Inter-chromophore interactions in pigment-modified and dimer-less bacterial photosynthetic reaction centers

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Abstract

The magnitudes of inter-chromophore interactions in bacterial photosynthetic reaction centers are investigated by measuring absorption and Stark spectra of reaction centers in which monomeric chromophores are modified and in a novel triplet mutant which lacks the special pair. The circular dichroism spectrum of the triple mutant reaction center was also measured. Only small changes in the spectroscopic properties are observed, as has also been found for several types of reaction centers in which the absorption or chemical properties of a chromophore are altered by site-specific mutations. We conclude that the electronic absorption, circular dichroism and Stark features of the special pair and the monomeric chromophores in the reaction center are relatively insensitive to inter-chromophore interactions.

Abbreviations: $B_{L,M}$ – the monomeric BChls for the active and inactive sides, respectively; BChl a – bacteriochlorophyll a; BPhe a – bacteriopheophytin a; CD – circular dichroism; Chl a – chlorophyll a; D – Debye; DEAE – diethylaminoethyl; EDTA – ethylene diamine tetraacetic acid; f – the local field correction; $H_{L,M}$ – the monomeric BPhes for the active and inactive sides, respectively; P – the primary electron donor; $P_{L,M}$ – the monomer BChls which comprise P, associated with the L and M subunit of the protein, respectively; Phe a – pheophytin a; Q_A – the first quinone electron acceptor; RC – reaction center; SDS – sodium dodecyl sulfate; Tris – tris-hydroxymethylaminomethane; WT – wild type; $\Delta\mu$ – difference dipole moment between the ground and excited state; ζ_a – angle between the difference dipole moment and the transition moment

Introduction

The ground state electronic absorption spectrum and transient changes in absorption are the primary observables used to characterize the pigment composition, integrity and function of photosynthetic reaction centers (RCs). The near-infrared (700–1000 nm) absorption spectrum of the *Rb. sphaeroides* RC consists of three well-resolved bands as seen in Figure 1A. The lowest energy band is ascribed to an electronic transition of the special pair P, a dimer of bacteriochlorophyll (BChl) molecules. The close proximity of these chromophores and the nearly anti-parallel orientation of their transition dipole moments lead to an assignment of this feature as the lower exciton band of the two interacting monomers in the dimer.

The precise energy and intensity of the upper exciton band is less certain (Breton 1985) . The band at 800 nm is assigned to the overlapping absorptions of the two monomeric BChls (B_L and B_M), and the band at 760 nm is assigned to the overlapping absorptions of the two monomeric bacteriopheophytins (BPhe, H_L and H_M).

A key unresolved question that impacts nearly every debate on RC function is the degree to which the absorption features are affected by inter-chromophore interactions. For example, transient changes in absorption, which are usually ascribed to the loss or appearance of species whose concentration changes, can be complicated by transient modifications in the spectral overlap of absorption bands, electrochromic bandshifts that result from the creation, loss or move-
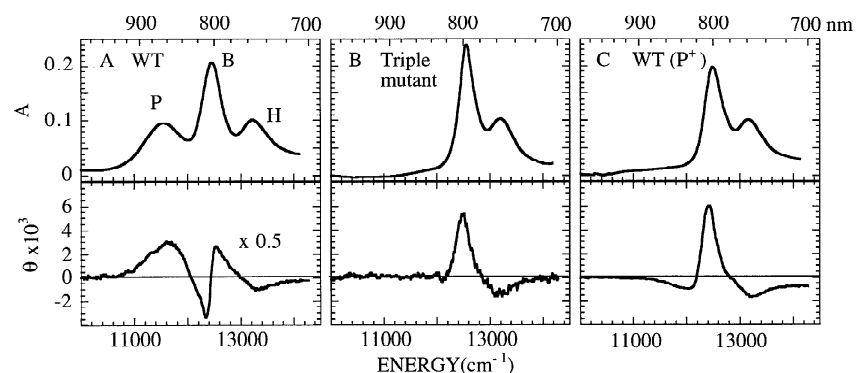
Figure 1. Room temperature absorption (upper panel) and circular dichroism (lower panel) spectra of (A) WT, (B) triple mutant L168HG/L173HG/M202HG, and (C) oxidized (P$^+$-containing) WT reaction centers. The absorption and CD spectra are normalized to an OD of 0.1 at 760 nm. The WT CD spectrum was multiplied by a factor of 0.5 to increase visibility of the other spectra.

