Characterization of Bacterial Reaction Centers Having Mutations of Aromatic Residues in the Binding Site of the Bacteriopheophytin Intermediary Electron Carrier†

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ABSTRACT: We report the initial characterization of a series of reaction centers (RCs) from the photosynthetic bacterium *Rhodobacter capsulatus* having single or double mutations of phenylalanines 97 and 121 on the L polypeptide. Substitution of these aromatic amino acids, which may interact with the photoactive bacteriopheophytin associated with the L polypeptide (BPh_L), was carried out to examine their possible roles in electron transfer, charge stabilization, and/or BPh_L binding. In some mutant RCs, the wild-type pigment content is obtained while in certain others a bacteriochlorophyll (BChl) replaces BPh_L. The mutant RCs with wild-type pigment content are found to have overall photochemistry effectively identical to that of wild-type RCs. This indicates that aromatic residues at L97 and L121 are not critical factors in the charge separation process, although an approximate 2-fold increase in the rate of electron transfer from BPh_L^- to Q_A is observed in two mutants where residue L121 is leucine. In two double mutants where L121 is histidine and L97 is either valine or cysteine, BPh_L is replaced with a BChl (denoted β). This pigment content is surprising since in the native RC structure amino acid L121 is not in optimum geometry for coordination to the Mg in the center of the pigment macrocycle. Charge separation takes place in the β-containing mutants with an ~70% yield of $P^+Q_A^-$ at 285 K compared to ~100% for wild-type. The photochemistry of these new beta-type RCs is very similar to that reported previously for the beta RC from *Rhodobacter sphaeroides* wherein the same pigment change was induced by a mutation in the M polypeptide.

The bacterial reaction center (RC) is the membrane-bound pigment−protein complex within which light energy is converted to chemical energy via a series of fast and highly specific electron transfer reactions that separate charge with near-unity quantum yield (Deisenhofer & Norris, 1993). Excitation energy is delivered to a dimer of bacteriochlorophyll (BChl) molecules called P, raising P to its lowest energy excited singlet state, P*. Within about 3 ps, an electron arrives on the bacteriopheophytin molecule associated with the L polypeptide (BPh_L), forming $P^+BPh_L^-$. Although a neighboring $BChl_L$ almost certainly is involved in this process, the precise mechanism is a point of much controversy (Breton et al., 1990; Finkele et al., 1990; Bixon et al., 1991; Kirmaier & Holten, 1991; Nagarajan et al., 1993; Jia et al., 1993; Peloquin et al., 1994; Marchi et al., 1993; Warshel et al., 1994). The BPh_L anion transfers an electron to the primary quinone, Q_A, in about 200 ps. Clearly underlying the unity quantum yield of $P^+Q_A^-$ formation is the fact that these charge separation steps are much faster than any charge recombination or other photophysical processes. On a broad level, there are three, likely interrelated, unresolved issues fundamental to understanding the photochemistry of the RC: (i) the mechanism(s) of forward electron transfer, including the role of $BChl_L$; (ii) the origin of the directionality of electron transfer essentially exclusively via the cofactors on the so-called L-side of the RC; and (iii) the means by which the deactivation processes that would wastefully return the photoenergized RC to the ground electronic state are inhibited.

These issues have been addressed by an already extensive body of experimental investigations of RCs having one or more mutations at various sites near the pigments (Bylina & Youvan, 1988; Kirmaier et al., 1988, 1991; Bylina et al., 1988; Finkele et al., 1990; Coleman & Youvan, 1990; Robles et al., 1990; McDowell et al., 1991; Taguchi et al., 1992; Murchison et al., 1993; Nagarajan et al., 1993; Jia et al., 1993). In this paper, we examine *Rhodobacter capsulatus* RCs having single or double mutations of two phenylalanines in the vicinity of the binding pocket of BPh_L at positions 97 and 121 on the L polypeptide (Figure 1). F(L97) is conserved in *Rb. capsulatus*, *Rhodobacter sphaeroides*, *Rhodopseudomonas viridis*, and *Chloroflexus aurantiacus*, while the symmetry-related residue M124 (M126 in *Rb. sphaeroides*) near BPh_M is either Val, Leu, or Ile in each of the four bacterial species (Komiya et al., 1988; Ovchinnikov et al., 1988). F(L121) also is conserved in all four species, but in this case, a Phe also appears and is species-conserved near BPh_M at the symmetry-related M148 site (M150 in *Rb. sphaeroides*). The plane of the aromatic ring of F(L121) is nearly parallel to, and only ~3.7 Å away from, ring V of BPh_L (Michel et al., 1986; Yeates et al., 1988; El-Kabbani et al., 1991; Ermler et al., 1994). F(L97) is twisted and further away (~6 Å closest approach), but it lies between BPh_L and the phytyl tail of the L-side BChl ring of P. Both F(L97) and F(L121) have been noted as being of interest due to their aromaticity and proximity to BPh_L [see, e.g., Michel-Beyerle et al. (1988)].

