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Investigation into the Source of Electron Transfer Asymmetry in Bacterial Reaction Centers†

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ABSTRACT: We have investigated the primary photochemistry of two symmetry-related mutants of *Rhodobacter sphaeroides* in which the histidine residues associated with the central Mg^{2+} ions of the two bacteriochlorophylls of the dimeric primary electron donor (His-L173 and His-M202) have been changed to leucine, affording bacteriochlorophyll (BChl)/bacteriopheophytin (BPh) heterodimers. Reaction centers (RCs) from the two mutants, (L)H173L and (M)H202L, have remarkably similar spectral and kinetic properties, although they are quite different from those of wild-type RCs. In both mutants, as in wild-type RCs, electron transfer to BPh_L and not to BPh_M is observed. These results suggest that asymmetry in the charge distribution of the excited BChl dimer (P*) in wild-type RCs (due to differing contributions of the two opposing intradimer charge-transfer states) contributes only modestly to the directionality of electron transfer. The results also suggest that differential orbital overlap of the two BChls of P with the chromophores on the L and M polypeptides does not contribute substantially to preferential electron transfer to BPh_L.

The three-dimensional structure of reaction centers (RCs) from both *Rhodopseudomonas viridis* and *Rhodobacter sphaeroides* reveals two possible electron-transfer pathways emanating from the bacteriochlorophyll (BChl) dimer (P) that serves as the primary electron donor (Deisenhofer et al., 1984; Allen et al., 1986; Chang et al., 1986). Much recent work has focused on determining the molecular factors responsible for the observed unidirectional charge separation via only one of these pathways, namely electron transfer from the excited primary donor (P*) to the bacteriopheophytin associated with the L polypeptide (BPh_L) and then to the primary quinone (Q_A) (Figure 1). Many structural and energetic factors have been considered for this directionality. This work addresses the extent to which charge separation exclusively via the L branch may be due to an asymmetric charge distribution in P*. Such charge asymmetry could be induced by differences in the structure and/or protein environment of the two BChl macrocycles of the dimer, which lead to unequal contributions of two intradimer charge-transfer (CT) configurations ($[BChl_{LP}^{+}BChl_{MP}^{-}]$ and $[BChl_{LP}^{-}BChl_{MP}^{+}]$). Net CT character in P* has received widespread attention from a number of experimental and theoretical studies (Meech et al., 1986; Parson & Warshel, 1987; Lockhart & Boxer, 1987; Lockhart et al., 1988; Warshel et al., 1988; Lösche et al., 1987; Scherer & Fischer, 1989; Boxer et al., 1989; Friesner & Won, 1989; Parson et al., 1990; DiMagno et al., 1990; McDowell et al., 1990; Thompson et al., 1991).

The importance of the intradimer CT configurations in defining the optical and photochemical properties of the excited primary donor has been explored in the (M)H200L mutant of *Rhodobacter capsulatus* in which the His residue coordinated to the central Mg^{2+} of $BChl_{MP}$ has been changed to Leu. This mutant RC contains a $BChl_{LP}BPh_{MP}$ heterodimer (D)[1]

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[1] In the original work on the primary photochemistry in *Rb. capsulatus* (M)H200L RCs, the nomenclature of D (for donor) was adopted to denote the heterodimer (Kirmaier et al., 1988). We will use D here in a generic sense and to simplify notation of states $D^{+}BPh_L^{-}$ and $D^{+}Q_A^{-}$ for *both* the M- and L-side *Rb. sphaeroides* heterodimers. However, for the M- and L-side mutants, D corresponds to $[BChl_{LP}BPh_{MP}]$ and $[BPh_{LP}BChl_{MP}]$, respectively, and D^{+} to the respective radical cations. In both cases the hole in the cation is expected (Bylina & Youvan, 1988; Kirmaier, et al., 1988) to reside largely on the BChl moiety due to its lower oxidation potential than BPh in vitro (Fajer et al., 1975). This has been demonstrated by recent ESR and ENDOR measurements (Bylina et al., 1990; Huber et al., 1990).

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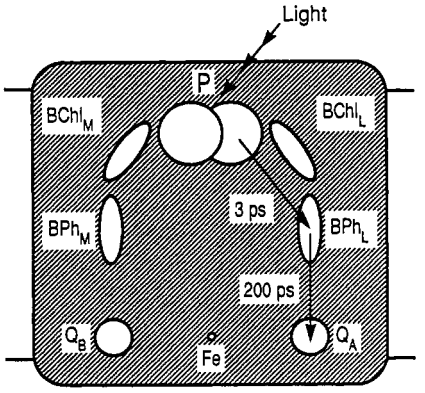

FIGURE 1: Schematic representation of the photochemistry and arrangement of the chromophores in wild-type RCs.

in place of the $BChl_{LP}BChl_{MP}$ homodimer (P) in wild-type RCs (Bylina & Youvan, 1988) and thus possesses inherent electronic asymmetry. The transient state observed immediately after subpicosecond excitation of (M)H200L RCs is very different from P* in wild-type RCs and is believed to have mainly $[BChl_{LP}^{+}BPh_{MP}^{-}]$ intradimer CT character (Kirmaier et al., 1989; McDowell et al., 1990). Unidirectional electron transfer to BPh_L is preserved in the (M)H200L mutant, but the yield of charge separation (to form $D^{+}BPh_L^{-}$) is reduced to about 50% due to a decreased rate of electron transfer to BPh_L and an enhanced rate of deactivation of $[BChl_{LP}^{+}BPh_{MP}^{-}]$ to the ground state.

