Excited State Energy Transfer Pathways in Photosynthetic Reaction Centers. 4. Asymmetric Energy Transfer in the Heterodimer Mutant

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In bacterial photosynthetic reaction centers, ultrafast singlet excited state energy transfer occurs from the monomeric bacteriochlorophylls, B, and bacteriopheophytins, H, to the homodimer special pair, a pair of strongly interacting bacteriochlorophylls. In the M202HL mutant, one of the bacteriochlorophylls comprising the special pair is replaced by a bacteriopheophytin, and this is called the heterodimer special pair or D. We report the direct observation of spontaneous fluorescence from ^{1}B in the heterodimer mutant. In contrast to results for the homodimer special pair where ^{1}B decays with a rate constant of $(\sim 160\ \text{fs})^{-1}$ (King, B. A.; McAnaney, T. B.; de Winter, A.; Boxer, S. G. *J. Phys. Chem. B* **2000**, *104*, 8895−8902), ^{1}B decay in M202HL exhibits two components with rate constants $(\sim 700\ \text{fs})^{-1}$ and $(\sim 190\ \text{fs})^{-1}$; these are similar to what we reported earlier for the rise of ^{1}D spontaneous fluorescence (King, B. A.; Stanley, R. J.; Boxer, S. G. *J. Phys. Chem. B* **1997**, *101*, 3644−3648). In the double mutant M202HL/M182HL, where the accessory bacteriochlorophyll on the M side is replaced by a bacteriopheophytin, the absorption bands corresponding to the chromophores in the B_L and B_M binding sites are quite well resolved, and it is possible to preferentially excite the chromophore on either the L or the M side. Analysis of the rise of ^{1}D fluorescence in the double mutant supports the earlier assignment of the slower ~700 fs energy transfer component to $^1B_L \rightarrow D$, while the faster ~190 fs energy transfer component is assigned to $^1B_M \rightarrow D$. Replacement of bacteriochlorophyll by bacteriopheophytin in the B_M binding site does not alter the time constants of the two energy transfer pathways. Excited state energy transfer to D is the same in Q_A-depleted and Q_A-reduced reaction centers, suggesting that electron transfer processes that be might sensitive to a charge on Q_A, such as $^1B_L \rightarrow B_L^+H_L^-$, do not compete with relatively slow $^1B_L \rightarrow D$ energy transfer. The results support earlier findings that singlet energy transfer from the monomeric chromophores along the L and M branches to the heterodimer special pair is asymmetric and is faster along the M side, in contrast to the homodimer special pair in wild type where the energy transfer rates along the two branches are very similar. Thus, conversion of the special pair homodimer to a heterodimer breaks the symmetry of ultrafast energy transfer along the two branches of chromophores. These findings may provide information on differences in the electronic interactions on the L vs M sides of the RC that is relevant to unidirectional *electron* transfer.

The bacterial photosynthetic reaction center (RC) is responsible for the initial light-driven charge separation events in photosynthesis. Light energy absorbed by antenna complexes is funneled to the special pair (P) in the RC; ^{1}P then transfers an electron to an electron acceptor, rapidly trapping the excitation energy in a transient charge-separated species. A schematic diagram based on the X-ray structure illustrating the arrangement of the relevant chromophores is shown in the upper part of Figure 1 and a putative singlet excitation energy transfer scheme paralleling the structure is shown in the lower part of Figure 1. In isolated RCs, excitation of the special pair can be achieved by rapid and efficient singlet excited state energy transfer from the monomeric bacteriopheophytins and bacteriochlorophylls. The chromophores labeled B_L and B_M are monomeric bacteriochlorophyll *a* on the functional and nonfunctional sides, respectively, of the RC; the chromophores labeled H_L and H_M are monomeric bacteriopheophytin *a* on the functional and nonfunctional sides, respectively. Functional is used here to denote the electron transfer process $^1P \rightarrow P^+H_L^-$ which is found to occur almost exclusively in normal RCs at all temperatures, despite the structural symmetry of the RC which suggests that $^1P \rightarrow P^+H_M^-$ might be equally likely to occur.

