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# Temperature Dependence of Electron Transfer to the M-Side Bacteriopheophytin in *Rhodobacter capsulatus* Reaction Centers

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Subpicosecond time-resolved absorption measurements at 77 K on two reaction center (RC) mutants of *Rhodobacter capsulatus* are reported. In the D<sub>LL</sub> mutant the D helix of the M subunit has been substituted with the D helix from the L subunit, and in the D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> mutant, three additional mutations are incorporated that facilitate electron transfer to the M side of the RC. In both cases the helix swap has been shown to yield isolated RCs that are devoid of the native bacteriopheophytin electron carrier H<sub>L</sub> (Chuang, J. I.; Boxer, S. G.; Holten, D.; Kirmaier, C. *Biochemistry* **2006**, *45*, 3845–3851). For D<sub>LL</sub>, depending whether the detergent Deriphat 160-C or *N*-lauryl-*N,N*-dimethylamine-*N*-oxide (LDAO) is used to suspend the RCs, the excited state of the primary electron donor (P\*) decays to the ground state with an average lifetime at 77 K of 330 or 170 ps, respectively; however, in both cases the time constant obtained from single-exponential fits varies markedly as a function of the probe wavelength. These findings on the D<sub>LL</sub> RC are most easily explained in terms of a heterogeneous population of RCs. Similarly, the complex results for D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> in Deriphat–glycerol glass at 77 K are most simply explained using a model that involves (minimally) two distinct populations of RCs with very different photochemistry. Within this framework, in 50% of the D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> RCs in Deriphat–glycerol glass at 77 K, P\* deactivates to the ground state with a time constant of ~400 ps, similar to the deactivation of P\* in the D<sub>LL</sub> mutant at 77 K. In the other 50% of D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> RCs, P\* has a 35 ps lifetime and decays via electron transfer to the M branch, giving P<sup>+</sup>H<sub>M</sub><sup>−</sup> in high yield (≥80%). This result indicates that P\* → P<sup>+</sup>H<sub>M</sub><sup>−</sup> is roughly a factor of 2 faster at 77 K than at 295 K. In alternative homogeneous models the rate of this M-side electron-transfer process is the same or up to 2-fold slower at low temperature. A 2-fold increase in rate with a reduction in temperature is the same behavior found for the overall L-side process P\* → P<sup>+</sup>H<sub>L</sub><sup>−</sup> in wild-type RCs. Our results suggest that, as for electron transfer on the L side, the M-side electron-transfer reaction P\* → P<sup>+</sup>H<sub>M</sub><sup>−</sup> is an activationless process.

## Introduction

Bacterial photosynthetic reaction centers (RCs) perform highly efficient, unidirectional electron transfer. For each photon absorbed by the primary electron donor pair (P), a dimer of bacteriochlorophyll (BChl) molecules, an electron is transferred via the cofactors on the so-called L branch (also referred to as the A branch) to quinone Q<sub>A</sub>, as indicated with the arrows in Figure 1A. Subsequent electron transfer then occurs to the final acceptor Q<sub>B</sub>. The presence of two possible electron-transfer pathways related by a local C<sub>2</sub> axis of symmetry is evident in the RC structure, but the pathway symmetry is broken by differences in amino acids around the cofactors.<sup>1–6</sup> These differences inhibit charge separation to the cofactors on the M branch (also referred to as the B branch) in the wild-type RC, while facilitating electron transfer to the L-side bacteriopheophytin (H<sub>L</sub>) in ~2.8 ps in *Bastochloris viridis* and *Rhodobacter (Rb.) sphaeroides* and ~4.2 ps in *Rb. capsulatus* RCs.<sup>7–10</sup> At cryogenic temperatures (77 K and lower) the reduction of H<sub>L</sub> is even faster, ~0.7, ~1.2, ~2.0 ps, in RCs from these same

three species, respectively.<sup>7,10–14</sup> Subsequent P<sup>+</sup>H<sub>L</sub><sup>−</sup> → P<sup>+</sup>Q<sub>A</sub><sup>−</sup> electron transfer exhibits a similar temperature dependence with the time constant decreasing from ~200 ps at room temperature to ~100 ps at low temperature.<sup>10,15</sup>

Although a given RC sample (wild type or mutant) is commonly evaluated as a homogeneous population, many experiments have suggested otherwise. A variety of results have been discussed in terms of heterogeneous populations of RCs composed of a distribution of forms, and in some cases these data have been analyzed most simply in terms of two forms of RCs. Evidence for this heterogeneity has come from a variety of measurements exploring parameters such as buffer or detergent properties, pH, or temperature. RC properties found to be affected include the position of the long-wavelength absorption band of P, the electronic structure of P<sup>+</sup>, the P/P<sup>+</sup> midpoint potential, the P\* lifetime, the rate of P<sup>+</sup>H<sub>L</sub><sup>−</sup> → P<sup>+</sup>Q<sub>A</sub><sup>−</sup> electron transfer, and the rate of P<sup>+</sup>Q<sub>A</sub><sup>−</sup> → ground state charge recombination.<sup>14,16–49</sup> For example, although a single value for the P\* lifetime is often quoted for wild-type and mutant RCs the decay kinetics are more complex and no obvious correlations have been found between mutational perturbations made in the RC and the range or temperature dependence of the observed time constants.

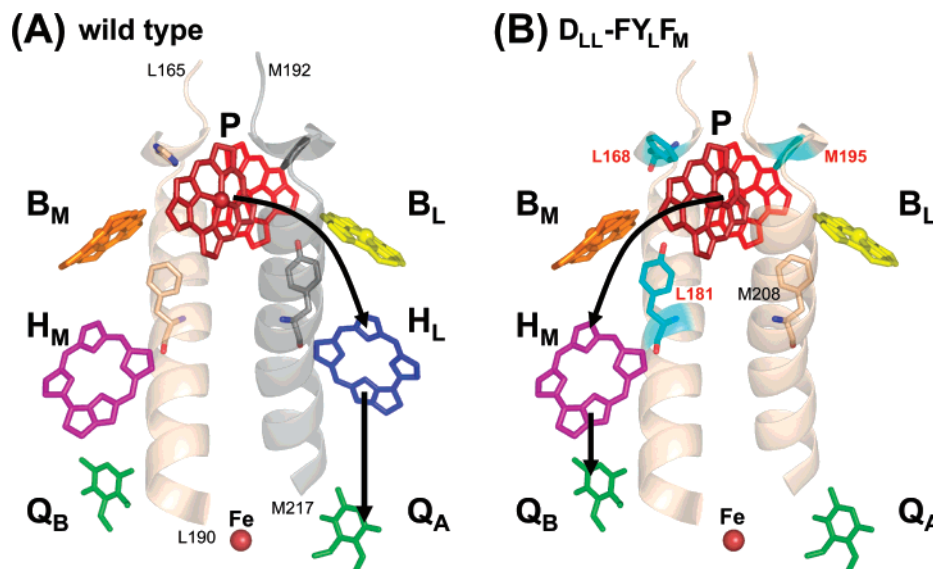
Unidirectional electron transfer in wild-type RCs is thought to be governed by differences between the free energies of the

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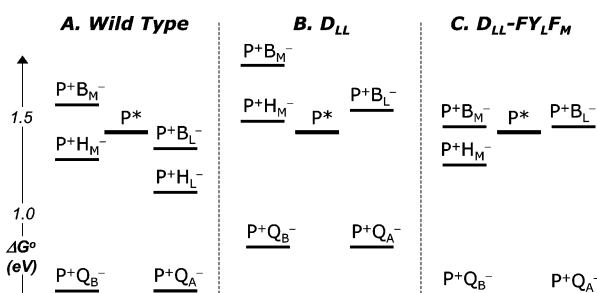
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**Figure 1.** (A) Arrangement of the wild-type L- and M-branch cofactors and the portion of the L polypeptide D helix (left helix) that is duplicated as the M polypeptide D helix (right helix) in  $D_{LL}$ . Key  $C_2$  symmetry-related amino acid pairs, His–L168 and Phe–M195, and Phe–L181 and Tyr–M208, are located as shown. (B) In  $D_{LL}$   $H_L$  is missing and M208 is changed to Phe as a result of the helix swap. Additionally, in the mutant  $D_{LL}$ -FY $_L$ F $_M$  residues L168 and M195 were mutated to Phe and L181 to Tyr.



**Figure 2.** Working models proposed for the relative free energies of the charge-separated states in RCs from (A) wild type, (B)  $D_{LL}$ , and (C)  $D_{LL}$ -FY $_L$ F $_M$ . See text for further details.

charge-separated states on the L and M branches and differences in electronic couplings between the cofactors on the two branches. A working model for the relative free energies of the charge-separated states in wild-type RCs is given in Figure 2A and is derived from the following considerations. Calculations that account for the combined effects of a multitude of amino acid residues place  $P^+B_L^-$  lower in free energy than  $P^+B_M^-$  and similarly for  $P^+H_L^-$  and  $P^+H_M^-$ .<sup>50–54</sup> Stark-effect and resonance Raman measurements indicate that a difference in the dielectric screening on the two branches also may contribute.<sup>55–57</sup> Additionally, calculations generally place the  $P^+B^-$  state higher in free energy than the  $P^+H^-$  state on both L and M branches by 0.15–0.25 eV, consistent with the difference in redox potentials of bacteriochlorophyll and bacteriopheophytin in vitro.<sup>58–61</sup> Calculations and experiments have provided estimates or bracketed ranges for the free energies of the charge-separated states in the wild-type RC:  $P^+B_L^-$  0.05–0.1 eV below  $P^*$ ;<sup>29,50,51,62–70</sup>  $P^+H_L^-$   $\sim$ 0.25 eV below  $P^*$  when relaxed;<sup>71–75</sup>  $P^+B_M^-$  0.1–0.2 eV above  $P^*$  and  $P^+H_M^-$  below  $P^*$  by no more than  $\sim$ 0.15 eV and probably within 0.1 eV.<sup>50–52,76–79</sup>

