Phototrapping of Doubly Reduced Monomeric Bacteriochlorophyll in the Photoreaction Center of *Ectothiorhodospira* sp.†

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ABSTRACT: The photoreaction center from the purple sulfur bacterium *Ectothiorhodospira* sp. was illuminated in the presence of reduced cytochrome *c* or dithionite under anaerobic conditions. This treatment first caused the monoelectronic reduction of both molecules of bacteriopheophytin (Bph), Φ_A and Φ_B, as witnessed by the appearance of EPR and optical signals typical of singly-reduced bacteriochlorins. Continued illumination under the same reducing conditions caused both of these signals to disappear. Such disappearance was accompanied by a complete bleaching of the Q_x and Q_y absorption bands of Bph but not of the corresponding transitions of bacteriochlorophyll (Bchl). These phenomena are interpreted by a double reduction of Φ_A and Φ_B. As long as the medium remained reducing and anaerobic, these changes were stable. Prolonged illumination under the same reducing conditions finally led to the bleaching of the Q_x (600 nm) and Q_y (800 nm) bands of Bchl but not of the 880-nm band. This generated no EPR or 645-nm absorption signals due to singly-reduced Bchl. The bleaching kinetics of the 800-nm band was biphasic and paralleled a shift of the peak wavelength. This is interpreted by a double reduction of both molecules of monomeric Bchl B_A and B_B in an undetermined order. After bleaching of the 800-nm band has reached saturation, the absorbance ratio of the 800/880-nm absorption bands remains constant, as would be expected if the ultimate spectrum was that of the primary electron donor. These experiments demonstrate the photoreduction of Bchl and allow the absorption spectrum of the primary donor to be measured for the first time. This spectrum is unusual in that the bandwidth of the lower-energy Q_y transition of the Bchl dimer is broader than that of its high-energy counterpart. This broadening can be explained by a higher charge-transfer character of the low-energy excitonic band.

Ectothiorhodospira sp. is a purple sulfur photosynthetic bacterium whose photoreaction center (RC)[1] is comprised of four polypeptide subunits, one of which is a four heme cytochrome *c*. Subunits L and M carry the binding sites of four molecules of bacteriochlorophyll *a* (Bchl), two molecules of bacteriopheophytin *a* (Bph), one molecule of spirilloxanthin, one iron atom, and only menaquinone as electron acceptor (Lefebvre et al., 1984). As was demonstrated in other RCs [reviewed in Deisenhofer and Michel (1989)], the photochemical process involves the transfer of an electron from a primary donor (P), made of a pair of Bchl molecules, through one of two nearly symmetric Bph molecules, Φ_A, to a terminal menaquinone acceptor, Q_A. A still unresolved question is whether this chain of events involves the physical oxidoreduction of the Bchl molecule (B_A) that is situated between P and Φ_A in the *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* RCs (Holzapfel et al., 1990; Chan et al., 1991; Vos et al., 1991, 1992). As in these two RC preparations (Shuvalov et al., 1976; Prince et al., 1977; Okamura et al. 1979), illumination of the *Ectothiorhodospira* sp. RC in the presence of reducing agents allows the trapping of intermediaries of the primary photochemical reaction. Indeed, in *Ectothiorhodospira* sp. RC, such experiments have led to the trapping of Q_A^-, of Q_AH_2, and of both Φ_A^- and Φ_B^-, with a 275-fold faster rate for the former (Mar & Gingras, 1990). Evidence for the trapping of anionic Bph in *Ectothiorhodospira* RC rests on the following transient events: a bleaching of the Q_x and Q_y absorption bands of Bph concomitant with the appearance of a broad absorption band at 645 nm and of an EPR absorption line (Mar & Gingras, 1991) that are characteristic (Fajer et al., 1975) of this anion. In that respect, the *Ectothiorhodospira* RC behaves like its counterparts from other bacteria (Shuvalov & Klimov, 1976; Tiede et al., 1976; Okamura et al., 1979). However, and this has not been observed yet in other RCs, prolonged illumination of the lowest-energy band of the *Ectothiorhodospira* RC leads to the disappearance of the 645-nm band and of the EPR signal, accompanied by a stable bleaching of the Q_x and Q_y bands of Bph. The initial absorption of these bands of Bph can be recovered only when an oxidizing agent is introduced in the preparation. We interpret these observations by the formation under reducing and anaerobic conditions of the doubly reduced forms of Φ_A and Φ_B (Mar & Gingras, 1991). The present article is an extension of this work and provides evidence for the trapping of doubly reduced Bchl by *Ectothiorhodospira* RC. The finding of such doubly reduced Bchl affords supportive evidence for the involvement of B_A^- in the normal primary photochemistry.