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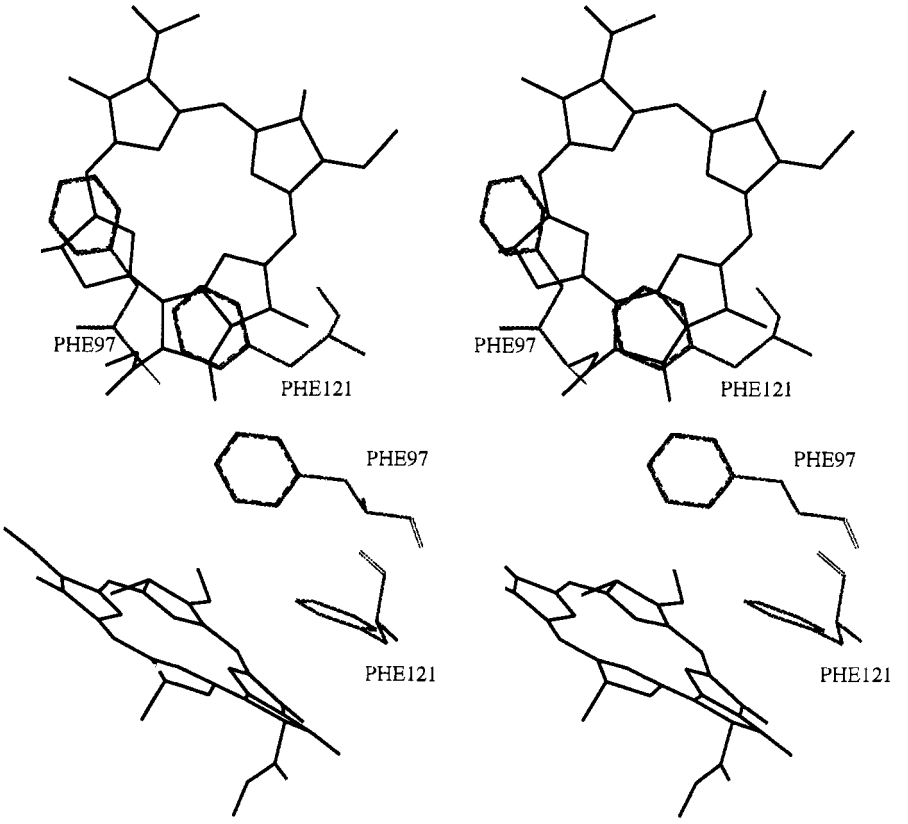

FIGURE 1: Stereoviews of BPh_L and neighboring Phe L97 and Phe L121, from *Rps. viridis* crystal structure coordinates provided by J. Deisenhofer.

We have changed these residues with the idea of exploring one or more of several topics including: (1) their possible importance, particularly L121 (and by analogy M148), in the binding of the BPh_L (BPh_M) pigment; (2) their possible role in stabilizing the charge-separated intermediate $P^+BPh_L^-$; (3) their possible importance in mediating electron transfer. The last two ideas have analogies in roles proposed for Y(M208), W(M250), and Y(L162) in the RC, ideas that have been explored both via experiments and via calculations in a number of labs (Plato et al., 1989; Parson et al., 1990; Finkele et al., 1990; Coleman & Youvan, 1990; Nagarajan et al., 1993; Jia et al., 1993).

MATERIALS AND METHODS

Site-directed mutations were made using essentially the system originally developed by Youvan and more recently used by Woodbury and co-workers: *Rb. capsulatus* U43 background deletion strain and plasmid pU2924, mated into U43 using *Escherichia coli* strain S-17 (Bylina & Youvan, 1988; Taguchi et al., 1992). Plasmid pU2924, carrying the *puf* operon and generously provided by Dr. D. Youvan, is a derivative of pU2922. It differs from pU2922 in that the *Bam*HI and *Hin*dIII restriction sites outside the *puf* operon are removed (Youvan et al., 1985; Bylina et al., 1986, 1989). Thus, in pU2924 the L gene fragment is carried on a unique *Hin*dIII−*Kpn*I fragment, and the M gene fragment is carried on a unique *Kpn*I−*Bam*HI fragment. Mutations in the L gene were made with plasmid pBSL using the Bio-Rad Muta-Gene Phagemid *In Vitro* Mutagenesis Version 2 kit. Plasmid pBSL, which has the L gene *Hin*dIII−*Kpn*I fragment cloned into Stratagene's Bluescribe vector, was a gift from N. Woodbury. The oligonucleotide-mediated mutagenesis utilized oligos that carried wild-card replacements. For F(L97), the oligo altered the Phe codon TTC to nnC where n is a wild-card for any of the four nucleotides. The primer also incorporated a silent mutation of the Cys 98 codon TGC to TGT, which maintained Cys but removed a *Pst*I restriction enzyme recognition site. For F(L121), two oligos were used where the Phe TTC codon was changed to CnC in one and GnC in the other. These oligos added an *Age*I restriction site in the codon for Ile 117 (a silent mutation of ATT to ATA). Candidate mutants were identified by testing for the alteration of the restriction enzyme site added or removed in the mutagenesis step. The nature of the mutation was then identified by dideoxy DNA sequencing through the region of interest. The mutated *Hin*dIII−*Kpn*I L gene fragments from pBSL were shuttled directly into the *Rb. capsulatus puf* operon in pU2924 in S-17, and this construction was conjugated with the *Rb. capsulatus* U43 background deletion strain. For ultimate verification of the presence of the target mutation(s) and the fidelity of the neighboring DNA, pU2924 was recovered from each mutant *Rb. capsulatus* strain, the L gene fragment was ligated into pTz18u (Bio-Rad), and ~200 base pairs upstream and ~200 base pairs downstream of the mutation site were resequenced.

Isolation and purification of RCs from cultures grown semiaerobically 72−96 h largely followed established procedures [see, e.g., Taguchi et al. (1992)]. In brief, following cell breakage at 20 000 psi in a French pressure cell and removal of debris via low-speed centrifugation, chromatophores were spun down at 45K rpm in a Beckman Ti70 rotor. The chromatophore pellets were homogenized in 10 mM potassium phosphate buffer, pH 7.6, and the concentration was adjusted to OD ~25 at 880 nm in 1 cm. LDAO (lauryldimethylamine *N*-oxide) was added to the chromato-

phore solution to a final concentration of 1.5%. Following incubation at 37 °C for 5 min, the chromatophores were spun down again (2 h at 45K rpm) and discarded. RCs were precipitated from the supernatant via addition of solid ammonium sulfate. In 1.5% LDAO, RCs generally began precipitating near 0.28 g of $(NH_4)SO_4$/mL of solution. Low-cut precipitates obtained up to that point were discarded. The final concentration of $(NH_4)SO_4$ was typically 0.32−0.35 g/mL. The precipitated RCs were resuspended in 10 mM potassium phosphate buffer, pH 7.6, dialyzed against buffer C [10 mM potassium phosphate (pH 7.6)/0.05% LDAO/1 mM EDTA/100 μM sodium ascorbate], and finally purified via two rounds of chromatography on DEAE anion-exchange gel (Bio-Gel Agarose, 100−200 mesh, Bio-Rad Laboratories). Ubiquinone UQ_{10} was added to a final molar ratio of 2:1 (UQ_{10}:RC) after each chromatography, and the RCs were maintained in buffer C for storage (at −80 °C) and for all experiments. Absorption spectra were recorded on a Perkin−Elmer 330 spectrometer. The purity of RCs was judged from the ratio of absorbances at 280 and 800 nm, with a general finding of A_{280}/A_{800} ~1.6. An identical level of purity is obtained with wild-type *Rb. capsulatus* using the method outlined above. RC yields were measured in units of ODVs, defined as the volume of the sample in milliliters multiplied by its optical density at 800 nm in a 1 cm path length.

