Effects of Asp Residues Near the L-Side Pigments in Bacterial Reaction Centers†

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Received June 10, 1996; Revised Manuscript Received September 16, 1996⊗

ABSTRACT: The primary photochemistry in *Rhodobacter capsulatus* reaction centers (RCs) containing the Phe to Asp mutation at L polypeptide residue 121 near the photoactive bacteriopheophytin (BPh_L) is characterized using ultrafast transient absorption spectroscopy. At 285 K, initial charge separation from P* proceeds with essentially unity quantum yield in ∼6 ps to form a transient denoted P^+I^-. This transient is proposed to involve $P^+BPh_L^-$ and probably $P^+BChl_L^-$ as well ($BChl_L$ is the L-side bacteriochlorophyll molecule). P^+I^- decays in ∼150 ps both by electron transfer to give $P^+Q_A^-$ (∼78% yield) and by charge recombination to the ground state (∼22% yield). These results indicate that the F(L121)D mutant is closely related, in terms of its electron transfer properties, to previously reported RCs in which BPh_L is replaced with a bacteriochlorophyll (beta-type RCs) or a pheophytin. However, the native BPh_L pigment is retained in the F(L121)D mutant. We propose that the Asp at L121 raises the free energy of $P^+BPh_L^-$, thereby giving rise to the altered photochemistry. At 77 K, the P^+I^- lifetime is shortened slightly to ∼120 ps and the yield of $P^+Q_A^-$ is increased to ∼88%. This result is somewhat different from that obtained for beta-type RCs at low temperature, where the P^+I^- lifetime lengthens and the yield of $P^+Q_A^-$ diminishes or stays about the same compared to the values near room temperature. We exploit these differences in developing a model for the charge separation process in the F(L121)D mutant. The effects of introducing an Asp near BPh_L are compared to those obtained previously in two mutants in which an Asp is introduced near $BChl_L$.

Upon absorption of light by the bacterial photosynthetic reaction center (RC), an excited singlet state (P*) of a dimer of bacteriochlorophylls (P) is formed. This primary electron donor state transfers an electron in about 3 ps to a neighboring bacteriopheophytin molecule (BPh_L) that in turn transfers an electron in about 200 ps to its neighboring quinone (Q_A). The quantum yield of this overall process is ∼1. $P^+Q_A^-$ is stable for several hundred milliseconds in RCs depleted of a secondary quinone. The molecular mechanism of $P^* \rightarrow P^+BPh_L^-$ conversion, and in particular the role of an intervening $BChl_L$ molecule, has been a controversial subject ever since the crystal structure of the RC was reported (Deisenhofer et al., 1984), and even to some extent before that. At the heart of the issue are the relative free energies of states P*, $P^+BChl_L^-$, and $P^+BPh_L^-$ and the mechanism(s) that applies (Kirmaier & Holten, 1987; Deisenhofer & Norris, 1993; Woodbury & Allen, 1995).

Information regarding the free energy of $P^+BChl_L^-$ has been derived from a number of efforts. Among these are studies on RCs in which BPh_L is exchanged for another pigment molecule. This change was first accomplished in the L(M214)H mutant of *Rhodobacter sphaeroides*, where a His residue is introduced over one face of BPh_L at position 214 on the M polypeptide (Kirmaier et al., 1991). This mutation results in incorporation of a bacteriochlorophyll (denoted β) in place of BPh_L, yielding the so-called beta-type RC. More recently, beta-type RCs in *Rhodobacter capsulatus* have been produced by introduction of a His residue on the L polypeptide over the opposite face of the BPh_L macrocycle (Heller et al., 1995a). Chemical substitution of BPh_L with several different tetrapyrroles has also been achieved (Shkurapatov & Shuvalov, 1993; Schmidt et al., 1994; Huber et al., 1995). This entire class of RCs has the same general response to absorption of light (Figure 1B). P* decays with, typically, a somewhat longer lifetime than in wild-type RCs to give a P^+I^- transient from which electron transfer onto Q_A proceeds with a diminished overall yield of $P^+Q_A^-$. In all cases, this branching of the decay of P^+I^-, in which 20−40% of the RCs return to the ground state, has been ascribed to increased involvement of $P^+BChl_L^-$ in supporting the competitive charge recombination reaction. Whatever the pigment substituted for BPh_L, β or another tetrapyrrole, the proposal is that $P^+\beta^-$ is at a higher free energy than $P^+BPh_L^-$ (due to the greater reduction potential of the substituted pigments compared to that of BPh_L) and so is positioned to allow for either a quantum mechanical or thermal mixture of $P^+BChl_L^-$ and $P^+\beta^-$.[1] It is this mixture that we denote P^+I^-. The relevant point is that, while the details of this mixed composition intermediate are not fully elucidated, the involvement of both basis states follows from the existing room- and low-temperature data and is consistent with the expected increase in the free energy of $P^+\beta^-$ relative to that of $P^+BPh_L^-$. Additionally, since this model implicates $P^+BChl_L^-$ as a key component of the transient intermediate P^+I^-, a general conclusion is that $P^+BChl_L^-$ probably lies below P*, and estimates of 55−75 meV for the P* − $P^+BChl_L^-$ free energy gap have been used in modeling the results on a number of these pigment-modified RCs and other mutants (Bixon et al., 1992, 1995; Nagarajan et al., 1993; Schmidt et al., 1994; Kirmaier et al., 1995).

Changing the redox properties of the active components in the RC, either by changing a pigment itself or by

† This work was supported by Grant MCB-9405248 from the National Science Foundation and by an Individual National Research Service Award to B.A.H. from the National Institutes of Health.

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⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

S0006-2960(96)01362-1 CCC: $12.00

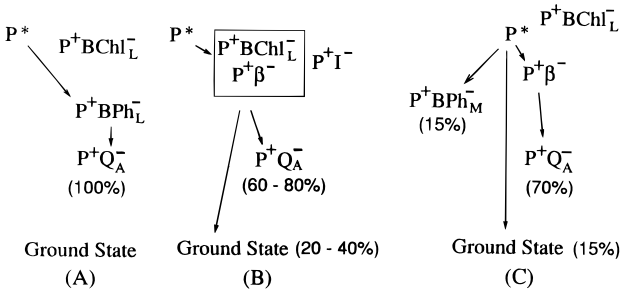

FIGURE 1: State diagrams comparing the proposed free-energy ordering of the states in wild-type RC (A), beta-type RCs such as L(M212)H (B), and the G(M201)D/L(M212)H RC (C). The free-energy gaps are not shown to scale. The box enclosing $P^+BChl_L^-$ and $P^+\beta^-$ denotes the fact that these two states are presumed to be very close in free energy and that the energy ordering of these two states may in fact vary from one beta-type mutant to another, as developed in the text.

