

## Excited State Energy Transfer Pathways in Photosynthetic Reaction Centers. 2. Heterodimer Special Pair

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Ultrafast singlet excited state energy transfer occurs from the monomeric chromophores to the primary electron donor or special pair in photosynthetic reaction centers. The mechanism of this process has been investigated using reaction centers whose special pair absorption is severely perturbed by removal of the Mg atom of one of the bacteriochlorophylls (the heterodimer mutant (M)H202L). This was achieved by observing the rise time kinetics and induced anisotropy in the spontaneous fluorescence from the heterodimer special pair following excitation of the monomeric chromophores using fluorescence up-conversion at 85 K. The rise is characterized by two time constants. By scanning the excitation wavelength across the absorption bands of the monomeric bacteriochlorophyll and bacteriopheophytin, it is demonstrated that the two components correspond to energy transfer along the L (functional) and M (nonfunctional) branches of chromophores. This contrasts with the more symmetric native special pair, where energy transfer along the two branches has been shown to have comparable rates (Stanley et al. *J. Phys. Chem.* **1996**, *100*, 12052–12059). Thus, the electronic asymmetry of the heterodimer special pair alters the interactions on the functional and nonfunctional sides of the reaction center that are responsible for singlet energy transfer.

The bacterial photosynthetic reaction center (RC) is designed to efficiently accept excitation energy from antenna complexes and rapidly transfer energy internally to the special pair primary electron donor, P. <sup>1</sup>P then transfers an electron within a few picoseconds to an electron acceptor, irreversibly trapping the excitation energy in a transient charge-separated species. A schematic diagram illustrating the chromophores that are relevant to both the energy and electron transfer processes in isolated RCs is shown in Figure 1 based on the X-ray structure.<sup>1</sup> The chromophores labeled B<sub>L</sub> and B<sub>M</sub> are monomeric bacteriochlorophylls on the functional and nonfunctional sides, respectively, of the RC; the chromophores labeled H<sub>L</sub> and H<sub>M</sub> are monomeric bacteriopheophytins on the functional and nonfunctional sides, respectively. Functional denotes the electron transfer process <sup>1</sup>P → P<sup>+</sup>H<sub>L</sub><sup>−</sup> which is found to occur in normal RCs at all temperatures. The structural symmetry of the RC suggests that <sup>1</sup>P → P<sup>+</sup>H<sub>M</sub><sup>−</sup> might be equally likely to occur, but it does not to any appreciable extent. In earlier work we showed that energy transfer from <sup>1</sup>B → P and from <sup>1</sup>H → P is very fast and, by using mutant (M)L214H (the β mutant where a bacteriochlorophyll β<sub>L</sub> replaces H<sub>L</sub>) in which selective excitation of β<sub>L</sub> or H<sub>M</sub> is possible, showed that the rates of energy transfer along the L and M branches are comparable.<sup>2</sup>

P is a homodimer of bacteriochlorophylls. If either of the coordinating histidine ligands to the central Mg atoms (histidines M202 or L173 in *Rb. sphaeroides*, see the expanded view of the special pair in the lower part of Figure 1) is replaced with leucine, the coordinated Mg atom is lost from the bacteriochlorophyll, and a bacteriopheophytin with two hydrogens replacing the Mg is found in its place. These mutant RCs have been named the heterodimer ((M)H202L)<sup>3</sup> and reverse heterodimer ((L)H173L).<sup>4</sup> The three-dimensional structure of the heterodimer-containing RC shows little change in the organization of the chromophores aside from the loss of electron density ascribed to the central Mg atom and the replacement of histidine M202 with leucine.<sup>5</sup> The special pair absorption is severely perturbed compared to the homodimer in wild type as shown