Since the electronic charge distribution in P* in wild-type RCs may be imbalanced toward half of the dimer (Plato et al., 1988; Scherer & Fischer, 1989; Parson et al., 1990; Thompson et al., 1991), it is desirable to investigate the symmetry-related L-side heterodimer mutant in which $BChl_{LP}$ is converted to BPh. To this end we have prepared both the L- and M-side heterodimer mutants of *Rb. sphaeroides* by changing the histidines associated with the central Mg^{2+} ions of $BChl_{LP}$ and $BChl_{MP}$ (residues L173 and M202, respectively) to Leu. If the direction of electronic asymmetry in the excited primary donor underlies the differentiation of the charge separation pathways, then preferential $[BPh_{LP}^{-}BChl_{MP}^{+}]$ intradimer CT character in the initial transient state of the L-side heterodimer might promote electron transfer to BPh_M or diminish the yield of electron transfer to BPh_L. Here we report comparative studies of the primary photochemistry of the two symmetry-related heterodimer mutants [see Schenck et al. (1990) for a preliminary report of this work]. We discuss the importance of electronic asymmetry in the excited primary donor in determining the directionality of charge separation and the effects of intradimer CT character on the photophysical properties of the dimer.

Experimental Procedures

The codons corresponding to amino acid 202 of the M polypeptide and 173 of the L polypeptide were changed from CAC (His) to CTG (Leu). The methodologies for creating the complementing plasmids bearing the mutant *puf* alleles have been described, as have the conditions for cell growth and RC purification (Nagarajan et al., 1990). (M)H202L RCs were present in chromatophores at concentrations approximately equal to that of wild-type RCs, whereas the concentration of (L)H173L RCs was diminished 5–10-fold. These concentrations were determined by quantitative densitometry of SDS–PAGE gels of chromatophore membranes. Neither heterodimer strain is capable of photoheterotrophic growth. The purest RC fractions gave A_{280}/A_{800} ratios of 1.21 for wild type and 1.10 for both heterodimers. (A_x is the absorbance at the peak of the bands near 280 and 800 nm). The methods for determination of pigment ratios and Mg^{2+} content have been described (Straley et al., 1973; van der Rest & Gingras, 1974; Kirmaier et al., 1988, 1991). For the metals analysis it was assumed that the mutations did not affect the extinction coefficient near 280 nm.

Subpicosecond transient absorption studies were performed as previously described and utilized either 50-μJ 582-nm excitation flashes having a duration of 150 or 350 fs or 15-μJ 150-fs 870-nm excitation flashes (focused to 1–2 mm) and white-light probe pulses polarized at 45° to the excitation (Kirmaier & Holten, 1991). These measurements were made at 275 K with RCs (in 10 mM Tris-HCl, pH 8/0.05% *N,N*-dimethyldodecylamine-*N*-oxide/1 mM EDTA) contained in a cooled reservoir and flowed through a 2–4-mm path length cell. Slower time scale measurements were performed with 50-mJ 10-ns pulses at 532 nm for excitation (Tait & Holten, 1983). For these measurements, the RCs were in glycerol/buffer mixtures (50–60% glycerol by volume).

Results

Ground-State Absorption Spectra and Pigment Content. Ground-state absorption spectra of (M)H202L (dashed), (L)H173L (dash-dotted), and wild-type (solid) RCs are shown in Figure 2. Compared to wild type, the spectra of (M)H202L and (L)H173L RCs show a diminished amplitude of the long-wavelength band of the primary donor (near 865 nm) and decreased absorption in the Q_x region of the BChls (near 600 nm), as has been found for the analogous (M)H200L and (L)H173L mutants of *Rb. capsulatus* (Bylina & Youvan, 1988, 1990; Kirmaier et al., 1988). It is interesting that in both *Rb. capsulatus* and *Rb. sphaeroides* the long-wavelength absorption of the L-side heterodimer displays a more readily discernible shoulder (near 865 nm in Figure 2) than that seen in the M-side heterodimer absorption spectrum. Likewise, there seems to be a stronger similarity between the (L)H173L and wild-type spectra in both the Q_x and Q_y regions. These findings may be related to protein-induced differences in the energies and coupling of the CT and locally excited states of the two heterodimers.

Pigment extractions yield a BChl:BPh ratio of 0.9 ± 0.1 for both (M)H202L and (L)H173L RCs, compared to 1.9 ± 0.1 for wild type. Metals analysis gives a Mg^{2+} content of 3.0 ± 0.3 Mg^{2+}/RC for both mutants, compared to 3.9 ± 0.4 for wild-type RCs. These results indicate a pigment content of 3 BChls and 3 BPhs in the two mutant RCs compared to 4 BChls and 2 BPhs in wild-type RCs. Thus, the pigment analyses are consistent with the ground-state absorption spectra and, considering the site of the mutations, indicate that (M)H202L RCs contain a $BChl_{LP}BPh_{MP}$ heterodimer, while (L)H173L RCs contain a heterodimer with opposite molecular asymmetry, i.e., $BPh_{LP}BChl_{MP}$.

Primary Photochemistry. Transient absorption studies on the *Rb. sphaeroides* M- and L-side heterodimer mutants reveal differences from wild-type RCs in the nature and decay pathways of the initial transient states. In (M)H202L RCs, the spectrum observed shortly after excitation with a 150-fs flash contains bleaching in the broad long-wavelength heterodimer absorption, bleaching in the BPh Q_x region near 550 nm, and a broad but pronounced absorption band centered near 655 nm (solid spectra in Figures 3A and 4A). The latter feature is similar to the large absorption band centered near 650 nm in the spectrum of the initially observed transient of the *Rb. capsulatus* (M)H200L mutant (Kirmaier et al., 1989; McDowell et al., 1990). By analogy with that work, we assign the state observed immediately after excitation as having