introducing or removing hydrogen bonds, has proven to be a very useful tact (Kirmaier et al., 1988, 1991, 1995; Bylina et al., 1988; McDowell et al., 1991; Shkurapatov & Shuvalov, 1993; Murchison et al., 1993; Williams et al., 1992; Schmidt et al., 1994; Peloquin et al., 1994; Heller et al., 1995a; Woodbury et al., 1995.). A different strategy involving neither a pigment nor a hydrogen bond change pertains to mutations at Tyr M210/M208 (*Rb. sphaeroides*/*Rb. capsulatus* numbering) and Phe L181 (Finkele et al., 1990; Nagarajan et al., 1990, 1993; Chan et al., 1991a; Jia et al., 1993; Beekman et al., 1995). Calculations indicating that this Tyr residue modulates the free energy of $P^+BChl_L^-$ provided a guiding concept behind these mutants (Parson et al., 1990). Seeking to specifically perturb the free energy of $P^+BChl_L^-$ in beta-type RCs, we have recently reported on a double mutant where we introduced an Asp residue at M201 near the ring V keto group of $BChl_L$ using the *Rb. capsulatus* L(M212)H beta-type mutant as the background (Heller et al., 1995b). This double mutant manifests a complete reversal of the branching of the P^+I^- decay otherwise found in the normal beta-type mutants (Figure 1C). In G(M201)D/L(M212)H RCs, the $P^+I^- \rightarrow P^+Q_A^-$ electron transfer process is restored to a yield of ~1. Following our hypothesis that holds that the reduced yield of this process in the beta-type single mutants is due to a higher free energy of $P^+\beta^-$ and resulting mixing between $P^+\beta^-$ and $P^+BChl_L^-$, we have proposed that in the double mutant this interaction is greatly diminished. In particular, we have suggested that the Asp at M201 raises the free energy of $P^+BChl_L^-$, very likely ensuring a position above P*. Support for these ideas also comes from perturbations to the initial charge separation events. $P^* \rightarrow P^+I^-$ is significantly slower (~20 ps) and has a yield of only ~70%. The remaining 30% decay of P* is divided approximately equally between internal conversion to the ground state and apparent electron transfer to the M-side BPh. Again, these results are consistent with the hypothesis that $P^+BChl_L^-$ is higher in free energy in the double mutant compared to the beta-type single mutants (Heller et al., 1995b).

We report here the results of placing an Asp residue near ring V of BPh_L, specifically at position L121 in *Rb. capsulatus* RCs. While the F(L121)D mutant retains the native BPh_L pigment, the electron transfer properties of this mutant are essentially the same as those described above for the simple beta-type RCs. We will also describe work on the L(M212)H mutant of *Rb. capsulatus*. Studies on this mutant were undertaken for comparison purposes, and we find some small differences between the L(M212)H mutant of *Rb. capsulatus* and the analogous L(M214)H mutant of *Rb. sphaeroides*. Finally, we also describe the G(M201)D mutant, which was made for the parallel purposes of providing an internal control for the G(M201)D/L(M212)H mutant and for comparison with the G(M203)D mutant of *Rb. sphaeroides* (Williams et al., 1992). A broader goal sought with these mutants is a general understanding of the consequences of placing an Asp residue near a photoactive chromophore.

EXPERIMENTAL PROCEDURES

The F(L121)D mutant is one of a series of mutants at this position that were obtained by oligonucleotide-mediated site-directed mutagenesis using a wild-card oligo (Heller et al., 1995a). In conjunction with this previous work, we also obtained the F(L97)V/F(L121)D double mutant, also to be reported on here. The same methods were used to prepare the L(M212)H and G(M201)D mutants. The oligos for these two single mutants are the same ones used to fabricate the G(M201)D/L(M212)H double mutant (Heller et al., 1995b). As described previously, all of the *Rb. capsulatus* mutant strains were verified by recovering and deconstructing the mutated pU2924 plasmid and resequencing the L or M gene through the targeted site(s) plus ~200 bp both upstream and downstream. Simple substitution of an Asp residue at L121 into the crystal structure coordinates of *Rhodopseudomonas viridis* yields a distance of closest approach between the introduced Asp and BPh_L of about 3.5 Å. A similar distance is obtained for the closest point of contact between an Asp at M201 and $BChl_L$. In both cases, the Asp lies near ring V of the respective chromophore (Figure 2). The renderings in Figure 2 do not represent reoptimized structures, merely substitutions, and are offered as reference points.

RC isolation and purification followed standard procedures, and the final A_{280}/A_{800} ratio was between 1.4 and 1.7 for all samples. The mutants all give comparable yields of RCs per volume of cells grown, and in all cases, this amounts to approximately 60−80% of the yield that is obtained for the same volume of wild-type cells. RCs were solubilized in a standard buffer solution [10 mM potassium phosphate (pH 7.6)/0.05% LDAO/1 mM EDTA/100 μM sodium ascorbate] and stored at −80 °C. Each of the mutant RCs

[1] Mixing between $P^+\beta^-$ and $P^+BChl_L^-$ in the beta-type RCs has been discussed previously (Kirmaier et al., 1995). The combined contribution of the two states to P^+I^- arises in our description because $P^+\beta^-$ and $P^+BChl_L^-$ are nearly isoenergetic in these mutants. By thermal mixing, we mean simple thermal repopulation of the upper state of the two states from the lower. By quantum mechanical mixing, we mean that the two states lose their identities and P^+I^- must be described in terms of two new states that are linear combinations of $P^+\beta^-$ and $P^+BChl_L^-$. In the simplest description, this mixing would be characterized by an electronic coupling term (related to the orbital overlap of β and $BChl_L$) and the energy separation between $P^+\beta^-$ and $P^+BChl_L^-$. This quantum mechanical mixing could be quite substantial if the energy gap goes to 0, even if there is only modest electronic coupling (which is probably at most a few tens of wavenumbers on the basis of current electronic structure calculations on the RC). The two linear combination states would be quite close together since the splitting between them would be twice the electronic coupling. Hence, if quantum mechanical mixing is substantial, one would view the wave function of the reduced acceptor in P^+I^- as being spread over both β and BChl. It is at present not clear whether thermal equilibration or quantum mechanical mixing contributes most substantially to the properties of P^+I^- in the beta-type mutants.

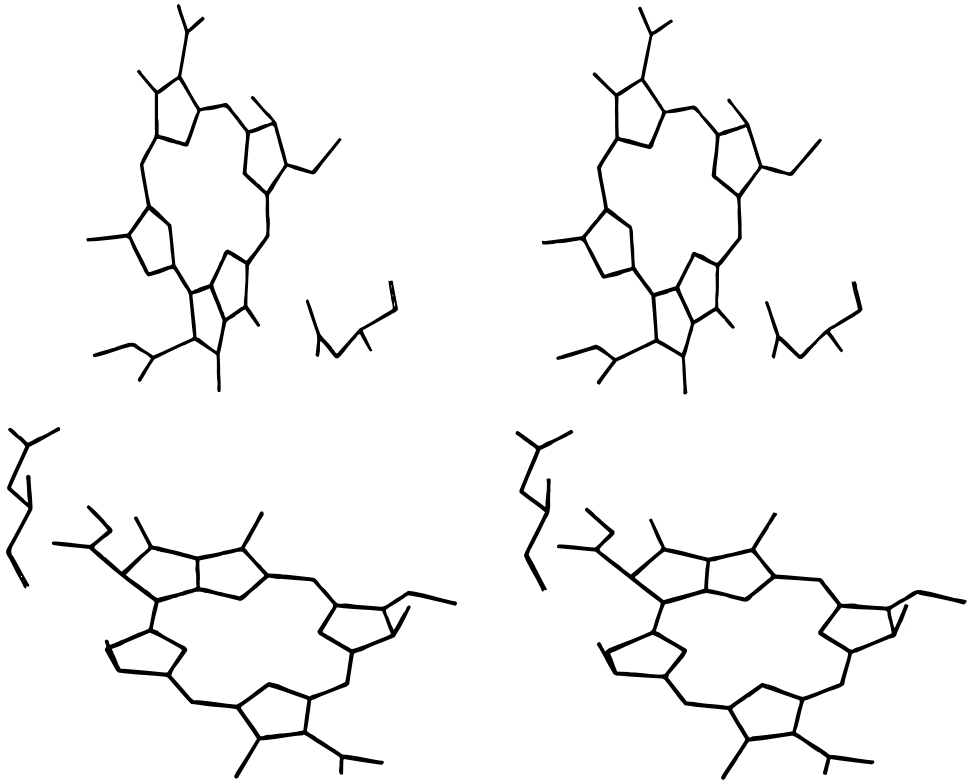

FIGURE 2: Stereoviews of BPh_L with an Asp residue at L121 (top) and $BChl_L$ with an Asp at M201 (bottom). Both cases represent simple replacement with Asp of the native residue in the crystal structure of the *Rps. viridis* RC.

has been kept for up to 6 months in storage with no changes or only slight changes in the absorption spectra found upon thawing the samples. In the latter cases, the samples were rechromatographed before measurements were performed. There were no differences in the results of any measurements for samples studied immediately following initial purification versus those kept first in storage. For measurements at 285 K, RCs were contained in an ice-cooled reservoir and flowed through a 2 mm path length cell using a peristaltic pump. Measurements at 77 K utilized RCs in 60/40 (v/v) glycerol/standard buffer and an Oxford Instruments cryostat.