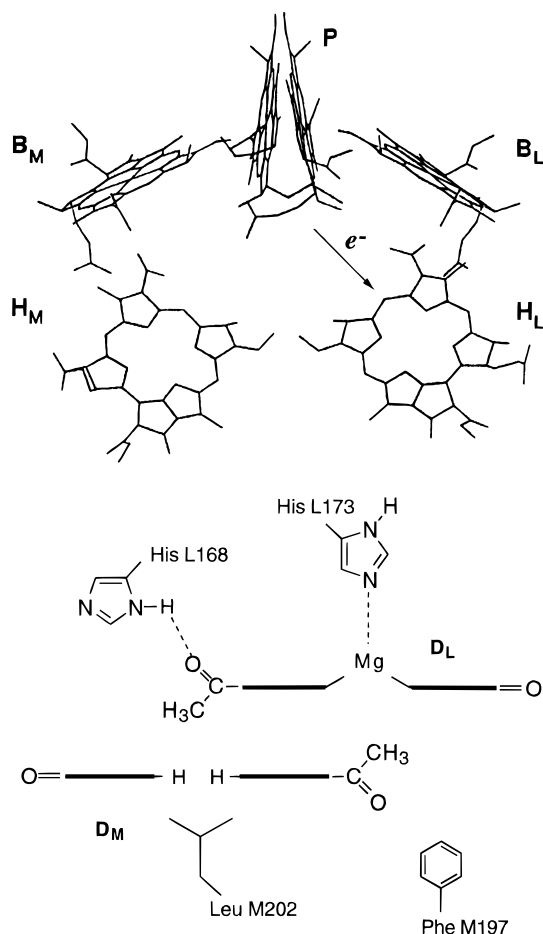
in Figure 2. The single Q<sub>Y</sub> absorption band of P is replaced by a broad absorption at room temperature, which appears as two relatively broad bands at cryogenic temperatures centered at 850 and 920 nm. This heterodimer special pair is denoted D to distinguish it from P. Both bands of D exhibit a large Stark effect,<sup>6–8</sup> suggesting substantially more charge transfer character than for the homodimer. The electron transfer dynamics are also perturbed. Laporte et al. report <sup>1</sup>D lifetimes of 37 ps at room temperature and 51 ps at 80 K.<sup>9</sup> No evidence for stimulated emission from <sup>1</sup>D was found at 273 K;<sup>3,10</sup> some evidence for stimulated emission between 920 and 950 nm was later observed at 77 K.<sup>9</sup> Steady state and time-resolved fluorescence from <sup>1</sup>D have not been previously reported. As shown in the following, <sup>1</sup>D fluorescence is peaked at 970 nm at 77 K (Figure 2B), and the rise time of its emission on the femtosecond time scale can be used to measure energy transfer from the monomeric bacteriochlorophyll and bacteriopheophytin chromophores along the L and M sides.

### Experimental Section

The low-temperature fluorescence up-conversion spectrometer has been described in detail previously,<sup>11,2</sup> along with methods for measuring fluorescence anisotropies and analyzing the data.<sup>2</sup> The spectral bandwidths of the excitation pulses were measured at each excitation wavelength, and typical full widths at half-maximum are indicated by the horizontal bars in Figure 2B (typically 150–200 cm<sup>−1</sup>). For room temperature measurements, the sample was rapidly stirred in a 1-mm path length quartz cuvette and was rastered between scans (every 1–3 min). Low temperature was achieved by using a miniature Joule-Thompson refrigerator (MMR Technologies, Mountain View, CA) and a very thin sample geometry.<sup>11</sup>

Wild-type (WT) and heterodimer (M)H202L *Rb. sphaeroides* were grown semiaerobically using the expression system described by McDowell et al.<sup>12</sup> RCs from WT were isolated and quinone depleted by the method of Okamura et al.;<sup>13</sup> quinone Q<sub>A</sub> was reduced in heterodimer RCs in triton X-100 using sodium dithionite. All samples were dissolved in 1/1 (v/