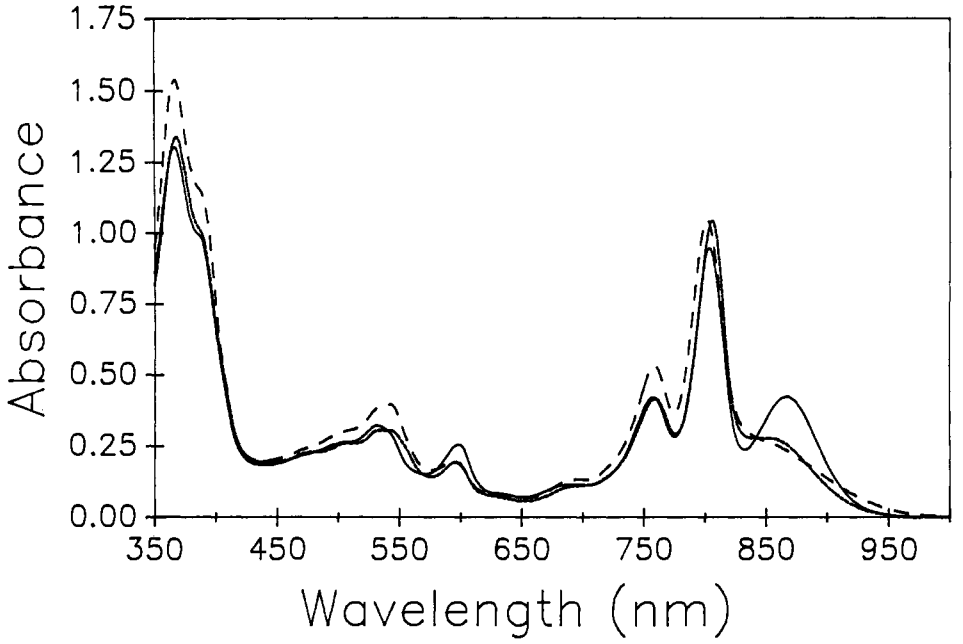

FIGURE 2: Ground-state absorbance spectra of wild-type (—), (M)H202L (---), and (L)H173L (····) RCs. The normalization was based on the protein absorbance near 280 nm.

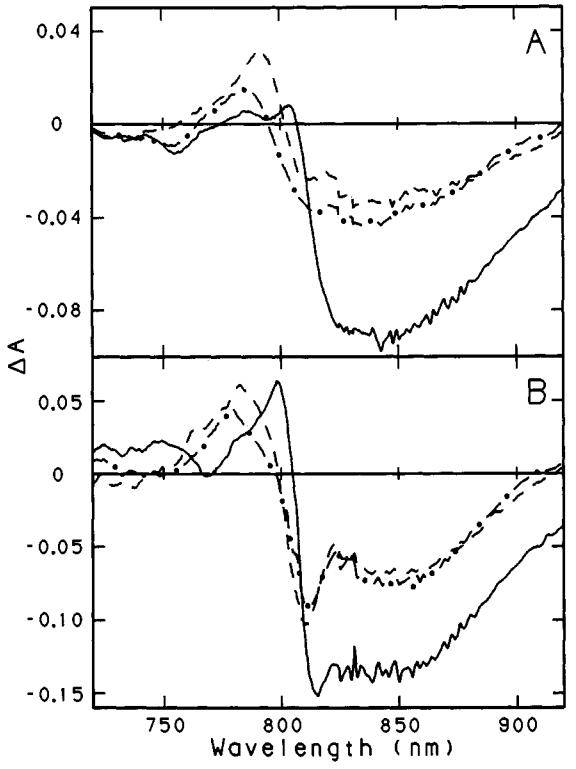

FIGURE 3: Transient absorption spectra in the Q_y region of the BChls and BPhs acquired at several times following a 150-fs excitation flash. Each spectrum represents an average of 600–1200 scans. The data were acquired with both 870-nm excitation (720–830 nm) and 582-nm excitation (825–920 nm) and normalized to the same ΔA in the region of overlap (825–830 nm). (A) (M)H202L RCs: effective $A_{801} \simeq 1.2$; times are 2 ps (—), 40 ps (---), and 1.5 ns (- - -). (B) (L)H173L RCs: effective $A_{805} \simeq 2$; times are 0.5 ps (—), 100 ps (---), and 1.5 ns (- - -).

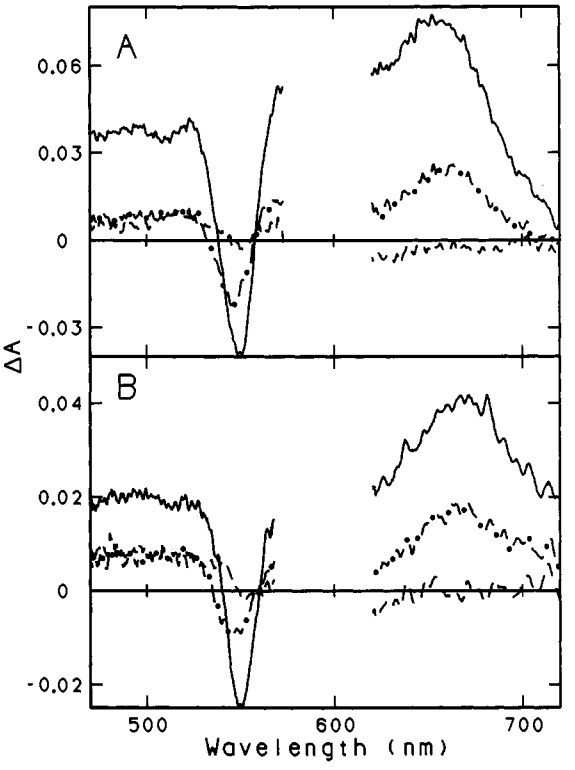

FIGURE 4: Transient absorption spectra in the BPh Q_x and anion regions acquired after a 150-fs 870-nm excitation flash. (A) (M)H202L RCs: $A_{801} \simeq 2.7$; excitation energy ~11 μJ; times are 0.3 ps (—), 42 ps (---), and 2 ns (- - -). (B) (L)H173L RCs: $A_{805} \simeq 2$; excitation energy ~21 μJ; times are 0.5 ps (—), 100 ps (---), and 1 ns (- - -).

substantial [$BChl_{LP}^{+}BPh_{MP}^{-}$] heterodimer CT character; the 655-nm absorption is key to this assignment, as it is indicative of BPh anion absorption. This assignment is also consistent with the initial bleaching near 550 nm in the BPh Q_x region, which we attribute to loss of absorption associated with the BPh_{MP} component of the heterodimer. It is important to note that although we assign the initial transient state as having mostly intradimer CT character, the state also likely contains some heterodimer exciton character (McDowell et al., 1990). However, to emphasize the dominant character of the state and to simplify nomenclature and comparisons with the L-side heterodimer, we will refer to the initially observed transient state in (M)H202L RCs as the intradimer CT state [$BChl_{LP}^{+}BPh_{MP}^{-}$].[2]