Isolated RCs were studied by time-resolved absorption difference spectroscopy employing either ~0.2 ps excitation pulses at 582 nm from a synchronously pumped laser system or ~0.1 ps flashes at 855 nm from a regeneratively amplified Ti:sapphire laser system. Both systems were operated at 10 Hz except for the low-temperature measurements where the synchronously pumped system was used at 5 Hz. Further details of both laser systems have been provided elsewhere, as have the methods of data acquisition and analysis (Kirmaier & Holten, 1991; Heller et al., 1995b). For all experiments, the excitation light was focused gently at the sample and attenuated with neutral density filters. It was confirmed that the RC response was subsaturating and linear. Typically, 10−30% of the RCs in the excitation region were excited on any one flash. Simulations of kinetics were carried out using the program KINSIM (Zimmerle & Frieden, 1989).

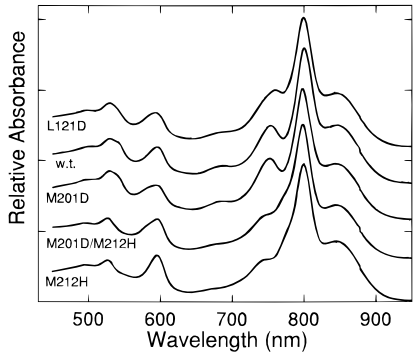

FIGURE 3: Room-temperature ground state absorption spectra of RCs from F(L121)D, wild type, G(M201)D, G(M201)D/L(M212)H, and L(M212)H.

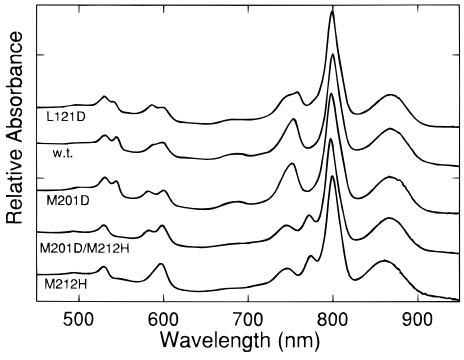

FIGURE 4: Ground state absorption spectra of mutant and wild-type RCs in 60% glycerol/40% buffer C (v/v) at 77 K.

RESULTS

Figures 3−5 show room- and low-temperature ground state spectra of F(L121)D, wild type, G(M201)D, G(M201)D/L(M212)H, and L(M212)H RCs. The spectrum of the F(L97)V/F(L121)D mutant is identical to that of the F(L121)D single mutant and is not shown. Pigment extractions were carried out on the F(L121)D mutant utilizing methods described previously (Van der Rest & Gingras, 1974). This assay gave 5.9 total pigments per RC and 2.1 for the BChl/

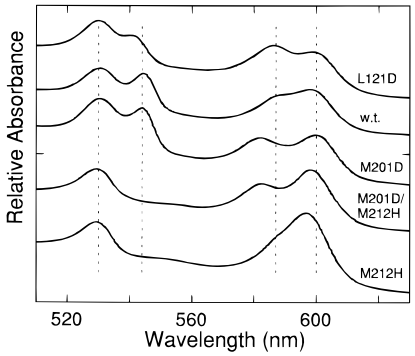

FIGURE 5: Expanded view of the 77 K ground state absorption spectra in the Q_X region. The dotted vertical lines are at 530, 544, 587, and 600 nm.

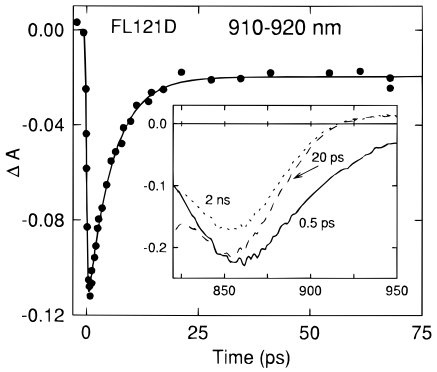

FIGURE 6: Representative decay kinetics of the P*-stimulated emission in F(L121)D RCs averaged between 910 and 920 nm. The solid line is a fit to the sum of an instrument function (the convolution of two pulses) plus a single exponential plus a constant, which returns a 5.6 ± 0.4 ps time constant. The average P* lifetime found between 870 and 930 nm is given in Table 1. The inset shows transient difference absorption spectra of F(L121)D RCs taken at the times indicated following excitation with ~0.2 ps flashes at 582 nm. This spectral region encompasses bleaching of the long-wavelength absorption band of P centered at ~850 nm. Stimulated emission from P* is present at longer wavelengths in the 0.5 ps spectrum.

BPh ratio, indicating this mutant retains the wild-type pigment content. On the basis of previous pigment extraction of the G(M201)D/L(M212)H RC (Heller et al., 1995b) and the similarities of the ground state spectra, it is assumed that the L(M212)H mutant has a BChl (β) in place of BPh_L. This pigment composition has been found for several other mutants where a His has been positioned over one face of the macrocycle (Kirmaier et al., 1991; Heller et al., 1995a). One of the main points of the work here is not to examine again beta-type RCs themselves but rather to pursue an understanding of the effects of locating an Asp residue near a pigment. The spectra in Figures 3−5 are collected for this purpose. Discussion of the spectra is deferred to the following section. However, as a general comment, the Asp at L121 has clear effects on both the ~540 nm Q_X and ~760 nm Q_Y bands of BPh_L, and the Asp at M201 has a clear effect in the Q_X region of the BChls. An effect of the G(M201)D mutation on the 800 nm BChl Q_Y region is not readily apparent.

The results of transient absorption measurements on the F(L121)D mutant are summarized in Figures 6−9. Formation of P* results in bleaching of the 850 nm absorption band of P (0.5 ps spectrum in Figure 6). Stimulated emission from P* appears as an additional absorption decrease on the long-wavelength side of this bleaching. The decay of the stimulated emission between 870 and 930 nm was fit to a single exponential plus a constant, yielding an average P* lifetime of 5.8 ± 0.6 ps; the data averaged between 910 and 920 nm and a corresponding fit are shown in Figure 6. Following decay of P*, the true shape of the bleaching of the dimer band is revealed in the 20 ps spectrum in Figure 6. On the blue side of the band, between 840 and 850 nm, stimulated emission does not contribute to the 0.5 ps

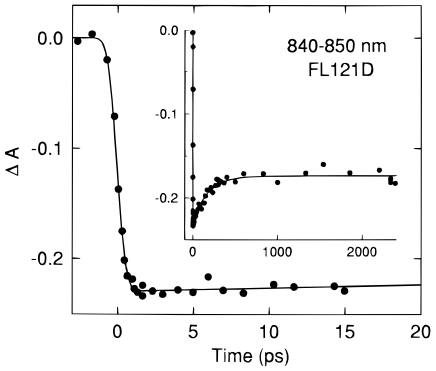

FIGURE 7: Decay of bleaching of P's band between 840 and 850 nm following excitation of F(L121)D RCs with a 0.2 ps 582 nm flash. The solid line is a fit to a single-exponential function (see the Figure 6 legend). As seen in Figure 6, there is no evidence for a fast component to the decay (main figure). Rather, the decay of P bleaching occurs essentially exclusively on a longer time scale with a time constant of ~160 ps.