The rate of singlet excited state energy transfer from the B and H chromophores to P can be probed by femtosecond transient absorption spectroscopy[1−5] or by measuring the rise of ^{1}P fluorescence using fluorescence upconversion. Using the mutants M214LH (the β mutant, where a bacteriochlorophyll, β_L, replaces H_L) and M182HL (where a bacteriopheophytin, θ_M, replaces B_M), in which selective excitation of the L- or M-side accessory chromophores is possible, we showed that the rates of energy transfer along the L and M branches of chromophores are comparable.[6,7] Specifically, ^1B_M and ^1B_L to P energy transfer occurs in about 160 fs. The rate of singlet energy transfer from the B and H chromophores to P can also be probed by measuring the rise and decay of ^{1}B emission using fluorescence upconversion. In wild-type and M182HL RCs, the excited state decay of ^{1}B closely matches the rise of fluorescence from ^{1}P, and following excitation of H, energy transfer occurs by a two-step sequential mechanism: $^1H \rightarrow B \rightarrow P$.[7]

In the M202HL (heterodimer) mutant, one of the coordinating histidine ligands to the special pair, histidine M202, is replaced

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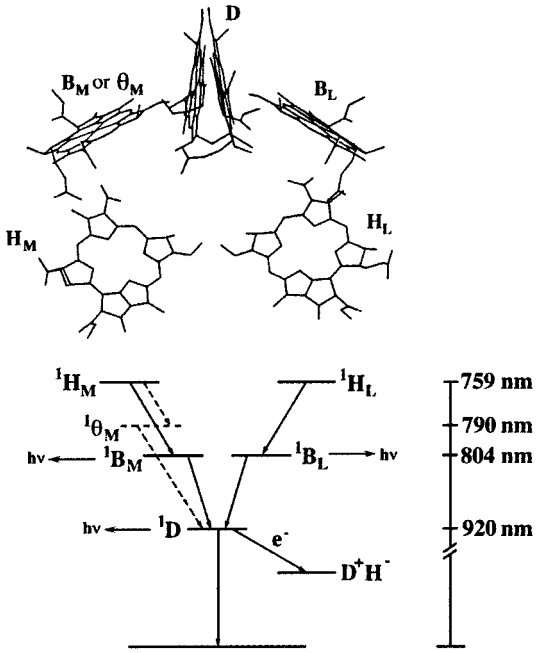

Figure 1. (top) Schematic diagram of the chromophores involved in the energy and electron transfer processes of isolated M202HL or M202HL/M182HL *Rb. sphaeroides* photosynthetic reaction centers taken from the X-ray structure.[44] (bottom) Schematic energy level diagram, using wavelengths from the absorption spectra at 77 K, illustrating possible energy transfer pathways paralleling the structure in the upper part of the figure.

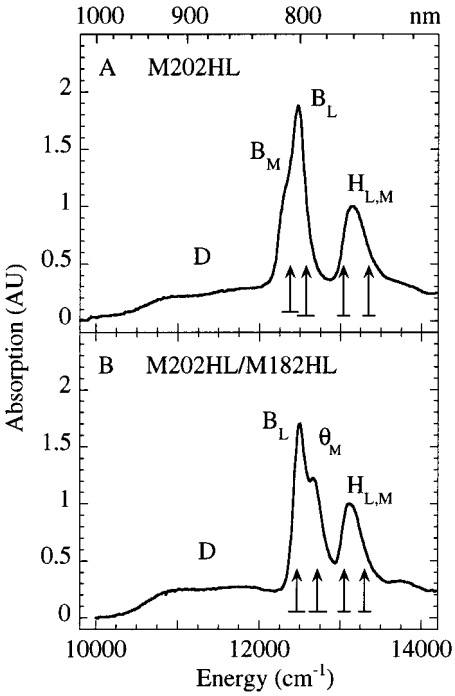

Figure 2. Absorption spectra in the Q_Y region at 77 K for *Rb. sphaeroides* M202HL (A) and Q_A-reduced M202HL/M182HL in which a bacteriopheophytin *a*, labeled θ_M, replaces a bacteriochlorophyll in the B_M binding site (B). The spectra have been normalized to their maximum absorption in the H band. The vertical arrows illustrate wavelengths used for excitation, and the horizontal bars indicate the spectral fwhm of the excitation pulse.