The primary electron transfer reactions were investigated at 285 K via transient absorption spectroscopy on a synchronously-pumped-laser-based system employing 10 Hz, ~150 fs excitation pulses at either 582 or 867 nm and ~150 fs broad band (i.e., "white light") probe flashes. In all experiments, the excitation flashes were defocused and/or attenuated so that 10−30% of the RCs were excited. A spectrum encompassing ~150 nm is obtained on each laser shot by utilizing a vidicon-based detection system. Further details of the transient absorption apparatus and data acquisition and analysis methods have been at described at length elsewhere (Kirmaier & Holten, 1991).

RESULTS

The ground state absorption spectra of mutants with a single change of Phe L97 or L121 to the aliphatic amino acids valine and leucine, F(L97)V and F(L121)L, respectively, are basically indistinguishable from wild-type. This is also true for the F(L97)V/F(L121)L double mutant where both changes are present. Figure 2 shows the spectrum (dashed) of the F(L121)L mutant. As representative of the group, F(L97)V RCs were examined along with wild-type RCs in side-by-side pigment extractions using procedures described previously (van der Rest & Gingras, 1974). A BChl:BPh ratio of 1.8 was found for both samples. Using the *Rb. sphaeroides* extinction coefficient at 800 nm of 288 mM^{-1} cm^{-1}, total pigment contents of 5.6 and 5.7 for F(L97)V and wild-type, respectively, were obtained. All three mutants grow photosynthetically and give RCs in yields roughly the same as wild-type (~10 ODVs per liter of culture).

From femtosecond transient absorption experiments of these three mutants, the native photochemistry, $P^* \rightarrow P^+BPh_L^- \rightarrow P^+Q_A^-$, was found to take place largely unperturbed. Figure 3 shows representative spectral data in the BPh_L Q_X and anion regions using the F(L97)V mutant. The spectrum observed at 0.3 ps is essentially identical to

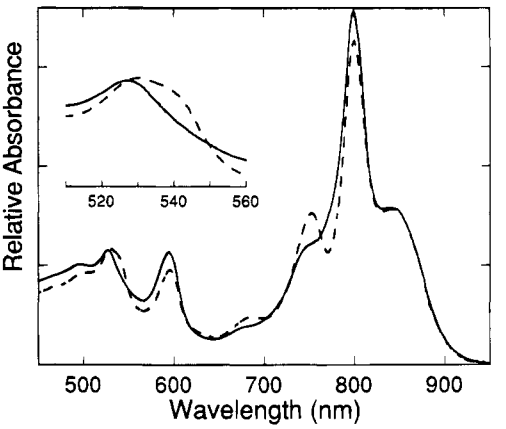

FIGURE 2: Room temperature ground state absorption spectra of RCs from the F(L121)L (dashed) and F(L97)V/F(L121)H (solid) mutants. The inset shows an expanded view of the same mutants in the region of BPh_L and BPh_M Q_X absorption.

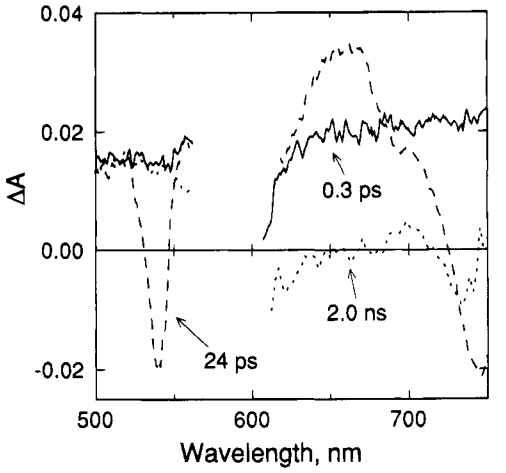

FIGURE 3: Transient difference spectra of F(L97)V RCs in the region of the Q_X bands of the BPhs and the transient BPh_L anion band. The spectra were taken at the time indicated following an 867-nm 0.15-ps excitation flash and can be assigned as follows: 0.3 ps to P*, 24 ps to $P^+BPh_L^-$, and 2 ns to $P^+Q_A^-$. Data were not obtained between ~560 and 610 nm due to cutoff of the dichroic filter employed in the probe light to remove the residual 582-nm flashes used to generate the continuum.

that observed at early time in wild-type RCs and can be assigned to P*. Similarly, the 24-ps spectrum can be assigned to $P^+BPh_L^-$ and the one at 2.0 ns to $P^+Q_A^-$. In the F(L97)V mutant, bleaching of the BPh_L Q_X band in state $P^+BPh_L^-$ occurs at ~542 nm, the same as found in wild-type RCs, as shown in Figure 3. This bleaching *may* occur 1−2 nm to shorter wavelength in F(L121)L and F(L97)V/F(L121)L RCs; however, low-temperature measurements are required to resolve this point unambiguously. Representative kinetic data for the appearance and subsequent decay of bleaching of the BPh_L Q_X band are shown in Figure 4. For all three mutants, the kinetics at 542 nm were fit to a function consisting of the cross-correlation of two pulses plus two exponentials plus a constant. The resulting time constants for the appearance of BPh_L bleaching (i.e., $P^* \rightarrow P^+BPh_L^-$) are 4.5 ± 0.3, 4.0 ± 0.6, and 4.4 ± 0.5 ps in the F(L97)V, F(L121)L, and F(L97)V/F(L121)L mutants, respectively, compared to 4.1 ± 0.4 ps obtained on control wild-type *Rb. capsulatus*. The time constants obtained for decay of BPh_L Q_X bleaching (i.e., for $P^+BPh_L^- \rightarrow P^+Q_A^-$) are 189 ± 12, 137 ± 11, and 110 ± 13 ps in F(L97)V, F(L121)L, and