Systematic efforts to manipulate the free energy differences of the L- and M-branch charge-separated states by site-directed mutagenesis of key amino acids, including those near  $B_M$  and  $B_L$ , have led to mutant RCs in which electron transfer to the M branch competes effectively with charge separation to the L branch, yielding  $P^+H_M^-$  (reviewed in ref 80). In one such

strategy in *Rb. capsulatus* RCs, the symmetry-related Phe at L181 and Tyr at M208 were switched, following calculations that suggested that the tyrosine hydroxyl dipole in a particular orientation stabilizes the  $B_L$  anion (and by analogy, would do the same for the  $B_M$  anion).<sup>50–52</sup> Coupled with the change of Leu at M212 to a His (causing a BChl to be incorporated into the RC in place of  $H_L$ ), this effort yielded the YFH mutant, in which  $P^+H_M^-$  is formed with  $\sim$ 30% yield.<sup>79</sup> Until recently this was the highest yield of this state obtained. Interestingly, the detergent-dependent heterogeneity of  $P^*$  decay mentioned above for wild type is even more pronounced in YFH. The  $P^*$  stimulated emission decay for YFH in LDAO can be reasonably fit by a monoexponential function yielding a 15 ps  $P^*$  lifetime; however, for YFH in Deriphat the kinetics require the use of a biexponential function with time constants of 35 and 220 ps. Regardless of whether the detergent LDAO or Deriphat is used, the yield of M-side electron transfer in YFH is  $\sim$ 30%. Subsequent  $P^+H_M^- \rightarrow P^+Q_B^-$  electron transfer proceeds with a yield of  $\sim$ 40% in the YFH mutant at room temperature.<sup>81</sup>

Efforts to characterize M-side electron transfer at low temperature have been limited, so we have little information on the temperature dependence of the rate or yield of  $P^* \rightarrow P^+H_M^-$ . In RCs that have various combinations of the mutations described above and also lack  $Q_A$ , low yields of  $P^+Q_B^-$  have been reported mainly in photoaccumulation experiments between 10 and 100 K.<sup>82–85</sup> These studies indicate that both  $P^* \rightarrow P^+H_M^-$  charge separation and  $P^+H_M^- \rightarrow P^+Q_B^-$  electron transfer can occur at low temperature. Others have found that a signal due to electron transfer to  $H_M$  seen at room temperature diminishes at low temperature and interpreted the finding in terms of perturbation of the L-branch energetics with temperature<sup>86</sup> or that  $P^* \rightarrow P^+H_M^-$  has an activation energy.<sup>87,88</sup> A weak temperature dependence of electron transfer to the M branch has been reported for the H(M182)L mutant, where a bacteriopheophytin denoted  $\phi_B$  replaces  $B_M$ .<sup>76</sup> In this mutant  $P^*$  decays to form  $P^+H_L^-$  and  $P^+\phi_B^-$  at both room and low temperature; electron transfer to  $P^+H_M^-$  does not occur because  $P^+\phi_B^-$  is apparently lower in free energy. The rate of  $P^* \rightarrow P^+\phi_B^-$  charge separation was found to be essentially the same at 295, 77, and 9 K.

RCs devoid of  $H_L$  provide the best platform from which to assess quantitatively electron transfer to the M branch. Recently, we reported that  $P^+H_M^-$  forms in  $\sim 70\%$  yield at room temperature in the  $D_{LL}$ -FY $_L$ F $_M$  mutant,<sup>89</sup> which is based on the  $D_{LL}$  construct first reported by Youvan and co-workers and shown in their chromatophore studies<sup>90,91</sup> and in isolated RCs in our work<sup>89</sup> to be devoid of  $H_L$ . In  $D_{LL}$ , the D-helix residues M192–M217 that make contact with most of the cofactors on the photoactive L branch are replaced with the residues from L165–L190 (Figure 1). A *Rb. sphaeroides* RC that lacks  $H_M$  can be generated by a single bulky mutation, A(M149)W,<sup>92</sup> but this strategy does not work to selectively remove  $H_L$  in *Rb. capsulatus* RCs when nearby residues are converted to Trp (e.g., A(L120)W, J. I. Chuang et al., unpublished results). Remarkably, the crystal structure of the A(M149)W mutant displays only minor changes in protein structure despite the absence of the large  $H_M$  cofactor<sup>93</sup> suggesting that the same could be true for the structure of  $D_{LL}$ , which is devoid of  $H_L$ .

We reported that the  $P/P^+$  potential in isolated  $D_{LL}$  RCs is at least 100 meV higher than in wild type.<sup>89</sup> On the basis of this finding, we proposed that all the charge-separated states (other than  $P^+Q_A^-$  and  $P^+Q_B^-$ ) are higher in frequency than  $P^*$  (Figure 2B). From this model it follows that in  $D_{LL}$  charge separation does not compete with internal conversion of  $P^*$ , which occurs with a time constant of  $\sim 180$  ps (Deriphat) or  $\sim 100$  ps (LDAO) at 295 K. We also showed in the  $D_{LL}$ -FY $_L$ F $_M$  mutant that two amino acid changes to  $D_{LL}$  [H(L168)F and H(M195)F; Figure 1B] together lower the  $P/P^+$  potential to  $\sim 470$  meV, which is comparable to the  $\sim 500$  meV wild-type value. The third amino acid change in the  $D_{LL}$ -FY $_L$ F $_M$  mutant is F(L181)Y (Figure 1B), which places a Tyr residue near  $B_M$ . Therefore, the  $D_{LL}$ -FY $_L$ F $_M$  RC has (1) a dimer P with an oxidation potential similar to the wild-type value, (2) a Tyr at L181 that may stabilize  $P^+B_M^-$ , and (3) a Phe at M208 that may destabilize  $P^+B_L^-$  (Figure 1B).

The proposed model for the free energies of the charge-separated states in  $D_{LL}$ -FY $_L$ F $_M$  is given in Figure 2C. Consistent with this model, we found that the  $P^*$  lifetime in  $D_{LL}$ -FY $_L$ F $_M$  at 295 K is 55 ps and that  $P^*$  decays via electron transfer to the M branch to give  $P^+H_M^-$  in 70% yield, with ground-state recovery (assumed to be by direct  $P^* \rightarrow P$  internal conversion) accounting for the remaining 30% of  $P^*$  decay. These data give a rate constant for electron transfer from  $P^*$  to  $P^+H_M^-$  of  $0.7/(55 \text{ ps}) = (78 \text{ ps})^{-1}$ , which is a factor of about 20 slower than the formation of  $P^+H_L^-$  in wild type and is presumed to be superexchange-mediated by  $P^+B_M^-$ . In the present paper we report measurements of  $D_{LL}$  and  $D_{LL}$ -FY $_L$ F $_M$  at 77 K that complement our previous room-temperature measurements. The complexity of the data leads us to consider a series of models involving homogeneous or heterogeneous populations of RCs in order to understand better the underlying physical phenomena. The goal of these studies is to obtain further information on the relative free energies of the charge-separated states in these mutants and the temperature dependence of electron transfer from  $P^*$  to the M-branch cofactors.

## Experimental Methods

**Reaction Center Growth and Purification.** Wild-type,  $D_{LL}$ , and  $D_{LL}$ -FY $_L$ F $_M$  RCs were expressed, isolated, and purified using the detergent Deriphat 160-C as described previously.<sup>89</sup> For measurements of  $D_{LL}$  RCs in LDAO, the detergent was exchanged to LDAO after initial Deriphat 160-C purification. Due to extremely low yields,  $D_{LL}$ -FY $_L$ F $_M$  was not subjected to buffer exchange and so was not examined in LDAO. Glycerol was added to RCs in 10 mM Tris pH 8, 0.05% Deriphat 160-

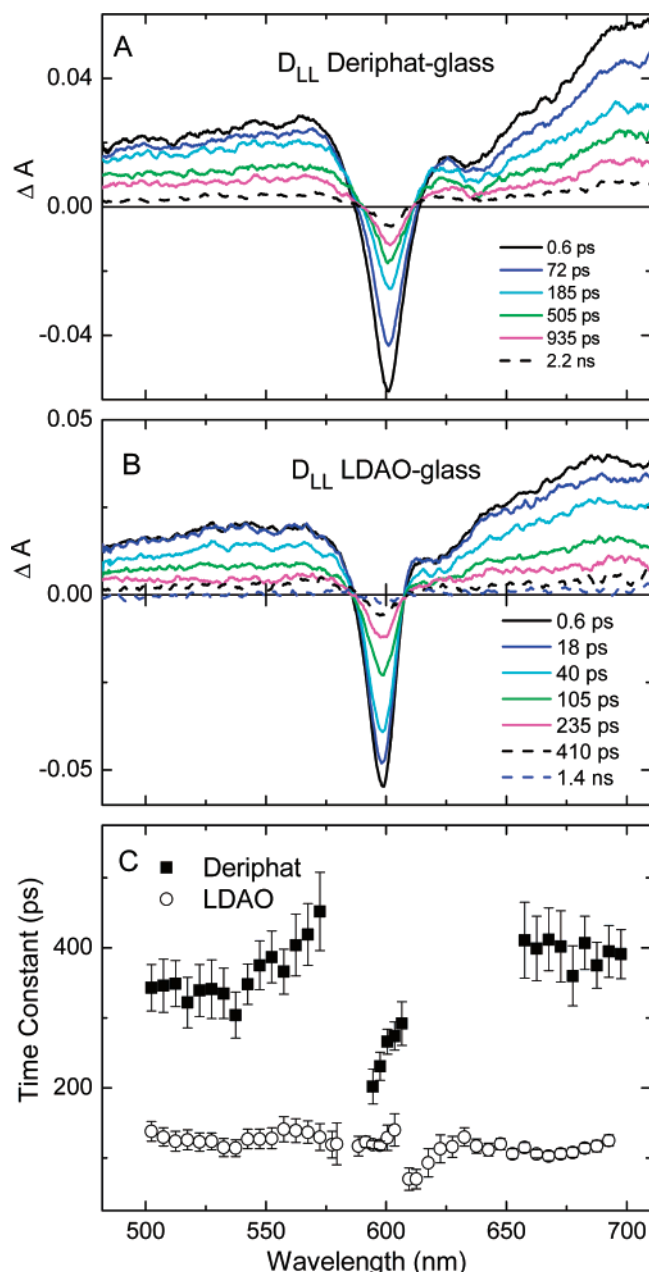
C, or 10 mM Tris pH 8, 0.05% LDAO, to a final concentration of 60% glycerol (v/v) for low-temperature experiments unless otherwise indicated and will be referred to as RC in Deriphat glass or LDAO glass, respectively.