State [$BChl_{LP}^{+}BPh_{MP}^{-}$] in (M)H202L RCs has a lifetime of 18 ± 5 ps. This is measured for both partial decay of bleaching of the 830–880-nm long-wavelength heterodimer absorption band (Figure 5) and for the first component of the dual-exponential decay measured between 630 and 680 nm in the anion region (Figure 6A). The spectrum remaining after decay of [$BChl_{LP}^{+}BPh_{MP}^{-}$] is that expected for state $D^{+}BPh_{L}^{-}$,[1] including the diagnostic BPh_L anion band centered near 665 nm and bleaching of the Q_x band of BPh_L near 545 nm (Figures 3A and 4A, dash-dotted spectra). The yield of $D^{+}BPh_{L}^{-}$ determined by the extent of bleaching in the long-wavelength band of the heterodimer remaining after the ~ 20-ps process is 41 ± 10%, while 34 ± 10% is obtained by comparing the preexponential factors of the dual-exponential fits of the anion-region decay kinetics. Similar results are obtained from the relative amplitudes of the two-component decay of bleaching in the BPh Q_x region.[3] The recovery of the 860- and 545-nm bleachings, together with the anion-region absorption decay, all indicate that [$BChl_{LP}^{+}BPh_{MP}^{-}$] decays via two routes: (i) rapid charge recombination to the ground state (~60% yield) and (ii) electron transfer to the normal BPh_L acceptor (~40% yield). At no point is bleaching near 530 nm observed, as would be expected if electron transfer to BPh_M had occurred (<10% BPh_M reduction, see e.g., Figure 4A).

State $D^{+}BPh_{L}^{-}$ in (M)H202L RCs decays with a time constant of 151 ± 16 ps, as measured from the second component of the dual-exponential decay in the anion-region absorption (Figure 6A). A similar time constant is found for the second component of the dual-exponential decay in the BPh Q_x bleaching. The spectrum following this decay is consistent with the formation of state $D^{+}Q_{A}^{-}$ (Figures 3A and 4A, dashed spectra). There is no appreciable decay of bleaching in the heterodimer long-wavelength absorption during the decay of $D^{+}BPh_{L}^{-}$ (Figure 3A), indicating a 100% yield of electron transfer from BPh_L^{-} to Q_A, as occurs in wild type and in *Rb. capsulatus* (M)H200L RCs (Kirmaier et al., 1988). Charge recombination returning $D^{+}Q_{A}^{-}$ to the ground state occurs with a time constant of 52 ± 4 ms, as measured by the decay of heterodimer bleaching at 860 nm following excitation with a 10-ns flash; this time constant is a factor of 2 shorter than the 115 ± 5 ms $P^{+}Q_{A}^{-}$ lifetime found here and similar values found previously (Okamura et al., 1982) for wild-type *Rb. sphaeroides* RCs. In *Rb. capsulatus*, (M)H200L and wild-type RCs have nearly the same rate of $D^{+}Q_{A}^{-}$ charge recombination at room temperature (Bylina & Youvan, 1988; Kirmaier et al., 1988; McDowell et al., 1991). These results collectively show that the primary photochemistry of the (M)H202L *Rb. sphaeroides* RC is very similar to that of the analogous M-side heterodimer from *Rb. capsulatus*; the only appreciable difference is the change in the charge-recombination rate of $D^{+}Q_{A}^{-}$.

We find similar primary photochemistry in the symmetry-related *Rb. sphaeroides* L-side heterodimer mutant, as the spectral data in Figures 3B and 4B indicate. A state we assign

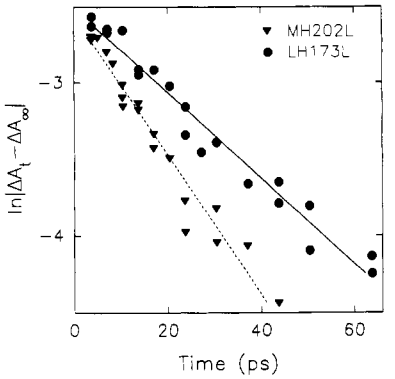

FIGURE 5: Semilogarithmic plot of the kinetics measured in the heterodimer absorption region (870–880 nm) following an 870-nm excitation flash [350 fs for (M)H202L, 150-fs for (L)H173L]. Both RC samples had a maximum absorbance near 800 nm of ~1.8. The ΔA_∞ values were determined by fitting an entire data set (which included data at longer delays than shown) to the function $\Delta A_t = \Delta A_\infty - \Delta A_0 \exp(-t/\tau)$. The lifetimes determined by use of the slope of the semilog plot are slightly larger (10–15%) than those obtained from the nonlinear fit. The lifetimes calculated from the data in this figure are 22 ps for (M)H202L RCs and 36 ps for (L)H173L RCs. The lifetimes determined by averaging all values obtained from the exponential fits in the 830–880-nm region are 20 ± 2 ps for (M)H202L and 36 ± 6 ps for (L)H173L. [For clarity, the (M)H202L data were offset by −0.4 along the ordinate.]

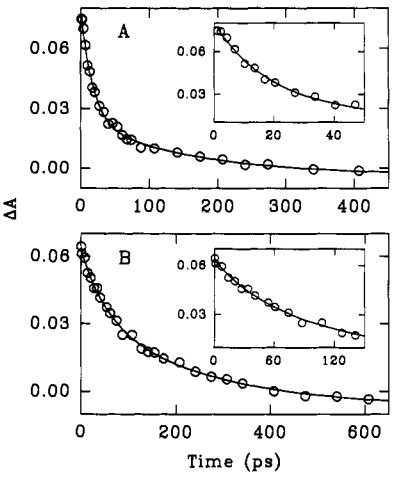

FIGURE 6: Anion-region kinetics following a 150-fs 870-nm excitation flash. The solid lines are fits of the entire data set (delay times to 2 ns) to a dual-exponential plus constant with no parameters fixed. The insets present an expanded view of the data at early times. (A) Data between 650 and 660 nm for (M)H202L RCs with $A_{801} \simeq 2.8$. The time constants for this particular data set are 17 ± 1 ps and 155 ± 17 ps, while the average time constants (630–680 nm) are 16 ± 2 ps and 151 ± 16 ps. (B) Data between 670 and 680 nm for (L)H173L RCs having $A_{805} \simeq 2.0$. The time constants determined from these data are 53 ± 9 ps and 253 ± 35 ps, and the average time constants (640–690 nm) are 47 ± 13 ps and 257 ± 43 ps.