ment of charge during electron transfer reactions, and a loss or gain of some inter-chromophore interaction. Other factors such as vibrational relaxation may affect the changes in the absorption on a short timescale, and all of these factors may contribute simultaneously. Irrespective of the precision of transient absorption data or the sophistication of methods used to analyze the data, the ultimate interpretation in terms of a molecular mechanism reduces to the assignment of the absorption features.

Several groups have performed quantum chemical calculations that model spectroscopic observables such as absorption, circular dichroism (CD), linear dichroism, triplet-minus-singlet absorption and Stark spectra (Parson et al. 1988; Scherer and Fischer 1987; Scherer and Fischer 1991; Parson and Warshel 1987) . These treatments suggest that inter-chromophore coupling is important for the characteristics of the absorption spectrum although they differ on precisely which interaction contributes to each band and the magnitudes of the interactions. The lowest energy dimer band has substantial charge transfer character (Lockhart and Boxer 1987), so coupling to intradimer charge transfer states such as $P_L^+P_M^-$ or $P_L^-P_M^+$ and interchromophore charge transfer states such as $P^+B_L^-$ are often considered in calculations or models of the data (Scherer and Fischer 1986, 1991; Thompson and Zerner 1991; Small 1995). There are only a few studies in which the magnitude of inter-chromophore coupling on the properties of the bands have been evaluated experimentally (Scheer and Struck 1993; Haran et al. 1996 but see Stanley et al. (1996) and Jonas et al. (1996) for different results and interpretation of this work). In the present paper we analyze the absorption and Stark effect spectra of pigment-modified RCs

and an RC mutant in which the special pair is missing (Moore and Boxer 1996). Combined with data in the literature on RC mutants in which the absorption of specific pigments changes (e.g. the beta, M214LH, and heterodimer, M202HL, mutants), we can estimate some limits on the degree of mixing between the monomeric chromophores or between the monomeric chromophores and the special pair as it affects the electronic absorption spectra.

Experimental

Rb. sphaeroides R26 RCs were isolated by standard methods (Okamura et al. 1975; Schenck et al. 1982). BChl *a* was isolated from lyophilized whole cells of wild type (WT) *Rb. sphaeroides* by methanol extraction and was purified on a DEAE Sepharose CL-6B column (Scherz and Parson 1984). 3^1-OH BChl *a* was prepared by sodium borohydride reduction of BChl *a* (Struck et al. 1992). Pheophytin *a* (Phe *a*) was prepared by acidification of chlorophyll *a* (Chl *a*) (Steffen 1995). Pigment exchange on R26 RCs was performed using the method pioneered by Scheer (Meyer and Scheer 1995; Struck and Scheer 1990). A triple mutant (L168HG/L173HG/M202HG) was created in which the axial histidine ligands to the Mg atoms in both halves of the special pair as well as the histidine that hydrogen bonds to the P_L acetyl carbonyl were removed and replaced by glycine using methods described by Goldsmith et al. (1996). A poly-histidine tagged version of this mutant was also created using a plasmid template described by (Goldsmith and Boxer (1996). The L and M genes were sequenced after conjugation of the plasmid into a *Rb. sphaeroides* deletion

strain (the antenna-containing, ΔLM1.1 (Murchison et al. 1993) or the antenna-less, ΔBALM1(Lee et al. 1989) strains) to verify the presence of the correct mutations.