MATERIALS AND METHODS

Ectothiorhodospira sp. (ATCC 31751) was cultured in 13-L cylindrical bottles as described before (Lefebvre et al., 1984). Its photoreaction center extracted according to Lefebvre et al. (1984) was suspended in 50 mM Tris-HCl/Triton X-100 (0.001 v/v, pH 7.4) containing sodium dithionite at concentrations ranging from 0.5 to 2 mM (see figure legends). Anaerobiosis was ensured by bubbling with nitrogen gas and

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[1] Abbreviations: Bchl, bacteriochlorophyll; Bph, bacteriopheophytin; RC, photoreaction center; B_A, B_B, Φ_A, and Φ_B, monomeric bacteriochlorophyll and bacteriopheophytin in the A and B branch of the photoreaction center; *Rb.*, *Rhodobacter*; *Rp.*, *Rhodopseudomonas*.

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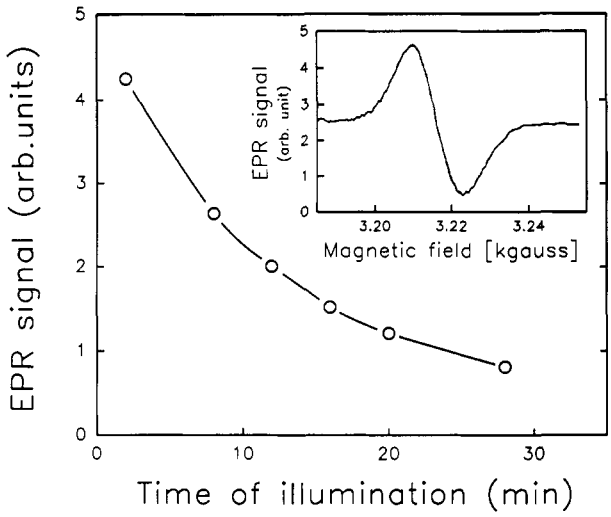

FIGURE 1: Decay of the EPR signal generated by illumination of the *Ectothiorhodospira* sp. photoreaction center in the presence of dithionite. The photoreaction center was suspended in 50 mM Tris-HCl (pH 8.0) containing 0.001 v/v Triton X-100 and 0.5 mM sodium dithionite. The EPR spectrum (inset) was recorded with a modulation frequency of 100 kHz, a microwave power of 1 mW, and a modulation amplitude of 4 G on a Varian E-104 A spectrometer operating at 9.01 GHz in a E231 cavity. Illumination was provided by a 500-W tungsten lamp focused on a 1-cm-diameter light-guide placed on an opening of the sample cavity.

then layering paraffin oil on top of the spectrophotometer cuvette. In the experiments involving horse heart cytochrome *c* as electron donor, anaerobiosis was ensured by adding glucose and glucose oxidase to the preparation. The hydrogen peroxide formed in the oxidation of glucose was removed by addition of reduced glutathione and glutathione peroxidase (see legend to Figure 6 for details). Horse heart cytochrome *c* and glutathione peroxidase were from Sigma Chemical Co. All the experiments were carried out at room temperature. Absorbance spectra and absorbance changes were measured in a Cary 2300 recording spectrophotometer. Actinic illumination was provided by an Oriel 250 W tungsten illuminator whose output was focused at the entrance of a 1 cm in diameter fiber-optic light guide. The light was filtered by a Baird Atomic broad-band interference filter centered at 930 nm (transmittance spectrum shown in Figure 3). Electron paramagnetic resonance measurements were carried out with a Varian E-104 A spectrometer operating at 9.01 GHz in a E231 cavity.