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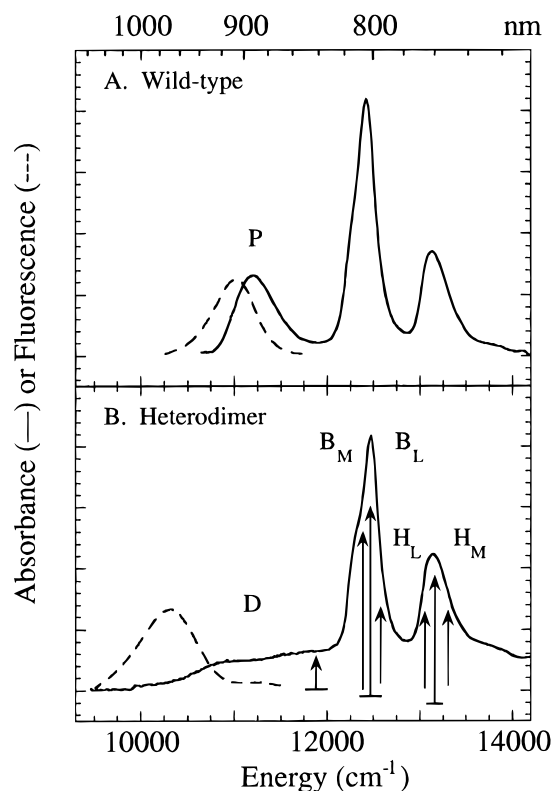


**Figure 1.** Schematic diagram of the chromophores involved in the initial energy and electron-transfer processes of photosynthetic reaction centers taken from the X-ray structure.<sup>1</sup> In the heterodimer mutant (M)H202L, the bacteriochlorophyll on the right of the special pair as illustrated is converted to a bacteriopheophytin, but the overall structure is otherwise conserved.<sup>5</sup> This is shown in greater detail in the lower diagram.

v) glycerol/buffer (10 mM Tris, pH 8.0) solution. The RCs were concentrated in order to achieve a sufficient optical density in the 25–70  $\mu\text{m}$  path length cell, typically 0.1–0.5 at 800 nm.

## Results

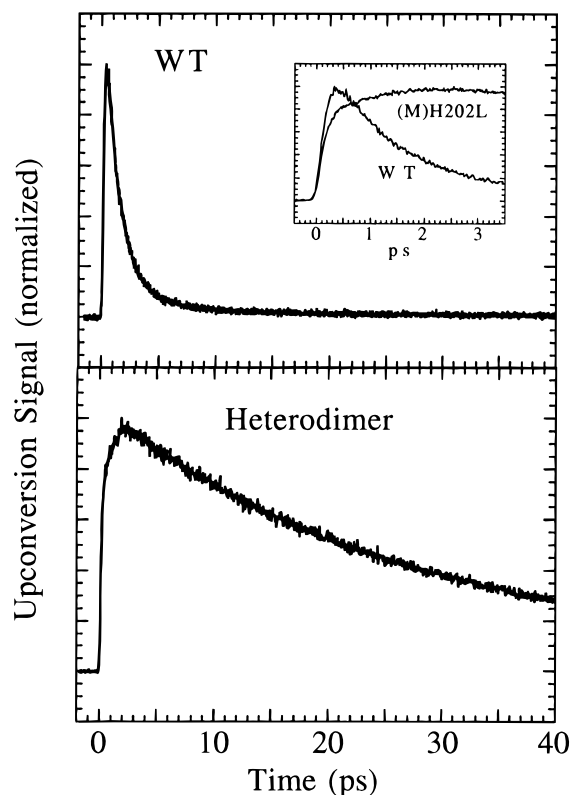
The uncorrected steady state fluorescence spectrum of heterodimer RCs at 77 K is shown in Figure 2B. The fluorescence is exclusively associated with the lower energy feature of the heterodimer special pair  $Q_Y$  absorption and is centered at about 970 nm. This wavelength was chosen for all subsequent upconversion experiments. The rise and initial decay of the fluorescence at 85 K following B excitation at 800 nm from  $^1\text{P}$  in wild-type and  $^1\text{D}$  in the heterodimer measured at 920 and 970 nm, respectively, are compared in Figure 3. The decay of the fluorescence from  $^1\text{D}$  is much slower than from  $^1\text{P}$ , reflecting the much slower rate of the initial electron transfer reaction.<sup>9</sup> The  $^1\text{D}$  decay required two exponential components to obtain a reasonable fit both at room temperature and at 85 K. At room temperature (data not shown), components with decay times of 10 and 25 ps were obtained with relative contributions of 45 and 55%, respectively; at 85 K the decay times were 24 and 71 ps with relative contributions of 66 and 34%, respectively. The anisotropy is  $0.274 \pm 0.002$  for excitation at 800 nm (at 85 K), corresponding to an angle of  $27 \pm 1^\circ$  between the transition moment at 800 nm and the emission transition moment of  $^1\text{D}$ . This is in good agreement with similar measurements made



