Biochimica et Biophysica Acta 810 (1985) 49–61
Elsevier

BBA 41864

Picosecond-photodichroism studies of the transient states in *Rhodopseudomonas sphaeroides* reaction centers at 5 K. Effects of electron transfer on the six bacteriochlorin pigments

Christine Kirmaier [a], Dewey Holten [a] and William W. Parson [b]

[a] *Department of Chemistry, Washington University, St. Louis, MO 63130, and* [b] *Department of Biochemistry, University of Washington, Seattle, WA 98195 (U.S.A.)*

(Received May 2nd, 1985)
(Revised manuscript received July 31st, 1985)

Key words: Photodichroism; Reaction center; Electron transfer; Bacteriochlorophyll; Bacteriopheophytin; (*Rps. sphaeroides*)

We have examined the dichroism of the visible and near-infrared absorption changes due to the early transient states in *Rhodopseudomonas sphaeroides* reaction centers imbedded in polyvinyl alcohol films at 5 K. The transient-state, ground-state and derivative spectra acquired under these conditions are highly resolved. Spectral features have been assigned to the bacteriochlorophyll (BChl) dimer (P) that serves as the primary electron donor, to each of the two additional BChls, and to the two bacteriopheophytin (BPh) molecules. The dichroism of the absorption changes, taken together with earlier results including our observation of a detection-wavelength dependence of the kinetics, argues that only one of the BPhs is a clearly resolved electron carrier prior to ubiquinone. The second BPh and the two BChls not constituting P display electrochromic effects and/or nuclear relaxations, possibly involving the protein, in response to the charge-separation process.

Introduction

Rhodopseudomonas sphaeroides reaction centers contain four molecules of bacteriochlorophyll (BChl), two of bacteriopheophytin (BPh), one tightly-bound ubiquinone (Q), a nonheme iron atom, and three polypeptides [1]. Photoexcitation prepares the excited singlet state (P^*) of the primary electron donor (P), a complex involving two of the BChls [2–4]. In 4–7 ps an electron from P^* arrives on an intermediary electron carrier (I) [5–10]. It has been suggested that I is a complex involving a BPh and one of the remaining two BChls, with most of the unpaired electron density probably located on the BPh [11–17]. The electron is transferred from I^- to a quinone with a time constant of approx. 200 ps at room temperature [5,6,8,17]. Unanswered questions remain as to the existence of an initial electron acceptor prior to BPh, the nature of the involvement (if any) of a BChl in I, and the roles of the second BPh, the fourth BChl, and the protein in the charge separation process.

The purpose of the present investigation was to address some of these questions. We have measured the dichroism of the absorption changes due to the formation of transient states P^+I^- and

Abbreviations: BChl, bacteriochlorophyll; BPh, bacteriopheophytin; P, a complex of bacteriochlorophylls; P^*, the excited singlet state of P; Q, a reaction-center quinone; I, a complex thought to consist of a BChl and a BPh.

P^+Q^- in polyvinyl alcohol films at low temperature. The difference spectra observed under these conditions are highly resolved, potentially allowing a clearer assignment of the absorption changes due to the pigments involved in or affected by the charge separation process. We have also examined the temperature dependence of the ground-state and transient-state spectra for *Rps. sphaeroides* reaction centers in the films. Finally, in order to learn more about possible medium effects, we compared the near-infrared absorption changes for reaction centers at 76 K in polyvinyl alcohol films and in glycerol/buffer mixtures.

Materials and Methods

The picosecond apparatus, methods for photodichroism studies, and facilities for low-temperature measurements have been described previously [17,18], except for two modifications. First, a Glan-Taylor prism was used to define the polarization of the 30-ps white-light probe pulse. Second, the laser was operated at 5 Hz instead of the 10 Hz repetition rate employed for the photodichroism studies on flowed reaction centers at room temperature [18]. The lower repetition rate was used to ensure that the reaction centers recovered between excitation flashes. At the temperatures used for the present measurements (5 and 82 K), the P^+Q^- recombination time is approx. 30 ms [19–21], and electron transfer from Q_A to Q_B is blocked [22]. Recovery of the reaction centers between excitation flashes was confirmed by the absence of absorption changes when the probe pulse was advanced to arrive at the sample approx. 100 ps before the excitation pulse. Most of the measurements reported here employed weak approx. 20-ps flashes at 867 nm that contained 1 μJ or less and were approx. 20% saturating. A few measurements employed somewhat stronger (up to approx. 100 μJ) 600-nm excitation flashes; photon densities were kept well below the level required to give the spectral distortions and the ≤ 30 ps kinetic component near 800 nm observed previously (and reproduced in our laboratory) when excessively strong excitation is employed [8,23]. Making use of a vidicon detector, picosecond transient difference spectra were acquired in 150-nm intervals, with typical standard deviations in ΔA of ± 0.005. Approx. 300–600-nm excitation flashes were used for the spectra in Figs. 2, 6 and 7, whereas 600–1000 867-nm flashes were used for the measurements from which the photodichroism spectra of Figs. 3–5 were calculated. Ground-state absorption and derivative spectra were measured on a Perkin-Elmer Model 330 spectrophotometer. *Rps. sphaeroides* reaction centers [24] and polyvinyl alcohol films [25] were prepared as described previously.

In the photodichroism spectra, the absorption changes parallel ($\Delta A_{\parallel}$) and perpendicular ($\Delta A_{\perp}$) to the 870-nm transition that was pumped were calculated from the absorption changes measured using parallel (ΔA_{vv}) versus perpendicular (ΔA_{hv}) relative pump/probe polarizations, according to the formulas derived by Verméglio et al. [26]: $\Delta A_{\parallel} = 2\Delta A_{vv} - \Delta A_{hv}$; $\Delta A_{\perp} = 3\Delta A_{hv} - \Delta A_{vv}$. The $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra are easier to interpret than the polarization ratio $p = (\Delta A_{vv} - \Delta A_{hv})/(\Delta A_{vv} + \Delta A_{hv}) = (2\Delta A_{\parallel} - \Delta A_{\perp})/(4\Delta A_{\parallel} + 3\Delta A_{\perp})$, which shows a sharp discontinuity near zero-crossing points in the transient-state difference spectra (i.e. where band-shift-like features occur). The angle θ between the net transition dipole giving rise to the absorbance change at a particular wavelength and the 870-nm transition can be calculated from the formula $\tan^2\theta = \Delta A_{\perp}/\Delta A_{\parallel}$.