RESULTS

Phototrapping of Doubly Reduced Bacteriopheophytin. When *Ectothiorhodospira* sp. RC, maintained at low redox potential, is subjected to a short period of actinic illumination with near-infrared light centered at 930 nm, it displays a new 645-nm absorption band (not shown) and a 13-G EPR signal (Figure 1, inset). This is accompanied by a transient bleaching of the 540- and 755-nm absorption bands of Bph (Mar & Gingras, 1991). These phenomena are characteristic of the formation of the Φ_A^--trapped state. Continued illumination results in the disappearance of the Φ_A^- EPR signal (Figure 1) and of the 645-nm absorption band (Mar & Gingras, 1991). As these signals disappear, one observes (Figure 2) the progressive bleaching of the Q_x and Q_y bands of Bph at 530 nm (due to Φ_B), 540 nm (due to Φ_A), and 755 nm (due to both Φ_A and Φ_B). This new form of Bph, generated by the photochemical activity of the primary donor, is stable as long as the conditions remain reducing and anaerobic. The original absorption bands of Bph are recovered only when oxygen or

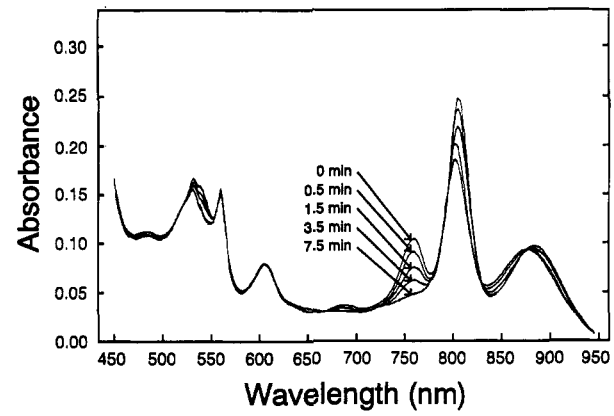

FIGURE 2: Bleaching as a function of the time of illumination in photoreaction center from *Ectothiorhodospira* sp. The photoreaction center (0.82 μM) was suspended in 50 mM Tris-HCl (pH 8.0) containing 0.001 v/v Triton X-100 and 0.5 mM sodium dithionite. Illumination was provided by a tungsten lamp filtered with a Baird Atomic wide-band interference filter centered at 930 nm. Intensity of illumination was 1.5×10^4 ergs cm^{-2} s^{-1}. The experiment was carried out at 20 °C. Illumination periods are indicated.

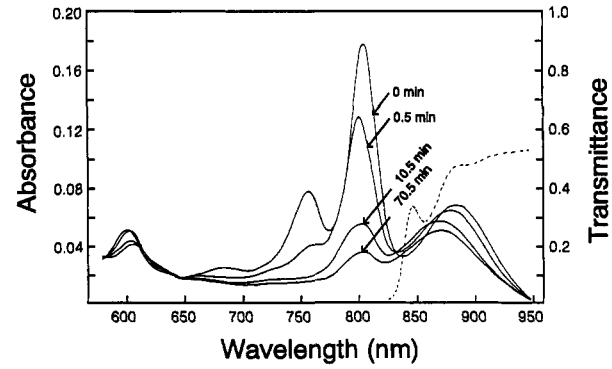

FIGURE 3: Absorption spectra of *Ectothiorhodospira* sp. photoreaction center after the trapping of doubly reduced B_A and B_B in the presence of dithionite. The sample (0.54 μM) was suspended in 50 mM Tris-HCl (pH 8.0)/0.001 v/v Triton X-100/2 mM sodium dithionite. Illumination was provided by a tungsten lamp through a Baird Atomic broad-band interference filter centered at 930 nm. Intensity of illumination was 1.2×10^5 ergs cm^{-2} s^{-1}. Illumination periods for phototrapping are indicated.

an oxidizing agent such as ferricyanide is introduced (a similar experiment for Bchl is shown below). The decline of the EPR and of the 645-nm signals and the concomitant buildup of a stable reduced species means that Φ_A^- and Φ_B^- are further reduced into a doubly reduced form of Bph. By selecting the proper dose of irradiation, it is possible, as shown in Figure 2, to bleach only the Q_x and Q_y bands of Bph at the exclusion of the Q_x band of Bchl at 600 nm, indicating that reduced Bchl is not trapped. The formation of this putative doubly reduced Bph affects the 800-nm and the 880-nm bands of Bchl: they both undergo a hypsochromic shift. However, the 800-nm band is affected the most by the bleaching of Bph, undergoing also a considerable hypochromic effect. We do not understand why the Q_y transition of Bchl is so much more sensitive than the Q_x transition to the trapping of doubly reduced Bph.