Rb. *sphaeroides* cells were grown semi-aerobically for 3–4 days in the dark in YCC medium and RCs were isolated using standard procedures (Paddock et al. 1988). The pigment content was analyzed using a methanol extraction method (van der Rest and Gingras 1974). Five extractions of the mutant RC sample and WT control were performed. An SDS polyacrylamide gel was run on the triple mutant to confirm the presence of the three protein subunits. The possibility of electron transfer in the triple mutant to produce a long-lived state (longer than μs) was investigated by monitoring the change in absorption in the Q band region upon excitation at 532 nm using a 10 ns pulse from a doubled Nd:Yag laser (532nm excitation light). 1 mM ubiquinone-10 was added to the triple mutant for these experiments, as under these conditions the Q_A binding site in native RCs would be fully occupied.

Low temperature absorption and Stark spectra were obtained on samples of the pigment-exchanged, triple mutant, WT and oxidized WT RCs in 50/50 glycerol/Tris buffer (0.1% LDAO, 10mM Tris, 1mM EDTA). 150 mM ferricyanide was added to a WT sample to oxidize P. Details of the methodology used to obtain and analyze Stark spectra is presented by Boxer (1993). Room temperature absorption and CD spectra were obtained on the triple mutant, WT, and oxidized WT using a Perkin Elmer Lambda 12 spectrophotometer and a Jasco J-500C spectropolarimeter, respectively.

Results

Triple mutant L168HG/L173HG/M202HG

The isolated yield of triple mutant RCs is quite low, ∼7 ODV per 100 g of cells. A relatively low yield was also observed earlier for the double mutant L173HG/M202HG which was isolated with a normal special pair (Goldsmith et al. 1996). Cell growth in the presence of imidazole did not improve the yield or change the spectral properties of the isolated RCs. The poly-histidine tagged version of the triple mutant which is isolated much more rapidly than a traditional RC preparation (Goldsmith and Boxer 1996) also produced a low yield. After isolation, the RCs appeared to be reasonably stable although they degraded at a faster rate than WT.

The room temperature absorption spectrum of the triple mutant is shown in Figure 1B, top panel; it is striking in that the absorption feature at 865 nm assigned to P is missing. No change was observed upon addition of sodium ascorbate suggesting that the absence of the P band is not due to P^+ formation. EPR and near-IR absorption (1000–1400 nm) spectra were also obtained to determine if the characteristic P^+ bands were present, but compared to a similar concentration of oxidized WT RCs they were absent in the mutant. Pigment analysis gave a 1.1 ± 0.1 ratio of BChl/BPhe content compared to 1.8 ± 0.1 for WT; the pigment content was determined to be 3.2 ± 0.5 pigments per RC, compared 5.5 ± 0.3 for WT. These results suggest that two BChl chromophores are missing in the triple mutant. The Stark spectrum of the carotenoid region was measured and found to be similar in lineshape and magnitude to that of WT (data not shown). This is significant as the Stark spectrum of the carotenoid band in the RC is very different from that of the isolated pigment due to specific interactions between the carotenoid and the protein surrounding the binding site (Gottfried et al. 1991). Thus, these interactions are retained despite the substantial cavity left behind by the loss of P. No bleach or shift of the Q_y bands of B or H were observed on the microsecond or millisecond time scale implying that no charge separation to form potentially stable species (e.g. $B_L^+Q_A^-$) takes place on these time scales.

The room temperature absorption and CD spectra of WT, the triple mutant and oxidized (P^+-containing) WT RCs are compared in Figure 1. The absorption spectra of the triple mutant and oxidized WT are similar in that both lack a P band. The B band of the triple mutant is blue-shifted by about 4 nm compared to that of oxidized WT. The CD spectra in the B band region of the triple mutant and oxidized WT are very different from WT: both have a positive signal of approximately the same magnitude, while (non-oxidized) WT has a negative component with a minimum around 810 nm. The triple mutant also lacks the negative feature that is found on the lower energy side of the B band in oxidized WT RCs.

