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Picosecond spectroscopy of the charge separation in reaction centers of *Chloroflexus aurantiacus* with selective excitation of the primary electron donor

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Absorbance changes in the picosecond region were studied in isolated reaction centers of the green photosynthetic bacterium *Chloroflexus aurantiacus* upon selective excitation of the primary electron donor, P, at 870 nm. The results indicate that the first observed state is an excited state of P (P*) which appears to transfer an electron to a bacteriochlorophyll *a* molecule absorbing at 812 nm (B_1) in 10 ± 2 ps as indicated by a bleaching at this wavelength. This reaction is followed by a rapid electron transfer (3 ± 1 ps) from B_1^- to bacteriopheophytin *a*, so that the fraction of reaction centers in the state $P^+B_1^-$ remains small during the experiment. An apparent bleaching at 925 nm is ascribed to stimulated emission from excited P, which emission disappears upon formation of P^+. The difference between these time constants for electron transfer and those observed for the same reactions in reaction centers of the purple photosynthetic bacterium *Rhodopseudomonas (Rhodobacter) sphaeroides* is discussed in terms of the energy difference between P* and $P^+B_1^-$, which appears to be larger for *C. aurantiacus*.

Introduction

Studies with isolated reaction centers of purple bacteria have shown that the charge separation that occurs upon excitation of the primary electron donor pair, P, is a very fast process. Time constants of 3–7 ps were observed for the formation of the charge pair $P^+H_1^-$ (where H_1 denotes the photoactive bacteriopheophytin) for *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* [1–7]. However, there is as yet no consensus with respect to the nature of the early processes that precede the reduction of H_1 and to the role of the so-called 'accessory' or monomeric BChl *a* that is located between P and H_1 [8]. The results of recent hole-burning experiments indicate the existence of a very short-lived excited state of P, the nature of which is not yet clear [9,10], but comparison of the rates of stimulated emission from excited P (P*) and of the formation of H_1^- indicates that both processes appear to have the same time constant, of approx. 4 ps [7]. Moreover, measurements of the kinetics of absorbance

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Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; B_1, BChl molecule absorbing at 812 nm; H_1, photoreactive BPh; P, primary electron donor; PMS, *N*-methylphenazonium methosulfate; Q, secondary electron acceptor (menaquinone).

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changes in reaction centers of *Rps. sphaeroides* after very short laster flashes gave no evidence for the existence of a charge-separated state involving the BChl *a* monomer [5].

In contrast to the latter results stand those obtained by kinetic analysis of absorption difference spectra during the early phases of exciting laser pulses. These spectra, measured with reaction centers of *Rps. sphaeroides*, showed a bleaching near 800 nm in addition to that caused by the formation of singlet excited P (P*) [1,3,6]. This bleaching at 800 nm was ascribed to an intermediate state that includes the radical pair $P^+B_1^-$ (where B_1 denotes the BChl *a* monomer). The time constant for electron transfer from B_1^- to H_1 was estimated to be 1.5 ps [6]. The reason for these apparent discrepancies are not yet understood (for a discussion of this and other conflicting evidence see Ref. 6), and further insight might be gained by more detailed examination of the kinetics near 800 nm after ultrashort flashes, preferably with reaction centers in which the second 'accessory' BChl *a* has been removed or modified [11,12].

In view of the above-mentioned uncertainties it seemed to interest to extend the experiments to the filamentous green photosynthetic bacterium *Chloroflexus aurantiacus*. Experiments with isolated reaction centers [13–16] as well as with membrane fragments [17] have shown that the primary photochemistry and secondary electron transport in this species are similar to those in purple bacteria. Although the pigment composition of the reaction center is different from that of purple bacteria (one BChl *a* molecule being replaced by BPh *a*), absorption difference spectroscopy of P^+ and H_1^- formation with polarized and non-polarized light as well as circular dichroism measurements [11,18] have indicated a similar arrangement of the chromophores as in purple bacteria [11,19]. Calculations based on the point-dipole approximation for exciton interaction to simulate the optical characteristics of the reaction center of *C. aurantiacus* [11,18] indicate that its structure is very similar [11,19] to that of *Rps. viridis* as determined by X-ray analysis [8].