**Picosecond Fluorescence Measurements.** The fluorescence decay of  $P^*$  was measured by fluorescence upconversion essentially as described.<sup>94</sup> Emission was detected at 890 nm at the magic angle relative to the excitation at 800 nm. RC samples had an OD of 0.6 at 800 nm in a 1 mm path length and contained 1 mM dithionite and terbutryn at 30 times the RC concentration to reduce  $Q_A$  and displace  $Q_B$ , respectively. Samples were held in a quartz cuvette in an MMR refrigerator (MMR Technologies) and cooled with argon to 140 K. Data were collected to 1.3 ns for each scan, and the cuvette position was rastered after each time scan. Between 10 and 40 scans were averaged for each sample. Each average was fit to the convolution of the pump–probe cross correlation as determined the same day (typically between 150 and 170 fs) and a sum of exponentials, which included a fixed rise time of 150 fs due to the energy transfer from  $B^*$  to  $P$ .<sup>94</sup>

**Picosecond Transient Absorption Spectroscopy.** The transient absorption measurements were conducted basically as described previously<sup>95</sup> and utilized a regeneratively amplified Ti:sapphire laser pumping an OPA (Spectra Physics) to generate 130 fs excitation and white-light probe flashes at 10 Hz. For experiments probing the visible region of the spectrum (500–700 nm) the RCs samples had an OD of 1 at 860 nm (at 295 K) in a 2 mm path length and were excited with  $\sim 20 \mu\text{J}$  excitation flashes at 870 nm. For experiments probing the near-infrared region (820–1000 nm), the RC samples had an OD of 0.5–0.6 at 860 nm (at 295 K) in a 2 mm path length and were excited with  $\sim 20 \mu\text{J}$  excitation flashes at 595 nm. The induced absorption changes for both 870 and 595 nm excitation were verified to be linear and subsaturating with 20% of the RCs typically excited. The  $D_{LL}$ -FY $_L$ F $_M$  samples contained terbutryn at 25–30 times the RC concentration, and sometimes additionally contained 1 mM sodium dithionite. No differences between the results were found as a function of the presence or absence of dithionite. The relative polarization of the excitation and probe flashes was at the magic angle. The RCs were held in a 2 mm plastic cuvette and cooled in the dark to 77 K in an Oxford Instruments cryostat. In some cases a small ( $\leq 5\%$ ) signal “before zero-time” was observed, representing a fraction of the RCs that had not recovered to the ground state between the 10 ms separation of successive excitation flashes (10 Hz repetition rate); the small pre-zero-time signal was ignored in data analysis.

The transient absorption changes were monitored simultaneously (on a dual diode array detector) over an  $\sim 220$  nm wide window following each excitation flash. The  $\Delta A$  spectra were recorded as a function of pump–probe delay time, with each spectrum representing the average of  $\sim 150$  excitation flashes. Such spectra were acquired as a function of pump–probe delay time from several picoseconds “before zero-time” to as long as 3.5 ns, with 60–100 spectra constituting a typical data set. Two or three such data sets, each one acquired on a fresh sample on a different day, were collected for both the 500–700 nm ( $Q_X$  and anion) probing region and the 850–1050 nm ( $Q_Y$  and stimulated emission) probing region. These data sets were then subject to several different fitting methods. First, kinetic data (time,  $\Delta A$  pairs) were generated by averaging the  $\Delta A$  over 3–5 nm intervals for each spectrum (as a function of delay time) in a data set. A time profile for the  $\Delta A$  within such a 3 or 5 nm interval is referred to herein as single-wavelength kinetics. These data were fit to either a single-exponential function (the





**Figure 3.** 77 K transient absorption difference spectra for  $D_{LL}$  Deriphat-glass RCs (A) and  $D_{LL}$  LDAO-glass RCs (B) acquired in the visible region spectrum at the times indicated following a 130 fs, 870 nm excitation flash. (C) Time constants resulting from fitting the single-wavelength kinetic data for  $D_{LL}$  Deriphat glass (solid squares) and  $D_{LL}$  LDAO glass (open circles) to a single-exponential function. See text for further details.

convolution of the instrument response with one exponential plus a constant) or a biexponential function (the convolution of the instrument response with two exponentials plus a constant). Second, the as-acquired spectra comprising the  $Q_X$  region and  $Q_Y$  regions were subjected to global kinetic and spectral analysis using routines written in Igor Pro 6.01 (Wavemetrics).

## Results

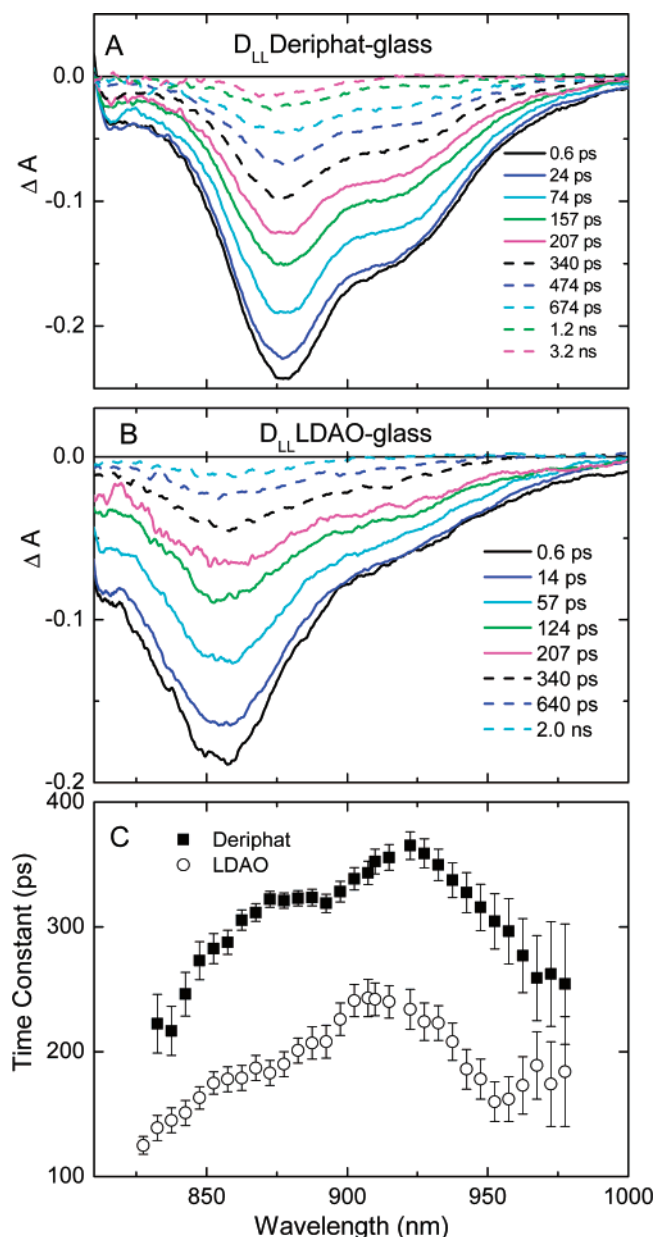
**$D_{LL}$  RCs in Deriphat Glass.** Excitation of  $D_{LL}$  in Deriphat glass or LDAO glass with 870 nm 130 fs flashes produces the lowest singlet excited state of the dimer,  $P^*$ , characterized in the visible region by the 0.6 ps spectrum shown in Figure 3, parts A and B, respectively. The transient absorption difference

spectrum of this state displays a broad, mostly featureless  $P^*$  absorption between 500 and 700 nm, bleaching of the  $Q_X$  band of  $P$  (maximum at 601 nm in Deriphat and 598 nm in LDAO), and a shallower dip just to the red of the  $Q_X$  bleaching (near 635 nm in Deriphat and 620 nm in LDAO). The 635 or 620 nm feature may represent bleaching of a weak ground-state absorption band of  $P$  (to a high-energy  $Q_Y$  excited state) that has been calculated to lie in this region.<sup>36</sup> These spectral features change shape little with time, including the persistence of two isosbestic points at  $\Delta A \sim 0$  flanking the  $Q_X$   $P$  bleaching for LDAO glass (but not Deriphat glass), and decay nearly to  $\Delta A = 0$  over the time course shown. These findings indicate that return of  $P^*$  to the ground state is essentially complete by 2 ns. There is perhaps a few percent yield of the triplet excited state of  $P$  ( $^3P$ ) as evidenced by the residual 600 nm bleaching at  $>1$  ns.

The single-wavelength  $\Delta A$  changes between 500 and 700 nm were individually fit to a function containing either one or two exponentials as detailed in the Experimental Methods section. For the single-exponential fits, the resulting time constants vary as a function of probe wavelength (Figure 3C), with average values of  $360 \pm 70$  ps in Deriphat glass and  $120 \pm 20$  ps in LDAO glass. The double-exponential fits gave average time constants of  $130 \pm 50$  and  $600 \pm 190$  ps in Deriphat glass and  $50 \pm 52$  and  $330 \pm 110$  ps in LDAO glass.