Results

Temperature dependence of the ground-state and transient-state spectra

Fig. 1A shows ground-state absorption spectra of *Rps. sphaeroides* R26 reaction centers in a polyvinyl alcohol film at 295 K (dashed) and 5 K (solid). The long-wavelength band of P at 855 nm in the 295-K film (compared to 865 nm in 295-K buffer) shifts to 880 nm as the temperature is lowered to 5 K. This red-shift is accompanied by an increase in peak absorbance and a decrease in bandwidth. These observations are similar to those made previously for *Rps. sphaeroides* reaction centers in gelatin films [27]. The inset to Fig. 1 shows the temperature variation of the peak position (in energy units) of the long-wavelength band in polyvinyl alcohol films (circles), and of the half-width-at-half-maximum for the red side of the band (crosses). The half-width-at-half-maximum

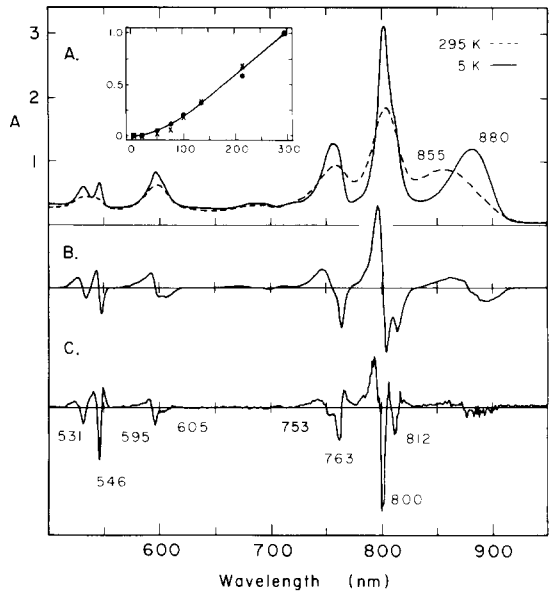

Fig. 1. (A) Ground-state absorption spectrum of *Rps. sphaeroides* reaction centers in a polyvinyl alcohol film at 295 K (— — —) and 5 K (————). First-derivative (B) and second derivative (C) also at 5 K. The inset shows the variation of the peak position (circles) and half-width-at-half-maximum for the red side of the long-wavelength band (crosses) plotted on a scale where each changes from 0 to 1 between 5 and 295 K. The peak position changes from 11696 cm^{-1} (855 nm) to 11364 cm^{-1} (880 nm), while the half-width-at-half-maximum changes from 485 cm^{-1} to 252 cm^{-1} between 295 and 5 K.

could be in error by 20% because of uncertainty in the contribution from underlying components to the peak amplitude, particularly at higher temperatures. Both the position and estimated half-width-at-half-maximum are normalized to a total change in each parameter from 0 to 1 between 5 and 295 K. (The similar temperature of both the peak position and bandwidth of P's band is unusual. A temperature dependence of vibronic coupling in the dimer, possibly involving a phorphyrin low-frequency deformation mode, could be the origin of this effect.) The other absorption bands in the spectrum also narrow and increase in peak amplitude as the temperatuare is reduced, but they show smaller shifts in peak positions. The Q_X bands of the two BPhs are resolved in the 530–550 nm region at low temperature (see also Fig. 6C), as reported previously in other media [26,28]. Shoulders are observed on the long-wavelength side of the BChl bands near 600 and 800 nm.

These spectral features are seen more clearly in the first (Fig. 1B) and second (Fig. 1C) derivatives of the 5-K spectrum. In the second-derivative spectrum, the Q_X and Q_Y bands of the 'short-wavelength' BPh are resolved at 531 and 753 nm; those for the 'long-wavelength' BPh are at 546 and 763 nm. Peaks at 595 and 605 nm are observed in the Q_X region of the BChls. In the Q_Y region of the BChls, peaks are resolved at 800 and 812 nm, as described previously from derivative spectra in other media [29,30].

Fig. 2 shows the temperature dependence of the near-infrared difference spectra acquired at 30 ps (solid) and 1.6 ns (dashed) with respect to the center of a 30-ps 600-nm excitation flash. The 30-ps spectrum is due mainly to P^+I^- and the 1.6-ns spectrum to P^+Q^-. The position, peak amplitude and bandwidth of bleaching in the long-

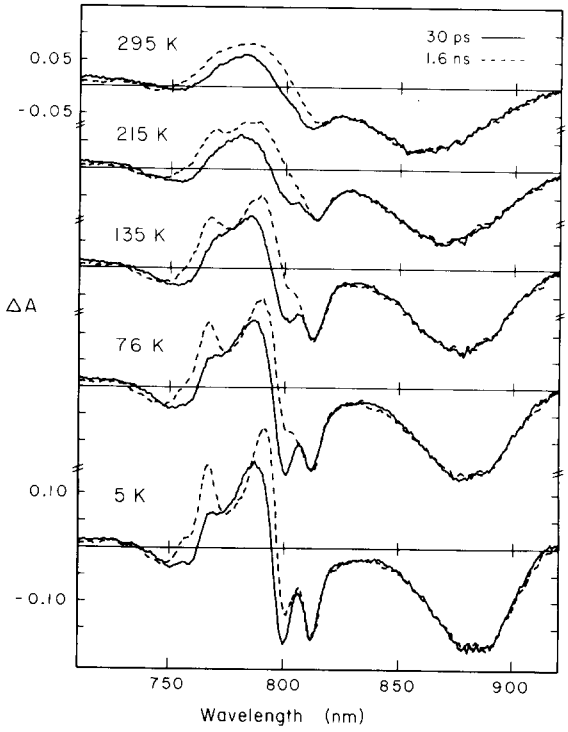

Fig. 2. Temperature dependence of the near-infrared transient difference spectra for *Rps. sphaeroides* reaction centers in polyvinyl alcohol film. Shown at each temperature are spectra for states P^+I^- at 30 ps (————) and P^+Q^- at 1.6 ns (— — —) with respect to the center of a 30-ps excitation flash at 600 nm. The excitation flash was polarized at 45° with respect to the white-light probe flash.