**Figure 2.** Electronic absorption spectra in the  $Q_Y$  region at 77K for *Rb. sphaeroides* (A) wild-type and (B) heterodimer mutant (M)H202L. The uncorrected fluorescence spectra of the special pair in both species are also shown with dotted lines. The vertical arrows indicate wavelengths chosen for excitation with the measured spectral full width at half-maximum of the excitation pulses indicated with the horizontal lines.

on WT RCs<sup>2</sup> and with linear dichroism measurements<sup>14</sup> on the heterodimer and demonstrates that the absolute direction of the transition moment of P is maintained when  $\text{P}_M$  loses a magnesium forming the heterodimer D.

It is evident from the inset in Figure 3 that the rise of the fluorescence of  $^1\text{D}$  following excitation of B is more complex than for  $^1\text{P}$ , with an obvious slower component. The effect of tuning the excitation wavelength across the absorption features associated with the monomeric B and H chromophores (cf. Figure 2B) on the rise of  $^1\text{D}$  fluorescence at 970 nm is shown in Figure 4. The decay of  $^1\text{D}$  fluorescence is not shown, but it was found to be independent of excitation wavelength. In contrast, the rise of the fluorescence of  $^1\text{D}$  does change with excitation wavelength. Qualitatively, tuning from low to high energy in the B band region leads to an increase in the relative contribution from the slower rising component, while tuning from low to high energy in the H band region leads to an increase in the relative contribution from the faster rising component. The data were analyzed quantitatively as described in detail by Stanley et al.<sup>2</sup> The rise time could be fit well in all cases with two components, and the results are summarized in Table 1. The listed values are averages of fit parameters for at least three data sets at each excitation wavelength. The individual data sets were collected with a step size of 21 fs/point over the first 7.5 ps of the decay, and the fits to the data generally yielded reduced  $\chi^2$  of less than 1.2. Within the experimental error it is evident that the time constants for the two rise components are independent of excitation wavelength,<sup>15</sup> but their amplitudes change substantially. The sum of the amplitudes of the rise components for each data set varies from 90 to 98%. The remaining amplitude is instrument