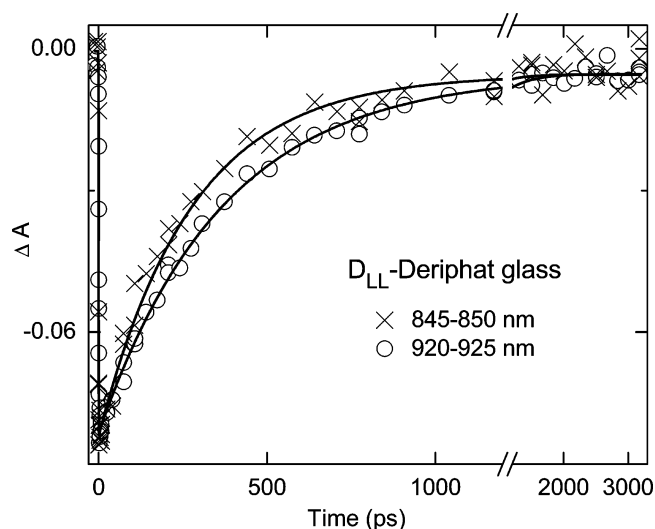
Figure 4, parts A and B, shows transient absorption difference spectra between 820 and 1000 nm for  $D_{LL}$  in Deriphat glass and LDAO glass, respectively, as a function of time following excitation with a 600 nm 130 fs flash. The 0.6 ps spectrum is characterized by bleaching of the long-wavelength absorption band of  $P$  at 875 nm with stimulated emission from  $P^*$  giving rise to a negative  $\Delta A$  at longer wavelengths. This overall spectral shape, including the prominent stimulated emission centered at 925 nm, persists as the features decay nearly to  $\Delta A = 0$ . As in the  $Q_X$  absorption region, these observations indicate that by 2 ns  $P^*$  has returned to the ground state, except for perhaps a few percent yield of  $^3P$  represented by the residual bleaching in the 3 ns spectrum.

The single-wavelength  $\Delta A$  time profiles spanning 825–980 nm were fit as above to a single-exponential function. The apparent  $P^*$  lifetime shows a significant variation as a function of wavelength (Figure 4C), ranging in this spectral region from 220 to 380 ps for  $D_{LL}$  in Deriphat glass and from 120 to 240 ps in LDAO glass. In Figure 5, two representative time profiles for the Deriphat-glass sample underscore the wavelength dependence reflected by the solid squares in Figure 4C. The fit time constant is  $273 \pm 13$  ps at 840–845 nm and  $365 \pm 11$  ps at 920–925 nm. At 885–890 nm an intermediate value of  $323 \pm 7$  ps is found. This value compares well to  $P^*$  lifetime determined in this medium by spontaneous fluorescence decay, which yields a time constant of 293 ps for the dominant (81%) phase of the observed biexponential decay at 890 nm and 140 K. A faster 12 ps component (19%) was also observed in the spontaneous fluorescence measurements but was not seen in the 77 K transient absorption measurements and so was not considered further. The average time constant from the single-exponential fits of the transient absorption data in Deriphat glass over the entire region between 825 and 980 nm is  $300 \pm 40$  ps, in good agreement with the  $360 \pm 70$  ps average  $P^*$  lifetime measured in the  $Q_X$  region. Table 1 (column 3) lists the  $330 \pm 60$  ps value that is the average of the single-exponential fits from the  $Q_X$  (500–700 nm) and  $Q_Y$  (825–980 nm) regions for  $D_{LL}$  in Deriphat glass. The average value for the  $Q_X$  plus  $Q_Y$  regions for LDAO glass is  $150 \pm 40$  ps.



**Figure 4.** 77 K transient absorption difference spectra for D<sub>LL</sub> Deriphat-glass RCs (A) and D<sub>LL</sub> LDAO-glass RCs (B) acquired in the region of the Q<sub>Y</sub> absorption band of P at the times indicated following a 130 fs, 595 nm excitation flash. (C) Time constants resulting from fitting the single-wavelength kinetic data for D<sub>LL</sub> Deriphat glass (solid squares) and D<sub>LL</sub> LDAO glass (open circles) to a single-exponential function. See text for further details.

The single-wavelength  $\Delta A$  time profiles from the Q<sub>Y</sub> region were also fit to a biexponential function. These fits for D<sub>LL</sub> in Deriphat glass give average time constants of  $180 \pm 60$  and  $430 \pm 60$  ps, which again compare favorably with the  $130 \pm 50$  and  $600 \pm 190$  ps determined from biexponential fits of the Q<sub>X</sub> data. The analogous fits for D<sub>LL</sub> in LDAO glass give average time constants of  $140 \pm 50$  and  $500 \pm 170$  ps, which also compare well with the biexponential fits of the Q<sub>X</sub> data. Averaging the time constants over the Q<sub>Y</sub> and Q<sub>X</sub> regions gives time constants of  $150 \pm 60$  and  $520 \pm 170$  ps for Deriphat glass and  $90 \pm 50$  and  $390 \pm 160$  ps for LDAO glass (Table 1, columns 4 and 5). The P\* lifetime for D<sub>LL</sub> in chromatophores at 80 K has been reported as  $500 \pm 100$  ps by transient absorption at 525 nm, and 440 (70%) and 840 ps (30%) by fluorescence at 920 nm,<sup>97</sup> in good agreement with the longer-



**Figure 5.** Decay of the P bleaching averaged between 845 and 850 nm (×) and decay of the stimulated emission averaged between 920 and 925 nm (○) for D<sub>LL</sub> Deriphat-glass RCs at 77 K. The data have been normalized to the same total  $\Delta A$  span for presentation purposes. The solid lines are fits to a single-exponential function giving time constants of  $273 \pm 13$  ps (845–850 nm) and  $365 \pm 11$  ps (920–925 nm).

**TABLE 1: Time Constants for P\* Decay in D<sub>LL</sub> RCs at 77 K**

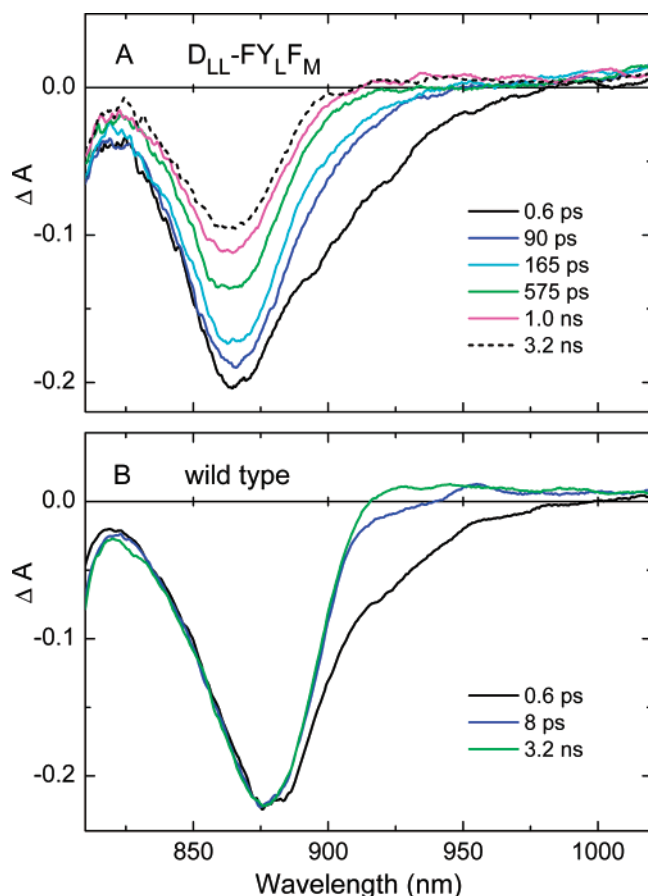
medium	analysis	1-exponential	2-exponential	
		$\tau$ (ps)	$\tau_1$ (ps)	$\tau_2$ (ps)
Deriphat	local <sup>a</sup>	$330 \pm 60$	$150 \pm 60$	$520 \pm 170$
	global <sup>b</sup>	$330 \pm 20$	$160 \pm 20$	$430 \pm 20$
LDAO	local <sup>a</sup>	$150 \pm 40$	$90 \pm 50$	$390 \pm 160$
	global <sup>b</sup>	$170 \pm 10$	$80 \pm 10$	$300 \pm 20$

<sup>a</sup> Average from individual single-wavelength fits (time profiles of  $\Delta A$  averaged over 3 or 5 nm intervals and spanning the visible (Q<sub>X</sub>) and Q<sub>Y</sub> regions; see the Results section). <sup>b</sup> From global fits of combined Q<sub>X</sub> and Q<sub>Y</sub> data.

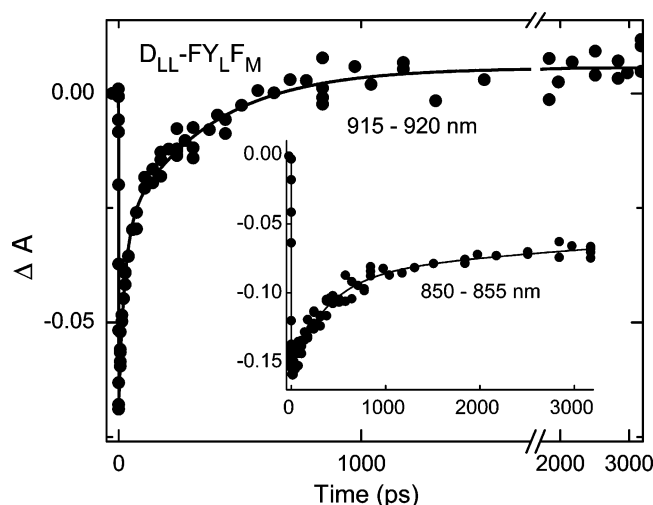
lived component observed here for D<sub>LL</sub> RCs in 77 K Deriphat glass.

**Global Analysis for D<sub>LL</sub> RCs at 77 K.** Global analysis was performed on the combined data from the Q<sub>X</sub> (500–700 nm) and Q<sub>Y</sub> (820–1000 nm) regions for D<sub>LL</sub> RCs in both Deriphat glass and LDAO glass. First, the combined data from the Q<sub>X</sub> and Q<sub>Y</sub> regions were fit globally to a single-exponential function. This analysis returned values of  $330 \pm 20$  ps for D<sub>LL</sub> in Deriphat glass and  $170 \pm 10$  ps for LDAO glass (Table 1, column 3). As expected, these values are essentially the same as the average values determined from the single-wavelength fits (Table 1, column 3). Second, the data were globally fit to a biexponential function, which gave time constants of  $160 \pm 20$  and  $430 \pm 20$  ps for D<sub>LL</sub> in Deriphat glass and  $80 \pm 10$  and  $300 \pm 20$  ps for D<sub>LL</sub> in LDAO glass (Table 1, columns 4 and 5). These pairs of values compare very well to the pairs of average values determined from the single-wavelength biexponential fits (Table 1, columns 4 and 5). Further analysis and modeling of D<sub>LL</sub> RCs at 77 K in terms of a heterogeneous population of two different forms of P\* are given in the Discussion section.