The 77 K absorption and Stark spectra of the triple mutant, WT and oxidized WT are compared in Figure 2. Here it is clear that the P band is entirely absent in the triple mutant. There are small differences among these three types of RCs in the B and H band regions. In WT, the monomer BChls have an absorption maximum at 803 nm with a shoulder at ∼810 nm, which are usually attributed to B_L and B_M, respectively (Bre-
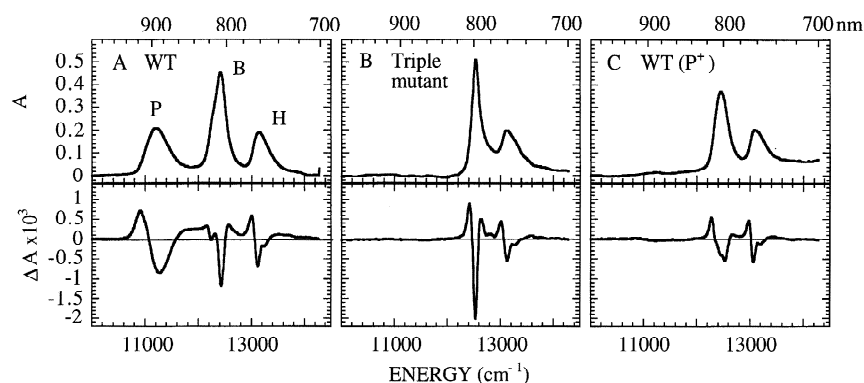
Figure 2. 77 K absorption (upper panel) and Stark (lower panel) spectra of (A) WT, (B) triple mutant L168HG/L173HG/M202HG, and (C) oxidized (P$^+$-containing) WT reaction centers. The Stark spectra are scaled to an applied electric field of 0.25 MV/cm and normalized to an OD of 0.2 at the maximum of the H band.

ton 1988). In the triple mutant, the B band absorption maximum is shifted to the blue by 5 nm, the band is narrower, and no distinct shoulder is present. The shoulder is also missing in the B band of the P$^+$ spectrum; however, the B band is considerably broader in the spectrum of oxidized WT than in the triple mutant. The H band maximum is the same for WT and the triple mutant, while it shifts 4 nm to the red in the spectrum of P$^+$-containing RCs. The ratio of the integrated area of the B and H bands is approximately the same for all three samples, with the area under the B band at most 10% less relative to the H band in the triple mutant.

The Stark spectra of the different RCs in the H band region have the same shape, a second derivative of the absorption band, and approximately the same magnitude. The Stark spectra of the B bands appear to be quite different in shape and magnitude. In all three cases the Stark spectra of the B bands are best described by the second derivative of their respective absorption spectra. The magnitude of the Stark effect of the B band in the triple mutant appears to be larger than that of WT or oxidized WT, however this is deceptive as the B band is narrower. The calculated difference dipole moment ($\Delta\mu$) for the triple mutant is actually slightly smaller than that of WT (Table 1). The angle ζ_a between the transition moment and $\Delta\mu$ is somewhat smaller in the B band of triple mutant than in WT. The magnitude of $\Delta\mu$ for the H band of the triple mutant is slightly smaller than that of the H band in WT and P$^+$ RCs.