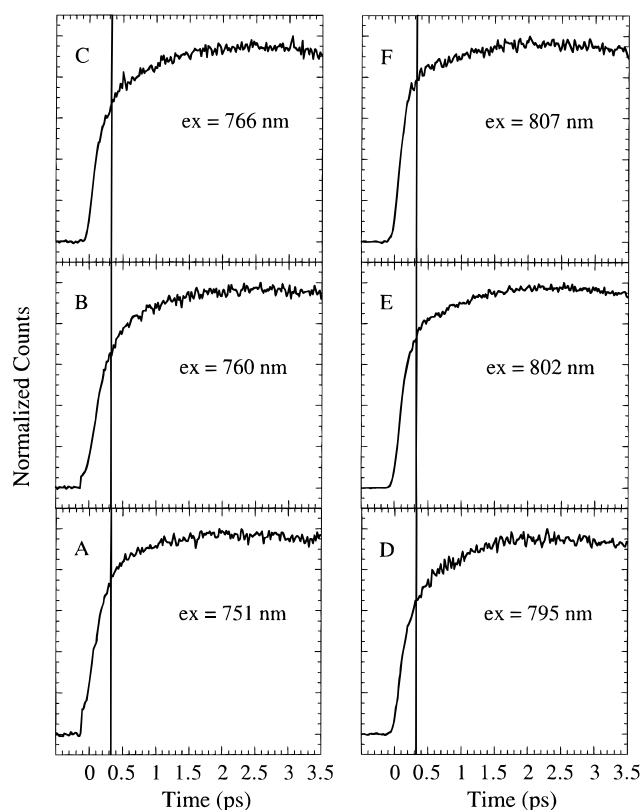
**Figure 3.** Spontaneous fluorescence at 85 K following excitation of B at 800 nm from  $^1P$  measured at 920 nm for wild-type RCs and at 970 nm for heterodimer (M)H202L RCs. The inset shows the rise times on an expanded time base.

response limited and may be fluorescence from  $^1D$  due to direct excitation of the underlying absorption of the broad heterodimer  $Q_Y$  band.

Excitation of the higher energy feature associated with D at 841 nm (cf. Figure 2B) gave a rise time for  $^1D$  fluorescence at 970 nm that was within the instrument response function followed by a similar but more complex decay than was observed for excitation into either B or H (data not shown). The model required a small-amplitude rapid decay component ( $<30\%$  with a decay time of  $108 \pm 9$  fs) in addition to the two components mentioned above. The associated fluorescence anisotropy was  $0.392 \pm 0.004$ . Thus, the transition dipole excited at 840 nm is nearly parallel to the emission dipole moment at 970 nm. This rapid decay component would not be detectable with excitation into the B or H bands as it is faster than the rise time of  $^1D$  fluorescence due to energy transfer from these chromophores. This data and the effects of exciting directly into the lower energy band will be discussed elsewhere.<sup>16</sup>

## Discussion

The observation of fluorescence from the heterodimer special pair  $^1D$ , whether steady-state (Figure 2B) or time resolved (Figure 3), is a significant result as it proved difficult to observe stimulated emission from the heterodimer special pair. Some evidence for stimulated emission was reported by LaPorte et al. at 77 K.<sup>9</sup> The presence of absorption peaked at 655 nm immediately after excitation has been reported.<sup>3,9,10,12,17,18</sup> Absorption in this region has characteristics similar to a bacteriopheophytin anion, leading to the suggestion that  $^1D$  has substantial charge transfer character. This is consistent with the observation of substantial charge transfer character for the  $Q_Y$  bands of D observed by Stark spectroscopy.<sup>6-8</sup>



**Figure 4.** Rise time of the spontaneous fluorescence at 85 K from  $^1D$  in heterodimer RCs measured at 970 nm following excitation at various wavelengths in the H (panels A–C) and B (panels D–F) band region (cf. Figure 2B). The vertical lines are guides for comparing the relative intensities at 0.3 ps for the various excitation wavelengths.

**TABLE 1: Amplitudes ( $A_1$  and  $A_2$ ) and Corresponding Rise Times ( $\tau_1$  and  $\tau_2$ , in fs) for Fluorescence from  $^1D$ , the Excited State of the Heterodimer Special Pair, following Excitation at Various Wavelengths across the H and B Bands at 85 K (cf. Figure 2B)<sup>a</sup>**