by leucine and the central Mg^{2+} ion is lost from the bacteriochlorophyll, resulting in the incorporation of a bacteriopheophytin.[8] The Q_Y absorption band of the special pair (designated D in the heterodimer mutant) is dramatically perturbed with respect to wild type as shown in Figure 2A. It appears as a much broader absorption with poorly resolved features at 840 and 920 nm at 77 K.[9,10] Electron transfer in the heterodimer mutant remains unidirectional; however, the excited state dynamics of ^1D are substantially different from wild type. The rate of electron transfer is nearly an order of magnitude slower than in wild type at both room and cryogenic temperatures, and substantial competing nonradiative decay occurs.[8,11−15] In contrast to wild type, by monitoring the rise of emission from ^1D at 970 nm and scanning the excitation wavelength across the partially resolved H_L/H_M absorption bands at 760 nm and the B_L/B_M absorption bands at 800 nm, we found that energy transfer along the L side is substantially slower. Specifically, it was found that $^1B_L \rightarrow D$ occurs in ∼840 fs, while $^1B_M \rightarrow D$ occurs in ∼110 fs. We denote this *asymmetric energy transfer*, in contrast with what is observed in homodimer-containing RCs.[16]

In the present work, we present further evidence for asymmetric energy transfer by direct observation of ^1B emission in the heterodimer mutant and by observation of the rise of ^1D emission following preferential excitation of θ_M or B_L in the M202HL/M182HL mutant. In addition, we extend our earlier measurements of the rise of ^1D fluorescence in Q_A-reduced (Q_A^-) heterodimer RCs using Q_A-depleted heterodimer RCs in order to investigate the suggestion by van Brederode et al. that our previous results were affected by Q_A reduction.[17] We find this not to be the case, and provide further support for our hypothesis that ultrafast singlet energy transfer can provide insight into the electronic interactions that are relevant to unidirectional electron transfer.

Experimental Section

The low-temperature fluorescence upconversion spectrometer has been described in detail, along with methods for measuring fluorescence anisotropies and analyzing the data.[6,18] Briefly, samples were excited using a mode-locked Ti:sapphire laser (Spectra Physics Tsunami) pumped by 6−10 W (all lines) from an argon ion laser (Spectra Physics Model 2080). For experiments exciting in the B band, the pulse widths were ∼80 fs with a time−bandwidth product typically less than 0.43. The maximum pulse width used in these experiments was ∼105 fs when exciting in the H band. The spectral bandwidths of the excitation pulses were measured at each excitation wavelength, and typical full widths at half-maximum (fwhm) are indicated by horizontal bars in Figure 2 (typically 150−200 cm^{-1}). Samples were excited at the magic angle with <250 pJ of energy per pulse.[19] Low temperature (85 K) was achieved by using a miniature Joule−Thompson refrigerator (MMR Technologies, Mountain View, CA) and very thin sample geometry. The individual data sets were collected with a delay line step size of 21 fs/point over the first 7.5 ps of the decay, and the fits to the data generally yielded reduced χ^2 of less than 1.3 and unstructured residuals. The values reported are averages of the fit values from three or more data sets, and the errors are the standard deviation of the averages.

The codon for the M202HL mutation was inserted into the poly-His M182HL background[20] to create the M202HL/M182HL double mutant. His-182 is the ligand for the central Mg^{2+} ion in the bacteriochlorophyll that occupies the B_M binding site. Upon replacing His with a nonligating Leu residue, the Mg^{2+} ion is lost, and a bacteriopheophytin *a* is found to occupy the B_M binding site;[21] this chromophore is designated θ_M. As seen in the low temperature absorption spectrum (Figure 2B), the absorption of this chromophore is significantly shifted

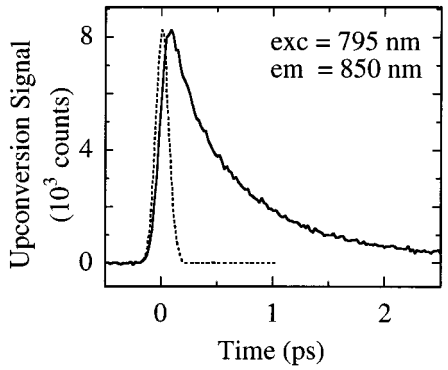

Figure 3. Spontaneous fluorescence from Q_A-reduced heterodimer RCs measured at 850 nm following excitation in the B band at 795 nm at 85 K. The cross correlation (dashed) is overlaid.

to higher energy relative to bacteriochlorophyll in the B_M site, so that θ_M and B_L are reasonably well separated at 77 K. Because of this and given the modest bandwidth of our excitation pulses (see horizontal bars in Figure 2), excitation can be launched with reasonably high selectivity along the L and M sides of the RC.

