Spectroscopic and Redox Properties of syml and (M)F195H: *Rhodobacter capsulatus* Reaction Center Symmetry Mutants Which Affect the Initial Electron Donor†

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ABSTRACT: The redox properties, absorption, electroabsorption, CD, EPR, and $P^+Q_A^-$ recombination kinetics have been measured for the special pairs of two mutants of *Rhodobacter capsulatus* reaction centers involving amino acid changes in the vicinity of the special pair, P. Both mutants symmetrize amino acid residues so that portions of the M-sequence are replaced with L-sequence: syml symmetrizes all residues between M187 and M203, whereas (M)F195H is a single amino acid subset of the syml mutation. (M)-F195H introduces a His residue in a position where it is likely to form a hydrogen bond to the acetyl group of the M-side bacteriochlorophyll of P. For both mutants compared with wild-type, (i) the redox potential is at least 100 meV greater, (ii) the $P^+Q_A^-$ recombination rate is about twice as fast at room temperature, and (iii) the large electroabsorption feature for the Q_Y band of P is shifted relative to the absorption spectrum. The comparison of the properties observed for the syml and (M)F195H reaction center mutants and the differences between these mutants and wild-type suggest that residue M195 is an important determinant of the properties of the special pair.

The availability of structural data for bacterial reaction centers (RCs) (Deisenhofer et al., 1984, 1985) laid to rest many questions which had been raised by earlier spectroscopic and kinetic investigations; however, it also raised many new issues. In particular, the X-ray structure reveals a local 2-fold axis of symmetry which includes the bound prosthetic groups and large segments of the polypeptide chains which bind the prosthetic groups. All available kinetic and spectroscopic evidence to date suggests that only one branch of prosthetic groups, designated the L- or A-branch, participates in physiological electron-transfer reactions. The molecular origin of this unidirectional electron-transfer reaction and the role of the inactive M- or B-branch of the prosthetic groups are thus open questions. Electroabsorption (Stark effect) measurements of the lowest electronic transition of the special pair primary electron donor demonstrate a substantial change in dipole moment upon photoexcitation (Lockhart & Boxer, 1987, 1988; Lösche et al., 1987, 1988) and, more importantly, that the vector characterizing this change in dipole moment does not lie along the local C_2 symmetry axis. A reasonable inference is that the local symmetry is broken at the level of the excited state(s) of the special pair, and this electronic asymmetry may have functional consequences and contribute to the unidirectionality of electron transfer. A central issue then is what structural features within the special pair or in its interactions with its protein environment lead to this symmetry breaking?

When two chromophores such as the bacteriochlorophylls in P are in close proximity, interchromophore charge-transfer excited states are expected to be close in energy to locally excited or exciton states and may mix with these states. This is equivalent to a molecular polarizability (Boxer et al., 1989), and the polarizability can be quite large (Middendorf, 1991). A matrix electric field, such as that provided by the organized interior of the RC, can then induce a substantial dipole moment in ^1P (Middendorf, 1991; Middendorf et al., 1992; Gottfried et al., 1991b). This provides an explanation for several aspects of the available electroabsorption data for the special pair, in particular the absence of a field-dependent lineshape (Scherer & Fischer, 1986).

The magnitude and molecular details of the matrix field in the vicinity of the special pair are not yet known. The amino acid sequences in the vicinity of the special pair do break the local 2-fold symmetry at several positions and so are obvious candidates for the origin of unidirectional electron transfer. A number of amino acids in the vicinity of the special pair have been simultaneously symmetrized (M187 to M203) to produce a mutant of *Rhodobacter capsulatus* (*Rb. capsulatus*) RCs, originally designated pAT-3 (Woodbury et al., 1990), and renamed syml (Taguchi et al., 1992). These RCs exhibit somewhat different kinetics and quantum yields for the initial steps in the primary charge separation process; however, no evidence has been found for charge separation down the normally inactive M- (B-) branch of electron acceptors (Taguchi et al., 1992).