Phototrapping of Doubly Reduced B_B and B_A. Figure 3 shows the spectra of an *Ectothiorhodospira* sp. RC preparation measured after different times of illumination in the presence of dithionite. Under the 8-fold more intense illumination than that used in the experiment of Figure 2, the complete bleaching of the Q_x and Q_y bands of both molecules of Bph, Φ_A, and Φ_B occurred after approximately 1 min. After 10.5 min of illumination, the 800-nm absorption band was nearly completely bleached and after 70.5 min this bleaching had reached

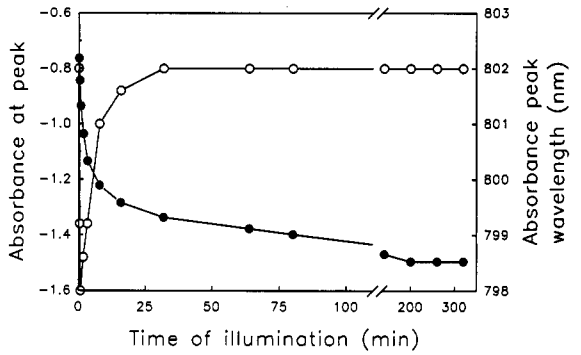

FIGURE 4: Time course of the peak wavelength shift (○) and absorbance decrease (●, log scale) with time of illumination plotted from the data of Figure 3.

a plateau. Figure 4 shows the time course of some of the spectroscopic changes that accompany this illumination. The absorption peak undergoes a wavelength shift that can be described by three kinetic components that we will designate as fast, slow, and very slow. Within the first minute, the peak is shifted from 802 to 798 nm, corresponding to the rapid and complete bleaching of the 530-, 540-, and 755-nm bands of Bph shown in Figure 2. This is the fast phase. Continued illumination causes a slow shift from 798 to 802 nm along with bleaching of the 800-nm band, this slow bandshift and bleaching ending after approximately 8 min. A very slow phase then takes place that is accompanied by a further bleaching of the 802-nm peak and by a small bandshift from 801 to 802 nm. After approximately 70 min, this very slow phase is ended: bleaching and bandshift have reached a plateau. The absorbance ratio of the 880/802-nm peaks is then 1.28. This ratio does not change even after an illumination of 6 h. Concomitant with the very slow phase, there occurs a loss in oscillator strength and a hypsochromic shift of the 880-nm band. We interpret the fast kinetic component as due to an interaction between monomeric Bchl and Bph that would be different according to whether the latter is nonreduced or doubly reduced. This effect cannot be due simply to the disappearance of the 755-nm band which overlaps to a very small extent with the 800-nm band. The slow and very slow phases are interpreted as being due to double reduction of one and then of the other of the monomeric B_A or B_B bacteriochlorophylls.

The Bleaching of the Bacteriochlorins Is Due to Photoreduction. If the photobleaching described in the two above paragraphs is really due to double reduction of Φ_A and Φ_B and of B_A and B_B, the original absorption spectrum would be expected to be recovered after an oxidation of these species. This is found indeed when ferricyanide (Figure 5) or oxygen (not shown) is added to the preparation. The Q_x and Q_y bands of Bph are then fully restored and the 800-nm band of Bchl is nearly completely restored. Addition of ferricyanide (or oxygen) does not completely reestablish the 800-nm and 880-nm bands. When the absorbance is normalized at 880 nm, the 800-nm band still shows a deficit in oscillator strength of about 10%. One possible explanation for this deficit is a protein conformational change induced by the double reduction of the monomeric bacteriochlorins.

Another question that we sought to answer concerning the phototrapping experiments reported above is how necessary is the use of a strong reducing agent like dithionite? The phototrapping experiment illustrated in Figure 6 used horse heart cytochrome *c* as an electron donor. In this experiment, the RC sample was first illuminated for 30 s with 930-nm light in the presence of dithionite to reduce the quinone primary acceptor and was immediately freed of dithionite by filtration