wavelength band of P have the same temperature dependence as the ground-state absorption band, as expected. The suggestion of a possible splitting of the long-wavelength band is probably experimental noise caused by Raman lines in the probe light in this region; it is not seen in the ground-state spectrum (Fig. 1). As one decreases the temperature, the absorption changes normally attributed to the BPhs (between 740 and 775 nm) are more clearly resolved from those normally ascribed to the BChls (between 780 and 830 nm). The increased resolution in the P^+Q^- difference spectrum at low temperature has been observed previously in other media (cf. Ref. 26). The two clear troughs seen at 800 and 812 nm in both the P^+I^- and P^+Q^- spectra at low temperature have not been described previously. This splitting is observed in the polyvinyl alcohol films [17], but the two bands coalesce to a single feature near 805 nm in high glycerol concentrations (see below). The major variations of the spectral features in both the ground-state (Fig. 1 inset) and transient-state spectra (Fig. 2) occur between 295 K and approx. 50 K.

Dichroism of the absorption changes in polyvinyl alcohol films at low temperatures

We have measured the dichroism of the visible and near-infrared absorption changes (500–850 nm) due to the formation of transient states P^+I^- and P^+Q^-. Measurements at 5 and 82 K were carried out in *Rps. sphaeroides* R26 reaction centers imbedded in polyvinyl alcohol films. The results are essentially the same at the two temperatures.

Fig. 3 shows the dichroism of the near-infrared absorption changes at 5 K for the formation of state P^+I^- at 20 ps (A) and state P^+Q^- at 1.6 ns (B) with respect to the center of a weak approx. 20-ps excitation flash at 867 nm. The $\Delta A_{\parallel}$ (solid) and $\Delta A_{\perp}$ (dashed) spectra represent the absorption changes parallel or perpendicular to the 870-nm transition (see Materials and Methods). The picosecond photodichroism spectra in the films at 5 and 82 K are much better resolved than those reported previously at 295 K in flowed buffer [18]. The $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra for state P^+Q^- are also better resolved than those reported previously from measurements on a slower time scale with

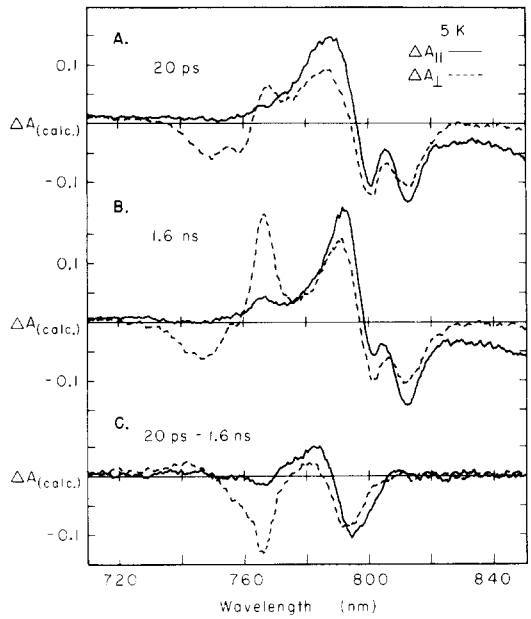

Fig. 3. Near-infrared photodichroism spectra of *Rps. sphaeroides* reaction centers in a film at 5 K for state P^+I^- (A) and P^+Q^- (B) induced by a weak 867-nm excitation flash lasting approx. 20 ps. The spectra show the absorption changes parallel ($\Delta A_{\parallel}$, ————) and perpendicular ($\Delta A_{\perp}$, — — —) to the 870-nm transition of P. These spectra and those in Figs. 4 and 5 were calculated from spectra acquired using parallel and perpendicular relative pump/probe polarizations according to the formulae given in the text [26]. Panel C shows the difference between the 20 ps and 1.6 ns spectra for the two polarizations.

reaction centers in 66% glycerol at 160 K [26], although overall the spectra in the two studies are similar. Besides the general sharpening of the features in the low-temperature films, the most notable difference from the earlier work is the resolution in the present study of the two troughs at 800 and 812 nm in the photodichroism spectra for both transient states (Fig. 3A and B). Fig. 3C shows the difference between the spectra at 20 ps (P^+I^-) and 1.6 ns (P^+Q^-) for both polarizations. Fig. 4 replots the data of Fig. 3A and B to show more clearly how the absorption changes for the two polarizations evolve between 20 ps (solid) and 1.6 ns (dashed). Comparison of the spectra in these various ways is useful for interpreting how the pigments are involved in, or affected by, the electron-transfer steps.

The bleaching observed between 830 and 850 nm is polarized parallel to the 870-nm transition in

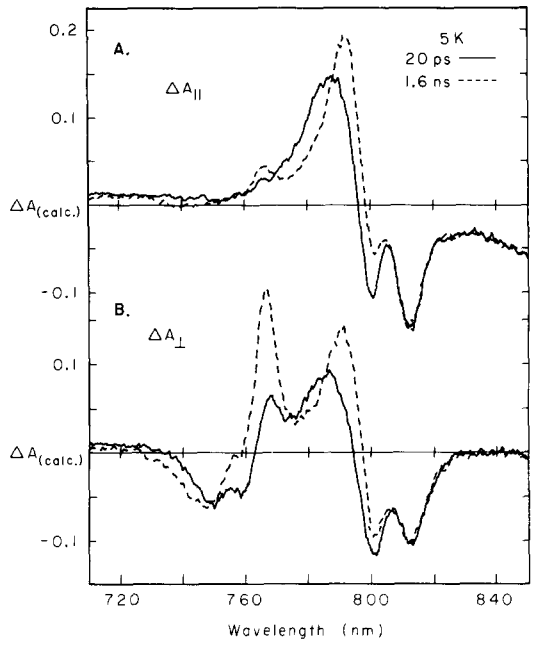

Fig. 4. Near-infrared photodichroism spectra from Fig. 3A and B replotted to show how the $\Delta A_{\parallel}$ (A) and $\Delta A_{\perp}$ (B) spectra evolve between 20 ps (————) and 1.6 ns (- - - - - -).