One striking and unexpected difference between the special pairs in wild-type and syml is that the potential of the P/P^+ couple in syml is substantially higher than in wild-type as described below. Interestingly, a similar increase in potential has been observed (J. Breton, personal communication) for

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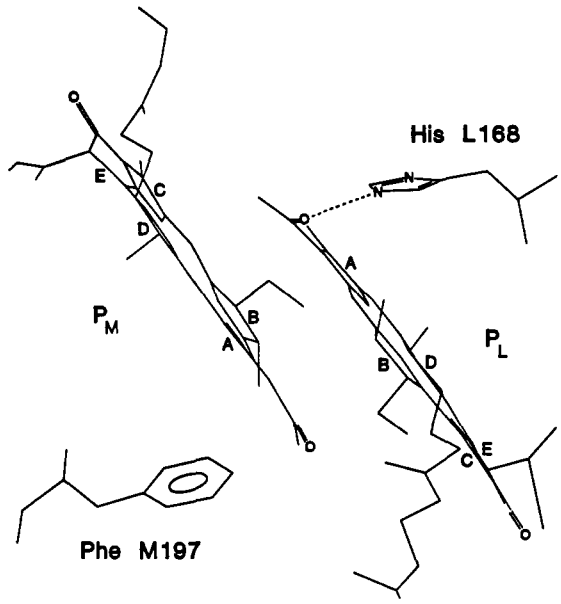

FIGURE 1: Schematic illustration of the special pair and symmetry-related residues His-L168 and Phe-M197 from the crystallographic coordinates for *Rb. sphaeroides* RCs (Allen et al., 1987). Phe-M197 is Phe-M195 in *Rb. capsulatus*.

a different symmetry mutant called D_{LL} (Robles et al., 1990a,b), in which residues M192 to M217 have been symmetrized, and for the heterodimer mutants (Bylina & Youvan, 1988; Kirmaier et al., 1988; Schenck et al., 1990; McDowell et al., 1991) in which one or the other of the macrocycles comprising the special pair apparently loses its central Mg atom. One of the most interesting amino acid changes which occurs in both the sym1 and D_{LL} mutants is at position M195 which is Phe in wild-type RCs. As shown in Figure 1, the symmetry-related residue His L168 is located near the acetyl group on ring A of the L-side bacteriochlorophyll *a* half of the special pair in of *Rhodobacter sphaeroides* (*Rb. sphaeroides*) RCs (Allen et al., 1987; El-Kabbani et al., 1991) and may form a hydrogen bond to this acetyl group. The other acetyl group cannot hydrogen bond to the symmetry-related Phe M197 (Phe M195 in *Rb. capsulatus*), but may be hydrogen bonded to Tyr M210 [M208 in *Rb. capsulatus* and *Rhodopseudomonas virdis* (*Rps. virdis*)] (Allen et al., 1987). A similar situation occurs in *Rps. virdis*, where His L168 hydrogen bonds to the acetyl group of the L-side Bch *b* of the special pair, whereas symmetry-related Tyr M195, not Tyr M208, is reported to be hydrogen bonded to the other acetyl group (Deisenhofer & Michel, 1989). It therefore seems reasonable that conversion of residue M195 from Phe to His in *Rb. capsulatus* introduces the possibility of a hydrogen bond to the acetyl group of the special pair. We have constructed the (M)F195H mutant, and in the following we report a comparison of some of the properties of the special pairs in sym1, (M)F195H, and the heterodimer strains.

MATERIALS AND METHODS

Plasmid Constructions. (M)F195H was prepared by changing the TTC codon for residue M195 to CAC by the polymerase chain reaction (PCR) (Kadowaki et al., 1989). Oligonucleotides (52 and 47 bases) were synthesized which flanked the region near the mutation and included unique restriction sites. The PCR was accomplished using GeneAmp (Perkin-Elmer Cetus), and the mutation was verified by independent DNA sequencing. Heterodimer strain (M)-H200L was generously provided by Professor Youvan. All RCs were prepared and purified as discussed elsewhere (Woodbury et al., 1990; Taguchi et al., 1992).

Redox Titrations. The procedure used to perform titrations of the P to P^+ redox couple in mutant and wild-type RCs will be described in detail elsewhere (Williams et al., 1992). Briefly, P^+ absorbance changes in the 750–1000-nm region were monitored in a flowing cuvette. $K_3Fe(CN)_6$ and ascorbate were used to vary the ambient redox potential which was measured with an electrode. Attempts were also made to utilize stronger oxidants [such as $Fe(phen)(CN)_4^-$] and achieve higher ambient potentials by adding known amounts of freshly prepared oxidant solution to either RCs or photosynthetic membranes and monitoring the spectral changes.

Spectroelectrochemical measurements of both wild-type and mutant RCs were also attempted to determine the P/P^+ redox couple using a thin spectroelectrochemical cell equipped with a gold mesh electrode as described by Moss et al. (1990). $K_3Fe(CN)_6$ was used as a mediator (Thibodequ et al., 1990).