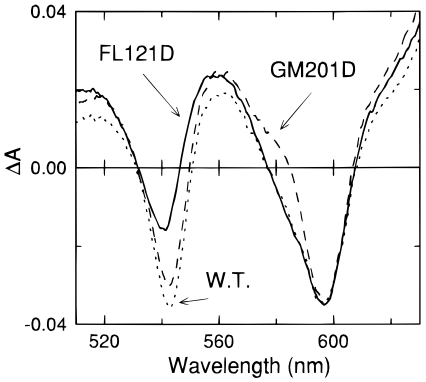

FIGURE 8: Transient absorption difference spectra encompassing the BPh and BChl Q_X region for F(L121)D (solid), G(M201)D (dashed), and wild type (dotted). The spectra were elicited with ~0.1 ps excitation flashes at 855 nm. See the text for delay times.

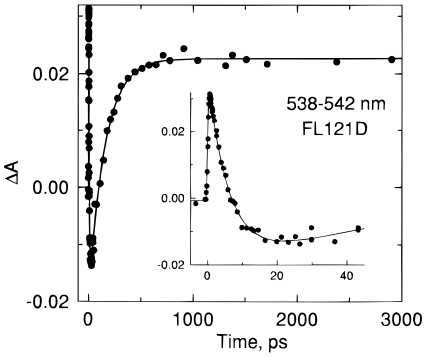

FIGURE 9: Kinetics of the appearance (inset) and decay of bleaching of the BPh_L Q_X band at 544 nm in F(L121)D RCs following excitation with an ~0.1 ps flash at 855 nm. The solid line is a fit to the sum of an instrument response (the convolution two pulses) plus two exponentials plus a constant. The time constants returned from the fit are 5.5 ± 0.3 and 155 ± 14 ps.

Table 1: Observed Lifetimes and Yields in Wild-Type and Mutant RCs[a]

sample	T (K)	τ for P* (ps)	ϕ for P^+I^-	τ for P^+I^- (ps)	ϕ for $P^+Q_A^-$
wild type	285	4.3 ± 0.2	1	176 ± 12	1.0
G(M201)D	285	7.6 ± 0.5	1	159 ± 14	1.0
F(L121)D	285	5.8 ± 0.6	1	155 ± 20	0.78
	77	4.1 ± 0.6	1	116 ± 10	0.88
L(M212)H	285	8.5 ± 0.8	1	212 ± 15	0.76
	77	6.5 ± 0.9	1	410 ± 60	0.76
L(M214)H[b]	295	5.8 ± 0.3	1	350 ± 50	0.60
	77	3.3 ± 0.3	1	605 ± 50	0.40

[a] Wild type and *Rb. capsulatus* mutants, except for L(M214)H which is a *Rb. sphaeroides* mutant. The error in the yields of states is ±0.05. [b] Data from Kirmaier et al. (1991, 1995).

spectrum, and it can be seen from the data in Figures 6 and 7 that bleaching in this region is constant on the ∼20 ps time scale. This result is routinely obtained for wild-type RCs, and the standard interpretation is that P* decay is not accompanied by a detectable return of RCs to the ground state and, therefore, that initial charge separation proceeds with (essentially) unity yield. At 2 ns in the F(L121)D mutant, bleaching of P's band is reduced to ∼78% of its initial magnitude (Figures 6 and 7). A fit of the bleaching decay at 840−850 nm yields a time constant of 158 ± 35 ps (Figure 7). In the simple kinetic model of Figure 1B, this decay time is assigned as the P^+I^- lifetime.

Initial electron transfer in the F(L121)D mutant is also accompanied by the formation of a prominent bleaching of the 540 nm Q_X band of BPh_L, as seen in the spectrum taken at 22 ps (Figure 8). Figure 9 shows the time course for the appearance and subsequent decay of bleaching of this band. The data were fit to a dual-exponential function that returned time constants of 5.5 ± 0.3 and 151 ± 14 ps. These values reproduce those measured in other wavelength regions for the P* lifetime (measured via the stimulated emission kinetics) and the P^+I^- lifetime (measured by decay of bleaching of P's band).

The bleaching at 540 nm demonstrates that BPh_L is a central constituent of the transient intermediate in the charge separation sequence in F(L121)D RCs. However, as we will discuss in the next section, the transient in this mutant may not be simply $P^+BPh_L^-$. For this reason, and for uniformity of discussion with the results of the beta-type RCs, we will refer to the intermediate species as P^+I^-. Collectively, then, the measurements presented here provide the following assessment of electron transfer in the F(L121)D mutant. P* → P^+I^- proceeds with 100% yield (within a few percent) and a time constant of about 6 ps. This step is followed by a branched decay due to competitive electron transfer, P^+I^- → $P^+Q_A^-$ (78% yield) and P^+I^- → ground state (22% yield). Based on these yields, the measured P^+I^- lifetime of ∼155 ps, and the most simple kinetic branching model (e.g., as in Figure 1B), one can derive inherent time constants for P^+I^- → $P^+Q_A^-$ and P^+I^- → ground state of ∼200 and ∼660 ps, respectively. (In the Discussion, we will present a more detailed kinetic model.)

Electron transfer in the F(L121)D mutant clearly has parallels to RCs in which BPh_L has been replaced with another chromophore. These RCs include the L(M214)H RC from *Rb. sphaeroides* (Kirmaier et al., 1991, 1995), other beta-type RCs recently prepared in *Rb. capsulatus* (Heller et al., 1995a), and pheophytin-substituted RCs (Shkurapatov & Shuvalov, 1993; Schmidt et al., 1994). To have another point of comparison, we have prepared and investigated the L(M212)H RC of *Rb. capsulatus*. No data are shown for the L(M212)H mutant; a summary of the kinetics and yields is given in Table 1. The basic picture is similar to that described for the F(L121)D mutant and for previous mutants having a BChl or pheophytin in place of BPh_L. The P* lifetime is 8.5 ± 0.8 ps, and decay of P* is not accompanied by a significant change in the magnitude of bleaching of the dimer band between 840 and 850 nm. As described above, these data give an apparent 100% yield of initial charge separation, P* → P^+I^-. Subsequent decay of P^+I^- occurs in 215 ± 25 ps to give $P^+Q_A^-$ in 76% yield and a 24% yield of deactivation to the ground state. As described above for the F(L121)D mutant, these yields are determined from the changes in magnitude of bleaching at 840−850 nm.

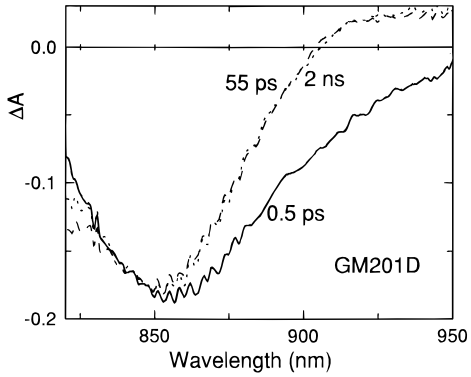

FIGURE 10: Transient absorption difference spectra of G(M201)D RCs taken at the times indicated following excitation with an ∼0.2 ps flash at 582 nm. The spectral region shown is described in the legend of Figure 6.