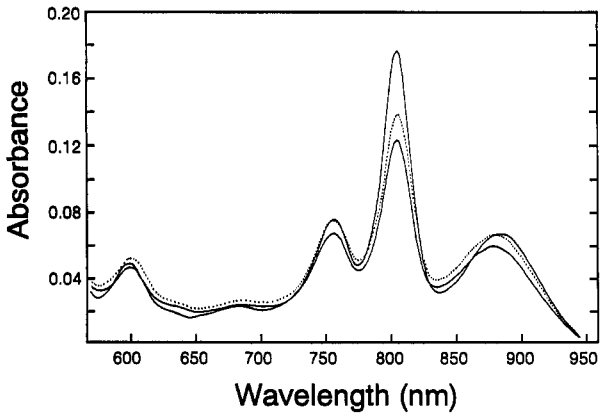

FIGURE 5: Reversibility of the absorption spectral changes induced by a prolonged illumination in photoreaction center of *Ectothiorhodospira* sp. (0.54 μM). Illumination conditions as in Figure 3. Continuous curves: (top) before actinic illumination, (bottom) 1 mM (final concentration) ferricyanide added to the 70.5-min illuminated sample. Dotted curve: bottom continuous curve normalized at the 880-nm peak of the top curve.

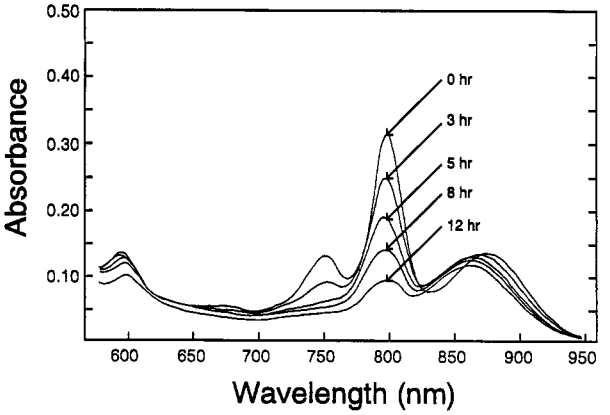

FIGURE 6: Absorption spectra of *Ectothiorhodospira* sp. photoreaction center after different light exposures in the presence of reduced cytochrome *c*. The concentrations of the RC and horse heart cytochrome *c* were respectively 1.06 μM and 46 μM. The sample was in 50 mM Tris-HCl (pH 8.0)/0.001 v/v Triton X-100/10 mM glucose/1.6 mM reduced glutathione. Anaerobiosis was ensured by the presence of 29.6 units of glucose oxidase and 34 units of glutathione peroxidase. Before this treatment, the sample was first illuminated for 30 s by 930-nm light in the presence of 2.3 mM dithionite to reduce the primary quinone acceptor and was immediately freed of dithionite by filtration on a NAP-25 (Pharmacia) column. The sample was then illuminated by heat-filtered white light of intensity 6×10^6 ergs cm^{-2} s^{-1}. Periods of illumination are shown.

on a NAP-25 (Pharmacia) column. The remaining part of the experiment was performed under anaerobic conditions maintained by the use of glucose and glucose oxidase plus reduced glutathione and glutathione peroxidase to remove the hydrogen peroxide generated by the oxidation of glucose. The sample was then illuminated by heat-filtered white light for periods of illumination shown in Figure 6. Except for the very long periods of illumination required with cytochrome *c*, the results are the same as those obtained with dithionite. Notice that complete photoreductive trapping of Bph took 5 h with cytochrome *c* under white light (Figure 6) compared to approximately 1 min under 930-nm light of a 50-fold lower intensity with dithionite (Figure 3). In this case too, addition of oxygen or ferricyanide restored the original spectrum of the *Ectothiorhodospira* RC preparation except for the 800-nm band that loses oscillator strength as was shown for dithionite in Figure 5. Similar results (not shown) were obtained using ascorbate as a reducing agent. The slowness of phototrapping with horse heart cytochrome *c* is explained

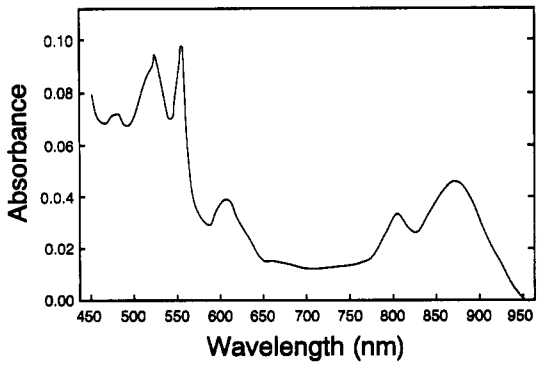

FIGURE 7: Absorption spectrum of the primary donor of the *Ectothiorhodospira* sp. photoreaction center. The RC sample (0.54 μM) was suspended in 50 mM Tris-HCl (pH 8.0)/0.001 v/v Triton X-100/2 mM sodium dithionite. It was illuminated for 130.5 min under the same conditions as in Figure 3.

by its slow electron donation to the four heme cytochrome of the *Ectothiorhodospira* sp. RC.