Electroabsorption Spectroscopy. The apparatus for obtaining electroabsorption (Stark effect) spectra has been described elsewhere (Lockhart & Boxer, 1987). It was found that sym1 RCs are not very stable in poly(vinyl alcohol) films; therefore, samples were also studied in frozen glycerol/buffer (50:50 v/v; 10 mM potassium phosphate, 0.05% LDAO buffer) glasses at 77 K as described in Hammes et al. (1990). Methods of analysis, which yield the magnitude of changes in dipole moment, $|\Delta\mu_A|$, polarizability, $|\Delta\alpha|$, and the angle ζ_A between $\Delta\mu_A$ and the transition dipole moment, are presented elsewhere (Lockhart & Boxer, 1987; Oh et al., 1991). The latter involves measuring electroabsorption spectra as a function of the experimental angle χ between the applied field and the electric vector of polarized light used to probe the effect. When $\chi = 54.7°$ (the magic angle), the magnitude of electroabsorption features is independent of the internal angle ζ_A.

Transient Absorption Spectroscopy. Time resolved light-minus-dark difference spectra on the microsecond to second time scale were obtained at 77 K using 50% glycerol/buffer samples in 4-mm path length cells. Transient spectra were constructed from single-wavelength measurements taken in a random order (1 point/4 nm). The samples were excited at 532 nm with 8-ns fwhm pulses from a Q-switched Nd:YAG laser. Samples were probed with light from a 250-W tungsten lamp passed through an 0.22-m monochromator (spectral bandwidth 4.5 nm). The raw data were converted to ΔA, and the data were analyzed using a singular value decomposition (SVD) program discussed in detail elsewhere (Lambright et al., 1991).

Electron Paramagnetic Resonance. EPR spectra were obtained using a Varian E-112 EPR spectrometer. Measurements were made on chromatophores suspended in 50% glycerol/buffer, cooled to 100 K, and excited with light from a 300-W xenon lamp passed through a $CuSO_4$ filter.

Circular Dichroism. CD measurements were taken with a Jasco J-500C spectropolarimeter in 50% glycerol/buffer samples at 4 K.

RESULTS

Redox Potential of the P/P^+ Couple. Figure 2 compares the absorption spectra of wild-type and sym1 RCs at 560 mV. At this potential, 90% of the Q_Y band of P is bleached in wild-type RCs, indicating that P has been oxidized to P^+ while only about 10% bleaching of the Q_Y band of P in sym1 RCs is observed. Subsequently, decreasing the ambient potential with buffered ascorbate resulted in re-reduction of P in both wild-type and mutant RCs, indicating that the observed

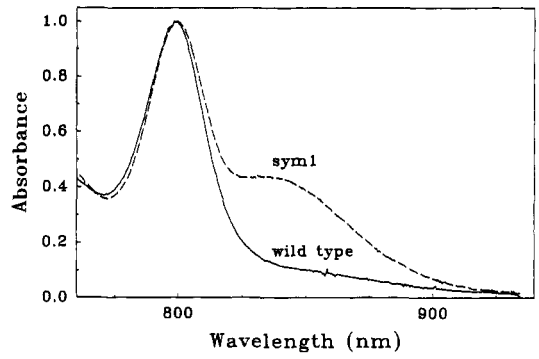

FIGURE 2: Steady-state absorption spectrum of wild-type and sym1 reaction centers at an ambient redox potential of 560 mV. The ambient potential was adjusted with $K_3Fe(CN)_6$ and ascorbate.

oxidation was largely reversible.[1] The analysis of wild-type *Rb. capsulatus* RCs titrated in this way gave a potential of 480 ± 15 mV. It was not possible to achieve greater than 10% reversible oxidation of sym1 RCs, but a lower limit could be placed on the redox potential of the P/P^+ couple for this sample of 590 mV. The (M)F195H RCs were also difficult to reversibly oxidize, and a similar lower limit of about 590 mV can be placed on the P/P^+ couple on the basis of partial titrations.

Attempts to achieve more complete oxidation of P in sym1 and (M)F195H samples were performed using whole photosynthetic membranes (chromatophores) and either higher $K_3Fe(CN)_6$ concentrations (up to 150 mM) or more powerful oxidants such as $Fe(phen)(CN)_4^-$ (E_M = 580 mV vs NHE; generously provided by C. Schenck, Colorado State University). However, these attempts were largely unsuccessful; more complete bleaching of the Q_Y band of P, though possible, was not reversible.