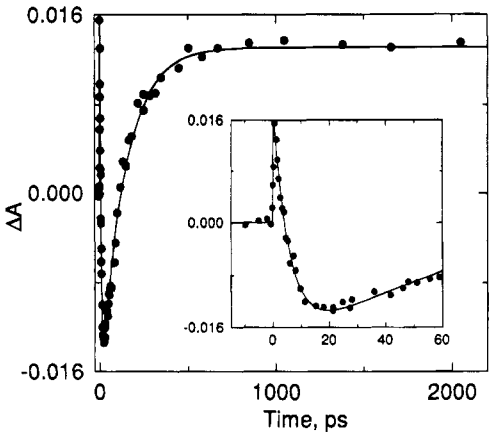

FIGURE 4: Kinetics of the absorption changes between 539 and 543 nm in the F(L121)L mutant following excitation with an 867-nm 0.15-ps flash. The inset shows a close-up view of the first 60 ps of the data and fits. The solid line is a fit to the cross-correlation of two pulses plus two exponentials plus a constant, returning time constants of 4.0 ± 0.6 ps for the appearance of BPh_L bleaching and 137 ± 11 ps for decay of this bleaching.

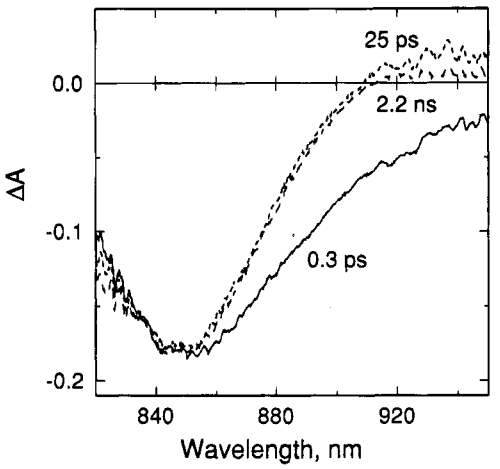

FIGURE 5: Transient difference spectra of F(L121)L RCs in the region of bleaching of the long-wavelength absorption band of P and stimulated emission from P*, taken at the times indicated following excitation with a 582-nm 0.15-ps excitation flash.

F(L97)V/F(L121)L RCs, respectively, compared to 194 ± 11 ps obtained for wild-type. There was no indication of bleaching of the Q_X band of BPh_M at ~530 nm in any of the mutants (see, e.g., Figure 3). Decay of the broad ~860–960 nm stimulated emission from P* (see spectra in Figure 5) was examined for the F(L121)L and F(L97)V/F(L121)L mutants. The time constants for decay of the P* stimulated emission obtained from single-exponential fits were within experimental error of those obtained for the appearance of BPh_L Q_X band bleaching in the respective mutants. As in wild-type RCs, the yield of $P^+Q_A^-$ appears to be essentially 100% for all three mutants. This is indicated by a constant amplitude of bleaching of the absorption band of P throughout the time course of a few hundred femtoseconds to several nanoseconds, as shown for the L(F121)L mutant in Figure 5.

The absorption spectrum of two double mutants having His at L121 and either Val or Cys at L97 is represented in Figure 2 by the (solid) spectrum of the F(L97)V/F(L121)H mutant. The Q_X absorption band of BPh_L near 542 nm appears to be absent [compare to F(L97)V RCs in Figure 2 inset], and the BPh Q_Y absorption band near 760 nm is

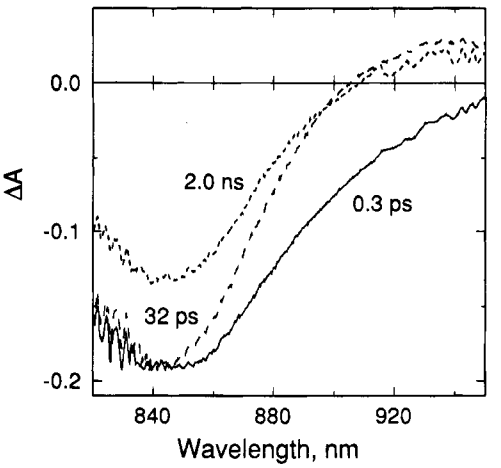

FIGURE 6: Transient difference spectra of F(L97)V/F(L121)H RCs taken in the same spectral region and under the same excitation conditions as in Figure 5.

reduced. In the regions of BChl absorption near 600 and 800 nm, there is a general broadening and/or increase in absorption with perhaps a slight new shoulder near 780 nm. This spectrum is noticeably different from wild-type, and the noted changes are very similar to those previously reported for the "beta" mutant from *Rb. sphaeroides*, L(M214)H, in which it was found that a BChl molecule replaces the native BPh_L acceptor (Kirmaier et al., 1991). Pigment extractions performed on the F(L97)C/F(L121)H mutant and wild-type RCs gave BChl:BPh ratios of 4.5 and 1.8 in the two RCs, respectively, and total pigment contents of 5.6 and 5.7, respectively. The values for the mutant are essentially identical to those reported previously for L(M214)H RCs, and, as for that mutant, indicate our new mutants contain 5 BChls and 1 BPh with the new BChl (β) presumably replacing BPh_L (see also Discussion). The F(L97)V/F(L121)H and F(L97)C/F(L121)H mutants grow photosynthetically (though more slowly than wild-type). The yields of RCs (from semiaerobic growth in the dark) are comparable to wild-type.