**Overview of Results and Analysis for D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> RCs.** Subpicosecond time-resolved transient absorption data for D<sub>LL</sub>-FY<sub>L</sub>F<sub>M</sub> at 77 K in Deriphat glass are shown in Figures 6–8. The data are more complex than those found previously for this mutant at room temperature.<sup>89</sup> cursory examination of the transient absorption spectra in Figure 8A reveals that a

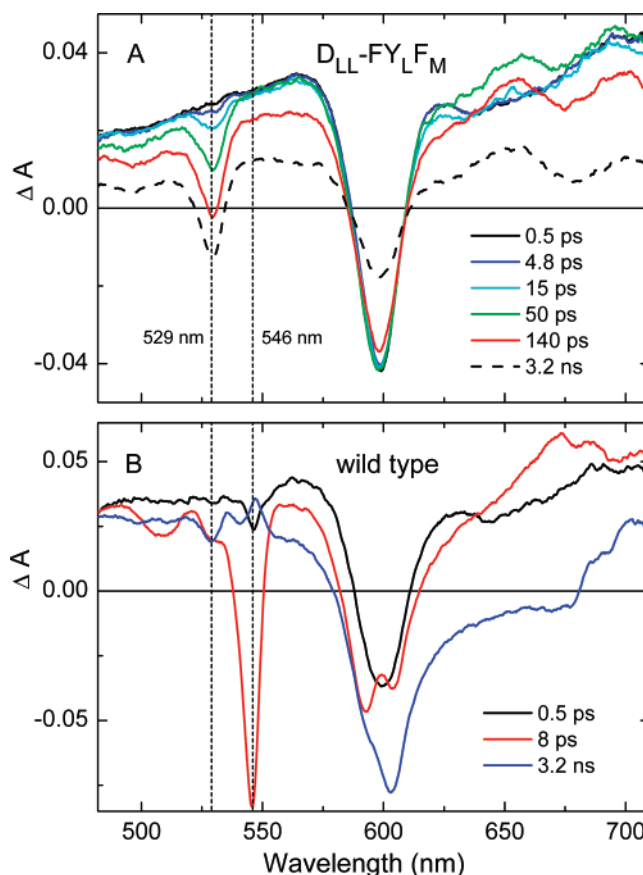


**Figure 6.** Comparison of the 77 K transient absorption difference spectra for  $D_{LL}$ - $FY_LF_M$  Deriphat-glass RCs (A) and wild-type Deriphat-glass RCs (B) acquired in the region of the  $Q_Y$  absorption band of P at the times indicated following a 130 fs, 595 nm excitation flash.



**Figure 7.** Kinetic data and fits for  $D_{LL}$ - $FY_LF_M$  Deriphat-glass RCs at 77 K. The main panel shows decay of the stimulated emission averaged between 915 and 920 nm (●) and fit to a biexponential function (solid line). The inset shows decay of the P bleaching averaged between 850 and 855 nm (●) and fit to a biexponential function (solid line). See text for further details.

significant bleach occurs in  $D_{LL}$ - $FY_LF_M$  in the  $Q_X$  band of  $H_M$  at 529 nm, whereas only a small negative signal, possibly a small bleach, is observed in this region in wild type; instead the well-known bleach of the  $Q_X$  band of  $H_L$  at 546 nm is observed (Figure 8B). The wild-type data reflect an essentially 100% yield of the  $P^+H_L^-$  state, whereas the relative yield (scaled

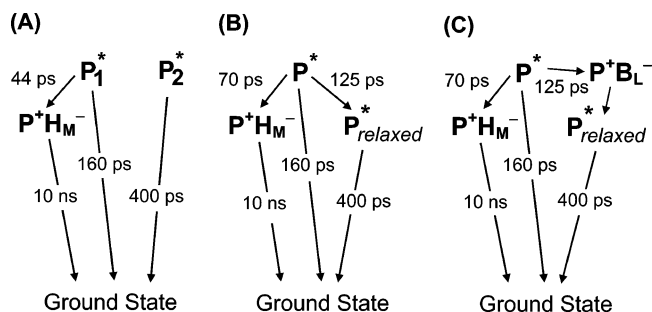


**Figure 8.** Comparison of the 77 K transient absorption difference spectra for  $D_{LL}$ - $FY_LF_M$  Deriphat-glass RCs (A) and wild-type Deriphat-glass RCs (B) acquired in the visible region of the spectrum at the times indicated following a 130 fs, 870 nm excitation flash.

to the bleach of the P  $Q_X$  band) is considerably smaller for  $P^+H_M^-$  in  $D_{LL}$ - $FY_LF_M$ . Furthermore, the bleach of the  $Q_X$  band of  $H_M$  in  $D_{LL}$ - $FY_LF_M$  is smaller than what was observed earlier at room temperature (Figure 4A in ref 89). Thus,  $P^+H_M^-$  is being formed at low temperature, but either its yield, per P excited, is lower than at room temperature or some fraction of  $P^*$  is not forming  $P^+H_M^-$  at all. These qualitative observations lead naturally to the consideration of models for heterogeneity that then permits quantitative analysis of the yields and rates. Following the introduction of two possible models, transient absorption data in the near-infrared  $Q_Y$  and then visible  $Q_X$  regions are presented along with detailed data analysis based on one of these models. The implications of this analysis for processes occurring in  $D_{LL}$ - $FY_LF_M$ ,  $D_{LL}$ , and wild type are described further in the Discussion section. It will be seen that regardless of the complexities and model chosen, electron transfer from  $P^*$  to the M side is found to have a weak temperature dependence and thus to be an effectively activationless process.

Two basic models are distinguished as heterogeneous and homogeneous with respect to the initial population of  $P^*$ . Both models have been discussed previously for the  $YFH$  mutant in Deriphat at 295 K.<sup>39</sup> In the heterogeneous-RC model of  $D_{LL}$ - $FY_LF_M$  RCs in Deriphat glass at 77 K, we propose there are at least two distinct and uncoupled populations having different forms of  $P^*$  whose fates are very different. These populations could differ in characteristics such as the electronic structure of  $P^*$ , the electronic couplings for electron transfer to  $H_M$ , and the free energies of the charge-separated states. With respect to this model, the data described below can be reconciled with





**Figure 9.** Models for charge separation in the  $D_{LL}$ -FY $_L$ F $_M$  RC in Deriphat glass at 77 K: heterogeneous-RC model (A) and two variants of a homogeneous-RC model (B and C). The internal conversion rate constant of  $(160\text{ ps})^{-1}$  for the photoactive  $P_1^*$  fraction in (A) and for the initial form of  $P^*$  in (B) and (C) is assumed to be the same as the shorter-lived component of the  $P^*$  decay for the  $D_{LL}$  RC in Deriphat glass at 77 K. Similarly, the rate constant of  $(400\text{ ps})^{-1}$  for the inactive  $P_2^*$  population in (A) and for the relaxed form of  $P^*$  in (B) and (C) is assumed to be the same as the longer-lived component of  $P^*$  decay and ground-state recovery for  $D_{LL}$ -FY $_L$ F $_M$  at 77 K. The parameters shown give a lifetime of  $P_1^*$  in (A) and  $P^*$  in (B) and (C) of 35 ps, which is the measured value. The calculated yield of  $P^+H_M^-$  using the parameters shown is 80% for the photoactive subpopulation ( $P_1^*$ ) in (A) and 50% for the entire  $P^*$  initial population in (B) and (C).

the following: (1) in approximately half of the RCs  $P^*$  decays by electron transfer to the M branch to give  $P^+H_M^-$ , and (2) in the remaining half,  $P^*$  decays by deactivation to the ground state. Within the heterogeneous model, the raw transient absorption spectra reflect the *sum* of the absorption changes arising from these two subpopulations in which very different and kinetically uncoupled photochemical events occur, as reflected in Figure 9A.

In alternative homogeneous models (Figure 9, parts B and C),  $P^*$  decays with roughly 50/50 yields via electron transfer to  $H_M$  and via formation of an inactive, or “dead-end” state. “Dead-end” implies that this state cannot do subsequent electron transfer to  $H_M$  (and clearly not to  $H_L$ , which is absent) but instead decays only to the ground state. An example of such an inactive state is a relaxed form of  $P^*$  produced from the initial form of  $P^*$  (Figure 9B) or produced from a charge-separated state such as  $P^+B_L^-$  (Figure 9C). In both the models shown in Figure 9, parts B and C, direct decay of the initial form of  $P^*$  to the ground state is less effective than the two alternative pathways.

Throughout the following, the results for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass are presented in terms of the heterogeneous-RC model. Doing so allows connections to be drawn between the data for both  $D_{LL}$ -FY $_L$ F $_M$  and  $D_{LL}$  and leads to a model that is consistent for both mutants. Further analysis and comparisons of these data in terms of the heterogeneous- and homogeneous-RC models are given in the Discussion section.