M202HL and M202HL/M182HL *Rb. sphaeroides* were grown semi-aerobically and isolated rapidly by the procedure designed to take advantage of the poly-His tag engineered into the RCs.[22] Yields of the detergent-isolated double mutant are very low, typically ~5% that of wild type and either the M202HL or H182HL single mutants. The 77 K absorption spectrum is found to be the summation of the distinct spectral characteristics of the M182HL spectrum in the B band region[20] and the M202HL spectrum in the D band region.[9,10] Q_A was either removed[23] or the detergent was exchanged for Triton X-100 and Q_A was chemically reduced by the addition of sodium dithionite just prior to the experiment. RCs were suspended in 10 mM Tris·HCl (pH 8.0), 0.1% Triton X-100 (0.1% LDAO for Q_A-depleted RCs), and 1.0 mM EDTA. All samples were dissolved in 1/1 (v/v) glycerol/buffer solution. The RCs were concentrated in order to achieve a sufficient optical density in the 25−50 μm path length cell, typically 0.1−0.2 at 800 nm.

Results

Time-Resolved Fluorescence from ^{1}B in M202HL RCs. Fluorescence from ^{1}B in heterodimer RCs at 85 K was measured for two different combinations of excitation and emission wavelengths: excitation at 795 nm (both B_L and B_M, but preferentially B_L) in Q_A-reduced and Q_A-depleted RCs with detection at 850 nm shown in Figure 3, and excitation at 751 or 766 nm (preferentially exciting H_M or H_L, respectively) in Q_A-reduced and Q_A-depleted RCs with detection at 815 nm shown in Figure 4. For excitation at 795 nm, the short wavelength limit of detection was 850 nm due to the background produced by the frequency-doubled gate beam at shorter detection wavelengths. For excitation in the H band, emission at 815 nm was chosen because it is accessible at both excitation wavelengths.

When B is directly excited at 795 nm, the rise of emission at 850 nm is instrument-response-limited and fitting the decay requires three time constants, the values of which are reported in Table 1. The time-resolved emission anisotropy in Q_A-reduced heterodimer RCs for direct excitation of B at 795 nm and emission at 850 nm is 0.352 ± 0.002, corresponding to an angle of 16.4 ± 0.3° between the absorption and emission transition dipole moments (data not shown). This is the same within experimental error to 16.8 ± 2.0°, the angle measured for B excitation and emission in wild-type RCs where, in the same work, the time-resolved emission spectrum of ^{1}B was also reported.[7]

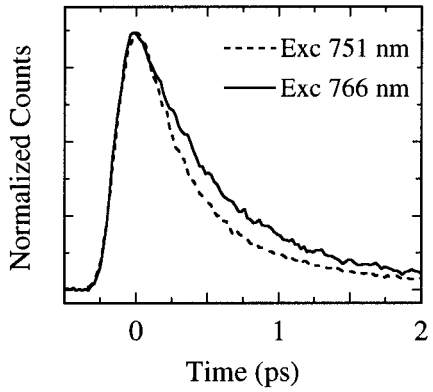

Figure 4. Spontaneous fluorescence from Q_A-reduced heterodimer RCs measured at 815 nm at 85 K following excitation in the H band at 751 (dotted) and 766 nm (solid). Note that the emission for excitation at 751 nm decays faster despite being convolved with a longer cross correlation (165 fs), and that emission for excitation at 766 nm decays slower despite being convolved with a shorter cross correlation (140 fs). Fits to the data are summarized in Table 1.

TABLE 1: Time Constants[25] and Amplitudes[a] (%) for Spontaneous Fluorescence from ^{1}B in Q_A-Reduced and Q_A-Depleted M202HL RCs at 85 K for Excitation on Either Side of the H Band and Detection at 815 nm, and for Excitation in the B Band at 795 nm and Detection at 850 nm

M202HL Q_A-Reduced RCs		
	H band	
	751 nm	766 nm
A_{rise}	−97 ± 1	−89 ± 2
τ_{rise}	85 ± 8 fs	84 ± 4 fs
A_1	20 ± 1	46 ± 3
τ_1	790 ± 41 fs	784 ± 33 fs
A_2	80 ± 1	54 ± 3
τ_2	232 ± 27 fs	284 ± 30 fs
A_1/A_2	0.25	0.85
	B band	
	795 nm	
A_1	52 ± 4	
τ_1	697 ± 25 fs	
A_2	45 ± 5	
τ_2	184 ± 8 fs	