the difference spectra at both delay times ($p = 0.50 \pm 0.05$). This result is in agreement with previous work [26] and is expected for pumping and probing the same transition or more than one parallel transition. For both transient states, the negative feature between 810 and 820 nm has $p = 0.20$, corresponding to a net transition dipole having an angle of approx. 40° with respect to the long-wavelength transition of P (compare $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra in Fig. 3A and B). An angle of about 45° is obtained for the 812-nm component, if one subtracts an extrapolated contribution to the absorption changes from the parallel-polarized component observed between 820 and 850 nm, but the validity of such a correction is questionable. The bleaching of the 812-nm component is essentially unchanged between the P^+I^- and P^+Q^- spectra (Fig. 4); it does not contribute to the difference-difference spectra of Fig. 3C. Therefore, this absorption change probably results mainly from the oxidation of P. It cannot be interpreted simply as bleaching of the higher-energy Q_Y exciton band of P, because that band should be polarized nearly perpendicular to the 870-nm band [26,29]. We therefore suggest that the 812-nm band is due mainly to one of the two BChls that are not part of P.

The absorption increase near 785 nm resembles the 812-nm trough in having $p \approx 0.20$, and in remaining largely constant as P^+I^- decays to P^+Q^- (Figs. 2–4). At intermediate wavelengths between 790 and 810 nm, the absorption changes are quite complex. In both the P^+I^- and the P^+Q^- spectra (Fig. 3A and B), the 800-nm trough contains larger perpendicular than parallel polarization, while the 790–795 nm absorption increase contains larger parallel polarization. This observation suggests that at least two components having different polarizations contribute to these absorption changes. Similar conclusions have been drawn previously regarding the 800-nm region of *Rps. sphaeroides* [18,26,29] and the 830-nm region of *Rps. viridis* [18,31–33].

Between 730 and 775 nm, where the BPhs absorb, the absorption changes are observed largely in the $\Delta A_{\perp}$ spectra. The P^+Q^- spectrum (Figs. 3B and 4) contains an absorption decrease centered near 750 nm and a sharp, strong absorption increase at 765 nm. The P^+I^- spectrum (Figs. 3A and 4) also contains the 750-nm trough, as well as a second trough near 760 nm; a relatively small absorption increase is seen near 765 nm. The dichroism of the absorption changes suggests that the Q_Y transitions of both BPh molecules are polarized essentially perpendicular to the long-wavelength transition of P, in agreement with previous work [18,26,34].

The difference between the 20-ps and 1.6-ns spectra (Fig. 3C) shows that electron transfer from I^- to Q results in net absorption increases centered near 765 and 795 nm, with a smaller net absorption decrease in between. The 765-nm feature contains essentially only perpendicular polarization. The sharp negative trough centered at 765 nm reflects bleaching in state P^+I^- of the Q_Y band of the long-wavelength BPh (see, e.g., Ref. 17). The broad negative shoulder to the blue may be due to the shorter-wavelength BPh, which appears to have its Q_Y band near 753 nm (Fig. 1C). On the other hand, the 795-nm trough contains nearly equal contributions from the two polarizations, although it appears that at least two components having

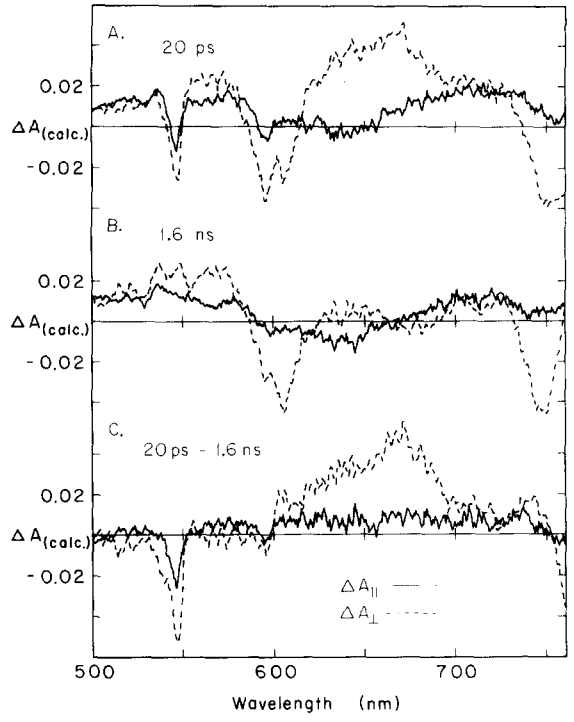

Fig. 5. Photodichroism spectra in the visible region at 5 K for *Rps. sphaeroides* reaction centers in a polyvinyl alcohol film at 20 ps (A) and 1.6 ns (B). The difference between the 20-ps and 1.6-ns spectra are shown in C. All three panels show the absorption changes parallel (————) and perpendicular (— — —) to the 870-nm transition of P.

different absorption maxima and different polarizations may contribute.

Fig. 5 shows the dichroism of the absorption changes in the visible region for states P^+I^- at 20 ps (A) and for P^+Q^- at 1.6 ns (B), polarized parallel (solid) or perpendicular (dashed) to the 870-nm transition. The spectra of Figs. 3 and 5 overlap between 710 and 760 nm. Fig. 5C shows the difference between the absorption changes at the two delay times for both polarizations. Some of the subtle features in the spectra in this region can be seen more clearly in the unpolarized spectra obtained previously using 600-nm flashes, which give absorption changes of larger amplitude (see below, and Ref. 17).