**Time Evolution of Near-Infrared Spectra for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat Glass at 77 K.** Spectra acquired at several delay times (0.6 ps to 3.2 ns) in the P-bleaching region for  $D_{LL}$ -FY $_L$ F $_M$  and wild type in Deriphat glass at 77 K are shown in Figure 6, parts A and B, respectively. The data for wild type reflect  $\sim 100\%$  conversion of  $P^* \rightarrow P^+H_L^- \rightarrow P^+Q_A^-$  with  $\sim 2$  and  $\sim 100$  ps time constants, respectively. Whereas the magnitude of P bleaching in wild type is constant on this time scale, in  $D_{LL}$ -FY $_L$ F $_M$  there is extensive decay of P bleaching (835–865 nm) at 3.2 ns compared to 0.6 ps. A representative  $P^*$  stimulated emission decay profile (data averaged between 915 and 920 nm) and fit to a biexponential function for  $D_{LL}$ -FY $_L$ F $_M$  are shown in Figure 7 (main panel). (Note that assignment of this data to stimulated emission is valid because little if any

contribution from P-bleaching decay is expected at these wavelengths, as is evident for wild type in Figure 6B.) Similar biexponential fits to the single-wavelength time profiles between 915 and 935 nm give average time constants of  $32 \pm 7$  and  $380 \pm 70$  ps. These results are in good agreement with the  $P^*$  kinetics measured independently by  $P^*$  spontaneous fluorescence, the majority of which decays with time constants of 22 and 282 ps at 890 nm and 140 K. Approximately 20% of the fluorescence amplitude arises from an additional 1.7 ps time constant that was not seen in the transient absorption data and was not considered further. Within the heterogeneous model, these observations are evidence for at least two populations of  $P^*$  in  $D_{LL}$ -FY $_L$ F $_M$ , one with a lifetime of several hundred picoseconds (similar to that observed for  $D_{LL}$  in Deriphat glass) and one with a lifetime that is only tens of picoseconds.

To examine the kinetics of P-bleaching decay in  $D_{LL}$ -FY $_L$ F $_M$ , the absorption changes on the blue side of the P bleaching between 835 and 865 nm were analyzed. At these wavelengths there is little if any contribution from  $P^*$  stimulated emission (see wild-type data in Figure 6B). A representative time profile (850–855 nm) is shown in Figure 7 (inset) along with a fit to a biexponential function. The constant value was fixed at  $\Delta A = 0$  since no long-term charge separation can occur; neither long-lived state  $P^+Q_A^-$  nor  $P^+Q_B^-$  can form in the absence of  $H_L$  and the presence of excess terbutryn, which displaces  $Q_B$ . The same results were obtained for  $D_{LL}$ -FY $_L$ F $_M$  RCs containing both a 25-fold excess of terbutryn and 1 mM sodium dithionite; the latter chemically reduces  $Q_A$  and  $Q_B$ . The average time constants determined from single-wavelength time profiles between 835 and 865 nm are  $410 \pm 60$  ps and  $\sim 10$  ns (range 5–15 ns), each with a  $\sim 50\%$  amplitude. Inclusion of a third exponential, either as a free parameter or with a fixed value between 20 and 50 ps, yielded only small amplitudes ( $\leq 5\%$ ) for this component and did not significantly improve the fits.

Within the heterogeneous-RC model these results indicate that in roughly 50% of the  $D_{LL}$ -FY $_L$ F $_M$  RCs in Deriphat glass, the ultimate fate of  $P^*$  is similar to that found in  $D_{LL}$ . In this fraction  $P^*$  decays to the ground state with an average time constant of  $\sim 400$  ps, as is evident from the P bleaching and  $P^*$  stimulated emission decay kinetics. In the other 50% of the RCs,  $P^*$  decays in  $\sim 35$  ps and produces a state with a lifetime of roughly 10 ns. This product is identified as  $P^+H_M^-$  based on the time evolution of the  $Q_X$  region spectra presented in the next section.

**Time Evolution of Visible Spectra for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat Glass at 77 K.** For  $D_{LL}$ -FY $_L$ F $_M$ , the spectrum acquired in the  $Q_X$  region 0.5 ps after excitation (Figure 8A) is again similar to the 0.6 ps spectrum of  $D_{LL}$  (Figure 3A) and wild type (Figure 8B), and assigned to  $P^*$ . [A small caveat is that in wild type the onset of  $H_L$  bleaching at 546 nm is captured at 0.5 ps because the  $P^*$  lifetime at 77 K is only  $1.9 \pm 0.2$  ps (kinetic data not shown).] In the spectra of  $D_{LL}$ -FY $_L$ F $_M$  at later times there is prominent bleaching at 529 nm, which can be assigned to bleaching of the  $Q_X$  ground-state band of  $H_M$ , and a prominent absorption band with a peak near 650 nm, which can be assigned to the  $H_M$  anion. These spectral features are consistent with formation of  $P^+H_M^-$ . They parallel, but are blue-shifted from, the 546 nm  $Q_X$  bleaching of  $H_L$  and 670 nm peak of the  $H_L$  anion band observed in the  $P^+H_L^-$  spectrum of wild type at 8 ps (Figure 8B). Note that there is no indication of  $H_L$  bleaching in  $D_{LL}$ -FY $_L$ F $_M$ , consistent with the absence of this pigment in these RCs. The blue shift of the  $Q_X$  and anion bands of  $H_M$  compared to  $H_L$  is expected and has been shown to derive largely from the hydrogen bond between the  $H_L$  ring-V keto



group and E(L104), whereas the symmetry-related V(M131) cannot donate an analogous hydrogen bond to  $H_M$ .<sup>98–101</sup> The  $Q_X$  bleaching of  $H_M$  in  $D_{LL}$ -FY $_L$ F $_M$  has a full width at half-maximum (fwhm) about 2 nm larger than that of the  $Q_X$  band of  $H_L$  in wild type (11 vs 9 nm). These width differences are consistent with the ground-state absorption features at 77 K, which have estimated fwhms of 11 nm (for  $H_M$  in  $D_{LL}$ -FY $_L$ F $_M$ ) and 8.5 nm (for  $H_L$  in wild type).

The bleaching at 529 nm develops with an  $\sim 35$  ps time constant (data not shown), which matches well the shorter component of the  $P^*$  stimulated emission kinetics ( $32 \pm 7$  ps). Within the heterogeneous model this 35 ps component can be assigned to the  $P^*$  lifetime for the 50% of the  $D_{LL}$ -FY $_L$ F $_M$  RCs in which electron transfer to the M side occurs, yielding  $P^+H_M^-$ . In contrast, there is no evidence ( $<10\%$  amplitude) for a 35 ps component in the decay kinetics of the 600 nm bleaching. Rather, the bleaching of the  $Q_X$  band of P decays biexponentially with time constants of  $\sim 380$  and  $\sim 10$  ns, both with  $\sim 50\%$  amplitude (data not shown). The same result is observed for P-bleaching decay in the  $Q_Y$  spectral region. Because P-bleaching decay represents ground-state recovery, a 35 ps kinetic component in the P ground-state absorption region would reflect competing  $P^*$  deactivation to the ground state in the photoactive population of  $P^*$ . Because the 35 ps component is absent from the  $Q_X$  and  $Q_Y$  P-bleaching decay kinetics, we conclude that  $P^* \rightarrow P^+H_M^-$  occurs with high yield in this 50% population. Estimates of the yield based on these and other data are presented in the Discussion section.

Comparing the spectra at 140 ps and 3.2 ns in Figure 8A, the magnitude of bleaching at 529 nm, when referenced to the positive absorption on either side of the peak, is little changed, indicating that  $P^+H_M^-$  is fully populated by 140 ps and persists for  $>5$  ns. This is consistent with  $P^+H_M^-$  formation with a 35 ps time constant and  $P^+H_M^-$  decay by charge recombination to the ground state with a time constant on the order of 10 ns. A qualitative examination of these two spectra also provides a useful consistency check for the heterogeneous model. Within this model, a given transient spectrum should be the sum of the predicted absorption changes due to the 50% population of RCs in which  $P^* \rightarrow P^+H_M^-$  has occurred in high yield and the 50% population of RCs in which  $P^*$  deactivates only to the ground state. A 140 ps delay time corresponds to four  $1/e$  times of the 35 ps  $P^*$  lifetime for the photoactive fraction and thus to a maximum population of  $P^+H_M^-$ . Therefore, one would expect maximal 529 nm bleaching of the  $Q_X$  band of  $H_M$  and maximal 650 nm absorption of the anion band of  $H_M$ . In contrast only minimal decay of the 600 nm P bleach is predicted because (1) the high yield of  $P^+H_M^-$  precludes significant P ground-state recovery in the photoactive fraction and (2)  $P^*$  decays to the ground state with a slow 400 ps constant in the nonphotoactive fraction. The 140 ps transient spectrum agrees with both of these predictions (Figure 8A).

The spectrum acquired at a 3.2 ns delay (Figure 8A) can be interpreted similarly. Within the heterogeneous model, by this time the absorption changes will have taken on different relative magnitudes of these same features. Both the 529 nm  $H_M$  bleach and the 650 nm  $H_M$  anion absorption band are of similar overall shape and amplitude (as measured against the overlapping positive absorption from  $P^*$ ) at 3.2 ns as at 140 ps, consistent with a  $\sim 10$  ns lifetime for  $P^+H_M^-$ . In contrast, between 140 ps and 3.2 ns significant fractions of both the broad, positive  $P^*$  transient absorption and the 600 nm P bleaching have decayed. This is consistent with  $P^* \rightarrow$  ground state deactivation with a

400 ps time constant in the 50% nonphotoactive fraction of  $P^*$ . Thus, the spectrum remaining at 3.2 ns arises exclusively from  $P^+H_M^-$ .

**Global Analysis for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat Glass.** Global analysis of the combined  $Q_X$  (500–700 nm) and  $Q_Y$  (825–1000 nm) data was performed as described for  $D_{LL}$ , except that the data were fit to three exponentials plus a constant (convolved with the instrument response). The analysis was carried out for the heterogeneous model described above and gave time constants of  $35 \pm 3$  ps,  $410 \pm 30$  ps, and  $10 \pm 1$  ns. As expected from the model, the species-associated spectra for the 35 ps and 410 ps components (two forms of  $P^*$ ) are similar overall to the raw spectrum (Figures 6A and 8A) at 0.5 or 0.6 ps except for differences in the relative amplitudes of the stimulated emission and bleaching features. The species-associated spectrum of the 10 ns component ( $P^+H_M^-$ ) is similar to the raw spectrum at 3.2 ns. Like the analysis in the preceding section, the global analysis is consistent with the presence of two roughly equal subpopulations of RCs. Both analyses support a model in which a photoactive fraction exhibits a 35 ps decay of  $P^*$  to  $P^+H_M^-$  in high yield and subsequent  $P^+H_M^- \rightarrow$  ground state charge recombination with an  $\sim 10$  ns time constant, whereas the other fraction is characterized by  $\sim 400$  ps deactivation of  $P^*$  only to the ground state.