The F(L97)C/F(L121)H and F(L97)V/F(L121)H mutants have photochemistry very similar to one another; representative data are shown in Figures 6–8 for the latter mutant. Figure 6 presents transient absorption spectra in the region of the long-wavelength absorption of P. As in wild-type RCs and the L97 and L121 mutants described above, stimulated emission from P* is clearly resolved in the 0.3-ps spectrum as the apparent extra absorption decrease to the red of the bleaching of the 850-nm ground state absorption band of P. Figure 7 (inset) shows the decay kinetics of the stimulated emission from P*, yielding a 5.5 ± 0.4 ps time constant when fit to a cross-correlation of two pulses plus a single exponential plus a constant. As seen in Figure 6, there is no decay of the bleaching of P absorption during the time of P* stimulated emission decay. However, bleaching of P's absorption does decay subsequently as shown. This decay was also fit to a single exponential plus a constant, yielding a time constant of 254 ± 35 ps at 845 nm (Figure 7). The yield of long-lived bleaching of P absorption, compared to the initial value, is 70% (determined from the kinetic fits and demonstrated via comparison of bleaching at 2 ns versus that at earlier times in the spectra in Figure 6). Figure 8 displays the transient absorption changes that occur in the Q_X and anion regions at the same key times.

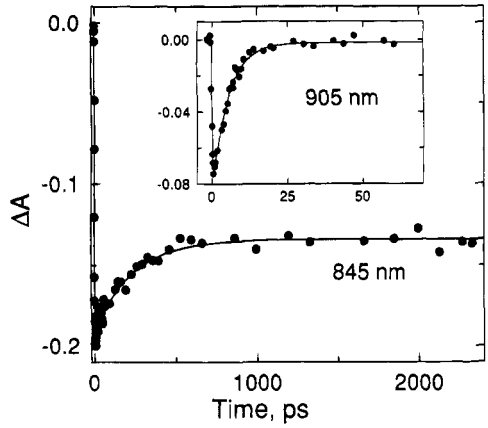

FIGURE 7: Kinetics of the absorption changes at 845 nm in F(L97)V/F(L121)H RCs following excitation with a 582-nm 0.15-ps excitation flash. The solid line is a fit to a function consisting of the cross-correlation of two pulses plus one exponential plus a constant. This fit returns a time constant of 254 ± 35 ps for decay of bleaching of P and an asymptotic bleaching 70% the magnitude of initial bleaching. In the inset, kinetic data for decay of stimulated emission from P* are shown, fit (solid line) to the same functional form as the 845-nm data, returning a lifetime for P* of 5.5 ± 0.4 ps.

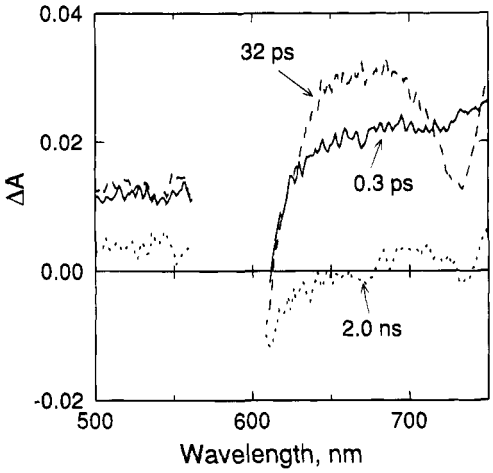

FIGURE 8: Transient difference spectra of F(L97V)/F(L121)H RCs taken in the same region and under the same conditions as in Figure 3.

[Data for wild-type RCs are not shown, but see the spectra for the F(L97)V mutant in Figure 3, and Kirmaier and Holten (1991).] The 0.3-ps spectrum is essentially identical to that of P* in wild-type RCs. Strikingly absent from the 32-ps spectrum in Figure 8 is bleaching at 542 nm, consistent with absence of the BPh_L pigment. The broad absorption band between 650 and 700 nm in the 32-ps spectrum is also distinct from the narrower BPh_L anion absorption band centered near 665 nm characteristic of state $P^+BPh_L^-$ in wild-type RCs and the L97 and L121 mutants described above (24-ps spectrum in Figure 3). The time constant measured for decay of the 650−700 nm transient absorption of the F(L97)V/F(L121)H mutant is 224 ± 18 ps, in agreement with the value found for the partial decay of P bleaching. The 2-ns spectrum is rather featureless and the same as that of $P^+Q_A^-$ in wild-type RCs.

Essentially identical spectral and kinetic results were obtained for the F(L97)C/F(L121)H mutant. The P* lifetime is 6.0 ± 0.5 ps as determined from decay of the stimulated emission from P*. The lifetime of the charge-separated intermediate was assayed by fitting the kinetics (as above) for decay of P bleaching at 840 nm (307 ± 27 ps) and from decay of the broad transient absorption between 640 and 720 nm (255 ± 30 ps). The yield of long-lived bleaching of P was again found to be 70%.

DISCUSSION

The relative geometries of Phe L97, Phe L121, and BPh_L are shown using the *Rps. viridis* crystal structure coordinates in two stereo perspectives in Figure 1. Phe L121 is the closer to BPh_L of the two amino acids and, as can be seen, potentially in position to π-stack with ring V of BPh_L. Phe L97 is positioned between BPh_L and the phytyl tail of the L-side ring of P (this detailing not shown). The initial goal of this work was to change these phenylalanines to aliphatic amino acids to investigate possible effects of their individual interactions with BPh_L, in the same vein as was done previously for Tyr M208 (M210 in *Rb. sphaeroides*) and Phe L181 with regard to their possible interactions with P and/or the monomeric BChls (Parson et al., 1990; Finkele et al., 1990; Nagarajan et al., 1993; Jia et al., 1993). Similarly, Trp M250 has been investigated with regard to its interactions with Q_A and possible involvement as a superexchange mediator of electron transfer from BPh_L^- to Q_A (Plato et al., 1989; Coleman & Youvan, 1990). The wild-card oligo used to produce mutations at L97 yielded Val and Cys upon initial sequencing of candidate mutants. Further mutants at this position were not sought since the primary goal was realized with either Val or Cys. DNA from each of these single mutants was used as template, in addition to wild-type, with two wild-card oligos to produce mutations at L121. The L121 oligos yield as possibilities Gly, Ala, Val, Leu, Arg, His, Asp, and Pro, a range of amino acids that includes not only aliphatics but also, *possibly*, both positively and negatively charged amino acids. Characterization of mutants with Asp or Arg at L121 is underway. Charged amino acids near the BPh_L acceptor would be very interesting (if charges are indeed obtained *in situ*) as they would almost certainly affect the free energy of the charge-separated state $P^+BPh_L^-$.