The results reported here show that the reduction of H_1 in *C. aurantiacus* reaction centers in which the menaquinone (Q) had been prereduced occurs with a time constant of 14 ps. Formation of P^+ is accompanied by a disappearance of stimulated emission from P*. As in *Rps. sphaeroides*, a transient bleaching of the monomeric BChl *a* band was observed that was attributed to reduction of B_1. Simulation of the kinetics suggested that the primary charge separation $P^*B_1 \rightarrow P^+B_1^-$ occurs in 10 ± 2 ps, followed by the reaction $P^+B_1^-H_1 \rightarrow P^+B_1H_1^-$. The latter reaction has a time constant of 3 ± 1 ps, which is close to that observed for the same reaction in *Rps. sphaeroides* reaction centers [6].

Materials and Methods

Reaction centers from *C. aurantiacus* were isolated by a method similar to that described in Ref. [15] using lauryldimethylamine *N*-oxide as a detergent, and sucrose gradient centrifugation and DEAE-cellulose chromatography for the purification [16]. The final preparation was dissolved in a buffer comprising 10 mM Tris (pH 8.0)/0.025% lauryldimethylamine *N*-oxide, and had an absorbance of 0.4 at 865 nm in a 2 mm cuvette.

The dual-beam spectrometer with 33-ps measuring and excitation pulses and equipped with an optical multichannel analyzer to register absorption difference spectra has been described in Ref. 6. Excitation at 815 nm and 870 nm was obtained using a parametric light generator pumped by the 35-ps pulses at 532 nm. The energy of the excitation pulse (about 6 mJ/cm^2) was sufficient to measure the absorbance changes when the center of the measuring pulse preceded that of the excitation pulse by up to 38 ps (indicated by negative numbers in the figures). At this 'negative delay', a time resolution of about 1.5 ps could be obtained [6]. A non-polarized measuring pulse was used in order to avoid photoselection effects. All experiments were done at room temperature.

The deconvolution of the kinetics of the changes of absorbance (ΔA) at wavelength λ, which are delayed with respect to the bleaching of the absorption bands of P, was done as discussed in Ref. 6, using the following expression:

$$\frac{\Delta A_\lambda(\tau)}{\Delta A_p(\tau)} = \frac{c\int_{-\infty}^{\tau} \frac{d(\Delta A_p)}{dt} \cdot e^{-k(\tau - t)}\, dt}{\Delta A_p(\tau)} \qquad (1)$$

where $\Delta A_\lambda(\tau)$ and $\Delta A_p(\tau)$ are the absorbance changes at wavelength λ and at the wavelengths of P absorption (at 600, 865 or 880 nm), respectively, at time τ; k is the rate constant for the rise of the absorbance change at wavelength λ; c is a normalization coefficient, which includes the ratio of extinction coefficients.

Results

The normalized kinetics of the absorbance changes (ΔA) at 600 nm, 865 nm and 880 nm are shown in Fig. 1, where the amplitudes of the bleachings at these wavelengths are plotted as a function of the time between the centers of the 33-ps probing and excitation pulses. Negative numbers indicate that the center of the probing flash preceded that of the excitation flash. The bleaching at 600 nm was measured as the depth of a trough on a broad absorbance increase (see Fig. 3B). The kinetics at the three wavelengths are very similar, when corrected for the shift in time due to chirping. This correction was about 3 ps between 600 and 880 nm and less than 0.5 ps between 760 and 815 nm. As will be discussed below, the bleaching in the region of 860–880 nm is initially mainly due to P* formation and at later times to formation of P^+. Since the conversion of P* to P^+ presumably does not cause marked absorption changes at these wavelengths, the kinetics reflect the rate of excitation of the reaction centers as a function of the time delay under our experimental conditions. The same is probably true for ΔA_{600}. Therefore these kinetics were used as a basis for deconvolution (see Eqn. 1) of slower phenomena due to electron transfer reactions to be described below.