The results of low-temperature measurements on the F(L121)D and L(M212)H mutants are summarized in Table 1. In contrast to the situation at 285 K, at 77 K the F(L121)D and L(M212)H mutants differ significantly. In the L(M212)H mutant at 77 K, the lifetime of P^+I^- doubles to ∼400 ps, but the yields of $P^+Q_A^-$ and the ground state stay about the same as at 285 K. This is unlike the situation for *Rb. sphaeroides* L(M214)H RCs, where at 77 K a similarly increased P^+I^- lifetime is accompanied by a significantly *reduced* yield of $P^+Q_A^-$ (Kirmaier et al., 1991, 1995). The F(L121)D mutant stands in contrast to both of these mutants, showing a decreased P^+I^- lifetime and an increased yield of $P^+Q_A^-$ at 77 K compared to that at 285 K. As we will discuss below, these results likely reflect small differences in the relative free energies of $P^+\beta^-$ and $P^+BPh_L^-$/$P^+\beta^-$ in these three mutants.

Finally, we present data for the G(M201)D mutant. As noted above, this mutant incorporates an Asp near $BChl_L$. Figure 10 shows 285 K transient spectra for the G(M201)D mutant in the region of bleaching of the long-wavelength absorption of P and stimulated emission from P*. There is a constant amplitude of bleaching of P (840−850 nm) as a function of time beginning immediately after excitation and out to 2 ns. This result is identical to that obtained for wild-type RCs and is taken as a demonstration of essentially unity yield of charge separation at each step of the process. For the G(M201)D mutant, the only readily apparent difference from wild type is a lengthening of the P* lifetime to ∼8 ps (Table 1). Measurements in the Q_X band of BPh_L (Figure 11) give time constants of 7.5 ± 0.3 and 159 ± 14 ps for

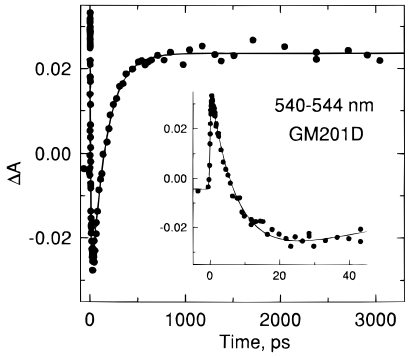

FIGURE 11: Kinetics of the appearance (inset) and decay of BPh_L Q_X bleaching at 544 nm in G(M201)D RCs following excitation with an ~0.2 ps flash at 855 nm. The solid line is a fit to a dual-exponential function (see the Figure 9 legend) and gives time constants of 7.5 ± 0.3 and 159 ± 14 ps.

the appearance and decay of bleaching at 540 nm, respectively. Again, these time constants can be associated with the P* and P^+I^- lifetimes, respectively.

The difference spectra in Figure 8 compare the Q_X-region data in wild-type, F(L121)D, and G(M201)D RCs. While taken at different absolute times, the spectra have been chosen at 3.5 lifetimes (1/e times) of P* decay for the respective samples.[2] The traces in Figure 8 were normalized on the basis of the *initial* bleaching at 600 nm at 0.5 ps. In other words, the spectra were normalized to reflect the same initial concentration of P*. The magnitudes of bleaching near 600 nm in these P^+I^- spectra, largely due to bleaching of P (plus possible contributions due to perturbations on the spectra of the assessory BChls), are the same in the three samples, but for G(M201)D, the bleaching appears to be slightly narrower. Bleaching of the BPh_L Q_X band at ~540 nm is of a similar magnitude for the wild-type and G(M201)D RCs, but in the F(L121)D mutant, the bleaching is smaller and at a slightly shorter wavelength.

DISCUSSION

Charge separation in the L(M212)H mutant of *Rb. capsulatus* is very similar to that reported previously in other RCs where BPh_L is replaced with a BChl (β) or with a pheophytin. The model we have used previously to depict electron transfer in beta-type RCs is given in Figure 1B. P* is somewhat longer lived than in the wild-type RC, but the yield of initial charge separation from P* is not diminished. The defining characteristic of beta-type RCs is the reduced yield of $P^+Q_A^-$ that results from competing deactivation processes of P^+I^-. The inherent rate for $P^+BPh_L^- \rightarrow$ ground state in wild-type RCs is about $(20\ ns)^{-1}$ (Woodbury & Parson, 1984; Ogrodnik et al., 1988; Goldstein & Boxer, 1989). However, in the L(M212)H RCs, as in other RCs of this class, the analogous decay of P^+I^- appears to be about 20 times faster (Kirmaier et al., 1991, 1995; Heller et al., 1995a). This means that deactivation to the ground state competes effectively with forward electron transfer to Q_A, resulting in a diminished yield of $P^+Q_A^-$. As indicated in Figure 1, we have suggested that an explanation for these results is that $P^+\beta^-$ is much closer in free energy to $P^+BChl_L^-$ than is $P^+BPh_L^-$ in wild-type RCs. [A similar situation is proposed for P^+Pheo^- in the case of the pheophytin-substituted RC (Schmidt et al., 1994).] This change in free energy results in a P^+I^- transient that is a mixture of two states and introduces a facile pathway to the ground state.[1] The inherent rate of $P^+BChl_L^- \rightarrow$ ground state is presumed to be on the order of $(1\ ns)^{-1}$ primarily due to the close proximity of P^+ and $BChl_L^-$ (Kirmaier et al., 1991, 1995; Bixon et al., 1992; Schmidt et al., 1994). This qualitative picture applies equally well to a number of different mutants or chemically substituted RCs yielding in place of BPh_L a pigment that has a greater *in vitro* reduction potential than does BPh (Kirmaier et al., 1991, 1995; Bixon et al., 1992; Shkurapatov & Shuvalov, 1993; Schmidt et al., 1994; Heller et al., 1995a; Huber et al., 1995).

Other than some small differences in lifetimes, at 285 K electron transfer in the F(L121)D mutant is the same as in L(M212)H RCs. In particular, the defining characteristic of these RCs is again the reduced yield of $P^+Q_A^-$ that is obtained from a branching point in the electron transfer pathway at P^+I^-. The most striking difference between F(L121)D and L(M212)H and other beta-type RCs is, obviously, the fact that the F(L121)D RC retains BPh_L. If we adopt the lines of reasoning applied to beta-type RCs, we come to the logical hypotheses that $P^+BPh_L^-$ is at a higher free energy in F(L121)D RCs than in wild-type RCs and that the Asp introduced at L121 is the cause of this change. Asp has the potential of being negatively charged, and introducing a negative charge near BPh_L would be expected to raise the free energy of $P^+BPh_L^-$. However, we do not know at present whether this Asp is indeed ionized, and it is quite plausible for a polar protonated carboxylic acid group to have a similar effect on the free energy of $P^+BPh_L^-$, though the magnitude would likely be smaller than if the acid group were charged. The important point here for the moment is the resultant end effect on electron transfer in this mutant and the parallel to the picture that has emerged for a number of other modified RCs.