The P^+I^- spectrum at 5 K (Fig. 5A) and the subtracted spectra of Fig. 5C show that the broad absorption band centered near 665 nm is polarized mainly perpendicular to the 870-nm transition. A similar result was obtained from linear dichroism studies on *Rps. viridis* oriented whole cells at 82 K [32] and reaction centers at 100 K [35]. In these earlier studies the reaction centers were converted to state PI^-Q^- by illumination at low potential. It was found that the broad absorption band of I^- centered near 690 nm in *Rps. viridis* is polarized essentially perpendicular to the 960-nm transition of P. The 665- or 690-nm band is usually ascribed to BPh^- [5,6,8,11,12,17,35], but both BPh^- and $BChl^-$ are expected to absorb in this region [12,36]. Ab initio calculations show that this absorption band for either anion radical should be polarized largely in the same direction in the macrocycle as the Q_Y transition in the ground-state spectrum of the neutral molecule [37,38]. On these grounds, the 665-nm band is consistent with BPh^-. The band would not appear to be due to the anion radical of the BChl giving rise to the trough at 812 nm, which appears to be polarized at an angle of approx. 40° with respect to P's long-wavelength band (Figs. 3 and 4). Because the photodichroism spectra are more complex between 790 and 810 nm, it is less clear whether the 665-nm band could contain a contribution from the anion radical of the other BChl. In the Discussion we suggest that Q_Y transition of this BChl probably makes an angle of less than 35° with the 870-nm band of P and, thus, does not contribute significantly to the 665-nm band via its anion radical. Our earlier kinetic results also argue against a significant contribution of $BChl^-$ to I^- and thus to the 665-nm band [17].

The oxidation of P results in a weak absorption increase between 690 and 730 nm containing both polarizations (Fig. 5B); this absorption appears to consist of several features that are better resolved when larger-amplitude signals are obtained with 600-nm flashes [17]. The P^+I^- and P^+Q^- spectra show absorption decreases at 595 and 605 nm that have mainly perpendicular polarization ($\theta > 70°$) and a weaker, broad absorption decrease between 620 and 650 nm that has mainly parallel polarization ($\theta < 30°$) with respect to the 870-nm transition. The troughs at 595 and 605 nm probably reflect bleachings and/or shifts of the absorption bands observed at these wavelengths in the second derivative of the 5-K ground-state absorption spectrum (Fig. 1C). All three troughs (595, 605

and 640 nm) probably result mainly from the oxidation of P, since they appear to be present in the spectra for both P^+I^- (Fig. 5A) and P^+Q^- (Fig. 5B). However, the apparent magnitudes of these features are influenced by the broad perpendicularly-polarized 665-nm band in the P^+I^- spectrum. The reduction of I or Q may cause an additional effect on the 595-nm band, since a small feature is observed at this wavelength in the spectra of Fig. 5C.

The finding of at least three components in the Q_X region of the BChls and the observed dichroism of these absorption changes are in agreement with previous work. Photodichroism measurements at 295 K of state P^+Q^- on a slower time scale in *Rps. sphaeroides* reaction centers [39] and chromatophores [40] revealed an absorption decrease at 600 nm that has mainly perpendicular polarization with respect to the long-wavelength transition of P and a weaker bleaching near 630 nm that has mainly parallel polarization. Photoselection studies on *Rps. sphaeroides* reaction centers at low temperature also have shown that the absorption changes around 605 nm are polarized largely perpendicular to the 870-nm transition [26]. It has been argued on the basis of magnetophotoselection measurements that the BChl Q_X region of *Rps. sphaeroides* contains contributions from at least three overlapping transitions [41]. One component appeared to exhibit a broad absorption between 620 and 660 nm that had polarization opposite to at least one of the components that showed maxima between 590 and 620 nm.

The P^+I^- spectrum exhibits a sharp bleaching in the 546-nm band of the long-wavelength BPh, which appears to be polarized at approx. 55° with respect to the 870-nm transition of P (Fig. 5A). This angle is in agreement with the value of 60° deduced from previous linear dichroism measurements [26] and magnetophotoselection studies [42] on *Rps. sphaeroides*. The same angle has been measured between the Q_X transition of the 545-nm BPh and the long-wavelength band of P in *Rps. viridis* reaction centers [35]. Fig. 5 shows that the 546-nm band recovers as the electron is transferred from I^- to Q, revealing a small feature near 530–540 nm in the P^+Q^- spectrum (Fig. 5B). The absorption changes are seen more clearly in Fig. 6A, which was obtained using 600-nm excitation flashes. We suggested previously from such spectra that the small 530–540-nm feature represents a red shift on the Q_X band of the shorter-wavelength BPh (see Fig. 1), resulting from the oxidation of P and/or the reduction of Q [17]. A red-shift of the 530-nm absorption could account for the apparent shoulder on the blue side of the sharp 546-nm trough in the subtracted spectra of Figs. 5C and 6B. (This shoulder is not likely to be a vibronic component of the 546-nm band, since the energy gap of approx. 500 cm^{-1} is much smaller than the vibronic spacing normally observed for porphyrins, chlorins and bacteriochlorins [43]. The small trough observed near 510 nm in Fig. 6B probably reflects bleaching of the first vibronic component of the 546-nm band, $Q_X(1,0)$, in state P^+I^-.) The asymmetry of the 530–555-nm negative feature (Fig.

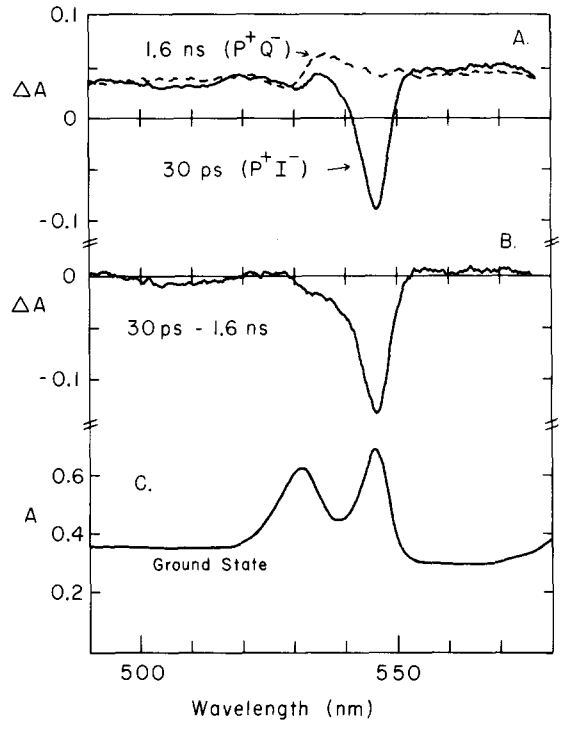

Fig. 6. (A) Transient-state difference spectra for *Rps. sphaeroides* reaction centers at 5 K in a film in the BPh Q_X region for P^+I^- at 30 ps (————) and P^+Q^- at 1.6 ns (— — —). These spectra were acquired using 30-ps, 600-nm excitation flashes. (B) Difference between the 30-ps and 1.6-ns spectra. (C) Ground-state spectrum of the same film in this region at 5 K.