In an effort to determine if RCs assemble with the special pair in membranes, antenna-less chromatophore membranes containing the triple mutant were examined by flash photolysis. A small bleach
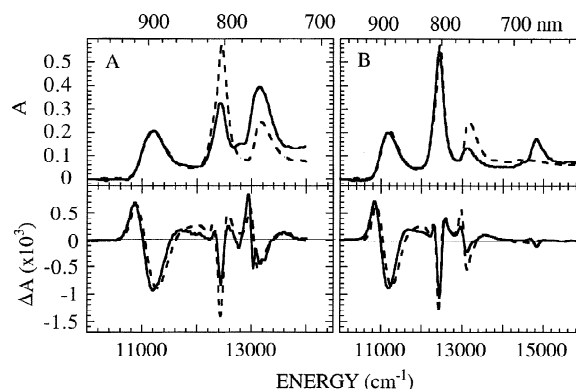

Figure 3. 77 K absorption (upper panel) and Stark (lower panel) spectra of (A) 3^1-OH BChl- and (B) Phe-exchanged reaction centers R26 reaction centers (solid lines) compared with native R26 reaction centers (dashed lines). The Stark spectra are scaled to an applied electric field of 0.25 MV/cm and normalized to an OD of 0.2 at the absorption maximum of the P band to facilitate comparison.

consistent with P$^+$Q$_A^-$ formation and decay was observed. Transient absorption spectra were obtained with a decay rate of 140 ms compared to a rate of 100 ms for WT RCs in antenna-less chromatophore membranes. The magnitude of the bleach in the mutant was the largest at ~830 nm. The amplitude of the bleach was substantially smaller than for a wild-type control sample with matched absorption, suggesting that some RCs containing a functioning special pair are present, though at substantially lower concentration. Given the large scattering background it was difficult to quantitate the RCs in the membranes; however, the data imply that at least some of the RCs in the membrane contain P, but upon isolation the dimer is completely lost.

Table 1. $\Delta\mu$ and ζ_a for modified reaction centers. $\Delta\mu$ is reported in Debye (D) divided by the local field correction (f)

Reaction center center	P		B		H		Phe a	
	$\Delta\mu$ (D/f)	$\zeta_a°$	$\Delta\mu$ (D/f)	$\zeta_a°$	$\Delta\mu$ (D/f)	$\zeta_a°$	$\Delta\mu$ (D/f)	$\zeta_a°$
WT	5.5±0.4	44±2	2.6±0.4	35±3	3.6 ±0.4	23±4	- - - - -	
Triple mutant	- - - - -		2.0±0.4	28±3	3.2±0.4	16±5	- - - - -	
Oxidized WT (P$^+$)	- - - - -		2.4±0.4	35±3	3.5 ±0.4	20±4	- - - - -	
R26	5.2±0.4	44±2	2.4±0.2	24±3	3.4±0.3	17±5	- - - - -	
Phe exchanged	5.2±0.4	42±2	2.2±0.2	29±3	3.2±0.3	17±5	1.6±0.3	34±2
3^1-OH BChl exchanged	5.7±0.5	41±2	2.8±0.2	28±3	n.d.		- - - - -	

Pigment exchanged RCs

The low temperature Stark and absorption spectra of pigment exchanged and R26 RCs are presented in Figure 3. Incorporation of 3^1-OH BChl, which absorbs approximately 40 nm to the blue of BChl, results in RCs which have an increase in absorption close to the H band and a decrease in the absorption of the B band (Figure 3A). The exchange in our samples is not as complete as in the best samples of Scheer and co-workers (Struck et al. 1990). Substitution of Phe a into the RCs results in the loss of intensity of the H band and the appearance of a new band at 14900 cm^{-1} (Figure 3B, top). The oscillator strength of the Q$_y$ band of Phe a is significantly smaller than that of BPhe a (Goedheer 1966) so the new band in the RC is less intense than band it has replaced. Direct integration of the H band in R26 and the remaining H band in the modified RCs shows that the incorporation of the new pigment is at least 70%.