The F(L121)D RC is the second mutant involving introduction of an Asp where a substantial change in overall yield of charge separation is obtained. The other example is the G(M201)D/L(M212)H RC (Heller et al., 1995b). We recently suggested that in this double mutant $P^+BChl_L^-$ is at a higher free energy than in the wild-type RC [or the L(M212)H single mutant], thus yielding the overall scheme of electron transfer shown in Figure 1C. G(M201)D/L(M212)H RCs are entirely different from L(M212)H RCs in that the branched electron transfer at P^+I^- is negated at room temperature. Following the logic presented above to explain the results on the beta-type single mutants, we have suggested that the findings on the double mutant have their origin in $P^+BChl_L^-$ being sufficiently above $P^+\beta^-$ in free energy that there is little quantum mechanical mixing or thermal equilibration involving these two states. Hence, in G(M201)D/L(M212)H RCs, P^+I^- is effectively $P^+\beta^-$. A branching of electron transfer at P* in the double mutant, including an apparent small yield of electron transfer to the M side, further attends the proposed upshift in the free energy of $P^+BChl_L^-$. Thus, there may be a consistent explanation for the results obtained for both F(L121)D and G(M201)D/L(M212)H RCs. Namely, in both cases, the Asp residue that has been introduced near a chromophore (BPh_L or

[2] This presentation does not represent a rigorous quantitative comparison of magnitudes since the subsequent ~170 ps kinetics will also modulate the maximal amplitude of $P^+BPh_L^-$ that is seen. However, the $P^+BPh_L^- \rightarrow P^+Q_A^-$ rates are so similar in the three RCs that Figure 8 suffices for the purposes intended here.

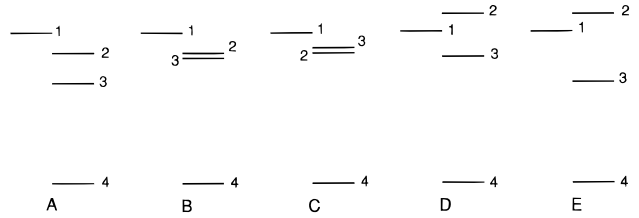

FIGURE 12: Energy level schemes. P* (1) and $P^+Q_A^-$ (4) have been kept at constant energy in these diagrams, and the relative energies of $P^+BChl_L^-$ (2) and $P^+BPh_L^-/P^+\beta^-$ (3) have been varied to display the orderings proposed for different RCs depending on the type of mutation. General classifications are (A) wild type, (B) F(L121)D, (C) L(M212)H, (D) G(M201)D/L(M212)H, and (E) G(M201)D. See the text for details.

$BChl_L$) appears to raise the free energy of the corresponding charge-separated state ($P^+BPh_L^-$ or $P^+BChl_L^-$).

Figure 12 depicts plausible schemes for the relative energy ordering of states in the various RCs we have discussed. Following from the above discussion, the scheme for F(L121)D (Figure 12B) is unlike that for the wild type (Figure 12A) in that $P^+BPh_L^-$ is at a higher free energy, and it is unlike that for the beta type (e.g., L(M212)H; Figure 12C] in that $P^+BPh_L^-$ is lower in free energy than $P^+\beta^-$. These relative energy positions are hypotheses, of course, but they are consistent with both the room- and low-temperature data and can be supported by kinetic modeling. At low temperatures, the yield of $P^+BPh_L^-$ is *increased* to almost 90% in the F(L121)D mutant. This finding is consistent with a thermal equilibrium model having $P^+BPh_L^-$ lower in free energy than $P^+BChl_L^-$. In the L(M212)H mutant, the yield of $P^+Q_A^-$ is about the same at 77 and 295 K (Table 1). The temperature dependence of the yield of $P^+Q_A^-$ in L(M214)H RCs is different still (Kirmaier et al., 1991, 1995). In the L(M214)H mutant, the yield is about 60% at room temperature and is *reduced* to 40% at 77 K (and to 25% at 5 K). These are real differences among the three mutants, which suggest the three RCs have slightly different free-energy gaps (and state orderings) between $P^+BChl_L^-$ and $P^+BPh_L^-/P^+\beta^-$. These differences in turn would dictate the contributions of these states to P^+I^- and thus give different overall rates of charge separation and charge recombination.

We have modeled the kinetics using different state orderings and free-energy gaps between P*, $P^+BChl_L^-$, and $P^+BPh_L^-/P^+\beta^-$. Figure 13 shows the designations of the various rate constants, and Table 2 gives some typical results. P* is placed highest in free energy always, with $P^+BChl_L^-$ 65 meV below P*, in accord with our work on the L(M214)H mutant, pheophytin-substituted RCs, and other mutants (Nagaragan et al., 1993; Bixon et al., 1992, 1995; Schmidt et al., 1994; Kirmaier et al., 1995).

What one might expect qualitatively and intuitively is borne out by this relatively simple kinetic analysis. First, $P^+BPh_L^-$ in the F(L121)D mutant must be below $P^+BChl_L^-$ in order to obtain a higher yield of $P^+Q_A^-$ at 77 K than at 285 K, but the free-energy gap must be very small (5−10 meV). With larger free-energy gaps, the yield of $P^+Q_A^-$ rapidly approaches 100% at low temperatures (other things being equal). This *trend* is demonstrated in the two pairs of 285 and 77 K simulations on the F(L121)D RCs in Table 2. For the L(M212)H mutant, a pair of 285 and 77 K simulations is shown with $P^+\beta^-$ above $P^+BChl_L^-$ by 10 meV. The simulation predicts a modest decrease in the $P^+Q_A^-$ yield

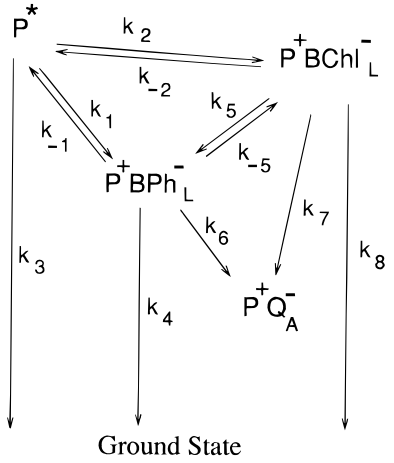

FIGURE 13: Kinetic scheme used in the simulations of the P^+I^- lifetimes and $P^+Q_A^-$ yields in F(L121)D and L(M121)H RCs. In the latter, $P^+BPh_L^-$ is replaced with $P^+\beta^-$. The results of a subset of the simulations are presented in Table 2.

as the temperature is reduced (78 to 69%), whereas little change is observed experimentally (78 to 76%; Table 1). These results suggest that the free-energy gap between $P^+BChl_L^-$ and $P^+\beta^-$ in L(M212)H RCs is even less than 10 meV. The difficulty with such a small free-energy gap (again, all other things being equal) is that the discrepancy between the predicted P^+I^- lifetime (Table 2) and the measured value of ∼400 ps (Table 1) becomes even larger.

Another puzzle is that, with such a small (<20 meV) free-energy gap between $P^+BPh_L^-$ and $P^+BChl_L^-$, the maximal transient population of $P^+BPh_L^-$ in the simulations on the F(L121)D RC ranges from 50 to 60% at 295 K, and there is a corresponding 40−50% transient population of $P^+BChl_L^-$. Since the F(L121)D mutant retains BPh_L, there is an opportunity to assess quantitatively BPh_L^- formation via bleaching of the BPh_L Q_X band. A similar assessment for β^- is not so straightforward in the L(M212)H or L(M214)H mutants, where the bleaching of the Q_X band of β overlaps with that of P as well as with electrochromic shifts or bleaching of the absorption of $BChl_L$. It can be seen in Figure 8 that the magnitude of the BPh_L Q_X bleaching in F(L121)D is smaller than that for the wild type or for G(M201)D. Consider, however, the ground state absorption spectrum (Figure 3). Even at room temperature, the magnitude of the 544 nm shoulder on the 530/544 manifold is noticeably smaller than in the wild type. This is clearly demonstrated in the spectra at 77 K (Figure 5). This apparent reduction in the strength of the BPh_L Q_X absorption may in and of itself account for the reduced magnitude of BPh_L bleaching at 544 nm in the F(L121)D mutant. Also, bleaching near 600 nm in the F(L121)D RC is the same as in the wild type and the G(M201)D mutant. In other words, the ground state and transient spectra suggest that there is a higher transient population of $P^+BPh_L^-$ and a lower transient population of $P^+BChl_L^-$ than the simulations predict.