[2] The state observed immediately following excitation of the heterodimers with a 150-fs flash, a state we assign as having mainly [$BChl^{+}BPh^{-}$] intradimer CT character, likely also contains some heterodimer exciton character. [Because of its mixed character, the initial transient state in *Rb. capsulatus* (M)H200L RCs has been referred to as D^{*} (McDowell et al., 1990).] It has been suggested that this state contributes to the long-wavelength edge of the near-infrared heterodimer absorption (Friesner & Won, 1989; McDowell et al., 1990; DiMagno et al., 1990) and thus may be produced directly upon excitation in this region. We have not yet resolved stimulated emission from D^{*}, due most likely to the substantial [$BChl^{+}BPh^{-}$] character of the state and/or the difficulty in distinguishing the stimulated emission from D's broad long-wavelength bleaching. The 865-nm excitation flashes may also excite the state referred to previously as D^{*}; this state has been proposed to have mainly exciton and some intradimer CT character and to lie above the [$BChl^{+}BPh^{-}$]-like state (Kirmaier et al., 1989; McDowell et al., 1990). Recent Stark measurements show that the state formed upon excitation in the near-infrared absorption of the heterodimers has more CT character than P^{*} in wild-type RCs (DiMagno et al., 1990; Hammes et al., 1990; Schenck et al., 1990).

[3] We assume that the anion region and BPh Q_x absorption each have comparable extinction coefficients in states [$BChl^{+}BPh^{-}$] and $D^{+}BPh_{L}^{-}$. As discussed in detail for *Rb. capsulatus* (M)H200L RCs (McDowell et al., 1990, 1991), decay of BPh Q_x bleaching occurs upon electron transfer from [$BChl^{+}BPh^{-}$] to BPh_L, which has a yield of ~50%, since (i) the Q_x bands of tetrapyrrole dimers are generally only weakly coupled and behave roughly as independent monomer bands and (ii) the hole in D^{+} in state $D^{+}BPh_{L}^{-}$ (or $D^{+}Q_{A}^{-}$) is preferentially localized on the BChl half of the oxidized heterodimer (see also footnote 1).

as having mainly intradimer CT character, [$BPh_{LP}^-BChl_{MP}^+$], is observed immediately after excitation of (L)H173L RCs. (Again, although we will refer to the initial transient state as [$BPh_{LP}^-BChl_{MP}^+$], it is likely that this state contains some heterodimer exciton character.) Facilitating this assignment, the spectrum of [$BPh_{LP}^-BChl_{MP}^+$] displays a prominant broad absorption band in the anion region. Appearing at 675 nm (Figure 4B, solid spectrum), the [$BPh_{LP}^-BChl_{MP}^+$] anion-region absorption band is red-shifted compared to the 655- and 665-nm positions of the [$BChl_{LP}^+BPh_{MP}^-$] and BPh_L^- bands, respectively. As for [$BChl_{LP}^+BPh_{MP}^-$] in (M)H202L RCs, the lifetime of [$BPh_{LP}^-BChl_{MP}^+$] in (L)H173L RCs can be determined from (i) the decay of D bleaching between 830 and 880 nm (Figure 5) and (ii) the first component of the dual-exponential decay in the anion region (640-690 nm, Figure 6B). We find the lifetime of [$BPh_{LP}^-BChl_{MP}^+$] to be 42 ± 10 ps, which is about a factor of 2 longer than the 18 ± 5 ps lifetime of [$BChl_{LP}^+BPh_{MP}^-$] in (M)H202L RCs. Similar to the M-side mutant, however, [$BPh_{LP}^-BChl_{MP}^+$] decays by about half (47 ± 10%) via rapid deactivation to the ground state, as reflected by the partial recovery of bleaching in the long-wavelength heterodimer absorption (Figure 3B) and of the BPh Q_x bleaching (Figure 4B).[3] The preexponential factors of the dual-exponential decay in the anion region also indicate a 47% yield for return to the ground state. The characteristic BPh_L anion absorption band at 665 nm and the 545-nm Q_x bleaching (Figure 4B, dash-dotted spectrum) indicate that the remainder of the [$BPh_{LP}^-BChl_{MP}^+$] decay proceeds via electron transfer to BPh_L, producing state $D^+BPh_L^-$. At no time is there any indication of bleaching near 530 nm (e.g., Figure 4B), indicating that in the L-side heterodimer mutant, as in (M)H202L and wild-type RCs, electron transfer occurs exclusively to BPh_L (<10% BPh_M reduction).

Electron transfer from BPh_L^- to Q_A in (L)H173L RCs is slower (257 ± 43 ps) than in the M-side heterodimer mutant. This time constant is derived from the second component of the dual-exponential decay observed in the anion region (Figure 6B). Again, this time constant for the decay of $D^+BPh_L^-$ reflects the rate of electron transfer from BPh_L^- to Q_A, since the absence of further decay of bleaching in the long-wavelength heterodimer absorption (Figure 3B, compare dash-dotted and dashed spectra) indicates that the yield of this process is essentially unity. $D^+Q_A^-$ charge recombination in (L)H173L RCs occurs with a time constant of 101 ± 9 ms.