It is evident from both the Stark and absorption spectra that the substitution of the different pigments has only a small effect on the position and band shape of the P band. In the Stark spectra, the minimum of the P band shifts to the red by approximately 100 cm^{-1} and 90 cm^{-1} in the Phe- and 3^1-OH BChl-substituted RCs, respectively; the band shape and the magnitude of the Stark effect are approximately the same as in R26 RCs. The bands other than P show some small differences. In the 3^1-OH BChl-substituted RCs, the remaining B band has the same shape and position as in R26 RCs. Any underlying absorption of P (e.g. the upper exciton band) is not revealed by the substitution with this exchange efficiency. The region in which the H and 3^1-OH BChl a bands are located is complicated by band overlap, so it is difficult to interpret. In the Phe-exchanged RCs, there is a slight decrease in the B band compared to R26, and the magnitude of the Stark effect is slightly smaller and the minimum of the spectrum is slightly shifted to the blue. The unexchanged BPhe remaining in these RCs has a Stark spectrum with the same shape and minimum as that of H for R26; this suggests that the exchange of H occurs with the same efficiency on both the L and M sides. The values of $\Delta\mu$ and ζ_a for the specific bands of the pigment-modified RCs are shown in Table 1. The difference dipole moments calculated for the remaining unexchanged pigments are also included. These molecular parameters are about the same for the P band, although there is a slight increase in the 3^1-OH BChl-substituted RCs. Likewise, ζ_a for the P band remains approximately constant. The $\Delta\mu$ for B decreases slightly in the Phe-exchanged RCs and the remaining B band in the 3^1-OH BChl-substituted RCs has a slight increase in difference dipole; ζ_a remains relatively constant in these bands. The difference dipole for the Phe a band is much smaller than that of the H band, consistent with the fact that $\Delta\mu$ for Phe a(or Chl a) is much smaller than that for BPhe a (or BChl a) (Krawczyk 1991; Steffen 1995). However, it is interesting that ζ_a is larger in the Phe a band, as isolated Phe a and BPhe a have comparable angles (Krawczyk 1991; Steffen 1995).

Discussion

All analytical and spectral data for the triple mutant suggest that P is entirely absent. By comparison, a special pair whose properties are indistinguishable from

P is assembled when both His ligands to the Mg are removed (Goldsmith et al. 1996) and in the single mutant L168HG where the hydrogen bond from HisL168 to the P_L acetyl carbonyl group is removed. Nonetheless, when all three His linkages to the special pair are removed, RCs are isolated that lack the special pair. Although the RC yield is quite low, suggesting that these RCs may not be stable *in vivo,* it is remarkable that the other chromophores, including the carotenoid, are present and quite normal as judged by their absorption and Stark spectra, and the assembly is reasonably stable after isolation. This suggests that the environmental effect of the protein on the remaining chromophores is not affected even though P is completely removed. While this work was in progress, Jackson et al. obtained an RC which also appears to lack P as a result of the insertion of a large charged residue in the special pair binding pocket (Jackson et al. 1997). Many of the properties of this dimer-less mutant and our triple mutant appear to be similar.

The removal of P excludes any possibility of inter-chromophore interaction with B and H, as well as any contribution from dimer/monomer charge transfer states. The elimination of the dimer has some effect on the B band which narrows, shifts to the blue and has a small decrease in the difference dipole. There are essentially no changes in the H band region. The CD spectrum of the triple mutant is similar to that of RCs in which P is oxidized, suggesting that the interactions of the B and H chromophores with the protein or with each other remain about constant whether P is oxidized or is physically removed. The differences observed may be due to changes in the protein caused by the absence of the dimer and not by electronic interaction with the dimer itself, or they may be due to the loss of spectral overlap between the vibronic progression of the lower exciton band and/or the upper exciton band with the B band. Other RC mutants, including the carotenoidless R26 RC itself, show variations in the shoulder on the low energy side of the B band (e.g. see Lin et al. (1994), although none, other than the other mutant in which P is missing (Jackson et al. 1997), exhibits such a large blue shift and narrowing of the band. Even these effects are relatively minor, and because only small changes are observed in the magnitude of the Stark effect, the results suggests that inter-chromophore interactions have only a small influence on the properties of the monomers.