## Discussion

**Heterogeneous-RC Model for  $D_{LL}$ .** Complex spectral evolution in bacterial RCs, both wild type and a variety of mutants, is well documented, taking different forms in different types of measurements. These include the following: (1) *detection*-wavelength-dependent time constants determined from transient absorption measurements for both  $P^* \rightarrow P^+H_L^-$  charge separation (involving  $P^+B_L^-$  as a discrete or superexchange intermediate or both) and  $P^+H_L^- \rightarrow P^+Q_A^-$  electron transfer in wild-type RCs at both room and low temperature,<sup>15,16</sup> (2) transient absorption measurements showing *excitation*-wavelength-dependent kinetics,<sup>37,102,103</sup> (3) biexponential  $P^*$  stimulated emission decay kinetics as measured by transient absorption,<sup>14,17,18,20,22,31,35,46</sup> and (4) biexponential or multiexponential  $P^*$  spontaneous fluorescence decay kinetics in wild type and mutants.<sup>19,23,24,30,35,45</sup> Various models for these phenomena have been discussed, often in terms of multiple RC forms or a distribution of RC forms, each form having a slightly different  $P^*$  lifetime (or rate of  $P^+H_L^- \rightarrow P^+Q_A^-$  electron transfer), such that the ensemble yields complex kinetic profiles. Static heterogeneity could arise from RCs that differ in distances between cofactors, the nature and strengths of cofactor–protein interactions, or a host of similar factors that affect either the electronic or Frank–Condon contribution (free energy gaps between the relevant states) to the rates of the electron-transfer processes. Contributions to complex kinetic profiles from dynamic effects involving motions of the chromophores, the protein, or both accompanying or following the decay of  $P^*$  or  $P^+H_L^-$  also have been discussed.<sup>18,22,65,74,104–107</sup>

In our previous work, we proposed the scheme for  $D_{LL}$  given in Figure 2B, wherein the charge-separated intermediates (states other than  $P^+Q_A^-$  and  $P^+Q_B^-$ ) lie above  $P^*$  in free energy. This scheme derived from (1) the current view of the free energies of the intermediates in wild type, (2) the measured  $P/P^+$  potential in  $D_{LL}$  being at least 100 meV higher than in wild type, and (3) the lack of spectral signatures for formation of a charge-separated state from  $P^*$  in  $D_{LL}$  at 295 K. In the simplest interpretation, the data presented here for  $D_{LL}$  in either Deriphat glass or LDAO glass at 77 K are similar to the results for  $D_{LL}$

at 295 K and can be reconciled with the model shown in Figure 2B, namely, with the charge-separated intermediates lying above  $P^*$  and  $P^* \rightarrow$  ground state (via internal conversion) being the only decay route of  $P^*$ . This simple framework for the time evolution of the spectra in Figures 3 and 4 follows from the clear persistence of  $P^*$  stimulated emission throughout the entire several hundred picoseconds during which the RC returns to the ground state. In this regard the  $D_{LL}$  RC presents an unprecedented opportunity to examine the spectral evolution of  $P^*$  decay across the entire spectrum precisely because it is not capable of electron transfer; the  $P$  bleach decay and  $P^*$  stimulated emission decay are indicative of the *same* process,  $P^* \rightarrow P$ .

Within the energy scheme illustrated in Figure 2B, the observed probe-wavelength dependence of the  $P^*$  lifetime in  $D_{LL}$  at 77 K (Figures 3C and 4C) requires, at the least, a heterogeneous model involving two subpopulations of  $P^*$  with different lifetimes. As noted in the Introduction, the possibility that there may be different forms of  $P^*$  and/or  $P$  in an RC sample is reasonable and supported by observed changes in various RC or cofactor properties as a function of milieu. For example, the long-wavelength absorption band of  $P$  in  $D_{LL}$  is  $\sim 20$  nm blue-shifted in LDAO compared to Deriphat at both room and low temperature. The near-infrared absorption band of  $P$  in wild type and many mutants has similarly been shown to vary widely with conditions (such as pH or detergent) indicating that the protein environment can affect the spectral/electronic properties of  $P$ . Even in the absence of a wavelength-dependent  $P^*$  lifetime, the difference in the average  $P^*$  lifetime of  $D_{LL}$  in LDAO versus in Deriphat (110 vs 180 ps at 295 K<sup>89</sup> and 150 vs 330 ps at 77 K) strongly suggests that different forms of  $P^*$  (and/or  $P$ ) exist in these samples.

The results of global analysis using a biexponential fit to the combined visible and near-infrared data sets for  $D_{LL}$  in Deriphat glass or LDAO glass are consistent with a heterogeneous-RC model, in which at least two subpopulations of  $P^*$  decay in parallel with distinct lifetimes (Figure 9A). These two subpopulations are denoted as the shorter-lived and longer-lived forms of  $P^*$ . Noteworthy aspects of the global analysis are as follows: (1) in Deriphat glass all the species-associated spectra show the same prominent features evident in the as-acquired spectra (Figures 3A and 4A), and the same holds true in LDAO glass (Figures 3B and 4B); (2) the amplitude ratio of  $P^*$  stimulated emission versus  $P$  bleaching is smaller for the shorter-lived form of  $P^*$  than for the longer-lived form of  $P^*$ , and this effect is more pronounced for  $D_{LL}$  in LDAO than in Deriphat; (3) both forms of  $P^*$  have a shorter lifetime in LDAO than in Deriphat, with the difference more pronounced for the shorter-lived form (Table 1); (4) a larger fraction of the sample (an additional  $\sim 15\%$ ) is in the shorter-lived  $P^*$  form than in the longer-lived form in LDAO versus Deriphat. In summary, these differences can be understood if the shorter-lived  $P^*$  form in either detergent is less emissive than the longer-lived form, if nonradiative deactivation of  $P^*$  is enhanced for  $D_{LL}$  in LDAO compared to Deriphat, and if the fraction of  $P^*$  in the shorter-lived form is larger in LDAO than in Deriphat.

The temperature dependence of the  $P^*$  decay profiles also likely is rooted in differences in the relative populations of different forms of  $P^*$ . We note that the 180 ps  $P^*$  lifetime for  $D_{LL}$  in Deriphat at 295 K is comparable to the 160 ps lifetime of the shorter-lived  $P^*$  form in Deriphat glass at 77 K (Table 1). Similarly, the 110 ps lifetime for  $P^*$   $D_{LL}$  in LDAO at 295 K is comparable to the 80 ps lifetime of the shorter-lived  $P^*$  form in LDAO glass at 77 K. Longer-lived  $P^*$  forms were not

resolved at room temperature. One possibility is that both  $P^*$  forms are present at 295 K but have similar photophysical properties such that they are not readily distinguished in the transient absorption measurements. At low temperature then, the shorter-lived form retains a lifetime comparable to, but perhaps slightly faster than, at room temperature, while the lifetime of the longer-lived form is substantially lengthened. The relative proportions of the two subpopulations may also change as a function of temperature, and one possibility consistent with the data is that the proportion of the longer-lived form increases at low temperature. In the extreme case,  $P^*$  exists in only one form or rapidly interconverting forms at room temperature but freezes into two different forms at low temperature where barriers for interconversions between the forms cannot be overcome, as has been postulated for other RCs.<sup>24,25,43,100</sup>

Finally, we consider possible physical differences between the two forms of  $P^*$ . One is medium-induced alterations in the electrostatic environment (or structure) of  $P$  that modulate its electronic character, including the admixtures of the charge-transfer and exciton configurations of  $P^*$ . There is good evidence that the electronic composition of  $P^*$  affects its internal conversion rate.<sup>108–111</sup> To explain the detergent-dependent  $P^*$  lifetime in  $D_{LL}$ , the contributions of one or both charge-transfer configurations would be enhanced in LDAO compared to in Deriphat, especially for the shorter-lived  $P^*$  subpopulation. Additional factors may distinguish the different  $P^*$  forms and may vary over a distribution of values. However, the simple heterogeneous-RC model for  $D_{LL}$  at 77 K involving just two independent forms of  $P^*$  is straightforward and provides a useful and consistent platform for understanding the even more complex data for  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> discussed in the next section.

**Heterogeneous-RC Model for  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> in Deriphat Glass.** In the Results section, the time-resolved absorption measurements on  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> in Deriphat glass were presented in terms of a static-heterogeneity model involving two subpopulations of RCs that have very different photophysics/photochemistry (Figure 9A). The  $P^*$  lifetimes in these roughly equal fractions are 35 and 410 ps based on global analysis. In the longer-lived fraction,  $P^*$  deactivation to the ground state dictates the 410 ps  $P^*$  lifetime. This value is essentially the same as the longer  $P^*$  lifetime (430 ps) derived from the biexponential global analysis of  $D_{LL}$  in Deriphat glass (Table 1). It is not evident a priori that the longer-lived form of  $P^*$  in  $D_{LL}$  and the nonphotochemically active form of  $P^*$  in  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> should be equated and have the same lifetime since the two RCs differ in three key amino acids that interact with  $P$ . However, the agreement between these two kinetics constants is a compelling parallel and supports the conclusion that nearly half the population of  $P^*$  in  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> in Deriphat glass decays by  $P^* \rightarrow$  ground state with a time constant of  $\sim 400$  ps.

The remaining 50% of  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> is photochemically active. In this subpopulation  $P^*$  has a significantly shorter lifetime (35 ps), and  $P^* \rightarrow P^+H_M^-$  charge separation occurs with  $\geq 80\%$  yield. This estimate comes from several observations. First, neither the  $P$   $Q_Y$  bleaching decay (835–865 nm) nor the  $P$   $Q_X$  bleaching decay (590–610 nm) exhibits a 35 ps ground state recovery component. This indicates that  $P^*$  internal conversion has a yield of  $< 10\%$  (based on the signal-to-noise), implying a yield of electron transfer to  $H_M$  of  $> 90\%$ . A second estimate comes from considering competing  $P^*$  decay to the ground state, using the shorter, 160 ps  $P^*$  lifetime for  $D_{LL}$  in Deriphat glass (Table 1). Using 35 ps for the  $P^*$  lifetime in  $D_{LL}$ -FY<sub>L</sub>F<sub>M</sub> and 160 ps as the inherent time constant for competing  $P^* \rightarrow$  ground

state gives a 45 ps time constant for  $P^* \rightarrow P^+H_M^-$  and a corresponding yield of 77%. A high yield of  $P^+H_M^-$  is also consistent with the substantial amplitude of  $H_M$  bleaching at 529 nm in  $D_{LL}$ -FY $_L$ F $_M$  (140 ps spectrum in Figure 8), although quantitation is difficult due to the complexities of the spectral data. On the basis of these collective findings, we conclude that the  $P^+H_M^-$  yield is  $\geq 80\%$  in the 50% subpopulation that is photochemically active.