Similar results were obtained measuring the P/P^+ couple using a spectroelectrochemical cell. Wild-type RCs yielded a P/P^+ redox potential of 475 ± 20 mV vs NHE. However, the same procedure applied to sym1 and (M)F195H RCs resulted in large amounts of sample degradation indicated by the concerted reduction of the Q_Y bands associated with the special pair, monomer Bchs and monomer Bphs, before oxidation of the special pair was observed.

Electroabsorption (Stark Effect) Spectroscopy. Figure 3 shows the absorption and electroabsorption spectra in the Q_Y region for both wild-type and sym1 RCs. The absorption spectra were normalized at 800 nm (the feature at 800 nm has a nearly identical lineshape in wild-type and sym1). The special pair Q_Y absorption band is somewhat broader than for wild-type. As expected, the change in absorption in an applied electric field, ΔA, was found to be quadratic with the applied field strength, and the ΔA spectra for both have been scaled to the same applied field strength. The position and amplitudes (per unit A at 800 nm) of the electroabsorption features of both the monomeric Bchs and Bphs are very similar in sym1 and wild-type. The magnitude, overall shape, and width (reflected by the zero-crossing points) of the electroabsorption feature associated with the special pair of sym1 and wild-type are also very similar. However, there is a significant difference in that the minimum of ΔA of the sym1 special pair is shifted to the red by slightly more than 200 cm^{-1} as compared to

[1] All RC absorption spectra taken as a function of ambient potential were normalized at 800 nm to correct for RC degradation during the measurement. Both wild-type and sym1 samples showed a decrease of approximately 5–8% in the 800-nm band during the course of the experiment.

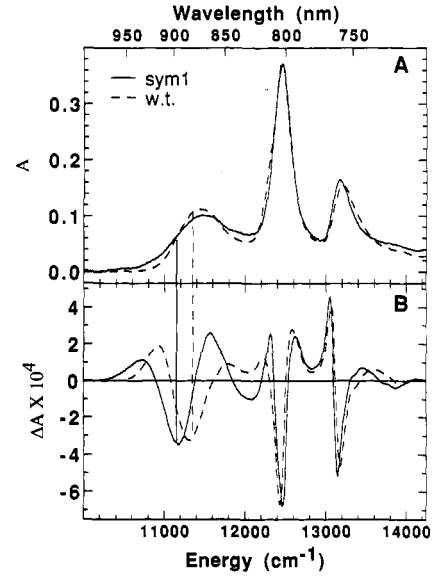

FIGURE 3: Absorption (A) and electroabsorption (B) spectra for sym1 and wild-type reaction centers in 50% glycerol/buffer at 77 K. The absorption spectra were scaled to be identical at 800 nm (see text). The electroabsorption spectra were taken with an applied field of 3.4 × 10^5 V/cm.

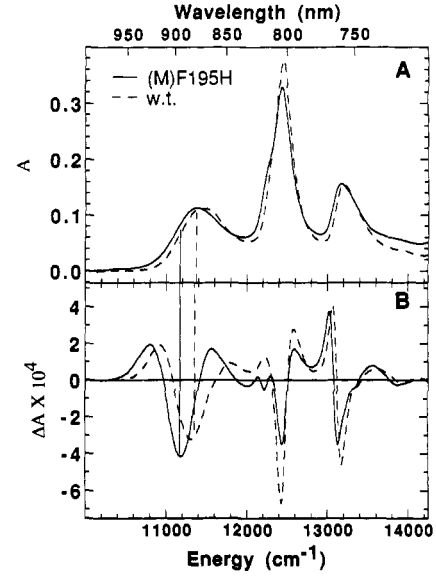

FIGURE 4: Absorption (A) and electroabsorption (B) spectra for (M)F195H and wild-type reaction centers in 50% glycerol/buffer at 77 K. The absorption spectra were scaled to be identical at 757 nm (see text). The electroabsorption spectra were taken with an applied field of 3.4 × 10^5 V/cm.

wild-type, even though the absorption maxima of the two are essentially identical at this temperature (Figure 3). Note that this shift separates the features at around 870 nm from those around 800 nm, so that a trough with negative ΔA is seen at around 840 nm in sym1, implying the presence of no other electronic feature or vibronic structure from P. The angle, ζ_A, between $\Delta\mu$ and the transition dipole moment was measured to be 42 ± 3° for the special pair of the sym1 mutant as compared with 38 ± 2° for the special pair of wild-type measured under the same conditions (in PVA films).