Fig. 2A shows the absorption difference spectrum of reaction centers of *C. aurantiacus* measured upon excitation with a 33-ps pulse at 870 nm. The spectrum was recorded at 55 ns after the excitation pulse, in the presence of dithionite to keep the quinone acceptor Q in the reduced state. We attribute this spectrum to the formation of P^T, the triplet state of the primary electron donor, which is probably formed by the radical pair mechanism [20]. The spectrum is characterized by a bleaching of the P band at 865 nm, by a blue shift of the B_1 band at 812 nm and by the development of a band at 765 nm, probably related to a red shift and an increase of dipole strength of the BPh *a* transition. The spectral features around 812 and 765 nm appear to be due to a loss of the exciton interaction of P with B_1 and BPh *a* when P is excited to the triplet state. These observations are at least qualitatively in agreement with model exciton calculations [19]. It is of interest to note here that there is only little net increase of dipole strength in the region 790–820 nm. This suggests that, in contrast to what was observed at 1.2 K [20], the triplet is delocalized over both molecules of the dimer.

The spectra shown in Figs. 2B and C and Fig. 3 were measured at much shorter times. Spectrum 2B was measured at −32 ps, i.e., at 32 ps before the center of the excitation flash. This spectrum is very similar to that of Fig. 2A and therefore may be attributed to the formation of the singlet ex-

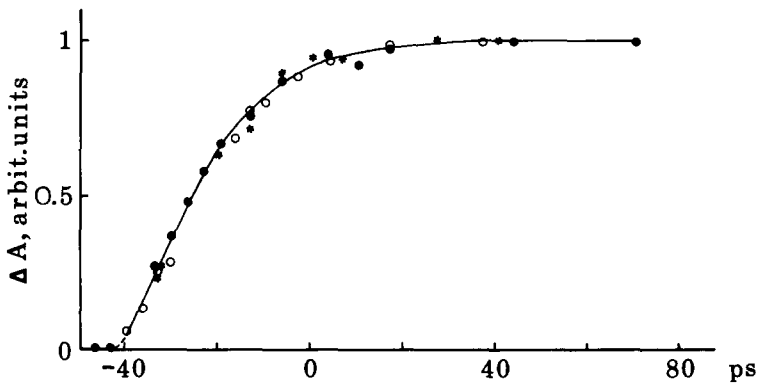

Fig. 1. Normalized kinetics of absorbance changes, ΔA at 600 nm (*) (measured as the depth of a trough on a broad absorbance increase, see Fig. 3B), at 865 (●) and 880 nm (○) of *C. aurantiacus* reaction centers in the presence of ascorbate (10 mM), PMS (10 μM) and continuous illumination to keep Q in the reduced state; 33-ps pulses at 870 nm (about 6 mJ/cm^2) were used for excitation. The horizontal scale gives the time difference between the excitation and measuring pulses; negative numbers indicate that the center of the measuring pulse preceded that of the excitation pulse. The measurements were corrected for chirping.