M202HL Q_A-Depleted RCs		
	H band	
	751 nm	766 nm
A_{rise}	−94 ± 3	−97 ± 4
τ_{rise}	75 ± 5 fs	91 ± 8 fs
A_1	17 ± 3	26 ± 1
τ_1	846 ± 124 fs	854 ± 51 fs
A_2	82 ± 3	72 ± 1
τ_2	246 ± 24 fs	276 ± 35 fs
A_1/A_2	0.21	0.36
	B band	
	795 nm	
A_1	41 ± 3	
τ_1	709 ± 18 fs	
A_2	55 ± 3	
τ_2	198 ± 12 fs	

[a] All data sets had an additional decay time constant with an amplitude of less than 10% that was unresolvably long given the 7.5 ps data collection window.

For the second set of experiments, exciting the high- and low-energy sides of the H band and detecting ^{1}B emission at 815 nm, we observe two major decay time constants that closely

TABLE 2: Rise Time Constants[25] and Amplitudes (%) for Spontaneous Fluorescence from ^{1}D Measured at 1040 nm for Excitation in the B Bands in Q_A-Reduced and Q_A-Depleted M202HL and Q_A-Reduced M202HL/M182HL RCs at 85 K

M202HL Q_A-Reduced RCs		
	795 nm	807 nm
A_1	-57 ± 1	-27 ± 3
τ_1	778 ± 7 fs	960 ± 25 fs
A_2	-40 ± 2	-68 ± 2
τ_2	158 ± 6 fs	138 ± 5 fs
A_1/A_2	1.43	0.40
M202HL Q_A-Depleted RCs		
	795 nm	807 nm
A_1	-51 ± 2	-24 ± 1
τ_1	784 ± 69 fs	849 ± 80 fs
A_2	-44 ± 5	-71 ± 2
τ_2	148 ± 7 fs	121 ± 6 fs
A_1/A_2	1.16	0.34
M202HL/M182HL Q_A-Reduced RCs		
	787 nm	802 nm
A_1	-29 ± 1	-66 ± 2
τ_1	976 ± 54 fs	830 ± 36 fs
A_2	-69 ± 1	-29 ± 1
τ_2	179 ± 3 fs	84 ± 18 fs
A_1/A_2	0.42	2.28

match those observed at 850 nm for direct excitation of B at 795 nm. The ratio of the amplitudes of these two components varies for excitation at different wavelengths within the H band. Additionally, when H is excited, a negative amplitude component is necessary to fit the rise of ^{1}B emission, indicative of resolvable energy transfer to the emitting state, as was observed previously in wild type.[6] The results for Q_A-reduced and Q_A-depleted heterodimer RCs are summarized in Table 1. The time-resolved anisotropy in Q_A-reduced heterodimer RCs for excitation of the H band at 761 nm and emission at 815 nm is 0.054 ± 0.003, corresponding to an angle of $49.4 \pm 0.3°$ between the absorption transition dipole moment of H and the emission transition dipole moment of the emitting species (data not shown). This is close to the angle of $45.8 \pm 1.9°$ between the absorption and emission transition dipole moments of H and B, respectively, reported in WT RCs.[7] The anisotropies reported here provide additional confirmation that the emission between 815 and 850 nm in heterodimer RCs is from ^{1}B.

Time-Resolved Fluorescence from ^{1}D in M202HL RCs. Previous experiments[24] exciting Q_A-reduced heterodimer RCs in the H and B bands and detecting the rise of emission at 970 nm were repeated in both Q_A-reduced and Q_A-depleted heterodimer RCs using a detection wavelength of 1040 nm. Specifically, for excitation in the B band at 795 and 807 nm and for excitation in the H band at 751 and 766 nm, the rise of ^{1}D emission was detected at 1040 nm in Q_A-reduced and Q_A-depleted heterodimer RCs. The reasons for monitoring emission at 1040 nm instead of 970 nm, as was used in ref 24, are explained in the Discussion. The results are summarized in Tables 2 and 3.