We have demonstrated here that the single mutants F(L97)V and F(L121)L and the double mutant F(L97)V/F(L121)L retain both the native pigment content and the native photochemistry. Native photochemistry is retained in the sense that there is initial charge separation forming $P^+BPh_L^-$ within a few picoseconds, electron transfer from BPh_L^- to Q_A on the 100−200 ps time scale, and an overall yield of $P^+Q_A^-$ formation of ~100% (as judged from constant bleaching of P at 840−850 nm throughout the time scale of 0.1 ps to several nanoseconds). One hypothesis discounted by our findings is that Phe L121, and by inference the species-conserved and symmetry-related Phe on the M polypeptide (M148), is essential for binding BPh_L (BPh_M). This is in contrast to the result obtained when the aromatic Trp M250 was changed to an aliphatic amino acid. In that case, removal of the putative π-stacking interaction between W(M250) and Q_A affected the binding of Q_A (Coleman & Youvan, 1990). The two phenylalanines investigated here, L121, in particular, and the previously investigated Glu L104 (Blyina et al., 1988), clearly are not individual determinants in binding BPh_L. Both BPh_L and BPh_M have a hydrogen bond from a Trp to the carbonyl oxygen of the methyl ester of ring V (Ermler et al., 1994), the potential importance of

which has yet to be investigated, as have cumulative effects of two or more of the direct interactions between BPh_L and the protein.

On the basis of the results obtained here, it also seems apparent that neither of these aromatics individually or acting together are key to efficient charge separation. Essentially wild-type photochemistry, most importantly an ~100% yield of $P^+Q_A^-$, is obtained in the cases of the aliphatic substitutions reported here, although there is a notable shortening of the time for decay of BPh_L bleaching in the F(L121)L and F(L97)V/F(L121)L mutants. In the latter mutant, the time constant for this process, nominally a measure of the rate of electron transfer from BPh_L^- to Q_A, is almost a factor of 2 shorter than in wild-type. On the most simple level, this would seem to indicate an enhancement of the rate of electron transfer from BPh_L^- to Q_A when Phe (L121) is an uncharged aliphatic residue. It is normally considered that the rate versus free energy balance for this reaction is already optimal in wild-type RCs. However, many mutants have displayed 2−3-fold changes, sometimes increases, in various electron transfer rates; for example, an ~100 ps time constant for BPh_L^- to Q_A electron transfer has also been found in the *Rb. capsulatus* L(M200)H heterodimer mutant (Kirmaier et al., 1988). It is not always clear what conclusions are most appropriate in these cases of relatively small changes in rates. Perturbation of both structure and energetics must be considered. With these caveats in mind, we consider briefly that, all other things being equal, the aromatic Phe L121 probably should have a stabilizing influence on a negative charge on BPh_L. Removing this Phe would, then, result in $P^+BPh_L^-$ being at a higher free energy with a correspondingly larger driving force for $P^+BPh_L^- \rightarrow P^+Q_A^-$ and a slower or at least comparable rate of electron transfer. Perhaps $P^+BPh_L^- \rightarrow P^+Q_A^-$ electron transfer in wild-type is very slightly toward the so-called normal region of the rate versus free energy relationship (where ΔG is less than the reorganization energy), and an improved Franck−Condon factor is obtained in the mutants. Low-temperature measurements may shed further light on potential interactions between Phe L121 and BPh_L (via changes in absorption spectra) and the influence of Phe L121 on the rate of electron transfer from BPh_L^- to Q_A.

The mutants in which His replaces Phe L121 and Val or Cys replaces Phe L97 yield new examples of "beta-type" mutants, those where BPh_L is replaced by a BChl (denoted β). The only other known RC of this type is the previously reported L(M214)H mutant of *Rb. sphaeroides* (Kirmaier et al., 1991). Three pieces of evidence support the conclusion that the F(L97)V/F(L121)H and F(L97)C/F(L121)H mutants have a BChl in place of BPh_L. First, as discussed under Results, the ground state absorption spectrum is consistent with the altered pigment content. Second, pigment extractions on F(L97)C/F(L121)H RCs give a BChl:BPh ratio of 4.5. This is only ~10% low compared to the 5.0 expected for replacement of BPh_L by a BChl and compares to 1.8, again ~10% low, obtained for wild-type. Also, as mentioned above, a BChl:BPh pigment ratio of 4.5 was obtained previously for L(M214)H RCs. Third, and perhaps the most compelling and interesting, the photochemistry of the two double mutants parallels almost exactly that of the original L(M214)H mutant (discussed further below). This point is relevant to sample composition in that the result of 4.5 for a BChl:BPh ratio could indicate a 50/50 mixture of RCs having lost a pigment at the BPh_L site and RCs with a substitution of BChl for BPh_L. However, RCs from the D_{LL} mutant (Robles et al., 1990), where BPh_L is known to be absent, are unstable toward isolation (at least to date). Additionally, it would be highly fortuitous for L(M214)H and the beta RCs here to give heterogeneous samples of precisely the same composition. Moreover, an RC with no pigment at the BPh_L site would not yield charge separation (as exemplified by the D_{LL} mutant). A sample containing 50% this kind of RC could give at most a 50% yield of $P^+Q_A^-$, inconsistent with the 70% found. Another possibility, discussed previously for the L(M214)H mutant, is that the samples contain 85% beta-type RC and 15% native (BPh_L containing) RC. However, again it would be highly fortuitous for L(M214)H and both F(L97)/F(L121)H double mutants to be heterogeneously identical, and there is no indication of native pigment photochemistry in any of the beta RCs (i.e., no indication of bleaching at 542 nm for a BPh_L Q_X band). Overall, the chemical and photochemical data taken as a whole point to high sample integrity.