	excitation wavelength		
	751 nm	760 nm	766 nm
H-band			
$A_1$	$-36.64 \pm 1.53$	$-47.67 \pm 4.68$	$-48.40 \pm 2.36$
$A_2$	$-52.87 \pm 5.84$	$-47.66 \pm 7.81$	$-43.16 \pm 2.97$
$A_1/A_2$	$0.69 \pm 0.08$	$1.00 \pm 0.25$	$1.12 \pm 0.04$
$\tau_1$	$824 \pm 31$ fs	$813 \pm 45$ fs	$778 \pm 45$ fs
$\tau_2$	$157 \pm 9$ fs	$153 \pm 24$ fs	$118 \pm 21$ fs
	excitation wavelength		
	795 nm	802 nm	807 nm
B-band			
$A_1$	$-51.65 \pm 1.88$	$-40.42 \pm 0.76$	$-33.72 \pm 1.32$
$A_2$	$-44.76 \pm 0.95$	$-55.78 \pm 1.51$	$-63.68 \pm 0.69$
$A_1/A_2$	$1.15 \pm 0.06$	$0.72 \pm 0.02$	$0.53 \pm 0.03$
$\tau_1$	$784 \pm 46$ fs	$866 \pm 104$ fs	$873 \pm 41$ fs
$\tau_2$	$139 \pm 8$ fs	$101 \pm 11$ fs	$99 \pm 3$ fs

<sup>a</sup> The amplitudes are given as negative as these are rising components; the decay of the  $^1D$  fluorescence due to electron transfer occurs on a much longer time scale and is independent of excitation wavelength.

The weighted average of the  $^1D$  decay times we observe is approximately the same as those measured by LaPorte et al.<sup>9</sup> using transient absorption changes. Those investigators fit their data to a single exponential, appropriate given the signal-to-noise and the complication of fitting further the secondary decay of  $D^+H_L^-$  to  $D^+Q_A^-$  in competition with recombination to the neutral ground state. The fluorescence up-conversion signals are not complicated by overlapping transient absorption features or subsequent dark chemistry. In this regard our observation of multiexponential  $^1D$  decay parallels the history of WT RC

kinetics, where all investigators initially reported a single-exponential  $^1\text{P}$  decay,<sup>19–21</sup> and it was later observed, first by fluorescence up-conversion<sup>22,23</sup> and later as the signal-to-noise improved by transient absorption,<sup>24</sup> that the  $^1\text{P}$  decay is somewhat heterogeneous.

It is interesting and significant that the transition dipole moment associated with the heterodimer higher energy absorption band at 850 nm is parallel, within the experimental error, to that of the heterodimer emission dipole moment at 970 nm. This indicates that the 850 nm transition is not the upper exciton band of D which is expected to be roughly orthogonal to the lower exciton band. A related result is that selective excitation of the 920 nm band in  $\text{Q}_\text{A}$ -containing heterodimer RCs leads to bleaching of both the 920 and 840 nm bands,<sup>6</sup> demonstrating that they share a common ground state. Thus, the band at 850 nm is not a contaminant or degradation product. The precise origin of the higher energy band is not known. It was not considered in the relatively detailed simulations of the absorption and Stark spectra of the 920 nm band.<sup>25</sup> Recent work from our group leads to the simple suggestion that the 850 and 920 nm bands arise from splitting of the  $\text{Q}_\text{Y}$  transition by a nearly degenerate charge transfer state.<sup>26</sup> In this interpretation the transition dipole moments are expected to be parallel.