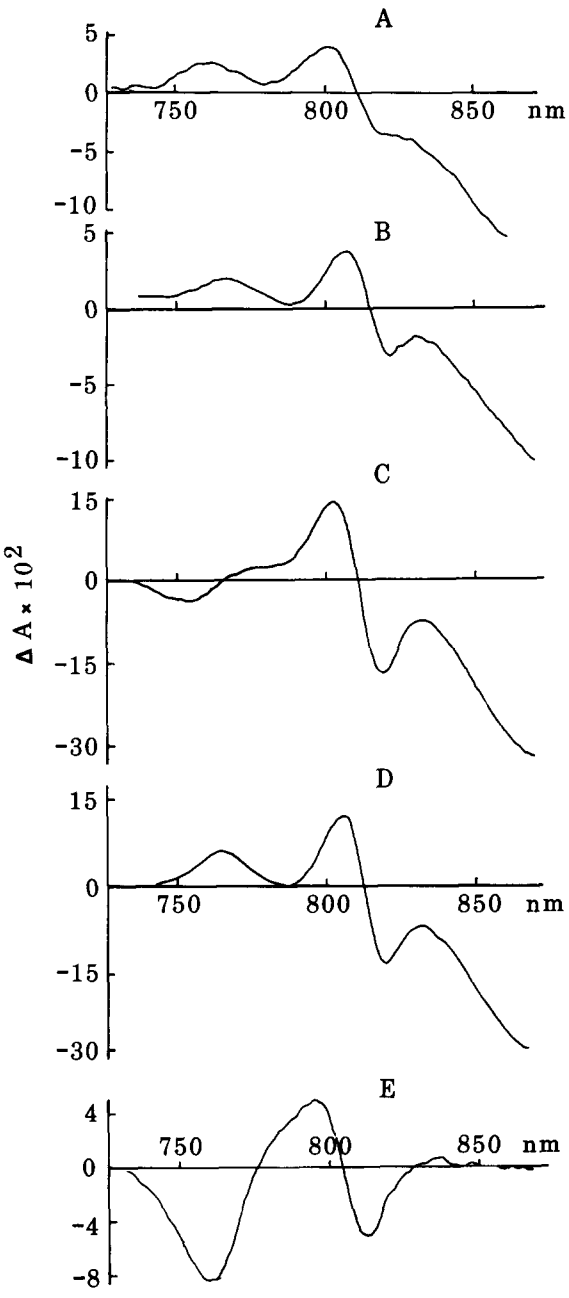

Fig. 2. Absorption difference spectra of *C. aurantiacus* reaction centers induced by 33-ps pulses at 870 nm and measured at different delays. Q was reduced by $Na_2S_2O_4$ (A) or continuous background illumination in the presence of ascorbate (10 mM) and PMS (10 μM) (B, C). (A) Triplet-minus-singlet spectrum measured at 55 ns after the center of the excitation pulse. (B) Spectrum of P*, measured at −32 ps, i.e. 32 ps before the center of the excitation pulse. (C) Spectrum of $P^+H_1^-$ measured at 71 ps. (D) Spectrum of P^+Q^- measured at 2.9 ns in reaction centers in which Q was oxidized before the measurements. (E) Absorption difference spectrum obtained by subtracting spectrum D from spectrum C.

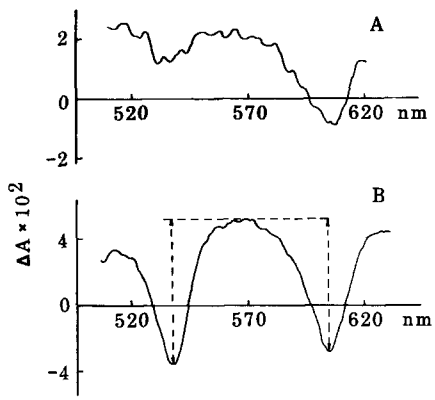

Fig. 3. Absorption difference spectra induced by 870-nm pulses, measured at −32 ps (A) and at 71 ps (B). Q was reduced by continuous background illumination in the presence of ascorbate (10 mM) and PMS (10 μM). The dashed vertical lines in Fig. 3B show the amplitudes of ΔA at 600 and 540 nm used in the plots of Figs. 1 and 5, respectively.

cited state of P, since the loss of exciton interaction between P, B_1 and BPh *a* may be expected to be the same when P is excited to the singlet or to the triplet state. In the visible region the spectrum shows a bleaching of the Q_x band of BChl *a* at 600 nm (Fig. 3A). As in the near-infrared region, there is no evidence for a significant reduction of BPh *a*. The spectrum measured at 71 ps (Figs. 2C and 3B) is clearly different from those attributed to the formation of singlet or triplet excited P. In contrast to the other spectra it shows, in addition to the bleaching of the P band at 865 nm, a bleaching of the BPh *a* bands at 755 and 540 nm. There is a shift of the isobestic point from 815 to 811 nm, together with an increase of the amplitudes of the negative and positive bands near 810 nm with respect to the spectrum measured at −32 ps. The spectrum is similar to that observed by Kirmaier et al. [13] and may be attributed to formation of the state $P^+H_1^-$.