Time-Resolved Fluorescence from ^{1}D in Q_A-Reduced M202HL/M182HL RCs. Emission from ^{1}D at 1040 nm was observed for the M202HL/M182HL mutant for excitation at 802 and 787 nm (preferentially B_L and θ_M, respectively) and for excitation at 766 and 751 nm (preferentially H_L and H_M, respectively). Figure 5 shows the rise of ^{1}D fluorescence at 1040 nm as the excitation wavelength is tuned to the low- or high-energy side of the B and H absorption bands. The full decay of ^{1}D fluorescence was not measured since the focus of these

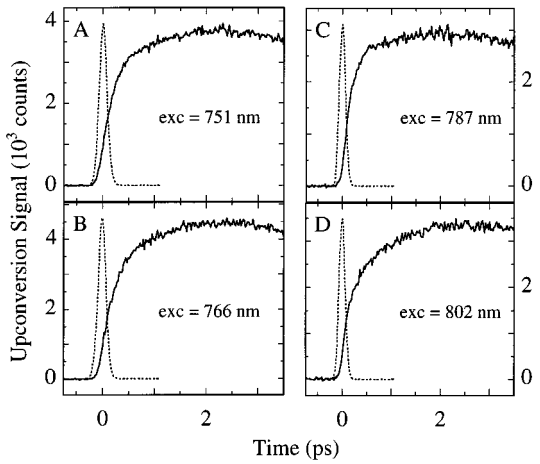

Figure 5. Rise of spontaneous fluorescence from ^{1}D in Q_A-reduced M202HL/M182HL RCs at 85 K measured at 1040 nm following excitation in the H band at (A) 751 and (B) 766 nm, and for excitation in the B band at (C) 787 and (D) 802 nm. Cross correlations (dashed) are overlaid, and fits to the data are summarized in Tables 2 and 3.

TABLE 3: Rise Time Constants[25] and Amplitudes (%) for Spontaneous Fluorescence from ^{1}D Measured at 1040 nm for Excitation in the H Bands in Q_A-Reduced and Q_A-Depleted M202HL and Q_A-Reduced M202HL/M182HL RCs at 85 K

M202HL Q_A-Reduced RCs		
	751 nm	766 nm
A_1	-30 ± 2	-54 ± 5
τ_1	1016 ± 66 fs	870 ± 89 fs
A_2	-68 ± 3	-44 ± 5
τ_2	222 ± 17 fs	250 ± 19 fs
A_1/A_2	0.44	1.23
M202HL Q_A-Depleted RCs		
	751 nm	766 nm
A_1	-31 ± 1	-50 ± 2
τ_1	1054 ± 71 fs	948 ± 32 fs
A_2	-67 ± 2	-46 ± 2
τ_2	196 ± 3 fs	196 ± 12 fs
A_1/A_2	0.46	1.09
M202HL/M182HL Q_A-Reduced RCs		
	751 nm	766 nm
A_1	-38 ± 3	-47 ± 4
τ_1	835 ± 63 fs	812 ± 49 fs
A_2	-58 ± 3	-50 ± 5
τ_2	216 ± 11 fs	215 ± 22 fs
A_1/A_2	0.65	0.94

experiments is the early-time dynamics. Over the 7.5 ps window of these measurements, ^{1}D decay was found to be independent of excitation wavelength. In contrast, the *rise* of ^{1}D fluorescence does change with excitation wavelength. Qualitatively, tuning the excitation from high to low energy across the B or H bands (θ_M to B_L or H_M to H_L) leads to an increase in the relative contribution from the slower-rising component. The rise of ^{1}D fluorescence was best fit in all cases with a fast and a slow component. The results are summarized and compared with those for M202HL in Tables 2 and 3. It is evident that the time constants of the two rise components are independent of excitation wavelength,[25] but their amplitudes change substantially.

Discussion

Singlet Energy Transfer in M202HL and M202HL/M182HL Mutant RCs. In previous work, we reported different

energy transfer rates for $^1B_L \rightarrow D$ and $^1B_M \rightarrow D$ based on the observation that ^1D emission rises with two well-resolved components. Tuning the excitation wavelength across the H and B bands changes the relative contributions of these components in a manner consistent with faster energy transfer along the M branch and slower energy transfer along the L branch.[24] In the present work, we confirm and extend this work by directly measuring the rise and decay of ^1B emission and by working with the M202HL/M182HL double mutant where excitation of the L and M sides of the B band is achieved with greater selectivity than in M202HL.

