the 100 $\pm$ 10 ps time constant for electron transfer from I^- to Q, as measured from the absorbance changes at 795 nm at 76 K [5]. Bleaching in the long-wavelength band of P (red-shifted to ~890 nm at 76 K) is identical at 30 ps and 1.6 ns. The spectra for P^+I^- and P^+Q^- differ substantially between 740 and 820 nm, particularly in the regions centered at 765 and 795 nm. These differences are emphasized in fig.1B, which plots the absorption changes at 30 ps minus those at 1.6 ns. Similar spectra are obtained when excitation flashes in the long-wavelength band of P are employed, except that photoselection must be taken into account [5,9].

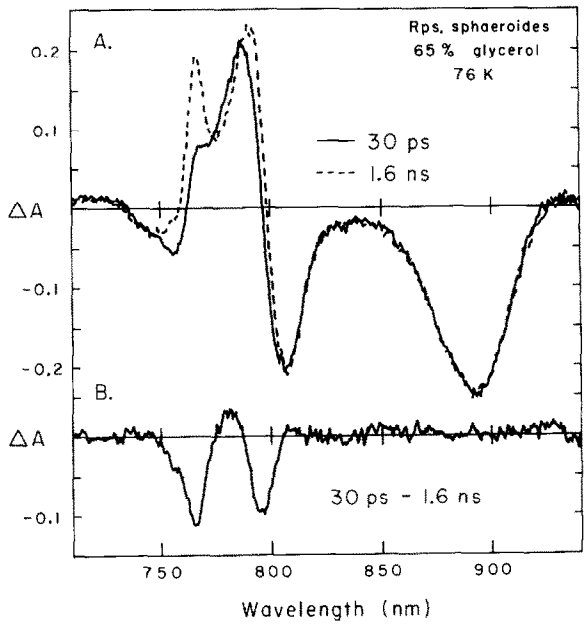

Fig.1. (A) Near-infrared absorption changes at 76 K for *Rps. sphaeroides* reaction centers in 65% glycerol at 30 ps (——) and 1.6 ns (– – –) with respect to a 30-ps 600-nm flash. Each spectrum was acquired in two 150-nm slices, that agreed within experimental error in the regions of overlap; each spectrum represents the average of spectra acquired using 300 excitation flashes. (B) Difference between the 30-ps and 1.6-ns spectra in (A).

The detailed spectral features for states P^+I^- and P^+Q^- depend on temperature (and in some regions on medium [5]), but under all conditions subtraction of a P^+Q^- difference spectrum from a P^+I^- difference spectrum gives a sharp trough in the 795-nm region (fig.1B). The relative amplitude of this feature compared to that near 765 nm depends on the pump/probe polarization for near-infrared excitation. The absorption changes near 765 nm for both P^+Q^- and P^+I^- are perpendicularly polarized with respect to the 870-nm transition of P; those near 795 nm contain substantial parallel polarization with respect to the 870-nm transition [9,29]. If the excitation flash is at 870 nm, and the probe beam is polarized parallel to the excitation, the absorbance changes in the

795-nm region will be emphasized compared to those near 765 nm. (See fig.4C in [9].)

Considering the 100 ± 10 ps decay kinetics [5], the spectrum at 85 ps used by Shuvalov and Klevanik [22] as the correction spectrum contained nearly equal contributions from P^+I^- and P^+Q^-. Thus, their calculated spectrum can be accounted for in the 800-nm region by the subtraction (after scaling) of a spectrum due partially to P^+Q^- (85 ps) from a P^+I^- spectrum ($t \sim 0$). The relatively small absorption changes in the 765-nm region of their spectrum are accounted for by their use of parallel pump/probe polarization. Their calculated bleaching at 880 nm derives from the fact that the scaling factor was based on a wavelength (785 nm) where the growth of the absorption changes lags those at 880 nm (see below).