Differences were also observed in the region above 880 nm (Fig. 4). The spectrum measured at 71 ps (solid curve) showed an isobestic point at 925 nm as compared to 950 nm for that measured at −32 ps (dashed curve). When normalized at 880 nm, the latter spectrum has an additional bleaching in the region of 880–970 nm with a relative amplitude of 0.16 at 925 nm. A similar apparent bleaching in the long-wave region in

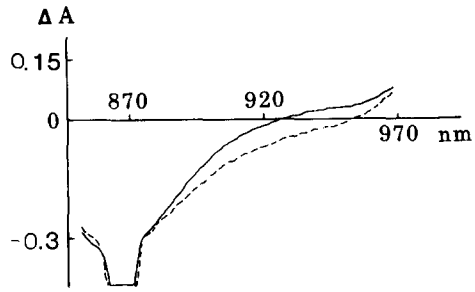

Fig. 4. Absorption difference spectra obtained upon excitation at 870 nm and measured at −32 ps (- - - - -) and 71 ps (———). Conditions as for Fig. 3. The spectra were normalized at 880 nm. The scale applies to the solid curve.

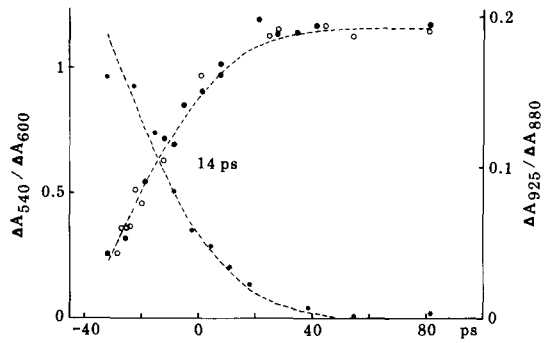

Fig. 5. Kinetics of the ratios of $\Delta A_{540}/\Delta A_{600}$ ($P^+H_1^-$ formation) and $\Delta A_{925}/\Delta A_{880}$ (*) (stimulated emission from P*) for *C. aurantiacus* reaction centers, with excitation at 870 nm (● and *) and at 815 nm (○). Conditions as for Fig. 3. Dashed curves show the calculated kinetics assuming that the rise time for $P^+H_1^-$ formation and the decay time for P* are the same (14 ps).

reaction centers of *Rps. sphaeroides* was attributed to stimulated emission form P* [4–7], which emission disappeared when the electron was transferred to H_1.

If Q is in the oxidized state before the excitation, the state $P^+H_1^-$ decays to the state P^+Q^-, the difference spectrum of which is shown in Fig. 2D. This spectrum is again characterized by the bleaching of the P band at 865 nm. Like the spectra of Figs. 2A and B it shows an increase in the dipole strength of BPh *a* at 765 nm, but the blue shift of the band of B_1 is more pronounced and shows an isobestic point at 813 nm. However, the blue shift is somewhat smaller than in the $P^+H_1^-$ spectrum. When the spectrum attributed to P^+Q^- is subtracted from that of $P^+H_1^-$, the resulting difference spectrum shows a bleaching at 760 nm and a blue shift centered at 805 nm (Fig. 2E). A similar spectrum was reported by Kirmaier et al. [13,14].

To study the conversion of the state P* to the state $P^+H_1^-$, absorption difference spectra like those of Figs. 3 and 4 were measured at various times between −40 and 80 ps for reaction centers in which Q was reduced before excitation. The ratio $\Delta A_{545}/\Delta A_{600}$ in these spectra was taken as a measure of the fraction of excited reaction centers that had been converted to the state $P^+H_1^-$, whereas the ratio $\Delta A_{925}/\Delta A_{880}$ was used to determine the fraction of excited reaction centers which showed stimulated emission from P*. Both processes showed essentially the same time-course (Fig. 5). By deconvolution of the kinetics of H_1 reduction and of the stimulated emission by means of Eqn. 1 on basis of the kinetics at 860 nm, identical time constants of 14 ± 2 ps for the formation of $P^+H_1^-$ and the decay of P* were obtained. The kinetics of the formation of $P^+H_1^-$ were the same upon excitation at 870 nm (filled circles) and at 815 nm (open circles).