The resolution of this is not readily apparent but leads us to examine alternatives to the simple picture considered thus far. One possibility is that the $P^+BPh_L^-$ → ground state deactivation process has a much faster rate (k_4) in the F(L121)D mutant than in the wild-type RC. This could occur, for example, if the Asp at L121 significantly affects the reorganization energy for the process. In the limit that an Asp at L121 has *no* effect on the free energy of $P^+BPh_L^-$,

Table 2: Simulations of P^+I^- Lifetimes and $P^+Q_A^-$ Yields in F(L121)D and L(M212)H RCs[a]

sample	T (K)	ΔG (meV) P*−$P^+BChl_L^-$	ΔG (meV)[b] P*−$P^+BPh_L^-$	τ_{-1}	τ_{-2}	τ_5	τ_{-5}	τ for P^+I^-	ϕ for $P^+Q_A^-$
F(L121)D	285	−65	−75	625 ps	100 ps	0.5 ps	0.7 ps	174 ps	0.88
	77	−65	−75	2.6 μs	115 ns	0.5 ps	2.3 ps	145 ps	0.94
F(L121)D	285	−65	−70	526 ps	100 ps	0.5 ps	0.6 ps	181 ps	0.84
	77	−65	−70	1.2 μs	115 ns	0.5 ps	1.5 ps	161 ps	0.90
L(M212)H	285	−65	−55	285 ps	100 ps	0.7 ps	0.5 ps	209 ps	0.78
	77	−65	−55	125 ns	115 ns	2.3 ps	0.5 ps	278 ps	0.69

[a] The τ_i ($1/k_i$) values are the inherent time constants for the corresponding processes given in Figure 13. The following were held constant: $\tau_1 = 7$ ps, $\tau_2 = 34$ ps, $\tau_3 = 200$ ps, $\tau_4 = 20$ ns, $\tau_6 = 125$ ps, $\tau_7 = 700$ ps, and $\tau_8 = 800$ ps. Also, τ_5 for F(L121)D and τ_{-5} for L(M212)H were set at 0.5 ps. τ_{-1}, τ_{-2}, and τ_{-5} were calculated from $\tau_{-i} = \tau_i e^{\Delta G/kT}$. The P^+I^- lifetime (τ) in column 9 is the value obtained by fitting the simulated time evolution of $P^+Q_A^-$ formation or ground state repopulation. The yield of $P^+Q_A^-$ (ϕ) in column 10 is also derived from the fits and represents the final concentration of $P^+Q_A^-$ relative to P*. [b] The free energy gap between P* and $P^+BPh_L^-$ in F(L121)D RCs and between P* and $P^+\beta^-$ in L(M212)H RCs.

an increase in the rate of $P^+BPh_L^-$ deactivation from about $(20\ ns)^{-1}$ to about $(800\ ps)^{-1}$ would be required in order to reproduce the 78% yield of $P^+Q_A^-$ at 285 K. An increased rate of $P^+BPh_L^- \rightarrow P^+Q_A^-$ with a reduction in temperature could then explain the results at 77 K (Table 1). A more plausible scenario is that the Asp at L121 changes both the free energy of $P^+BPh_L^-$ and the reorganization energy for deactivation. To test this case, we performed simulations using the same parameters as in the top 285 and 77 K pair given in Table 2 ($P^+BPh_L^-$ 10 meV below $P^+BChl_L^-$) but with k_4 set at $(2\ ns)^{-1}$. This simulation yields P^+I^- lifetimes of 167 and 137 ps at 285 and 77 K, respectively, and corresponding $P^+Q_A^-$ yields of 82 and 88%. These values are in reasonable agreement with the results on the F(L121D) RC (Table 1).

It can be seen from these comments that various combinations of free energies and rates can yield reasonably successful simulations of the observed P^+I^- lifetimes and $P^+Q_A^-$ yields. Some variation in the microscopic rate constants with mutant and/or temperature also may be expected because of factors not included in the simple thermal equilibrium model (Kirmaier & Holten, 1990; Kirmaier et al., 1991, 1995; Jia, 1993; Laporte et al., 1993; Woodbury & Parson, 1994; Peloquin et al., 1994; Ogrodnik et al., 1994; Bixon et al., 1995). For example, structural parameters, the electronic interactions between the relevant states, reorganization energies, and the proposed heterogeneous distributions of free energies and electron transfer rates may change with RC and temperature. Although such factors may be important for reproducing the results in detail, they do not change the basic conclusions from the simple zeroth order model presented in Figures 1 and 12. In particular, the major conclusion is that the experimental results obtained here on the F(L121)D, L(M212)H, and G(M201)D mutants and previously on the G(M201)D/L(M212)H, L(M214)H, and L(M214)H/E(L104)V mutants (Kirmaier et al., 1991, 1995; Heller et al., 1995b) and pheophytin-substituted RCs (Shkurapatov et al., 1993; Schmidt et al., 1994) are most consistent with the idea that the differences in the primary photochemistry among these RCs, including the temperature dependence of the $P^+Q_A^-$ yield, are governed largely by the relative free-energy positions of P*, $P^+BChl_L^-$, and $P^+BPh_L^-$/$P^+\beta^-$.

The obvious inference is that the free-energy gaps between these states are relatively small in all these RCs. The free-energy gap between P* and $P^+BPh_L^-$ in wild-type RCs is 250−280 meV (Chidsey et al., 1985; Goldstein & Boxer, 1989; Ogrodnik et al., 1988, 1994), although smaller values are proposed to be more appropriate immediately after excitation (Woodbury & Parson, 1984; Peloquin et al., 1994). If $P^+BChl_L^-$ lies ∼65 meV below P*, then $P^+BChl_L^-$ lies ∼200 meV or so above $P^+BPh_L^-$. In the model, the free energy of $P^+BChl_L^-$ with respect to $P^+BPh_L^-$/$P^+\beta^-$ is central since the degree of participation of $P^+BChl_L^-$ in the transient P^+I^- is the critical determinant of the rate of charge recombination that competes with electron transfer to Q_A. One expects $P^+\beta^-$ to be raised in the *Rb. capsulatus* L(M212)H and *Rb. sphaeroides* L(M214)H RCs by ∼200 meV relative to $P^+BPh_L^-$ in the wild type due to the 180−280 meV difference in reduction potentials of BChl and BPh *in vitro* (Fajer et al., 1975; Cotton & Van Duyne, 1979). Hence, $P^+\beta^-$ should be very close to $P^+BChl_L^-$ in free energy in the beta-type single mutants [within 20 meV or so; Table 2 and Kirmaier et al. (1995)]. This energy positioning is supported by the pronounced additional effect that removal of a hydrogen bond between Glu L104 and β has on the $P^+Q_A^-$ yield in the *Rb. sphaeroides* L(M214)H/E(L104)V double mutant (Kirmaier et al., 1995). Removal of the hydrogen bond is expected to increase the free energy of $P^+\beta^-$ by a modest ∼60 meV (values range from 30 to 80 meV; Hanson et al., 1987; Michel-Beyerle et al., 1988; Lin et al., 1994). However, an ∼60 meV increase in the free energy of $P^+BPh_L^-$ is not sufficient to have a measurable effect on the $P^+Q_A^-$ yield in the E(L104)V single mutant or the analogous E(L104)L *Rb. capsulatus* RC (Bylina et al., 1988; Kirmaier et al., 1995). The resulting ∼140 meV free-energy gap between $P^+BPh_L^-$ and $P^+BChl_L^-$ in the E(L104) mutants is clearly too large for $P^+BChl_L^-$ to be thermally populated from $P^+BPh_L^-$ or for one to consider electronic mixing to be of any consequence in altering the characteristics of the two states.