Discussion

Initially Observed Transient State and Its Decay Pathways. The primary photochemistry of the two BChl/BPh heterodimer-containing RCs is summarized in Figure 7 and can be compared to the photochemistry of wild-type RCs given in Figure 1. Electron transfer in the L- and M-side heterodimer RCs is strikingly similar, with the principal difference from wild-type photochemistry being found in the nature and decay pathways of the initially formed state, as has been described in detail for the (M)H200L RC of *Rb. capsulatus* (Kirmaier et al., 1988, 1989; McDowell et al., 1990, 1991). The assignment of the initial state as having mainly [$BChl^+BPh^-$] intradimer CT character, wherein there is a net shift in electron density from the BChl to the BPh moiety of the heterodimer, is based on (i) the broad but well-resolved absorption band between 600 and 700 nm that is characteristic of tetrapyrrole anions, (ii) the lack of any such feature in the P* spectrum of wild-type RCs, which displays only a flat featureless absorption in this region (Kirmaier et al., 1989; Kirmaier & Holten, 1991; McDowell et al. 1990), and (iii) the fact that

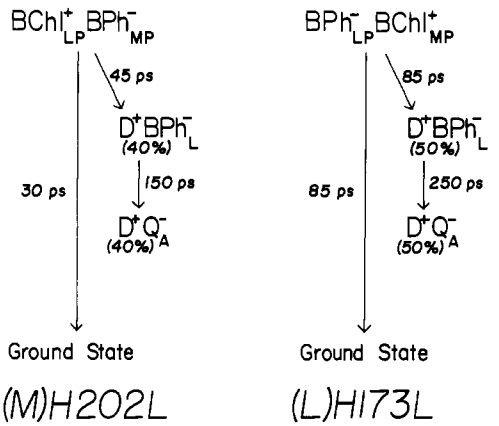

FIGURE 7: Schematic of the photochemistry observed in (M)H202L and (L)H173L RCs, comparing the rates and yields of electron transfer. The states observed immediately after a 150-fs flash and denoted simply as $BChl_{LP}^+BPh_{MP}^-$ and $BPh_{LP}^-BChl_{MP}^+$ for the two mutants, respectively, are likely not pure intradimer CT states but also contain some heterodimer exciton character. In each mutant, the 865-nm excitation flashes may excite this state directly and/or a higher energy state (not shown) having mostly exciton and some intradimer CT character. See text for further details.

BPh is easier to reduce than BChl in vitro by 200–300 meV (Fajer et al., 1975; Cotton & Van Duyne, 1979). This assignment is also consistent with the dichroism of the anion band observed at 0.5 ps in the *Rb. capsulatus* (M)H200L RC (Kirmaier et al., 1989). [As noted above, the initial CT-like state likely has some heterodimer exciton character, and it also may contribute to the near-infrared heterodimer absorption (Won & Friesner, 1989; McDowell et al., 1990; DiMagno et al., 1990).][2] The 655- and 675-nm positions of the anion-region absorption of [$BChl_{LP}^+BPh_{MP}^-$] and [$BPh_{LP}^-BChl_{MP}^+$], respectively, straddle the 665-nm position of BPh_L^- and likely reflect the known differences in the protein environments of the two macrocycles of P (Michel et al., 1986; Yeates et al., 1988). For example, hydrogen bonding to the BPh_L ring V keto group is known to affect the position of the BPh_L anion band (Bylina et al., 1988).

The decreased quantum yield in the heterodimer mutants is associated not with electron transfer from BPh_L^- to Q_A,[4] but rather with less efficient initial electron transfer to BPh_L. Two effects contribute to this: (i) a decreased rate of electron transfer to BPh_L and (ii) an increased rate of deactivation to the ground state of the heterodimer state [$BChl^+BPh^-$]. Regarding the first point, changes in energetics (and thus potentially in the Franck–Condon factor) and electronic coupling must be considered. Recent studies on wild-type and mutant RCs suggest that the rate of the electron transfer initiated from P* is not particularly sensitive to energetics, i.e., to the energy of $P^+BPh_L^-$ (Lockhart et al., 1990; Kirmaier et al., 1991). For example, in the *Rb. sphaeroides* mutant (M)L214H in which the BPh_L acceptor is replaced with a

[4] There is no ground-state recovery or other evidence for a loss of quantum yield accompanying electron transfer from BPh_L^- to Q_A. Nonetheless, the time constants of 150 and 250 ps for this process in the two *Rb. sphaeroides* heterodimer mutants, and of 100 ps in *Rb. capsulatus* (M)H200L RCs (Kirmaier et al. 1988, 1989; McDowell et al., 1990, 1991), are somewhat different from one another and from the value of 200 ps found in wild-type RCs. The origin of the difference in the time constants is not known but may result from a secondary structural change in the BPh_L site caused by the mutation in the vicinity of P. A perturbed BPh_L site is also indicated by the observation that the width of the BPh_L Q_x bleaching at low temperature is different in both *Rb. capsulatus* (McDowell et al., 1991) and *Rb. sphaeroides* (L.M.M., D.G., C.K., D.H., and C.C.S., unpublished results) heterodimers from that in wild-type RCs.

BChl (β_L), the time constant for $P^+\beta_L^-$ formation is only about a factor of 2 longer than for $P^+BPh_L^-$ formation in wild-type RCs (6.5 versus 3.5 ps) despite a substantially reduced free energy gap with P* (~75 versus ~160 meV) (Kirmaier et al., 1991). In a complementary manner, the free energy gap for $D^+BPh_L^-$ formation in the heterodimer mutants is reduced (likely more than in the (M)L214H RCs) due to two factors associated with the primary donor: (i) $D^+BPh_L^-$ should lie higher in free energy than in wild-type RCs since D is harder to oxidize than P by over 150 meV (i.e., the D/D^+ potential is ≥600 meV; C.C.S., unpublished results), and (ii) the free energy of the precursor state [$BChl^+BPh^-$] may be lower than P*. This change in energetics has not pushed BPh_L reduction in the heterodimers into the activated region (where the free energy gap for the process is smaller than the reorganization energy), since in both *Rb. capsulatus* (M)H200L RCs and *Rb. sphaeroides* (M)H202L and (L)H173L RCs the rate of $D^+BPh_L^-$ formation is relatively insensitive to temperature (McDowell et al., 1991; L.M.M., D.G., C.K., D.H., and C. C.S., unpublished results). Physical implications of the insensitivity of the initial electron transfer in the RC to temperature and energetics have been discussed elsewhere (Bixon & Jortner, 1989; Lockhart et al., 1990; Parson et al., 1990; Kirmaier et al., 1991; McDowell et al., 1991). The results suggest that the slower rate of BPh_L reduction in the heterodimer mutants is primarily associated with diminished electronic coupling rather than a decreased free energy gap.