Therefore, we conclude that evidence for the intermediacy of P^+BChl^- from such calculated spectra is ambiguous. With the potential problems in mind, we compared spectra acquired early in the excitation flash (before $t = 0$) with each other and with a 30-ps spectrum in an attempt to obtain spectral evidence that could be assigned with a higher degree of confidence to P^+BChl^-. These 'negative' times correspond to temporal overlap of the tail of the probe pulse with the rising edge of the pump pulse at the sample. Spectra were acquired at the earliest time at which the absorption changes were measurable (−20 ps) and at ~7 ps increments during the remainder of the excitation flash. Fig.2 illustrates a typical result for reaction centers in 65% glycerol at 76 K using 600-nm excitation flashes. The 30-ps spectrum of fig.1A has been divided by 2.3 (dashed) for comparison with a spectrum taken near $t = 0$ (solid). The difference between these two spectra is shown in fig.2B. Most of the differences between the two spectra can be accounted for by the 25% P^+Q^- contribution to the longer-time (30 ps) spectrum (i.e., cf. fig.1B). Similar comparisons were made on reaction centers in flowed buffer at 295 K, in 56% glycerol/buffer glasses at 76 K, and in polyvinyl alcohol films at 76 and 5 K, using excitation flashes at 600 or 867 nm (both pump/probe polarizations). In some cases (fig.2B, for example), it was not completely clear whether the differences between spectra taken before $t = 0$ and at 30 ps could be accounted for quantitatively by the P^+Q^- contribution and/or photoselection effects.

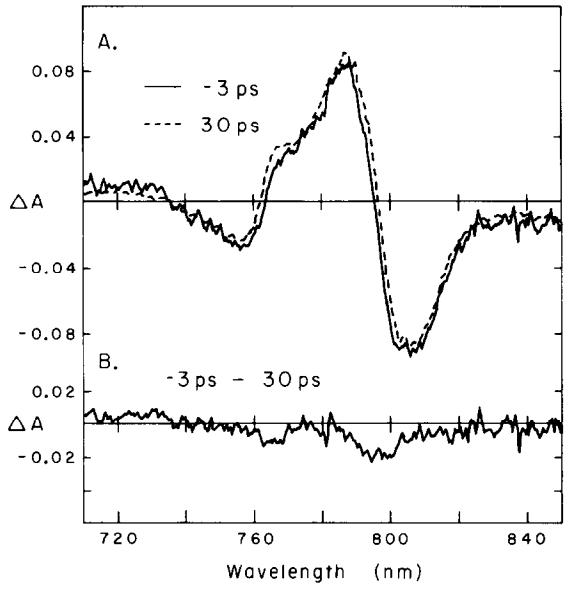

Fig.2. (A) Near-infrared absorption changes at −3 ps (——) and 30 ps (– – –). (B) Difference between the spectra of (A). Other conditions as in fig.1.

However, the residual differences are comparable to or less than the experimental error ($\Delta A = \pm 0.006$). Assignment of these small absorption changes to P^+I^-, P^+BChl^-, or another transient would be extremely speculative; some absorption changes might be expected in this region for P* itself. Therefore, to within our temporal and spectral resolution we find no clear spectral evidence for absorption changes early in the flash that can be assigned with any degree of confidence to P^+BChl^-.

We also examined the rise time of the absorption changes in the visible and near-infrared at 295 K. The open circles in fig.3 show the instrument-limited kinetics of bleaching of Kodak IR-132 dye in the 830–840-nm region produced by 867-nm excitation flashes. The pump and probe flashes in these experiments and those of fig.4 were polarized near 45° to each other. Potential problems due to chirp in the probe light (different arrival times at the sample of probe light at different wavelengths) are ruled out by agreement of these data with the rise of the transient absorption at 565–570 nm with the same dye (filled circles). With reaction centers, the bleaching in the 590–600-nm band (triangles) follows the instrument-limited response.

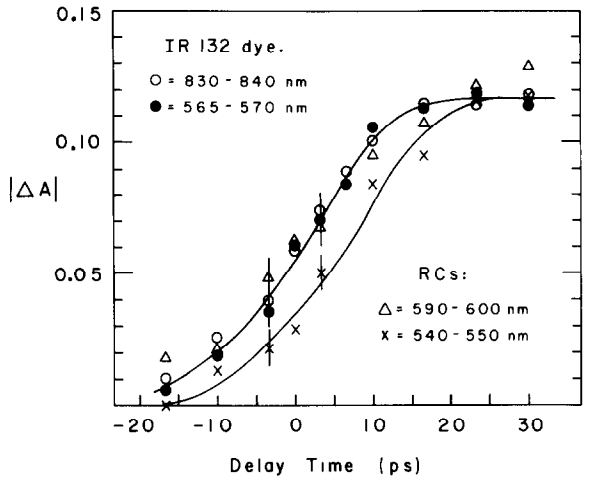

Fig.3. Instrument-limited growth of the bleaching at 830–840 nm (○) and transient absorption at 565–570 nm (●) for IR-132 dye, induced by excitation with weak 25-ps 867-nm flashes. Growth of bleaching in the 595-nm band (△) for flowed *Rps. sphaeroides* reaction centers in buffer follow the instrument-limited response, but the appearance of the bleaching in the 545-nm band (×) lags behind. These measurements were made at 295 K.