To study the kinetics at 812 and 760 nm a similar set of spectra was measured in the region 730–865 nm. The spectrum measured at −32 ps (Fig. 2B, attributed to P* formation) was subtracted from the spectra measured at later times normalized at 865 nm. Examples are shown in Fig. 6A–D. The spectrum of Fig. 6D, obtained as a difference between the spectra measured at 50 ps and −32 ps, represents the absorbance changes that occur upon conversion of P* to $P^+H_1^-$. If it is assumed that no other electron transfer reactions are involved, it is easy to see that the other spectra should be proportional to that of Fig. 6D. The difference spectrum measured at −32 ps $(\Delta A_1)_\lambda$ mainly represents the difference between the spectrum of $P^*(A_{P^*})_\lambda$ and that of the ground state $(A_G)_\lambda$:

$$(\Delta A_1)_\lambda = (A_{P^*} - A_G)_\lambda \qquad (2)$$

A spectrum normalized at 865 nm measured at later time $(\Delta A_2)_\lambda$ includes a fraction α of the spectrum of $P^+H_1^-$ and a fraction β of that of P*,

where $\alpha + \beta = 1$:

$$(\Delta A_2)_\lambda = (\alpha A_{P^+H^-} + \beta A_{P^*} - A_G)_\lambda \quad (3)$$

Subtraction gives:

$$\Delta_\lambda = (\Delta A_2 - \Delta A_1)_\lambda = (\alpha A_{P^+H^-} + \beta A_{P^*} - A_{P^*})_\lambda$$

$$= \alpha(A_{P^+H^-} - A_{P^*})_\lambda \quad (4)$$

$$(A_{P^+H^-} - A_{P^*})_{865} = 0 \quad (5)$$

The last expression shows that the normalization at 865 nm is based on the assumption that formation of $P^+H_1^-$ and of P^* are accompanied by the same bleaching at 865 nm.

From Eqn. 4 it follows that all spectra Δ_λ at different delays should have the same shape, with amplitudes which are proportional to α. Comparison of the spectrum of Fig. 6D with that obtained by photoacumulation of H_1^- [11] indicates that the bleachings at 760 nm and near 812 nm can indeed be attributed to the formation of H_1^-. The same features are observed in the other spectra, but it can be seen in the spectra measured at early times that the bleaching near 812 nm is relatively larger.

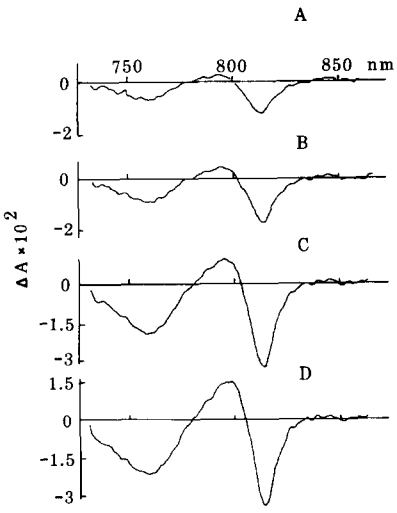

Fig. 6. Absorption difference spectra obtained by subtraction of the difference spectrum measured at −32 ps from spectra of ΔA measured at −22 ps (A), −12 ps (B), 5 ps (C) and 50 ps (D). Conditions as for Fig. 3. The spectra measured at −22, −12, 5 and 50 ps were normalized at 865 nm to the amplitude of the spectrum measured at −32 ps before the subtraction.