Electroabsorption data for (M)F195H are compared with those for wild-type in Figure 4. The special pair absorption of (M)F195H has about the same width as that for wild-type, in contrast with sym1. Also, there appears to be a slight improvement in the resolution of substructure within the 800-nm band relative to wild-type (and sym1); thus, the absorption

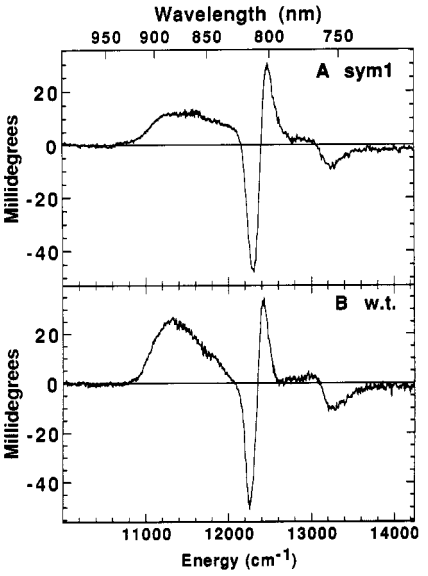

FIGURE 5: Circular dichroism spectra for both (A) sym1 and (B) wild-type reaction centers at 4.2 K in 50% glycerol/buffer in a 3-mm path length cell. The sample absorbances were scaled to 0.5 at 800 nm.

spectra were normalized for comparison at the maximum of the 757-nm band rather than at 800 nm (cf. Figure 3) to provide a more meaningful comparison of the ΔA spectra. As with sym1, the minimum of ΔA for the special pair in the (M)F195H is shifted approximately 200 cm^{-1} to the red of the absorption maximum at 11 380 cm^{-1}. Thus, the offset between ΔA and A observed for sym1 appears not to be correlated with the greater width of the absorption band; rather it is a specific result of the change at M195. The angle, ζ_A, was measured to be 36 ± 2° for the (M)F195H mutant (in PVA).

Circular Dichroism. The circular dichroism spectra for sym1 and wild-type RCs at 4 K are shown in Figure 5. The spectra are essentially identical in the monomeric Bch and Bph regions; however, the 820–950-nm regions are noticeably different. The positive feature arising from the special pair is somewhat broader and less intense in sym1 than its counterpart in the wild-type spectrum.

Absorbance Changes Associated with $P^+Q_A^-$ and $P^+Q_A^-$ Recombination Kinetics. Figure 6 shows time-dependent absorption spectra associated with $P^+Q_A^-$ recombination to the ground state for sym1 and wild-type RCs at 77 K. The decrease in absorbance seen in the region 810–920 nm is assigned to the bleaching of the absorption associated with the ground state of P. The features at 760 and 800 nm are electrochromic bandshifts of the monomeric Bphs and Bchs, respectively, caused by the formation $P^+Q_A^-$. Analysis by singular value decomposition yields only one major component (i.e., the rank of the W matrix is one) at all times observed. This indicates that the $P^+Q_A^-$ spectrum shows a homogeneous recombination rate throughout the 750–950-nm region for both wild-type and sym1. The rate of $P^+Q_A^-$ recombination to the ground state at room temperature at 850 nm was (145 ± 5 ms)$^{-1}$ for wild-type, (75 ± 5 ms)$^{-1}$ for sym1, and (70 ± 5 ms)$^{-1}$ for (M)F195H (Figure 6C). It is of interest to note that, at room temperature, both sym1 and (M)F195H do not fit as well to a single exponential as wild-type.

EPR of P^+. The light-induced P^+ EPR signal was measured for both wild-type and mutant photosynthetic membranes (chromatophores). The characteristic first derivative lineshape was seen for sym1, (M)F195H, wild-type, and (M)H200L