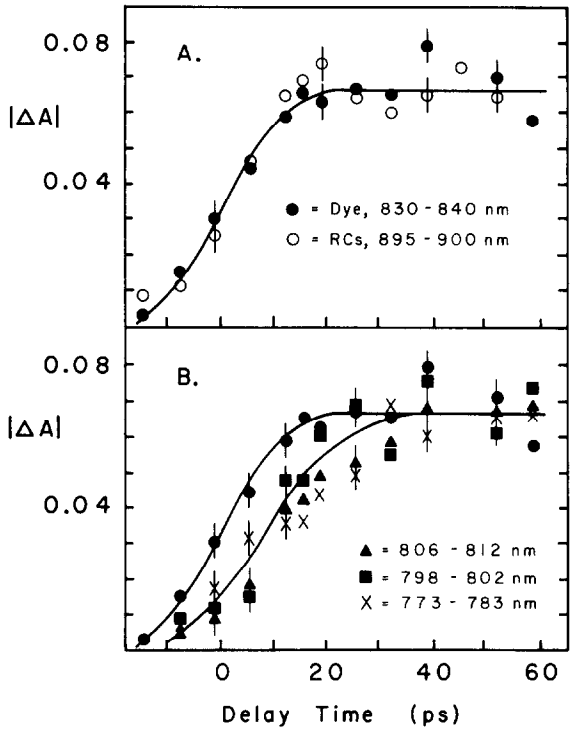

Fig.4. Instrument-limited growth of bleaching near 835 nm (●) for IR-132 dye (A and B). Growth of bleaching near 897 nm (○) for reaction centers follows the same response (A); growth of the absorption decreases near 808 nm (▲) and 800 nm (■) and of the absorption increase near 778 nm (×) lag behind. Other conditions as in fig.3.

On the other hand, development of bleaching in the Q_X band of BPh shows a lag (crosses in fig.3). The time-resolved absorption difference spectra over the 520–620-nm region corresponding to the kinetic data of fig.3 can be found in fig.2 of [9]. The data agree with earlier results placing a time constant of 4–7 ps on the reduction of BPh [9,17,19,22].

Corresponding data in the near infrared are shown in fig.4. The open circles in fig.4A show that the growth of bleaching in the long-wavelength band of P, as measured between 895 and 900 nm, follows the instrument-limited dye response (filled circles). Fig.4B compares the instrument-limited rise kinetics (filled circles) with the growth of the reaction center absorption changes in 3 other regions of the near-infrared. The crosses show the 773–783-nm absorption increase; squares, the absorption decrease at 798–802 nm and triangles, the absorption decrease at 806–812 nm. The data were normalized at 40–60 ps. (The 76 K difference spectra of figs 1 and 2 can be consulted for the major spectral features.) The first region is due to both BPh and BChl, while the longer-wavelength regions are due mainly to the BChls [1,5,9,29]. The reaction center absorption changes in all 3 regions rise with comparable rates and lag the instrument-limited response (and the bleaching of the 870-nm band of P). Similar data are obtained when 600-nm excitation flashes are used. These results, taken with the data of fig.3, suggest that 600-nm flashes mainly excite P, and/or that energy transfer from the other BChls to P is extremely rapid (possibly subpicosecond).

Borisov et al. [31] have argued previously against P^+BChl^- as an early intermediate. However, their argument was based mainly on their lack of finding a clear lag in the growth of the absorption decrease at 810 nm, as compared to the growth of bleaching at 840 nm (using 4-ps flashes at 870 nm). This observation is not consistent with

the lags observed in the visible and near-infrared in the present study (figs 3a and 4) and previously [9, 17,19,22].

In conclusion, the majority of evidence indicates that the time constant for the formation of P^+I^- is 4–7 ps. This step includes the reduction of BPh and is accompanied by absorption changes in the near-infrared normally attributed to BChl. We argue that no picosecond study to date has demonstrated convincingly that P^+BChl^- is a kinetically or spectrally resolved intermediate state. However, we cannot completely rule out some involvement of BChl in the early photochemical events. Additional studies employing flashes having duration shorter than the observed 4–7 ps rise times are needed to explore this point further and are underway.

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