At 50 ps the (negative) band near 812 nm is approximately 50% larger than that at 760 nm, whereas at −22 and −12 ps (Fig. 6A and B) it is about twice as large. Moreover, the contribution by a blue shift of the B_1 band, as indicated by the amplitude of the positive band near 795 nm relative to the negative one near 812 nm, is clearly smaller in the latter spectra. To check the contribution by two photon processes in the spectra measured at later times, absorption difference spectra were also measured at 3-times lower intensity of excitation. The ratio $\Delta A_{812}/\Delta A_{760}$ at 50 ps was found to be the same within 7% with respect to that shown in Fig. 6D, indicating that such a contribution, if any, was not significant.

Fig. 7 shows the kinetics of $\Delta A_{812}/\Delta A_{865}$ and of $\Delta A_{760}/\Delta A_{865}$. The rise time of $\Delta A_{760}/\Delta A_{865}$ obtained by deconvolution was approx. 14 ps. This number agrees very well with that for $\Delta A_{540}/\Delta A_{600}$, which shows that the bleaching at

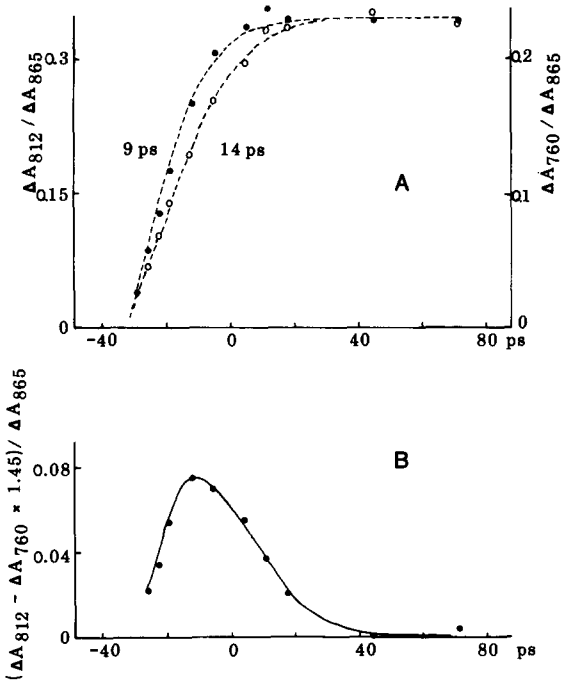

Fig. 7. (A) Kinetics of the ratios $\Delta A_{812}/\Delta A_{865}$ (●) and $\Delta A_{760}/\Delta A_{865}$ (○). Conditions as for Fig. 3. Dashed curves show the calculated kinetics for time constants of 9 and 14 ps, respectively. (B) Kinetics of the 'extra' bleaching at 812 nm, obtained as described in the text, and ascribed to reduction of B_1.

760 nm reflects the reduction of H_1 only. However, the rise time for $\Delta A_{812}/\Delta A_{865}$ was clearly shorter (about 9 ps), which indicates that some earlier process in the charge separation is observed in the kinetics at 812 nm. At 42 ps the ratio $\Delta A_{812}/\Delta A_{760}$ is 1.45. A plot of $(\Delta A_{812} - \Delta A_{760} \times 1.45)/\Delta A_{865}$ (Fig. 7B) shows the transient additional bleaching at 812 nm which does not directly correspond to the reduction of H_1. It reaches a maximum amplitude of 0.08 at -14 ps and subsequently decreases again. The nature of the reaction giving rise to this phenomenon will be discussed in the next section.

Discussion

The results presented in this paper show that the reduction of H_1 is significantly slower in *C. aurantiacus*, at least in the presence of Q^-, than in *Rps. sphaeroides*. A relatively slow reduction of H_1 was also observed by Kirmaier et al. [13]. The kinetics of H_1^- formation in *C. aurantiacus* were the same upon excitation at 870 nm (P* formation) and at 815 nm (B_1^* formation). Because excitation transfer from P* to B is not possible, this indicates that excitation of B_1 is followed by a very rapid energy transfer to P, and that only P*, but not B_1^* participates in the charge separation.