The emphasis of the work reported here is the asymmetry in arrival time of excitation energy along the L and M sides of the RC for the heterodimer special pair, in contrast with the homodimer in wild-type. The data in Figure 4 and Table 1 show that the two resolved rise times in the  $^1\text{D}$  population are present in different proportions as the excitation wavelength is tuned across the H and B bands. Neither the H nor B absorption feature centered at 760 and 802 nm, respectively, is well-resolved into two bands corresponding to the L and M side chromophores, though the resolution is substantially improved by working at low temperature. There is good evidence that the higher energy side of the H band is associated with  $\text{H}_\text{M}$  while the lower energy side is associated with  $\text{H}_\text{L}$ . This is particularly clear in the  $\beta$  mutant ((M)L214H) where  $\text{H}_\text{L}$  is converted to a bacteriochlorophyll<sup>27</sup> whose  $\text{Q}_\text{Y}$  absorption band shifts to lower energy, leaving the  $\text{H}_\text{M}$  absorption at 759 nm.<sup>28</sup> Likewise, in the  $\text{D}_{\text{LL}}$  mutant of *Rb. capsulatus*, which lacks the  $\text{H}_\text{L}$  chromophore altogether, both the absorption and Stark spectrum are consistent with loss of the lower energy band.<sup>29,30</sup> The assignments in the B band region are more problematic. Most data in the literature are consistent with  $\text{B}_\text{L}$  absorption at higher energy and  $\text{B}_\text{M}$  at lower energy, with the latter contributing most, if not all, of the intensity to the shoulder observed around 810 nm at low temperature.<sup>31</sup> This shoulder is more pronounced in WT, where carotenoid is positioned nearby  $\text{B}_\text{M}$ , than in the carotenoidless R-26 strain.<sup>32</sup> Transient linear dichroism measurements show that the shoulder does not change on going from  $\text{P}^+\text{H}_\text{L}^-$  to  $\text{P}^+\text{Q}_\text{A}^-$  in *Rb. sphaeroides* while the feature at higher energy around 800 nm does.<sup>33</sup> As  $\text{B}_\text{L}$  is positioned between P and  $\text{H}_\text{L}$ , its absorption is expected to be more sensitive to the change in electric field associated with this charge shift reaction than  $\text{B}_\text{M}$ . We are thus led to the energy ordering of the L and M side monomeric chromophores shown in Figure 2B.

These assignments give a context for analyzing the components of the rise in the fluorescence at 970 nm from the lower energy state of the heterodimer as the excitation wavelength is tuned across the B and H bands. Given the energy bandwidth of the laser and the degree of band overlap, pure excitation of the monomeric L and M side chromophores is never achieved; however, their relative contributions should change as the excitation wavelength is tuned across each band. Two com-

ponents are clearly resolved in the rise time of  $^1\text{D}$  emission, and their relative amplitudes change as the excitation energy is tuned across each monomeric absorption band. The contribution of the slower component increases as the excitation energy is tuned from the high to the low-energy side of the H band; the contribution of the faster component increases as the excitation energy is tuned from the high to the low-energy side of the B band. Because the energy ordering of the L and M side chromophores is reversed in the H and B band regions, the simplest interpretation of the results is that the energy transfer rates along the L and M sides are different, with the faster rise time associated with excitation along the M side and the slower rise time associated with excitation on the L side. This contrasts with the situation for wild-type homodimer RCs where energy transfer on the L and M sides from the monomeric chromophores to P is found to be comparable.<sup>2</sup> Interestingly, the rate of energy transfer along the M side in the heterodimer is comparable to that in the WT homodimer. The measured lifetime for the faster rising component of  $^1\text{H}$  to D is about 50% longer than that for  $^1\text{B}$  to D (see Table 1). If we assume a two-step model for energy transfer, first from  $^1\text{H}$  to B and second from  $^1\text{B}$  to D, then the rate of each step must be similar. In good agreement with this finding, Stanley et al. estimate that the lifetime for  $^1\text{H}$  to B is about 160 fs.<sup>2,34</sup> The lifetime of the slower rising component for  $^1\text{B} \rightarrow \text{D}$  is at least 4 times longer than that for  $^1\text{H} \rightarrow \text{B}$ . In this case the expected small change in the lifetime of this component for H excitation will not be measurable within the experimental uncertainty.