For excitation of B and H in the heterodimer mutant, the two major ^1B decay time constants correspond closely with the rise time constants for ^1D. The emission from ^1D is spectrally broad, and the values of the short and long rise time constants of ^1D are somewhat emission-wavelength dependent. The values measured at 1040 nm are somewhat longer than those measured at 970 nm[24] and better correspond to the ^1B decay time constants. As we have discussed previously,[24] there is evidence of complicated excited state dynamics in the heterodimer mutant when the higher energy absorption feature of D at 840 nm is excited. One explanation for this is that the 840 and 920 nm absorption features of D have associated emission bands, each with different dynamics, and the overlap of the two bands could give rise to the observed emission-wavelength dependence of the rise times. Detection at longer emission wavelengths, such as 1040 nm, would be expected to minimize contributions from the higher energy feature. Most importantly, from the perspective of energy transfer asymmetry, the ratios of the amplitudes of the slow/fast components follow the same trend when measured at 1040 nm as for emission at 970 nm.

The data in Table 1 show that the two decay time constants of the ^1B population are present in different proportions as the excitation wavelength is tuned to either side of the H band. The H absorption feature centered at 760 nm is not well resolved into two bands corresponding to the L- and M-side chromophores. However, evidence from many laboratories points to H_M absorbing at higher energy and H_L at lower energy.[26−29] The ratio of the amplitudes of the slow/fast components of the decay of ^1B emission measured at 815 nm progresses from 0.85 to 0.25 as the excitation wavelength is tuned from the L to the M side of the H band in Q_A-reduced RCs. This trend follows that for the rise of ^1D emission at 1040 nm, where the ratio progresses from 1.23 to 0.44 as the excitation wavelength is similarly tuned across the H band. These data demonstrate that the relative contributions to either ^1B or ^1D emission from the L- and M-side chromophores can be varied by preferentially exciting the low- or high-energy side of H.

There is better resolution of the θ_M and B_L absorption bands in the M202HL/M182HL mutant than in the M202HL mutant. Furthermore, the θ_M absorption is on the high-energy side of the B_L absorption band, energetically reversed from the B_L/B_M band order in heterodimer (or wild-type) RCs. Thus, in M202HL/M182HL, one would predict that the ratios of the amplitudes of the slow/fast components should follow the same trend as for excitation in the H band and be reversed in the B band, as the excitation wavelength is tuned from high to low energy, compared to M202HL. This is what we observe (see Table 2). Also, the time constants of the two components of the rise are similar to those for heterodimer,[25] indicating that the same energy transfer pathways exist.

It is significant that the rate of energy transfer from $^1\theta_M$ to D is the same in M202HL/M182HL relative to energy transfer from ^1B_M to D in M202HL. Given the ~18 nm shift to higher energy in absorption for the θ_M chromophore relative to bacteriochlorophyll in the B_M binding pocket, one might expect substantially different spectral overlap with whatever feature of the D band absorption is responsible for energy transfer in a Förster-type analysis.[30] In this case, the observed energy transfer time constant for $^1\theta_M \rightarrow D$ in M202HL/M182HL would be different from $^1B_M \rightarrow D$ in heterodimer. Assuming that the spectral overlap is significantly perturbed, the observation of unchanged rates of energy transfer supports the hypothesis that singlet energy transfer in the RC is not predominantly occurring by a conventional Förster dipole−dipole mechanism. Alternatively, it is possible that the broad heterodimer special pair absorption overlaps equally well with the θ_M fluorescence in M202HL/M182HL as with the B_M fluorescence in M202HL. Considering this in a conventional Förster analysis, it is not obvious what factors would be responsible for the differences in energy transfer rates along the L and M sides. Structurally, wild type and M202HL are similar.[31] In a general model that treats electronic energy transfer using a Coulombic interaction in the framework of the Fermi golden rule, what is required is a source of electronic coupling and a sufficient density of states to conserve energy. We suggest that the density of states is always sufficient and not rate limiting; further evidence supporting this suggestion will be presented in part 5 of this series, which describes ^1B energy transfer in P^+ and ^3P containing RCs.[32] We therefore conclude that the electronic interactions between chromophores are important. Such interactions along the functional and nonfunctional sides may be proportional to those for electron transfer between the same chromophores, in which case observations of ultrafast singlet energy transfer asymmetry may be relevant to electron transfer asymmetry. Calculations of energy transfer rates in the RC are in progress[33] and may be able to address the theoretical basis for our observations more directly.