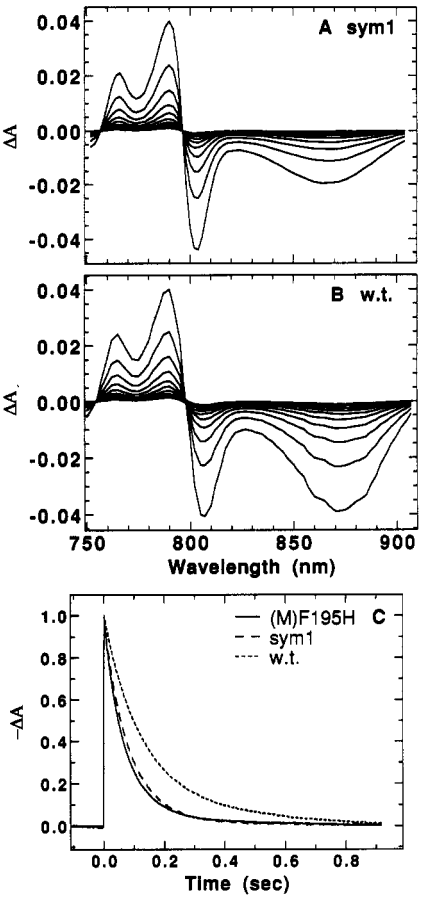

FIGURE 6: Time-resolved absorption spectra associated with $P^+Q_A^-$ recombination to the ground state in the 752–900-nm region for (A) sym1 and (B) wild-type reaction centers at 77 K in a 50% glycerol/buffer glass using a 532-nm excitation pulse. Kinetic traces were taken at a minimum of every 4 nm starting with 752 nm. (C) $P^+Q_A^-$ recombination kinetics measured at 850 nm for sym1, (M)F195H, and wild-type reaction centers at room temperature using a 532-nm excitation pulse.

heterodimer RCs, giving a peak-to-peak linewidth for sym1 of 10.5 ± 0.2 G and 9.3 ± 0.3 G for (M)F195H. The peak-to-peak linewidth measured for wild-type is 9.5 ± 0.2 G, and for the (M)H200L heterodimer mutant it is 11.8 ± 0.3 G, in agreement with previously published results (Bylina et al., 1990).

DISCUSSION

Comparison of sym1 and (M)F195H. The key differences between P in wild-type, sym1, and (M)F195H are the following: (i) the redox potential for P/P^+ is substantially higher in both mutants than in wild-type; (ii) the EPR linewidth of P^+ is larger in the sym1 mutant than in either (M)F195H or wild-type which have almost identical linewidths; (iii) the $P^+Q_A^-$ recombination rate speeds up by about a factor of 2 in sym1 and (M)F195H relative to wild-type at room temperature; (iv) the electroabsorption spectrum of the Q_Y band of the special pair, though of comparable absolute magnitude in the mutants and wild-type, is red-shifted in the mutants relative to the absorption band. It appears that the differences in the redox potential, $P^+Q_A^-$ recombination rate, and electroabsorption spectra between wild-type and sym1 RCs are largely the specific result of replacement of the Phe M195 with His.

Comparison of P/P^+ Redox Potentials and the Free Energy between P^* and $P^+Bph_A^-$. The delayed fluorescence measurements of sym1 reported in the preceding paper (Taguchi

et al., 1992) indicate that the driving force for the initial charge separation event is about 75 meV. This is considerably less than that of wild-type which has been measured to be about 120 meV using the same technique. Interestingly, the special pair of the (M)F195H mutant is also difficult to oxidize with ferricyanide. Because of the large shift, the actual reversible midpoint potentials for these mutants could not be measured; however, it is evident that the redox potentials for both syml and (M)F195H have increased dramatically. This suggests that most of the change in redox potential observed for syml is due to the His residue inserted at position M195.

The observed redox potential change in the P to P^+ couple of syml and (M)F195H RCs vs wild-type RCs is substantially larger than the change in the free energy gap between P^* and $P^+Bph_A^-$ as measured by delayed fluorescence. Similar observations have been made with other mutants that significantly alter the redox potential of the P to P^+ couple (Williams et al., 1992). One would not necessarily expect these values to be identical. First, there could be some contribution from a change in the redox potential of the $Bph_A \rightarrow Bph_A^-$ half-reaction in the overall ΔG estimations. There could also be changes in the electrostatic interaction between P^+ and Bph_A^-. More importantly, the P/P^+ redox titrations take place on a very long time scale relative to the free energy change measurements from the picosecond fluorescence decays. It has been postulated that the free energy gap between P^* and $P^+Bph_A^-$ changes significantly in the time following initial electron transfer (Woodbury et al., 1984). Finally, the energetics of the initial electron-transfer reaction as determined by delayed fluorescence is model dependent, and consequently, problems with the model would yield results which may not be accurate (Taguchi et al., 1992).

We note an interesting correlation between the increased midpoint potential of P and the measured electron-transfer rates. The P^*Bph_A to $P^+Bph_A^-$ reaction rate has slowed, whereas the rates of charge recombination for the states $P^+Bph_A^-$ and $P^+Q_A^-$ have sped up. The $P^+Bph_A^-$ to $P^+Q_A^-$ rate, whose driving force is unaffected by a change in the midpoint potential of P, is unchanged. These results are consistent with earlier electric field effect experiments (Lockhart et al., 1991; Franzen et al., 1990).