The mechanism of ultrafast singlet energy transfer in RCs has been the subject of considerable discussion.<sup>2,35–37</sup> As seen in Figure 2, the spectral overlap expected for the  $^1\text{B}$  fluorescence (assuming a small Stokes shift) with the special pair absorption in either the homodimer or heterodimer is small.<sup>38</sup> The distances between the chromophores in the heterodimer mutant are the same, within the experimental error, as in WT,<sup>5</sup> and the orientations of the transition moments are conserved.<sup>14</sup> These observations, along with estimates based on Förster dipole–dipole theory (applied to WT),<sup>35</sup> have led to the suggestion that the Förster dipole–dipole energy transfer mechanism does not dominate energy transfer from  $^1\text{B}$  to P. The precise role of the upper exciton band in mediating energy transfer is still an open question; however, the upper exciton component is expected to be quite different in position and intensity in the heterodimer compared with the homodimer,<sup>31</sup> and energy transfer, at least along the M side, is about as fast to the heterodimer special pair as to the homodimer. A resolved higher energy transition at 850 nm is observed in the heterodimer. The anisotropy of nearly 0.4 induced in emission at 970 nm following excitation of this feature rules out the possibility that this is the upper exciton band. These results and others have led to the consensus that a mechanism involving direct orbital overlap between  $^1\text{B}$  and P (or D) dominates the energy transfer process.<sup>2,35,36</sup>

It is evident from the absorption and Stark spectra that the relevant orbitals of the heterodimer are quite different from those of the homodimer. Likewise, the unpaired spin density distribution in the cation radicals  $\text{D}^+$  and  $\text{P}^+$  produced by the light-induced charge separation are quite different, with the hole localized to a greater extent on the bacteriochlorophyll part of the heterodimer.<sup>39,40</sup> Neither of these measurements can provide a precise description of the asymmetry of the wave function in D as compared with P which would permit a quantitative prediction of the asymmetry in the energy transfer rates to D from  $^1\text{B}_\text{M}$  vs  $^1\text{B}_\text{L}$ . Nonetheless, it is reasonable to suggest that the two observed singlet energy transfer rates result from the electronic asymmetry in the heterodimer special pair as com-

pared with the homodimer. As argued in ref 2, it is reasonable to correlate relative singlet energy transfer rates on the functional and nonfunctional sides with electronic coupling for energy transfer when Förster dipole–dipole transfer does not dominate and further correlate this relative electronic coupling with that which is relevant for singlet electron transfer. This suggests that observations of singlet energy transfer rate asymmetry in a variety of RC mutants where the special pair is electronically perturbed may prove to be useful in assessing the functional consequences of electronic asymmetry. Such studies are in progress.

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- The upper exciton band of P, sometimes denoted  $^1P_{Y+}$ , may also contribute to the absorption at 810 nm at low temperature in WT RCs<sup>32</sup> (Breton, J. *Biochim. Biophys. Acta* **1985**, *810*, 235–245. Knapp, E. W.; Fischer, S. F.; Zinth, W.; Sander, M.; Kaiser, W.; Deisenhofer, J.; Michel, H. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 8463–8467. Vermeglio, A.; Clayton, R. K. *Biochim. Biophys. Acta* **1976**, *449*, 500–515. Xie, X.; Simon, J. D. *Biochim. Biophys. Acta* **1991**, *1057*, 131–139). Because the two monomers comprising the special pair in the heterodimer are nondegenerate and the oscillator strength of bacteriochlorophyll *a* is smaller than that of bacteriochlorophyll *b*, the exciton splitting in the heterodimer is expected to be smaller than the in homodimer (all other things being equal). Consistent with this, the negative linear dichroism feature seen in WT at around 810 nm is absent in the heterodimer spectrum.<sup>14</sup> As seen in Figure 2, the 800 nm region of the absorption spectrum at low temperature is relatively unchanged when comparing WT with heterodimer, despite the large perturbation of the  $Q_Y$  absorption band of the special pair in the heterodimer. It is difficult to make quantitative comparisons of the absorption spectra of WT and heterodimer at the 10% level as this depends on an accurate base line which is difficult to obtain as the scattering of samples at low temperature is never identical. Nonetheless, if we argue that the upper exciton band intensity in the homo- and heterodimers should be very different, the data in Figure 2 indicate that the intensity of the upper exciton band in the 800 nm region in the homodimer makes only a very small contribution. A similar result emerged from quantitative band-shift analysis.<sup>28</sup>
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