Effects of Alternate Charge Separation Pathways from ^1B. Our group[34,35] and van Grondelle's[17,36] independently provided evidence for $^1B_L \rightarrow B_L^+H_L^-$ electron transfer in several RC variants; other groups had previously discussed the possibility of alternative electron transfer pathways.[5,37,38] Using advanced Stark spectroscopy techniques,[39] we discovered an unusually strong and broad signal associated with the B_L absorption band.[34] Analysis of this novel signal in wild-type and mutant RCs suggested that its origin was electron transfer from ^1B_L to H_L. This new method, called resonance Stark spectroscopy, provides information on the fundamental parameters that determine electron transfer irrespective of whether the rate is fast compared to that of competing processes such as energy transfer that are not expected to be very sensitive to an applied electric field. Van Brederode's analysis of ^1B electron transfer in the M210YW mutant was based on transient absorption studies using global analysis, and the observation of reduced H and B band intensity in the fluorescence excitation spectrum of ^1P compared with the absorption spectrum, while the $P^+Q_A^-$ action spectrum closely matched the absorption spectrum.[36,40] They performed a similar analysis on the heterodimer mutant[17] and observed a 340 fs monoexponential decay of ^1B, which contrasts with the biexponential kinetics in Q_A-reduced M202HL we had reported earlier.[24,41] To explain the discrepancy between their results and ours, van Brederode suggested that our measurements of the rise of ^1D might be affected by the presence of Q_A^- [17] since their measurements in M210YW were sensitive to a negative charge on Q_A. We have shown here that both the decay of ^1B_L and ^1B_M and the rise of ^1D are the same in Q_A-reduced and Q_A-depleted heterodimer

RCs.[42] If $^1B_L \rightarrow B_L^+H_L^-$ occurs on a time scale similar to energy transfer from ^1B_L, one would expect the decay time constant of ^1B_L to be sensitive to the presence or absence of a negative charge on Q_A.

It is important to consider whether the $^1B_L \rightarrow B_L^+H_L^-$ process competes with $^1B_L \rightarrow D$ (or P) energy transfer because this could affect the interpretation of the fluorescence upconversion data. The relative rates are also central to whether $^1B_L \rightarrow B_L^+H_L^-$ is physiologically important. Quantitative analysis of the resonance Stark spectra[35,43] suggests that $^1B_L \rightarrow B_L^+H_L^-$ is considerably slower than what is reported by van Brederode et al. Although the absolute rates are important and will be dealt with in detailed analyses of resonance Stark data,[35,43] we do not need to discuss the absolute rates here, because in any scenario energy transfer is slowed along the L side in the heterodimer mutant. Consider two limiting cases: (i) the observed time constants of ^1B_L decay and concomitant rise of ^1D are dominated by $^1B_L \rightarrow B_L^+H_L^-$, that is, electron transfer is much faster than singlet energy transfer; and (ii) the observed ^1B_L decay and concomitant rise of ^1D are dominated by energy transfer, that is, $^1B_L \rightarrow D$ is much faster than $^1B_L \rightarrow B_L^+H_L^-$. In case (i), $^1B_L \rightarrow D$ energy transfer would be much slower than 700 fs; in case (ii), $^1B_L \rightarrow D$ alone accounts for the observed 700 fs ^1B_L time constant. In *either* limit and, of course, any intermediary case, the conclusion is that $^1B_L \rightarrow D$ is substantially slower than $^1B_L \rightarrow P$ in wild-type homodimer-containing RCs, while $^1B_M \rightarrow D$ energy transfer is comparable to $^1B_M \rightarrow P$. Thus, the symmetry of singlet energy transfer is broken when the electronic structure of the special pair is changed from the homodimer to the heterodimer.

Conclusions

Further evidence for asymmetric energy transfer in the heterodimer mutant RC has been presented. The existence of a slower energy transfer pathway on the L side in the heterodimer mutant may provide a system to directly study alternative electron transfer pathways by fluorescence upconversion if they can be made competitive by further mutations in the heterodimer background. Additionally, we have significantly changed the spectral overlap between one of the accessory chromophores and D, without observing any effect on the energy transfer time constant. If the relative electronic coupling for singlet energy transfer along the functional and nonfunctional sides is proportional to that for electron transfer between the same chromophores, then observations of singlet energy transfer asymmetry may be relevant to electron transfer asymmetry. Since there is no evidence for appreciable M-side electron transfer in the heterodimer mutant, this leads to the suggestion that asymmetric electronic coupling is not a major factor leading to unidirectional electron transfer.

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