The finding that a His residue at L121 in these two double mutants results in incorporation of a BChl in place of BPh_L is, however, largely serendipitous and surprising in view of the geometry shown in Figure 1. Three residues away and located nearly directly over the center of the BPh_L macrocycle is Ala L124, which would appear to be a more appropriate candidate for replacement with His in an attempt to force a BChl pigment. The L124 position is much more amenable to His−Mg ligation, and is analogous to that occupied by M212 (M214 in *Rb. sphaeroides*), which is centered over the opposite face of the pigment. A(L124)H is another mutant we have made, and our preliminary finding is that BChl is incorporated in place of BPh_L in these RCs as well. (The photochemistry of this mutant is much like the other beta-type RCs and will be described in a future publication.) Upon examining the geometry of the RC crystal structure, it is very difficult to see how a His at L121 can be ligated to the Mg of β unless there is significant, even gross, local dislocation of the protein and/or significant displacement of the pigment itself. However, the former possibility is not without precedent. Hawkins et al. (1994) have recently reported finding in a cytochrome that replacement of Phe 82 with His causes displacement of the wild-type Met 80 ligand to the iron in favor of the new His 82 (even though the native wild-type Met 80 is still present). Seemingly significant local change of the protein structure would be necessary to accomplish this.

Unlike the work of Hawkins et al. (1994), it is not possible to ascertain at present the identity of the ligand to β in our new mutants. Water may serve such a function. There are no other obvious neighboring amino acids that could coordinate. On the basis of the *in vitro* chemistry of BChl, it would be surprising for β, or any of the BChls in the RC, to be four-coordinate (i.e., without axial ligation). Boxer and co-workers have recently made the H(M202)G mutant of *Rb. sphaeroides*, where the His ligand to one of the rings of P is replaced with a Gly. In this mutant, the native BChl−BChl dimer appears to be retained (S. Boxer, personal communication). This is a surprising result that stands in stark contrast to the several BChl−BPh heterodimer mutant RCs previously obtained from mutation at the same site but using Leu instead of Gly (Bylina & Youvan, 1988, 1990; Kirmaier et al., 1988; McDowell et al., 1991). Schenck and

Table 1: Yields and Lifetimes of States in Beta-Type RCs[a]

sample	P* lifetime (ps)	$\phi(P^+I^-)$ (%)	P^+I^- lifetime[b] (ps)	$\phi(P^+Q_A^-)$ (%)	τ(et)[c] (ps)	τ(cr)[d] (ps)
L(M214)H[e]	5.8	100	350	60	583	875
F(L97)V/F(L121)H[f]	6.1	100	241	70	344	803
F(L97)C/F(L121)H[f]	5.5	100	281	70	401	936

[a] All data at 285 K. The errors are ±10% for the lifetimes and ±5% for the yields. [b] The lifetimes collected here are the average of the time constant for decay of P bleaching at 840−850 nm and the time constant for decay of the 630−700 nm transient absorption assigned to I^-. [c] Time constant for electron transfer $P^+I^- \rightarrow P^+Q_A^-$, calculated from the data in columns 4 and 5, assuming the simple branching model described in the text. [d] Time constant for charge recombination of P^+I^- to the ground state, calculated from the data in columns 4 and 5, assuming the simple branching model described in the text. This time constant includes both direct recombination and possible indirect routes involving thermal repopulation of P*. [e] *Rb. sphaeroides* mutant; data taken from Kirmaier et al. (1991) and Kirmaier et al. (1995a,b). [f] *Rb. capsulatus* mutants; this work.

co-workers also have found an unexpected pigment content in the *Rb. sphaeroides* H(M202)L/L(M214)H double mutant, where instead of a heterodimer/beta RC only the beta phenotype is obtained (C. C. Schenck, personal communication). Again, the ligation to BChl is called into question. The results on the *Rb. sphaeroides* H(M202)L/L(M214)H and H(M202)G mutants and our *Rb. capsulatus* F(L97)/F(L121)H mutants suggest that the ligation of BChl and the presence of BChl vs BPh in the RC are not dictated in a simple fashion by the presence or absence of a His. Clearly this has important ramifications for PSII where there are not enough His residues to coordinate all the Chls thought to be present (Kuhlbrandt & Wang, 1991). This discussion is of course speculative as pertaining to the F(L97)/F(L121)H double mutants reported here because there is no way to rule out gross structural changes to accommodate a His L121 ligation to β, although the structural dislocations would have to be significant.

To further explore the effect of a His at L121, we also isolated the F(L121)H single mutant. This mutant grows photosynthetically, albeit slowly as do the two F(L97)/F(L121)H double mutants. Attempts to purify RCs from the F(L121)H mutant have yielded poor results: 1−5% the normal yield and protein of questionable content and fidelity. While other RC purification procedures may give better results, we have at the moment a negative finding that allows for speculation regarding amino acid size effect on RC stability and/or pigment content and/or ligand binding. Specifically, it may be speculated that either in the end-product RC or at some point during protein assembly His L121 must either be ligated to β or be otherwise important for incorporation of the β pigment, but that this can only be accommodated when Phe L97 (or perhaps another large neighboring residue) is replaced with a smaller amino acid. Cysteine is the fourth smallest amino acid (molar volume ~109 Å^3), and valine has a molar volume of ~141 Å^3 (Bylina & Youvan, 1987). This compares to a molar volume of ~188 Å^3 for Phe, the third largest amino acid. A similar line of reasoning regarding size constraints was invoked in the case of the D_{LL} mutant, where residues 192 through 217 comprising the D helix of the M polypeptide were swapped for residues 165 through 190 comprising the D helix of the L polypeptide (i.e., a mutant with two D helices of the L polypeptide was constructed). Attempts to isolate RCs from this mutant also have been frustrated, but in an antenna-less strain the D_{LL} RC was shown to be present in chromatophore membranes and missing the BPh_L pigment (Robles et al., 1990; Breton et al., 1990). Both the lack of RC stability and loss of the BPh_L pigment were hypothesized to be due to the overall larger molar volume of the amino acids of the D helix of the L polypeptide compared to those comprising the D helix of the M polypeptide. While such size considerations may pertain to secondary changes required for BChl incorporation when there is a His at L121, our results leave open to speculation why the simple result of BPh incorporation (stable wild-type RCs) is not obtained in the case of the single F(L121)H mutation.