Several factors could contribute to a reduced electronic coupling for $D^+BPh_L^-$ formation in the heterodimer mutants. One consideration is localization of the hole on the BChl half of the oxidized heterodimer in state $D^+BPh_L^-$ compared to the more delocalized hole distribution in the oxidized wild-type dimer in $P^+BPh_L^-$. Such hole localization has been found recently in the oxidized M heterodimer (Bylina et al., 1990; Huber et al., 1990) and in the L heterodimer (Huber et al., 1990). Another possibility is that small structural changes reduce the orbital overlap of the primary donor with BPh_L and/or $BChl_L$ in both heterodimer mutants. Finally, we consider decreased electronic coupling with $BChl_L$ due to the higher free energy of $D^+BChl_L^-$ in the mutants. This last point has different mechanistic implications depending on the free energy of $P^+BChl_L^-$ and its role in facilitating electron transfer from P* to BPh_L in wild-type RCs. For example, if $P^+BChl_L^-$ lies several *kT* above P* in wild-type RCs, then $D^+BChl_L^-$ would lie even farther above the initial transient state [$BChl^+BPh^-$] in the heterodimer mutants. $BChl_L$ could then serve in a one-step mechanism such as superexchange in both systems, albeit with a reduced quantum mechanical mixing and hence a reduced electronic coupling in the mutants due to a larger energy denominator. On the other hand, if $P^+BChl_L^-$ lies below or only slightly above P*, then in wild-type RCs $BChl_L^-$ could function as a chemical intermediate (Warshel et al., 1988; Marcus, 1988; Scherer & Fischer, 1989; Bixon et al., 1991). [The experimental evidence for such a two-step mechanism is the subject of considerable debate [see e.g., Breton et al. (1988), Lockhart et al., (1988), Holzapfel et al. (1990), and Kirmaier and Holten (1991).]] The increased energy of $D^+BChl_L^-$ in the heterodimer mutants would reduce or eliminate a contribution from a chemical intermediate mechanism and the overall electronic coupling for BPh_L reduction. A related, but mechanistically different, possibility follows from recent arguments that if $P^+BChl_L^-$ is isoenergetic with or below P* in wild-type RCs, it could serve in a coherent one-step mechanism with an overall rate faster than in the two-step process (Reimers & Hush, 1989). A reduced electronic coupling would be expected in the heterodimer mutants since $D^+BChl_L^-$ would be pushed above the initial transient state, although a one-step mechanism would remain operative.

Relevant to the question of energetics are recent studies on *Rb. sphaeroides* (M)L214H RCs, in which BPh_L is replaced by a BChl (β_L) (Kirmaier et al., 1991). A roughly order-of-magnitude increase in the charge recombination rate of $P^+\beta_L^-$ compared to that of $P^+BPh_L^-$ was proposed to arise largely from increased quantum mechanical mixing with $P^+BChl_L^-$. Since $P^+\beta_L^-$ in the mutant has a free energy about 85 meV higher than $P^+BPh_L^-$, the change in deactivation rate is most readily understood in terms of a reduced superexchange energy denominator if $P^+BChl_L^-$ lies fairly close to or below P* in energy (Kirmaier et al., 1991). However, if $P^+BChl_L^-$ lies much below P* in wild-type RCs, it would lie energetically close enough to $P^+BPh_L^-$ to increase deactivation of the latter and reduce the yield of electron transfer to Q_A (Kirmaier et al., 1991). The results on (M)L214H RCs thus suggest that $P^+BChl_L^-$ lies energetically close to P*, in agreement with recent electrostatic calculations (Parson et al., 1990). This conclusion is in accord with our hypothesis that the increased energy of $D^+BChl_L^-$ reduces the electronic coupling and the rate of BPh_L reduction in heterodimer RCs. It is interesting to point out that the time constants for electron transfer to BPh_L in the heterodimers (Figure 7) approach the time constant for electron transfer from BPh_L^- to Q_A, a process that occurs over a comparable distance (Deisenhofer et al., 1984; Allen et al., 1987) without the participation of $BChl_L$.

Regarding the second process contributing to the lower yield of BPh_L reduction in the heterodimer RCs, the rapid deactivation rates $(\sim 30\ ps)^{-1}$ and $\sim(85\ ps)^{-1}$ of the heterodimer CT-like state of (M)H202L and (L)H173L RCs, respectively, are both some 2 orders of magnitude faster than internal conversion of the excited singlet state of BChl in solution (Tait & Holten, 1983). The enhanced rate of radiationless decay in the *Rb. sphaeroides* and *Rb. capsulatus* heterodimer mutants is reasonably associated with the substantial intradimer CT character of the initial state (Kirmaier et al., 1988; McDowell et al., 1990, 1991; Thompson et al., 1991). Coupled electronic and nuclear motion (the failure of the Born–Oppenheimer approximation) may be enhanced in the heterodimers due to modulation by interring dimer modes (Warshel, 1980) of the energies and mixing of the *close-lying* states having CT/exciton character (McDowell et al., 1991). Hence, the reduced quantum yield of BPh_L reduction in the heterodimer mutants can be traced in large part to the increased intradimer CT character, and thus increased electronic asymmetry, of the initial transient state. It is reasonable to argue that some [$BChl_{LP}^+BChl_{MP}^-$] and [$BChl_{LP}^-BChl_{MP}^+$] character in P* in wild-type RCs is advantageous on the basis of energetics, redox properties, etc. [see e.g., Friesner and Won (1989)]. However, the results on the heterodimer mutants suggest that a reasonably symmetric primary electron donor is advantageous for maximizing the quantum yield of charge separation.