Protein Effects on the Redox Potential of P. Measured oxidation potentials for bacteriochlorophyll *a* in organic solvents range between +560 mV (methanol) and +760 mV [tetrahydrofuran; reviewed by Watanabe and Kobayashi (1991)]. One unusual property of the special pair in the bacterial RC is its low redox potential (about 480 mV), suggesting that some combination of the properties of the dimer itself and the influence of the protein environment results in a low oxidation potential for P in the RC.

One of the factors that may contribute is the fact that the special pair is a strongly coupled dimer. It has been postulated that this accounts for most of the decrease in the redox potential seen in the special pair of bacterial RCs vs monomeric bacteriochlorophyll *a* in organic solvents (Watanabe & Kobayashi, 1991). Thus, the redox potential of P should be sensitive to changes in the relative positions or orientations of the Bchs in the special pair or the orientation of Bch's side chains. One might expect that structural changes within the dimer itself may lead to a less strongly coupled dimer and would be accompanied by a blue shift in the excitonically generated Q_Y band of P (Thompson et al., 1991) and by an increase in the linewidth of the P^+ EPR spectrum (Bylina et al., 1990).

In syml, both a blue shift of the Q_Y band of P and increase in the EPR linewidth of P^+ are observed. The EPR linewidth of P^+, which is thought to be affected by the degree of delocalization of the unpaired electron on P^+, is significantly broadened and is closer to the value seen for Bch^+ monomers in solution than that of the special pair in wild-type RCs. This would be consistent with a more localized radical cation state in syml than in wild-type. It has been postulated that the increased redox potential and broadened EPR linewidth of the heterodimer suggest a decoupling of the Bch and Bph in its special pair and result in a P^+ state in which the positive charge resides mainly on what is effectively a Bch monomer (Bylina et al., 1990).[2] The pigment extraction results for syml indicate that the special pair is still composed of two Bchs as in wild-type; however, the large changes in the protein which serves as the physical scaffolding in which the special pair is imbedded could have caused changes in the relative geometries between the two Bchs comprising the special pair. Such a change could cause the increased redox potential and broadened EPR linewidth and the changes seen in the CD spectrum (see Figure 5). It should be noted that the ground-state absorption spectrum of P at room temperature is shifted to the blue by about 10 nm as compared to that of wild-type and could also be used to argue for a special pair in syml comprised of two Bchs which do not interact as strongly as those of the wild-type special pair.

Though a structural change within the dimer itself may be an explanation for the increase P/P^+ redox potential and is also consistent with the shift of the Q_Y band of P and the broadening of the EPR spectrum of P^+ in syml RCs, this is apparently not the case for (M)F195H RCs. In the single-site mutant, a significant increase in the P/P^+ redox potential is seen, but there is no observed increase in the EPR linewidth of P^+. This observation would argue against large structural changes within the special pair. Since (M)F195H is a subset of the syml mutation, it seems likely that the same mechanism causes the observed increase in the P/P^+ redox potential for both of the mutants. Thus, a significant change in the structure of the Bchs relative to one another in the special pair probably does not play a major role in the increase in the P/P^+ redox potential seen in these mutants.

Another possible mechanism for increasing the redox potential of the special pair is the alteration of the electrostatic environment. In both the syml and (M)F195H RCs, amino acid M195 has been changed from a phenylalanine to a histidine. In aqueous solution, histidine has a pK_A near neutral pH. Exchanging a very nonpolar amino acid like phenylalanine for a polar and potentially positively charged amino acid like histidine could account for an increased redox potential for P.

Additionally, changing M195 from Phe to His could directly affect the electronic structure of the special pair through hydrogen-bonding. The analogous histidine on the L side (L168) has been suggested to be involved in hydrogen-bonding interactions either with the C-2 acetyl group of ring 1 (Allen et al., 1987; El-Kabbani et al., 1991) or with histidine L173, the histidine most closely associated with the Mg atom of the L-side Bch of the special pair (Yeates et al., 1988). Hydrogen bonds to peripheral carbonyl groups involved in the conjugated

[2] We note that a careful EPR/ENDOR study of the *Rb. sphaeroides* heterodimer and its comparison with wild-type shows that this mutant gives results which might be expected for a Bch cation radical (Huber et al., 1991). Recent ENDOR studies of single crystals of both R26 and heterodimer *Rb. sphaeroides* RCs confirm the localization of the unpaired electron on the Bch of the heterodimer (Huber et al., 1992).

system are thought to significantly affect the π-electron system (Thompson et al., 1991). Altering the position or orientation of metal ligands (such as might be done if the newly introduced histidine on the M side in sym1 were to hydrogen-bond to histidine M200, the ligand to the Mg on the M side of the dimer) could change the overall coordination of the metal and possibly alter the interaction between the metal and the π-electron orbital system (Thompson et al., 1991). It is evident that although it is useful to dissect possible effects, many of these effects are interrelated. FTIR and resonance Raman experiments are currently in progress to provide more precise structural information.