We turn finally to discuss the photochemistry of the two new beta-type mutants, F(L97)V/F(L121)H and F(L97)C/F(L121)H. As mentioned above, overall charge separation (formation of $P^+Q_A^-$) takes place in these mutants and is very similar to that of the original M(L214)H beta-type mutant from *Rb. sphaeroides*. These similarities include the following. (1) Initial charge separation is slightly slower than in wild-type, forming P^+I^- with an ~100% yield. P^+I^- is the adopted nomenclature for the charge-separated intermediate in beta-type RCs, in analogy to $P^+BPh_L^-$ of the wild-type photochemistry (Kirmaier et al., 1995a,b; Laporte et al., 1995). (2) The transient spectrum of P^+I^- has a broad absorption band from ~630 to 700 nm that is consistent with the formation of a BChl anion. (3) Branching of the photochemistry occurs at P^+I^-, which decays 70% by electron transfer to form $P^+Q_A^-$ and 30% by deactivation to the ground state (at 285 K). Using this simple branching scheme, the lifetime of P^+I^- and yield of $P^+Q_A^-$ can be used to calculate the putative time constants for electron transfer to Q_A and deactivation to the ground state. These are collected for all three beta-type RCs in Table 1. Overall, the results are strikingly similar. It is additionally interesting that the same yield of overall charge separation, 70%, and similar inherent time constants have recently been reported in *Rb. sphaeroides* RCs where pheophytin is substituted for BPh_L (Shkurapatov & Shuvalov, 1993; Schmidt et al., 1994), even though the mechanism of electron transfer (possibly) and states involved (clearly) are different.

Since the differences between the various time constants collected in Table 1 are relatively small, it would be premature to tie them to any particular origin. Also beyond the scope of the work here is a discussion of the mechanism of initial electron transfer ($P^* \rightarrow P^+I^-$) or that of electron transfer from I^- to Q_A. At this point, it is the overall similarity between the three beta-type RCs that seems noteworthy since there are implications for further identifying the electron carrier in P^+I^-, a question fundamental to the mechanism of charge separation. In the initial work on the L(M214)H mutant, P^+I^- was appraised to be largely $P^+\beta^-$ but having enough $P^+BChl_L^-$ character to be responsible for the ~(1 ns)$^{-1}$ rate of charge recombination, $P^+I^- \rightarrow$ ground state. Recent exhaustive studies of L(M214)H and L(M214)H/E(L104)V RCs give serious consideration to the possibility

that P^+I^- may in fact have largely the character of $P^+BChl_L^-$ (Kirmaier et al., 1995a,b; Laporte et al., 1995). However, in the end, the assignment of P^+I^- is still complex and unresolved with regard to the precise relative involvements of $P^+\beta^-$ vs $P^+BChl_L^-$. For example, photodichroism measurements of the 630−700 nm transient anion absorption of I^- yield an angle of ~46° between this absorption transition and the 870 nm transition of P. This angle is roughly halfway between the ~65° expected for β^- and ~30° expected for $BChl_L^-$. We caution that this finding and analysis is distilled to a single sentence here with the intent of illustrating the level of complexity and to suggest that the new beta-type RCs may ultimately shed light on some of the outstanding questions. The possibility that P^+I^- is $P^+\beta^-$ likely requires that β is physically rotated in the binding pocket compared to the position occupied by BPh_L, thus yielding a different angle for the anion transition (Kirmaier et al., 1995a,b). It would be highly fortuitous for the same angle to be obtained for the new beta-type RCs owing to precisely the same rotation, especially considering the previous discussion regarding coordination of a His at L121. Potential structural changes associated with site-directed mutations are an insidious problem. Only recently has there been the first report of the crystal structures of some *Rb. sphaeroides* mutants, including the L(M214)H RC (Chirino et al., 1994). At the present level of resolution, there do not appear to be any significant changes in the positions or orientations of any of the pigments or protein in the mutants investigated (aside from the obvious ones expected for the mutation itself). This helps obviate the trivial explanation that the altered photochemistry in the beta-type mutants, compared to wild-type, would not be obtained if were it not for disruptive structural changes.

It seems probable that there is something different about the two beta-type RCs reported here and the L(M214)H beta-type RC, if not local structural changes associated with establishing ligation between His at L121 and β, then perhaps the difference in ligation mentioned earlier (water vs His). Changing the ligand to water is certainly less disruptive than the structural gyrations that may be necessary to ligate a His at L121 to the Mg of β, and a difference in ligand would not necessarily be accompanied by a significant change in redox potential. Meanwhile, a four-coordinate β probably would have a significantly different redox potential than a five-coordinate β, and significant differences in the rates and yields of the electron transfer processes might result. Whatever differences between the new F(L97)V/F(L121)H and F(L97)C/F(L121)H β RCs and the original L(M214)H β RC ultimately are found to exist, they will have to be reconciled with remarkably constant photochemistry. This should help shed light on the question of the relative involvements of $P^+\beta^-$ and $P^+BChl_L^-$ in charge separation in this interesting class of RCs, and, ultimately, on the free energy and role of $P^+BChl_L^-$ in the native photochemistry.

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