Directionality of Electron Transfer. A number of energetic, electronic, and structural factors have been considered to explain the directionality of electron transfer down the chain of chromophores on the L side of the RC. For example, on the basis of recent calculations it has been suggested that charge asymmetry in P* and differential orbital overlap between the macrocycles of P and $BChl_L$ versus $BChl_M$ make a major contribution to the ratio of the overall electronic couplings between P and BPh_L versus BPh_M (Michel-Beyerle et al., 1988; Plato et al., 1988; Bixon et al., 1989). Asymmetry

in the charge distribution in P* is presumably derived mainly from somewhat unequal contributions of the two opposing intradimer CT configurations [$BChl_{LP}^{+}BChl_{MP}^{-}$] and [$BChl_{LP}^{-}BChl_{MP}^{+}$]. As noted above, we believe that the initially observed transient state in the heterodimer mutants has mainly [$BChl^{+}BPh^{-}$] intradimer CT character and thus possesses significant inherent electronic asymmetry. In (M)H202L RCs, this asymmetry will tend to shift the electron density in the initial dimer transient state toward the M macrocycle (i.e., BPh_{MP}) and in (L)H173L RCs toward the L macrocycle (i.e., BPh_{LP}).

Our results show that charge asymmetry in P* is not critical for the directionality of electron transfer to BPh_L. We have found that state [$BChl_{LP}^{+}BPh_{MP}^{-}$] in the M-side heterodimer and [$BPh_{LP}^{-}BChl_{MP}^{+}$] in the L-side mutant both transfer an electron to BPh_L exclusively (>90%), and with rates that differ by only about a factor of 2 (Figure 7). When one considers the similar photochemistry resulting from mutations on opposite sides of the dimer, it seems unlikely that structural differences between the mutant and wild-type RCs significantly affect the rates and source of the directionality of electron transfer. Therefore, the data imply that differential orbital overlap involving the two macrocycles of P with $BChl_L$ versus $BChl_M$ (or with BPh_L versus BPh_M) is not likely to be a major source of directionality. If the difference in *direct* electronic couplings (orbital overlap) involving P were crucial, then one would expect very different rates of electron transfer in the two heterodimer RCs. The observed two-fold difference in the inherent rate of electron transfer to BPh_L in the two heterodimer mutants may approximate the degree to which electronic asymmetry in P* and differential orbital overlap involving P together contribute to unidirectionality. Our results on the heterodimer mutants thus do not support the idea that directionality of electron transfer is built into the excited primary electron donor.

What are other sources of the unidirectionality of electron transfer? Possibilities include (i) different energetics for electron transfer from P* to BPh_L versus BPh_M, (ii) differences in *indirect* electronic coupling between P* and $P^{+}BPh_L^{-}$ versus $P^{+}BPh_M^{-}$, couplings mediated, for example, by $P^{+}BChl_L^{-}$ versus $P^{+}BChl_M^{-}$ or by protein residues, and (iii) a combination of many small effects, some of which have been discussed above. Regarding the first point, recent experiments have shown that relatively large changes in the energy of state $P^{+}BPh_L^{-}$ only marginally affect the rate of electron transfer from P* to BPh_L and have no measurable effect on the unidirectionality of charge separation (Lockhart et al., 1990; Kirmaier et al., 1991). These observations imply that electron transfer to BPh_M is not prevented by differences in the free energies of $P^{+}BPh_L^{-}$ and $P^{+}BPh_M^{-}$ (or the relative Franck–Condon factors if the reorganization energies are comparable), unless interactions with the protein raise the free energy of $P^{+}BPh_M^{-}$ above P* and make electron transfer to BPh_M endergonic. In this regard, the only major difference in the protein residues that apparently interact directly with the two BPhs is a glutamic acid within hydrogen-bonding distance of the ring V keto group of BPh_L (Michel et al., 1986; Yeates et al., 1988). Genetic replacement of this residue in *Rb. capsulatus* with Leu has only a minor effect on the rate of electron transfer and no detectable effect on directionality (Bylina et al., 1989), in agreement with predictions (Hanson et al., 1987; Michel-Beyerle et al., 1988). Furthermore, calculations including the protein place $P^{+}BPh_M^{-}$ energetically close to $P^{+}BPh_L^{-}$ and hence below P* (Michel-Beyerle et al., 1988; Parson et al., 1990; Thompson & Zerner, 1991).

The ratio of the overall electronic factors for electron transfer from P* to BPh_L versus BPh_M may be enhanced by differences in the electronic contributions of $BChl_L$ and $BChl_M$ other than the direct orbital overlap between P and the BChls. For example, if $P^{+}BChl_M^{-}$ lies substantially above P* and $P^{+}BChl_L^{-}$ close to P*, as recent calculations suggest (Parson et al., 1990), then a larger overall electronic factor for reduction of the L-side BPh would arise either by a more substantial quantum mechanical mixing of $P^{+}BChl_L^{-}$ with P* and with $P^{+}BPh_L^{-}$ in a one-step process or by a contribution from a two-step mechanism (vide supra). It has also been suggested that the orbital overlap between $BChl_L$ and BPh_L is larger than between $BChl_M$ and BPh_M, which would also preferentially enhance the overall electronic coupling for $P^{+}BPh_L^{-}$ formation (Michel et al., 1986; Allen et al., 1987; Plato et al., 1988; Bixon et al., 1989, 1991). The energies of the ionic states and mediation of electron transfer from P* to the BPh(s) by the protein may be affected by differences in amino acid residues (conformation and identity) between the two branches.

In light of the findings on all mutant RCs to date, it seems that no once source of directionality dominates, but rather that preferential electron transfer via the L-branch prosthetic groups may be derived from a combined contribution of a number of small effects involving all six chromophores and many protein residues. From an evolutionary standpoint, this would be an obvious and effective way to ensure that preferential unidirectional electron transfer via the L-branch chromophores would be maintained despite mutations or environmental effects that might perturb individual pigment–pigment or pigment–protein interactions.

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