Electroabsorption Spectra of sym1 and (M)F195H. The difference in the electroabsorption spectrum of the Q_Y transition of the special pair in wild-type compared with sym1 or (M)F195H RCs is interesting and unusual. The standard approach to analyzing electroabsorption spectra is to decompose ΔA into contributions from the second, first, and zeroth derivatives of A. As shown in the classic work of Liptay (1974), these contributions arise largely from changes in dipole moment ($\Delta\mu_A$), polarizability ($\Delta\alpha$), and transition moment, respectively. When the second derivative of the absorption dominates the effect, the minimum in ΔA corresponds approximately to the absorption maximum, which is roughly correct for wild-type RCs at 77 K (see Figure 3B). A first derivative contribution to ΔA has the effect of shifting the electroabsorption band; however, it also changes the relative contributions of the positive lobes of the ΔA spectrum. This is why the observation for sym1 and (M)F195H that the ΔA spectrum is shifted, but retains essentially the identical shape, is so striking. If one were simply to use the measured values of the second derivative of the absorption as the basis for analyzing the sym1 or (M)F195H electroabsorption spectra, then $|\Delta\mu_A|$ for the special pair in the mutants would appear to be considerably larger than for wild-type; however, we do not believe this analysis is legitimate.

Our group (Lockhart & Boxer, 1987, 1988) and Feher's (Lösche et al., 1987, 1988) analyzed the electroabsorption spectrum of the special pair in wild-type RCs, and both concluded that the effect in poly(vinyl alcohol) matrices was dominated by the second derivative. Furthermore, it was found that ΔA is quadratic in the applied field, but more importantly the electroabsorption lineshape is independent of applied field over more than 2 orders of magnitude in applied field strength (the signal intensity changes by 10^4 over this range). More recently, we have investigated the effect in glycerol/buffer matrices at 1.5 K (Middendorf, 1991; Middendorf et al., 1992) because the inhomogeneous broadening of the P absorption appears to be considerably smaller in this medium (Johnson et al., 1989). Both at 77 K and especially at 1.5 K we found that the ΔA lineshape deviates substantially from the second derivative. Addition of a substantial first derivative contribution improved the fit, suggesting a substantial change in molecular polarizability for the special pair.

Even in the absence of this more recent data, it is surprising that the electroabsorption lineshape does not depend on applied electric field strength. If the origin of the large apparent change in dipole moment for P is mixing with charge-transfer states, then this mixing should depend strongly on the applied electric field strength because the charge-transfer states have a large electric dipole moment. This point was raised some time ago by Scherer and Fischer (1986), who demonstrated that because the mixing is field dependent, a substantial first derivative contribution and field-dependent lineshape should be observed, the latter contrary to experiment. A parallel situation has recently been found for carotenoids when they are associated with photosynthetic proteins (Gottfried et al., 1991a,b). Very large dipole moments were observed when the carotenoid was in the protein matrix, and a reasonable explanation, which we also believe is applicable to the special pair in the RC, is that the organized protein matrix interacts with the large intrinsic molecular polarizability to induce a permanent dipole moment. There may be many contributions to this matrix field, including charged, polar, and polarizable amino acids, helix dipoles, and buried waters. So long as the matrix field is large relative to the applied field, it dominates the mixing, which leads to the permanent dipole moment, and the external field simply probes this dipole. It can be shown (Middendorf, 1991) that deviations from the simple lineshape prediction of Liptay (1974) are readily possible with this scenario. In particular, electroabsorption lineshapes which have a second derivative shape, but shifted, can be obtained. This same characteristic feature of a shifted second derivative lineshape has been observed for a number of simple transition metal complexes (Oh et al., 1991; Hug and Boxer, unpublished results) and chlorophyll dimers (Middendorf, 1991). We conclude that differences in the electroabsorption spectra for both sym1 and (M)F195H relative to wild-type reflect a substantial change in the matrix electric field which can alter the mixing of charge-transfer states into P*. In principle, the molecular polarizability tensor for P could be measured and used to probe the topology of the matrix field. Experiments are in progress toward this goal.

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