As discussed above, H_1^- formation causes a bleaching at 812 nm in addition to that at 760 nm, presumably by loss of exciton interaction [11,19]), but one has to assume that at early times the bleaching at 812 nm includes also a fraction due to another process, since the kinetics at 812 nm are faster than those at 760 nm (Fig. 7). Since the reaction centers were excited at 870 nm, formation of the singlet excited state of B_1 can be excluded in this case. It is therefore more likely that the initial bleaching at 812 nm reflects a partial reduction of B_1. According to Fig. 7B, the maximum amount of bleaching near 812 nm that can be attributed to reduction of B_1 is 8% of that at 865 nm, which value was obtained at -14 ps. Taking into account that the amplitude of the absorption band at 812 nm is 1.35-times as large as that at 865 nm [18] and if we assume that the band at 812 nm is completely bleached upon reduction of B_1, this means that the fraction of excited reaction centers in the state $P^+B_1^-$ reaches a maximum value of 6% under our experimental conditions. A reasonable fit of the experimental data shown in Fig. 7A and B was obtained by a simulation with time constants of 10 ± 2 ps for electron transfer from P* to B_1 and of 3 ± 1 ps for electron transfer from B_1^- to H_1, respectively.

The similarity of the kinetics of the reduction of H_1 and of the decay of P* as measured from its stimulated emission (Fig. 5) should not be taken as evidence that stimulated emission does also occur from the state $P^+B_1^-$, since the amount of $P^+B_1^-$ at any time during the experiment is quite small. This means that the rate of decay of P* will deviate only slightly from that of the formation of $P^+H_1^-$. There is no evidence for a delay in stimulated emission with respect to $P^+H_1^-$ formation as was observed in reaction centers of *Rps. sphaeroides* [6], which delay can be explained by the slower energy relaxation of $P^+H_1^-$ as compared to the electron transfer reaction in this species.

The time constant for the electron transfer from B_1^- to H_1 (3 ± 1 ps) is in reasonable agreement with that estimated for *Rps. sphaeroides* R-26 reaction centers (about 1.5 ps [6]). However, the rate of electron transfer from P* to B_1 in reaction centers of *C. aurantiacus* is much slower than in *Rps. sphaeroides*. Apart from a possible effect due to the presence of Q^- [7], this may be caused by a relatively large difference in the energy levels of $P^+B_1^-$ and P*. The midpoint potential of the P^+/P couple in *C. aurantiacus* is about 0.07 V lower than that in *Rps. sphaeroides* [11,21]. The energy level of P* is the same in both species. If the redox potentials of the B_1/B_1^- couples are also approximately the same, then in *C. aurantiacus* the energy of the state $P^+B_1^-$ will be considerably lower than that of P*, in contrast to *Rps. sphaeroides* [6], where the close proximity of the energy levels of P* and $P^+B_1^-$ presumably leads to the quantum mechanic mixing of these states. Eqn. 7 of Ref. 22 then predicts that the rate of electron transfer from P* to B_1 will be considerably slower in *C. aurantiacus*, even for the same exchange interaction energy between P* and B_1^-, and consequently the maximum amount of $P^+B_1^-$ obtained during a flash much lower, in agreement with our experiments.

To explain the population of 0.35 of the state

$P^+B_1^-$ in *Rps. sphaeroides* reaction centers at an early time (about 1 ps), mixing of the states P* and $P^+B_1^-$, which differ in energy by about 0.01 eV, was proposed [6]. However, the results obtained with *Rps. sphaeroides* may also be described in a formal way using time constants of about 1.5 ps for both reactions: $P^*B_1H_1 \rightarrow P^+B_1^-H_1$ and $P^+B_1^-H_1 \rightarrow P^+B_1H_1^-$, corresponding to a maximal population of 0.35 of $P^+B_1^-$ and a time constant of about 4 ps for H_1^- formation. For *C. aurantiacus*, the difference spectrum in Fig. 7B indicates that the initial population of the state $P^+B_1^-$ due to mixing of P* and $P^+B_1^-$ is very small.

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