Absorption Spectrum of the Primary Electron Donor. As shown in Figures 3 and 4, an illumination period prolonged after all four bacteriochlorin monomers are trapped in their doubly reduced state affords no further change to the absorption spectrum of the RC. In particular, its 880/802-nm absorbance ratio does not change even after a very long period of illumination. We surmise that this unchanging spectrum (shown on Figure 7) is that of primary donor.

The only possibility of obtaining this spectrum is by eliminating, as we have done here for the first time, the spectral interference brought about by the four monomeric bacteriochlorins. Although the low-energy absorption (880 nm) band has been known for a long time to belong to the primary donor, because it is bleached upon oxidation of the latter, the very existence of the high-energy band has been in dispute. Its absorption spectrum (Figure 7) displays unexpected features: compared to the low-energy band, its oscillator strength is lower and its bandwidth is narrower (see Discussion). The Q_x band at 600 nm is more difficult to resolve precisely, because it overlaps with a large reduced cytochrome *c* band.

DISCUSSION

This work shows that photochemistry involving the primary electron donor placed under reducing conditions is conducive to the trapping of species other than the singly reduced Bph already observed in RC from other photosynthetic bacteria (Shuvalov & Klimov, 1976; Tiede et al., 1976; Okamura et al., 1976). RC preparations from *Ectothiorhodospira* sp., when illuminated by 930-nm light in the presence of artificial electron donors such as ascorbate, dithionite, or cytochrome *c*, first display a 645-nm absorption band and an EPR signal that are characteristic of bacteriochlorin anion radicals. Continuation of this actinic illumination entails the disappearance of both signals and of the Q_x and Q_y absorption bands of Bph. The 530-, 540-, and 755-nm bands of Bph are thus discolored and stay discolored as long as the suspension medium remains reducing and anaerobic. This leaves no doubt that such treatment leads to the formation, first of singly reduced Φ_A and Φ_B, followed by their further reduction to a doubly reduced form.

Consecutive to the trapping of doubly reduced Bph, the primary photochemistry under reducing conditions leads to the stable bleaching of the 600-nm and 802-nm bands of the RC's Bchl but not of its 880-nm band. The recovery of the original absorption spectrum of the RC after addition of oxygen or ferricyanide, with a renewed ability to photochemically reduce the primary acceptor, is strong evidence that the trapped species are indeed reduced. The bleaching of the 600- and 802-nm bands was not accompanied by the appearance of a band at 645 nm or by a new EPR signal that is typical of a bacteriochlorin π-anion (Fajer et al., 1975). This indicates that the new stable species of Bch is doubly reduced. We assume that the lifetime of trapped B_A^- and B_B^- is too short for these obligatory intermediates to be accumulated in any detectable quantity. In an analogous fashion, it is only at low temperatures that Φ_B^- could be detected (Mar & Gingras, 1990). The bleaching kinetics of the 800-nm band was biphasic, the changes being concomitant with peak wavelength bandshifts. This is interpreted by a double reduction of both molecules, B_A and B_B, of monomeric Bchl, one preceding the other in an undetermined order. After bleaching of the 800-nm band has reached saturation, the absorbance ratio of the 802/880-nm absorption bands remains constant, as would be expected if the ultimate spectrum was that of the primary electron donor. The 880-nm band which is clearly due to the primary donor is not bleached during this photoreductive trapping process.

The spectral isolation of the special pair of Bchl should allow us to better understand the mechanism of the primary charge-separation reaction. The first evidence for a high-energy band of the primary donor was obtained by low temperature absorption spectroscopy of the *Rb. sphaeroides* RC which, on oxidation, showed a bleachable shoulder at 812 nm (Feher, 1971). Because it is difficult to observe, the high-energy band has been postulated to be a forbidden band (Shuvalov & Asadov, 1979) and its existence has been very controversial at best (Parson, 1982). The main experimental reason for this uncertainty is the large hypsochromic shift undergone by the 800-nm band upon oxidation of the primary donor. Further evidence for a high-energy band in *Rb. sphaeroides*, *Rp. viridis*, and *Rhodospirillum rubrum* RCs was brought forth by photodichroism (Verméglio & Clayton, 1976; Breton, 1985) and photoselection experiments (Verméglio & Paillotin, 1982; Mar & Gingras, 1984a) which showed the existence of a weak absorption dipole differently oriented than the two strong dipoles of the 800-nm (or 830-nm) spectral region. However, this band could not be resolved.