6B) is similar to that observed here (Fig. 3C) and previously [17] in the 750–770-nm Q_Y region of the BPhs. Comparison of Fig. 6A and B with the 5-K ground-state spectrum in the Q_X region of the BPhs (Fig. 6C) shows that only the long-wavelength BPh is reduced significantly in I^-, as suggested from previous measurements [11,13,14, 17,44].

Comparison of the low-temperature transient-state spectra in polyvinyl alcohol films and glycerol / buffer glasses

Fig. 7 shows the near-infrared difference spectra for states P^+I^- (A) and P^-Q^- (B) for *Rps. sphaeroides* reaction centers at 76 K in a polyvinyl alcohol film (solid), and 65% glycerol (dashed). These spectra were acquired at 30 ps and 1.6 ns with respect to the center of a 30-ps 600-nm excitation flash. The bleaching maximum in P's long-wavelength absorption band occurs at 890 nm in both 65% glycerol (dashed) and 56% glycerol

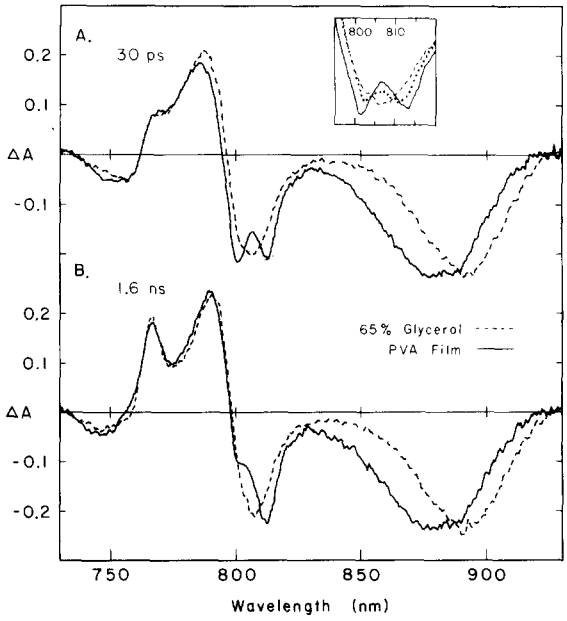

Fig. 7. Difference spectra at 76 K for states P^+I^- at 30 ps (A) and P^+Q^- at 1.6 ns (B) for *Rps. sphaeroides* reaction centers in a polyvinyl alcohol film (————) and in 65% glycerol/buffer (— — —). The inset shows the 795–820-nm region on an expanded scale for the film (————), 65% glycerol (— — —) and 56% glycerol (·····). These spectra were acquired using 30-ps, 600-nm excitation flashes.

(not shown), as compared to 880 nm in the film (solid). The splitting observed at 800 nm and 812 nm in the film in the P^+I^- spectrum is replaced by a single trough centered near 805 nm in 65% glycerol. These differences can be seen more clearly in the inset, which also shows that in 56% glycerol (dotted) the behavior is intermediate between that observed in the film and 65% glycerol. Similar effects are observed in the P^+Q^- spectra (Fig. 7B); the 56% glycerol spectrum is not shown. To shorter wavelengths, the absorption changes observed between 710 and 790 nm in either transient state are virtually identical for reaction centers in films or in the two glycerol concentrations.

Discussion

The results presented in this paper, taken together with the temperature and detection-wavelength dependence of the kinetics that we observed previously [17], suggest that it may be possible to distinguish the absorption changes and kinetics associated directly with electron transfer from those that may result from electrochromic effects, alterations in excitonic interactions, or movements of the pigments and/or protein. The enhanced resolution and spectral splittings observed in the low-temperature polyvinyl alcohol films make this a useful medium for such studies.

As pointed out by Rafferty and Clayton [39], the long-wavelength band of P in *Rps. sphaeroides* at room temperature varies between 850 and 870 nm depending on the environment of the reaction center. The absorption changes between 775 and 805 nm normally attributed to the BChls also appear to be sensitive to the medium, more so than those due to BPhs (Fig. 7). The peak wavelength of 855 nm observed in the polyvinyl alcohol films at 295 K (Fig. 1) is close to that observed at room temperature for reaction centers in gelatin films [27,40] and for chromatophores dried on a glass plate [29,39]. The width at half-height of the long-wavelength absorption band is comparable in the two types of films at both 295 K and 76–82 K, although the band is sharper and red-shifted in both media at the lower temperature. In the present study on polyvinyl alcohol films, as in the earlier studies on gelatin films [27,40], the full bandwidth is bleached upon photoexcitation. It

has been determined from linear dichroism measurements on *Rps. sphaeroides* chromatophores [29,39] and on reaction centers in gelatin films [27,40] or in glycerol/buffer mixtures [26] that the long-wavelength band of P is either a single transition or the sum of more than one parallel transition. In the present study, there is no indication of a shoulder on the long-wavelength side of P's band, as has been observed in *Rps. viridis* reaction centers at low temperature [9,32]. We measured the dichroism of the absorption changes by exciting with 867-nm flashes. This excitation wavelength is on the blue side of the long-wavelength absorption band at both 5 and 82 K, the temperatures at which photodichroism measurements were performed. The dichroism of the absorption changes in the Q_X band of the long-wavelength BPh (546 nm), the Q_X region of the BChls (590–650 nm), the Q_Y region of the BPhs (750–770 nm), and in the 820–850 nm region of P all are in excellent agreement with the results of earlier studies carried out under a variety of conditions. Also, the sums of the near-infrared $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ photodichroism spectra for states P^+I^- and P^+Q^- determined from our measurements (Figs. 3 and 4) are in excellent agreement, after scaling, with the (unpolarized) difference spectra obtained using 600-nm flashes (Fig. 2). All of these observations suggest that the 867-nm flashes have not selectively excited a component of the long-wavelength band which is somehow oriented differently in the film than in other media, or that results in different photochemical behavior.