On the basis of this logic, it would appear that the effect of the Asp at L121 in the F(L121)D mutant is closer to the effect of replacing BPh_L by a BChl (β) (∼200 meV) than it is to removing a hydrogen bond from the chromophore (∼60 meV). This conclusion is supported by the F(L121)D simulations (Table 2) and our recent results on the G(M201)D/L(M212)H mutant. In the latter RC, placement of an Asp near $BChl_L^-$ appears to raise the free energy of $P^+BChl_L^-$ by over 100 meV in order to cause the observed effects on the photochemistry (Figures 1 and 12D).

Thus, a consistent picture of the results on a number of mutants and chemically modified RCs can be obtained under the assumption that the predominant (but probably not sole) effect of the modifications is modulation of the relatively small free-energy gaps among P*, $P^+BChl_L^-$, and $P^+BPh_L^-$/

$P^+\beta^-$. The inference from this analysis that $P^+BChl_L^-$ lies below P* in the native RC is also in keeping with previous work on wild-type RCs and other mutants (Holzapfel et al., 1990; Parson et al., 1990; Finkele et al., 1990; Bixon et al., 1991, 1995; Nagarajan et al., 1990, 1993; Chan et al., 1991b; McDowell et al., 1991; Maiti et al., 1993). This conclusion supports but does not prove the idea that $BChl_L$ serves in a two-step process, $P^* \rightarrow P^+BChl_L^- \rightarrow P^+BPh_L^-$. However, if this mechanism operates, concurrently there must be a reasonable contribution of the one-step superexchange process $P^* \rightarrow P^+BPh_L^-$ in which $P^+BChl_L^-$ participates as a virtual intermediate. This requirement follows from the logic that if the mechanism were purely two steps then changing the energy of the final state should not affect the P* lifetime. Yet the P* lifetime *is* altered in all RCs where the $P^+BPh_L^-$ free energy is changed, and further, this perturbation persists at low temperatures where thermal repopulation of P* from the charge-separated intermediate should not significantly affect the P* kinetics. The parallel contributions of both mechanisms have been suggested previously (Bixon et al., 1991, 1995; Chan et al., 1991b; Nagarajan et al., 1993).

The results we have obtained for the single G(M210)D mutant are very similar to those obtained previously for the analogous *Rb. sphaeroides* G(M203)D mutant (Williams et al., 1992). In this earlier work, the P* lifetime was found to be ~11 ps and there was no effect on the yield of electron transfer. Assessment of electron transfer in the G(M203)D RC was based on the idea that the Asp residue is probably hydrogen bonded to the ring V keto group of $BChl_L$, thus lowering the free energy of $P^+BChl_L^-$. This is opposite to what we have proposed for the effect of the Asp in the F(L121)D and opposite to the effect we have proposed for the Asp in the G(M201)D/L(M212)H mutant. In both of the latter RCs, significant reductions in the yields of electron transfer have enabled us to gain more direct insight into the root origins of the effects. In the absence of such effects, the lengthened P* lifetime (i.e., slower initial charge separation) in the G(M201)D or G(M203)D mutant could be rationalized on the basis of either a slight increase or a slight decrease in the free energy of $P^+BChl_L^-$. It is also possible, of course, that the G(M201)D *Rb. capsulatus* and G(M203)D *Rb. sphaeroides* mutants are not identical in terms of the local effects of the introduced Asp. As we have seen here, there is a small difference in the temperature dependence of $P^+I^- \rightarrow P^+Q_A^-$ electron transfer in the L(M212)H and L(M214)H mutants of *Rb. capsulatus* and *Rb. sphaeroides*, respectively. A study of the MG203D/L(M214)H *Rb. sphaeroides* mutant would be useful in assessing the situation.

There have been fairly detailed and specific predictions made for the effects on the ground state absorption spectra when a bare charge is placed near a pigment (Eccles & Honig, 1983; Hanson et al., 1987a,b). A negative charge near ring V of BChl or BPh should red-shift the Q_Y band and blue-shift the Q_X band. In the ground state spectrum of the F(L121)D RC, there appears to be an ~2 nm blue shift of the Q_X band of BPh_L that is borne out by the transient bleaching in Figure 8. The 760 nm manifold is clearly split into two bands at 77 K, but it is not possible to say whether this splitting represents a blue-shifted or a red-shifted position for BPh_L compared to the wild-type RC. The Q_Y region of the G(M201)D and G(M201)D/L(M212)H mutants is rather silent with regard to spectral perturbations on $BChl_L$, but in the Q_X, region there is a well-resolved new band at 582 nm. This feature appears to be a new blue-shifted position of the band that is at ~587 nm in wild-type, F(L121)D, and L(M212)H RCs. Again, it is not possible to be certain of this assessment, but the spectra in Figure 5 would seem to strongly suggest this conclusion. The directions of the spectral shifts that are observed are in agreement with the theoretical predictions for the effects of charges near the pigments (Eccles & Honig, 1983; Hanson et al., 1987a,b) and in any case should provide further impetus for efforts to model the RC spectra under different conditions. As a final point, we note that the spectral effects shown in Figure 5 are opposite to what one would expect if the Asp in the G(M201)D mutant is simply hydrogen bonded to the ring V keto group of $BChl_L$. It has been shown previously in the E(L104)L and E(L104)V mutants that removal of the hydrogen bond between Glu L104 and the ring V keto group of BPh_L causes the 540 nm Q_X band of this pigment to shift to 530 nm (Bylina et al., 1988; Kirmaier et al., 1995). An analogous effect on the Q_X absorption of β has been found in the L(M214)H/E(L104)V double mutant (Kirmaier et al., 1995). Consequently, *addition* of a hydrogen bond to the same position of $BChl_L$ should cause a red shift in the Q_X band (other things being equal). There is no obvious sign of such an effect in the spectra of Figure 5. Indeed, the opposite appears to be true as described above.

This discussion of spectral effects and theoretical predictions for charged groups near the cofactors is not to be construed as proof or a preference on our part that either Asp L121 or Asp M201 is in fact charged. Polar and polarizable groups can be expected to exert an influence on the optical properties of BChl and BPh. Such effects have also been amply demonstrated in rhodopsin, where introduction of a single hydroxyl-bearing residue near the retinal chromophore can cause shifts in its peak wavelength as large as 500 cm^{-1} (Chan et al., 1992; Lin et al., 1994b). It would, therefore, seem reasonable that a protonated carboxylic acid group could exert a profound influence not only on the ground state spectra but also on the energies of the charge-separated intermediates and on the electron transfer rates. We are currently working toward testing these ideas with further mutations at both the L121 and M201 sites.

ACKNOWLEDGMENT

We acknowledge useful disscussions with Drs. D. Bocian and S. Boxer. We also thank Ms. G. Yeh for valuable assistance with the RC preparations.

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BI961362F