Substitution of altered monomer pigments into the B and H positions have no observable effect on the absorption band and only a small effect on the Stark spectrum of P. This is consistent with previous results that showed the room temperature CD of the dimer band was only altered slightly by the same modifications (Meyer and Scheer 1995; Scheer and Struck 1993; Struck et al. 1991). The substitution of Phe *a* into the H binding site also perturbs the B band slightly. One would expect that if excitonic coupling were important for the characteristics of these bands larger changes would be observed as both the energy and oscillator strength of Phe *a* are quite different from BPhe *a*. The energies of the inter-chromophore charge transfer states (e.g. $P^+B_L^-$ and $P^+H_L^-$) are also expected to change substantially with these modifications since 3^1-OH BChl *a* is 200 mV harder to reduce *in vitro* than BChl *a* and Phe *a* is 150 mV harder to reduce *in vitro* than BPhe *a* (Geskes et al. 1995). This suggests that both types of dimer/monomer interactions are not a significant factor in determining the absorption spectrum.

Other mutants in which specific chromophores are altered or removed likewise show little alteration in the absorption of the unchanged pigments. In heterodimer (M202HL) and reverse heterodimer (L173HL) RCs, one of the BChls in the dimer is replaced by a BPhe leading to a radically different special pair absorption. The B and H absorption bands and calculated difference dipoles are about the same in both the reverse heterodimer and heterodimer as in WT (Hammes et al. 1990; Schenck et al. 1990), suggesting that the dimer absorption and redox potential has little influence on the properties of H and B. β mutant RCs (ML214H) assemble with a BChl in the H_L binding site (Kirmaier et al. 1991). This has no effect on the P band properties and only small changes in the lineshape of B (in part because the BChl in the H_L binding site absorbs at 780 nm and overlaps somewhat with the B bands). Finally, the D_{LL} mutant of *Rb. capsulatus* assembles with H_L missing (Robles et al. 1990). There are small changes in the absorption bands of B and P which can be attributed to individual amino acid changes near these pigments (Boxer et al. 1992). However, consistent with the result for the Phe-substituted RCs, the absence of H_L has little effect on Stark spectrum of B or P (Boxer et al. 1992; Eberl et al. 1992).

The different pigments of the RC must interact to some extent as they carry out both efficient electron (L side only) and energy transfer (both L and M sides). It is likely that the interactions that are important for electron transfer are altered in some of these modified or mutated RCs since rate of electron transfer is slowed significantly (Finkele et al. 1992; Laporte et

al. 1993). However, the strength of the interactions between the pigments required for these processes to be very fast can be substantially smaller than the inhomogeneous linewidth of the absorption bands so their effect on the properties of the absorption bands is relatively insignificant. Most of the small changes we have observed could just as well be accounted for by changes in the spectral overlap of independent features. In contrast to these results, amino acid mutations around P leading to a heterodimer or the addition or deletion of a hydrogen bond to P can significantly alter the lineshape of the P absorption (Hammes et al. 1990; Zhou and Boxer 1997; Moore et al., manuscript in preparation). These differences point to what is important in determining the absorption spectrum of P. In the heterodimer and the hydrogen bond mutants, the intradimer as well as the inter-chromophore charge transfer state energies are expected to shift relative to their values in WT. In the pigment-modified RCs, only the inter-chromophore charge transfer states are altered. Because only small changes are seen in the P band of pigment-modified RCs, it appears that the coupling of the intradimer charge transfer states to ^{1}P has a more significant effect on the absorption band than the inter-chromophore charge transfer states. The removal of the dimer or replacement of one monomer with a different pigment also has only small effects on the monomer chromophores of the reaction center. From this, we conclude that the individual chromophores of the RC are weakly coupled so that alteration of the individual pigments has only minimal effects on the absorption of the other chromophores.

Acknowledgment

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