One of the two BPh molecules is an early, if not the initial, electron acceptor. Reduction of this 'long-wavelength' BPh causes bleaching in its 546-nm Q_X and 765-nm Q_Y bands. The dichroism of the absorption changes indicates that this BPh macrocycle is oriented so that its Q_Y transition is nearly perpendicular to the 870-nm transition of P, while the Q_X transition makes an angle near 60° with the 870-nm transition (Figs. 3–5 and Refs. 26,42,44). The anion radical of this BPh gives rise largley, if not completely, to the 665-nm absorption increase in the P^+I^- spectrum (Fig. 5A and C); the band has the proper polarization for this assignment at both 5 and 82 K, as discussed in Results. Development of the 665-nm absorption increase [8], the 546-nm bleaching [8,10,18] and the 765-nm bleaching [9,23,45] all appear to occur with a time constant of about 4–7 ps at room temperature. Decay of the 665-nm absorption increase and the 546-nm bleaching both occur with a time constant of approx. 100 ps at temperatures between 5 K and approx. 100 K in polyvinyl alcohol films [17]. At 5 K and 76 K the same time constant has been measured in the sharp trough at 765 nm (Fig. 3C) [17]. These results are fully consistent with the assignment of I as the long-wavelength BPh, and I^- as BPh^-.

The Q_X and Q_Y absorption bands of the second, or 'short-wavelength', BPh are resolved near 530 and 755 nm in the 5-K ground-state spectrum (Fig. 1). Electron transfer from the long-wavelength BPh^- (I^-) to Q_A appears to cause a net red-shift of the 530- and 755-nm bands of the short-wavelength BPh, giving rise to the negative shoulders at 530–540 nm and 750–760 nm in the spectra of Figs. 3C, 5C and 6B. The relaxation time of approx. 135 ps measured previously near 755 nm in the films at 5 or 76 K is longer than the decay time of approx. 100 ps measured in the 546-, 765- and 665-nm bands. At room temperature in flowed buffer, where the absorption bands of the two BPhs overlap, the decay kinetics measured previously in the 546-nm and 765-nm regions ($\approx$ 250 ps) are slower than those measured in the 665-nm band ($\approx$ 210 ps) [17]. These observations support the view that the short-wavelength BPh is not an electron carrier, but rather is affected by changes in the local pigment/protein environment caused by the charge separation, resulting in a 'slow' relaxation involving this BPh.

The short-wavelength BPh also has its Q_Y transition essentially perpendicular to the 870-nm transition of P (Figs. 3 and 4) [18,26]. We were unable to determine the orientation of the Q_X band of this molecule because of the small size of the absorption changes near 530 nm. Vermeglio et al. [26] found that the Q_X band of this BPh molecule makes an angle of approx. 50° with the 870-nm transition.

The Q_X region of the BChls contains three resolvable features at 595, 605 and approx. 640 nm (Fig. 5). The oxidation of P causes absorption decreases in all three bands, but only the 595-nm feature appears to be perturbed by the reduction of I (BPh). This observation, together with the

absorption changes in the near infrared (see below), suggests that the 800-nm BChl may have its Q_X transition at 595 nm. Whether the second BChl has its Q_X band near 595 or 605 nm is not clear, but the former position seems more likely. At least part of the 605-nm trough (Fig. 5A and B) probably represents bleaching of a Q_X band of P. Rafferty and Clayton [39,40] proposed that the absorption decreases observed near 600 and 630 nm in photodichroism spectra for P^+Q^- in gelatin films represented bleaching of two Q_X excitonic components of P. Although the respective perpendicular and parallel polarizations of the two bands are correct for this assignment, the observed energy splitting is rather large, about the same as that calculated from the wavelength of the proposed Q_Y excitonic components (810 and 860 nm). For most geometries of the dimer, one would expect that the splitting in the Q_X region would be less than 10% of that in the Q_Y region, since the exciton splitting is proportional to the square of the transition strength of the respective monomer bands. The Q_X band of BChl-*a* in organic solvents is about 1/5 as intense as the Q_Y band. An alternative view is that the dominant Q_X component of P is near 605 nm and that the broad, parallel-polarized absorption between 620 and 660 nm represents a charge-transfer state [46].

The absorption changes observed between 775 and 830 nm also are usually ascribed to BChl. We assign the two absorption bands at 800 and 812 nm (Fig. 1) mainly to the two BChls that do not constitute P. Shuvalov and Asadov [31] and Hoff et al. [47] have made a similar proposal that in *Rps. viridis* the two BChls that are not part of P give rise to the 830- and 850-nm bands observed at low temperature. (The crystal structure of *Rps. viridis* reaction centers shows a BChl and a BPh molecule situated on each 'side' of the reaction center with respect to the BChl macrocycles comprising P [48].) The two BChls may interact differently with P, the protein, or the nearby BPh molecules, resulting in the different peak positions. The excited states of the two BChls probably mix significantly with those of P, and can exchange intensity with them [46]. This transfer of dipole strength between the 870-nm and 800- or 812-nm bands would be lost when P is raised to an excited state or is oxidized. Such effects could result in a change in wavelength, intensity and polarization of the bands in the Q_Y region of the BChls. However, absorption changes of this nature seem likely to be less significant than those due to the Stark (electrochromic) effect of P^+ on the neighboring BChls.