Theoretical calculations based on the known structure of the *Rp. viridis* and *Rb. sphaeroides* RCs by Knapp et al. (1985) and Parson and Warshel (1987) led to the conclusion that the high-energy band results from mixed contributions from all six chromophores. This interpretation is justified by work on the *Chloroflexus aurantiacus* RC which contains only three Bchl molecules (Vasmel et al., 1986). Its absorption spectra show three distinct bands with a high-energy band clearly seen at 792 nm. However, if this band was due to the high-enery exciton band of the special pair, its rotatory strength should be negative as opposed to the positive rotatory strength of the low-energy band. But it is in fact positive, implying that the 792-nm band is the high-energy band of a trimer rather than that of the special pair. To observe the high-energy band of the special pair, all the other interacting chromophores must be removed. This is what we have achieved by reducing all the bacteriochlorins except the special pair [Fig 7; see also Mar and Gingras (1988)].

As shown in Figure 7, the Q_y transition of the special pair is split into a high- and a low-energy component, the former with a lower oscillator strength than the latter. This is in line with a dimer formed by the excitonic interaction between two not quite parallel Bchl molecules, as shown by the X-ray crystallography of two other RCs (Deisenhofer et al., 1985;

Chang et al., 1986; Allen et al., 1987). For an excitonically coupled dimer, the bandwidths of the two transitions should be smaller by a factor of $1/\sqrt{2}$ than that of the corresponding monomer band (Heminger, 1977). However, the spectrum of the special pair (Figure 7) shows that the bandwidths of the high-energy (full width at half-maximum height = 28 nm) and of the low-energy (full width at half-maximum height = 81 nm) Q_y bands are respectively narrower and broader than that of monomeric Bchl in solution [full width at half-maximum height in acetone = 44 nm, according to Philipson and Sauer (1972)]. We previously have rationalized this difference by an asymmetric exciton model (Mar & Gingras, 1984b). But pure exciton models cannot account for the large change in the dipole moment of the lower-energy band as found in Stark effect experiments (Losche et al., 1987; Lockhart & Boxer, 1987). A charge-transfer state may be involved. Parson and Warshel (1987) have investigated in detail the charge-transfer states in the special pair. They concluded to two charge-transfer states, the lower-energy one having larger mixing with its corresponding excitonic state. Plato et al. (1988) also found the excited state charge-transfer distribution to be asymmetric. Because the first singlet excited state of the primary donor is the only electronic excited state with charge-transfer character, linear electron–phonon coupling effects could be responsible for the observed large bandwidth of the lower-energy band (Wiersma, 1988). In line with this, Won and Friesner (1987), using vibronic-coupling calculations, found that the first singlet excited state of P has a larger bandwidth than monomeric Bchl. Although charge transfer has been implicated, it has not been directly measured for P in its reduced state. Our experimental spectrum of the special pair should help refine the calculations based on dynamic models. If it is granted that the band-broadening of the primary donor is due to an exciton with mixed-in charge-transfer character, then our results clearly show that charge transfer is generated within the special pair before charge is transferred to its neighboring bacteriochlorins.

The other main contribution of this work is the trapping of doubly reduced B_A and B_B and the inference, on the basis of the monoelectronic nature of the primary photochemistry, of B_A^- and B_B^- as obligatory intermediates in this reduction. Since these species are made by the photochemistry of the primary electron donor placed under reducing conditions, they also are possible intermediates under the conditions of normal electron transfer. On the other hand, Φ_A^- has long been recognized as an intermediary state in the reduction of the primary quinone acceptor, Q_A [reviewed in Parson and Ke (1982)]; since B_A is physically situated between P and Φ_A, it is the most probable intermediate in the transmembrane electron transfer chain between P and Q_A. Therefore, and despite the presently controversial nature of the conclusions drawn from femtosecond kinetic work (Holzapfel et al., 1990; Chan et al., 1991; Vos et al., 1991, 1992), we feel the trapping of doubly reduced Bph and Bchl to be consistent with B_A^- as an intermediate in the primary reduction of Φ_A.

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