The absorption decrease at 812 nm is reasonably well separated from other absorption changes in the region in the 5-K films. The dichroism of the absorption changes observed between 780 and 820 nm suggests that the Q_Y transition of the 812-nm BChl, apparently oriented at an angle of approx. 40° with respect to the 870-nm transition of P, is blue-shifted to the 780–790-nm region upon oxidation of P. This view is consistent with the results of photodichroism measurements of state P^+Q^- on a slower time scale by Vermeglio et al. [26]. They concluded that the absorption changes in the 780–820-nm region include a blue-shift of a component(s) making a net angle of about 39° ($p = 0.23$) with respect to the 870-nm transition. The 812-nm BChl molecule probably is the one closer to the short-wavelength (530/755 nm) BPh, rather than to the long-wavelength (546/765 nm) BPh that serves as the electron carrier in I^-. This conclusion is based on the observation that the 812-nm trough is unchanged between the P^+I^- (P^+BPh^-) and P^+Q^- spectra, as discussed in Results (Figs. 2–4); there are no absorption changes observed near 812 nm in the difference-difference spectra of Fig. 3C. The 812-nm trough is not likely to be the higher-energy Q_Y excitonic component of P, because it does not have the proper polarization. Perhaps the 812-nm BChl is the one that is modifiable by sodium borohydride [49] and apparently not involved in the primary charge separation process [50].

Assignment of the 800-nm absorption, at least partially, to the second BChl seems reasonable. The 800-nm band evidently blue-shifts and/or loses intensity upon oxidation of P, as the 812-nm band does. The second BChl molecule is probably a near neighbor to the long-wavelength BPh molecule that is reduced in I^- and the absorption bands of this BChl could be perturbed by the reduction of the BPh, as well as by the oxidation of P. Part of the 795-nm absorption decrease in the $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra of Fig. 3C could reflect a loss of intensity in the Q_Y absorption of this

BChl, and/or an enhancement of a shift to shorter wavelengths, due to reduction of the nearby BPh.

However, one cannot account for the absorption changes between 780 and 820 nm entirely on the basis of a blue-shift or loss of intensity in the 800- and 812-nm bands of the BChls, because the polarization varies markedly across this region of the transient-state difference spectra (Figs. 3A, 3B and 4). There also is difficulty in reconciling the transient spectra with the average polarization ratio of $p \approx 0.3$ ($\theta = 30$–$35°$) across the 800-nm region, as determined by Vermeglio et al. [26] and Mar and Gingras [51] from measurements of the excitation spectrum for bleaching in the long-wavelength band of P. This result, together with $\theta \approx 40°$ for the 812-nm component determined above, suggests that the Q_Y transition of the second BChl makes an angle of less than 35° with respect to the 870-nm transition. In contrast, the dichroism in the 800-nm trough in the transient-state spectra (Figs. 3A and B) gives $\theta \approx 51°$ for the 800-nm transition. Thus, if the Q_Y transition of the second BChl is at 800 nm, then either another component having more perpendicular polarization also contributes to the 800-nm trough, or another component having more parallel polarization gives rise to an absorption increase in this region. Either of these additional components would also account for the variation in polarization across the photodichroism spectra (Figs. 3A, 3B and 4).

It has been proposed that the higher-energy excitonic component of P is near 805 nm [26] or 815 nm in *Rps. sphaeroides* [29]. This band would be polarized perpendicular to the 870-nm transition. Perhaps bleaching of such a component occurs near 800 nm in the low-temperature polyvinyl alcohol films. An additional possibility is that part of the absorption increase that is centered near 790 nm is a new band in the P^+ spectrum. This component could be the absorption band of the (neutral) monomer-like BChl in P^+, as proposed previously by Vermeglio and Clayton [29]. It would be expected to have mainly parallel polarization with respect to the 870-nm transition. At least part of the variation in polarization across the 795-nm region in the difference-difference spectrum (Fig. 3C) could be explained by assuming that the P^+ absorption band is located near 785 nm in the 20 ps (P^+I^-) spectrum and near 795 nm in the 1.6 ns (P^+Q^-) spectrum. A shift of the band to longer wavelengths could result from movements in nearby charged groups on the protein. Another possibility is that the pigments themselves move in response to charge separation. For example, if the 800-nm BChl moves in response to the formation of P^+, there could a change in the orientation of its Q_Y axis relative to that of P. Vermeglio and Paillotin [33] proposed from photodichroism measurements at 4.2 K that the two BChls of P may change orientation upon the formation of P^+Q^- in *Rps. viridis* reaction centers. Kleinfeld et al. [52] recently proposed that a conformation change occurs upon the formation of P^+Q^- in *Rps. sphaeroides* reaction centers.

We recently proposed that the detection-wavelength dependence of the kinetics in *Rps. sphaeroides* reaction centers may reflect movements of the pigments and/or protein [17]. The changes in the BChl Q_Y bands between 20 ps and 1.6 ns apparently are not closely linked to the transfer of the electron from I^- (BPh^-) to Q. This follows from our previous observation that the decay kinetics measured near 795 nm ($\approx$ 70 ps at 5 and 76 K; $\approx$ 150 ps near 295 K) are faster than those measured in the 665-nm band of BPh^- ($\approx$ 100 ps below 100 K; $\approx$ 210 ps near 295 K) [17]. The 795-nm kinetics could reflect a nuclear relaxation involving the BChls and/or P^+, following the initial formation of P^+I^- (P^+BPh^-). This interpretation is similar to that used above to describe the kinetics associated with the shorter-wavelength BPh, except that in that case the relaxations may occur after electron transfer from the long-wavelength BPh^- to Q.

The temperature dependences of the kinetics [17] and of certain features in the ground-state and transient-state spectra (Figs. 1 and 2) exhibit the largest changes between 295 K and 50–100 K, below which they become independent or much less dependent on temperature. There may be a common origin for the effects, as discussed previously [17,53,54]. Nuclear (vibrational) motions of the electron carriers and the protein may manifest themselves in the rate of electron transfer, features of the ground-state electronic absorption spectrum, and in the absorption changes that are observed. Such considerations, together with the discussion of the dichroism of the near-infrared ab-

sorption changes, suggest that the dynamics of the pigments and/or the protein could play an important role in the photophysical and photochemical properties of the reaction center. Small movements of the pigments and/or protein, alterations in interactions between pigments, and changes in the local pigment/protein environment resulting from electron transfer could provide mechanisms for preventing unwanted back reactions.

Acknowledgements

This work was supported by grants PCM-8302477 (D.H.) and PCM-8312371 (W.W.P.) from the National Science Foundation. We thank Paul Linnemeyer for preparing the reaction centers.

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