Once  $P^+H_M^-$  forms from the photoactive  $P^*$  population in  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass, its spectrum persists past 3.2 ns (Figures 6A and 8A), indicating that the  $P^+H_M^-$  lifetime is on the order of 10 ns. This lifetime is at least a factor of 2 longer than the  $P^+H_M^-$  lifetime of  $3.0 \pm 0.8$  ns found previously from nanosecond pump–probe experiments on the YFH RC at 295 K.<sup>81</sup> In both cases this lifetime reflects charge recombination since electron transfer from  $H_M^-$  to  $Q_B$  is blocked by addition of terbutryn. Charge recombination may return  $P^+H_M^-$  to the ground state, or lead to formation of the triplet excited state of P ( $^3P$ ) following spin rephasing from the singlet to triplet forms of  $P^+H_M^-$ . For the YFH RC at 295 K, the yield of  $^3P$  was found to be  $< 5\%$ , so the  $\sim 3$  ns lifetime of  $P^+H_M^-$  reflects charge recombination to the ground state. For  $D_{LL}$ -FY $_L$ F $_M$  at 77 K, the somewhat longer  $P^+H_M^-$  lifetime should afford a higher triplet yield (e.g.,  $\sim 10\%$ ). If so, future analysis of the temperature dependence of the  $^3P$  decay could give the free energy of  $P^+H_M^-$  relative to  $P^*$  ( $^1P$ ),<sup>112</sup> but this measurement is beyond the scope of the present study. The two potential decay pathways of  $P^+H_M^-$  are analogous to the well-studied decay pathways of  $P^+H_L^-$  in wild-type RCs with  $Q_A$  removed or pre-reduced. There  $P^+H_L^-$  decays with a lifetime of  $\sim 10$  ns at 295 K and  $\sim 20$  ns at 77 K, although the precise lifetime as well as the fraction of charge recombination that leads to the ground state versus  $^3P$  depends on conditions.<sup>26,71,113–116</sup> Overall our results here indicate that the temperature dependence of  $P^+H_M^-$  decay is similar to that of  $P^+H_L^-$  in showing a small decrease between 295 and 77 K.

A heterogeneous-RC model similar to the one developed here for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass at 77 K has been applied previously to YFH in Deriphat at 295 K.<sup>39</sup> The YFH mutant has two of the same changes incorporated in the  $D_{LL}$ -FY $_L$ F $_M$  mutant, namely, Tyr substituted at L181 (“Y $_L$ ”) and Phe substituted at M208 (“F $_M$ ”). The third mutation in YFH is a His substituted at M212 that results in the incorporation of BChl (denoted  $\beta$ ) in place of  $H_L$ ;  $D_{LL}$ -FY $_L$ F $_M$  lacks a cofactor at this position. The time evolution of the absorption changes for YFH in Deriphat at 295 K exhibit complexities similar to those found here for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass at 77 K. The heterogeneous model for YFH includes two distinct subpopulations of RCs. In a 35% subpopulation no charge separation occurs, and  $P^*$  decays to the ground state with a  $\sim 200$  ps time constant. This 200 ps time constant matches well the 180 ps (average)  $P^*$  lifetime found previously for  $D_{LL}$  in Deriphat at 295 K and the shorter-lived  $P^*$  component (160 ps) found here for  $D_{LL}$  in Deriphat glass at 77 K. In the remaining 65% of the YFH RCs,  $P^*$  has a 30 ps lifetime and decays by charge separation to both the L and M sides, yielding a mixture of  $P^+\beta^-$  and  $P^+H_M^-$ .

Although different charge-separated states are formed in YFH versus  $D_{LL}$ -FY $_L$ F $_M$ , there are clear similarities between the behavior of YFH in Deriphat at 295 K and of  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass at 77 K. The similarities include the following: (1) there appear to be two populations of RCs with different photochemical behavior, (2) at the least, biexponential fits to the data are required, (3) in a significant fraction of the RCs,  $P^*$  decays to the ground state with little or no competing electron

transfer, (4)  $P^*$  stimulated emission is observed over several hundred picoseconds, and (5) the simplest model consistent with all the spectral and kinetic data involves static heterogeneity with two distinct subpopulations of  $P^*$ . YFH in Deriphat at 295 K and  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat glass at 77 K therefore represent extreme examples of RC heterogeneity, in which subpopulations of  $P^*$  are distinguished not by different rates of electron transfer, as has previously been observed for other RCs, but the capacity to perform electron transfer at all.

**Homogeneous-RC Models for  $D_{LL}$  and  $D_{LL}$ -FY $_L$ F $_M$  at 77 K.** In the prior analysis of YFH in Deriphat at 295 K,<sup>39</sup> additional models were considered that involve a homogeneous population of RCs. The models all involved the formation of a photochemically inactive state that does not perform electron transfer to either  $H_M$  or  $\beta$  but, rather, decays only to the ground state. The inactive state considered most likely was a “relaxed” form of  $P^*$ , which is produced directly from the initial form of  $P^*$  or indirectly from an intermediate such as  $P^+B_L^-$ .  $P^+B_L^-$  itself was not considered a likely candidate for the inactive state because  $P^+B_L^-$  would not give rise to the observed long-lived stimulated emission unless it thermally repopulated the initial form of  $P^*$  or decayed by charge recombination to a “relaxed” form of  $P^*$  like the one considered above.

Similar reasoning applies to homogeneous-RC models for  $D_{LL}$  and  $D_{LL}$ -FY $_L$ F $_M$  at 77 K. In order to be consistent with the data for both mutants the model must adhere to additional constraints that help identify the photophysical processes underlying the observed kinetics. The observation of a long-lived component of  $P^*$  stimulated emission ( $\sim 400$  ps) for both mutants in Deriphat glass supports the assignment of this signal to a relaxed form of  $P^*$  that is incapable of electron transfer and deactivates only to the ground state (Figure 9B).

It is improbable that the decay of a charge-separated state such as  $P^+B_L^-$  (formed from  $P^*$ ) could be directly responsible for the  $\sim 400$  ps component in either  $D_{LL}$  or  $D_{LL}$ -FY $_L$ F $_M$ , since this state would not give stimulated emission. Furthermore, it is improbable that, if formed,  $P^+B_L^-$  would decay primarily by uphill thermal repopulation of the initial form of  $P^*$  at 77 K unless the energy gap in both  $D_{LL}$  and  $D_{LL}$ -FY $_L$ F $_M$  RCs is less than 0.005 eV. This is a highly unlikely coincidence since the oxidation potential of P is  $> 100$  meV higher in  $D_{LL}$  than in  $D_{LL}$ -FY $_L$ F $_M$ . In addition, for both mutants the as-acquired and the global-analysis-derived spectra of the 400 ps component lack spectral signatures that can be readily attributed to  $P^+B_L^-$ . Given these constraints, a homogeneous-RC model consistent with both  $D_{LL}$  and  $D_{LL}$ -FY $_L$ F $_M$  RCs at 77 K most likely involves the initial  $P^*$  form decaying to a relaxed  $P^*$  form, either directly or via  $P^+B_L^-$ , that then deactivates to the ground state in  $\sim 400$  ps (Figure 9, parts B and C).

The fundamental difference between the heterogeneous- and homogeneous-RC models is whether the two principal kinetic components observed derive from (1) two initial, kinetically uncoupled forms of  $P^*$  or (2) an initial and a relaxed form of  $P^*$ . Fortunately, in the case of  $D_{LL}$ -FY $_L$ F $_M$  the same general conclusions emerge from both models: the short-lived (initial)  $P^*$  form is capable of electron transfer to  $H_M$ , whereas a longer-lived  $P^*$  form is not.

**Temperature Dependence of M-Side Charge Separation in  $D_{LL}$ -FY $_L$ F $_M$ .** We previously showed that for  $D_{LL}$ -FY $_L$ F $_M$  in Deriphat at 295 K,  $P^*$  decay is well described by a single exponential ( $\sim 55$  ps time constant) and gives a 70% yield of  $P^+H_M^-$  and a 30% yield of deactivation to the ground state.<sup>89</sup> These values give a rate constant for electron transfer from  $P^*$  to  $P^+H_M^-$  of  $0.7/(55 \text{ ps}) = (78 \text{ ps})^{-1}$ . For  $D_{LL}$ -FY $_L$ F $_M$  in



Deriphath glass,  $P^*$  in the photoactive RCs has a 35 ps lifetime and  $P^* \rightarrow P^+H_M^-$  electron transfer occurs with high yield ( $\geq 80\%$ ) based on the heterogeneous-RC model (Figure 9A). These values give a rate constant of at least  $0.80/(35 \text{ ps}) = (44 \text{ ps})^{-1}$ , indicating that the  $P^* \rightarrow P^+H_M^-$  charge separation process is roughly a factor of 2 faster at 77 than at 295 K. As described in the Introduction, this same roughly 2-fold increase at 77 versus 295 K is well documented for the overall L-side process  $P^* \rightarrow P^+H_L^-$ . If we also consider some simple homogeneous-RC models for  $D_{LL}\text{-FY}_L\text{F}_M$  a range of rates and yields is consistent with the data; some typical values for rate constants are detailed in Figure 9, parts B and C. On the basis of these values the rate constant for M-side electron transfer in  $D_{LL}\text{-FY}_L\text{F}_M$  at 77 K differs from that at 295 K by no more than a factor of 2. Thus, the general conclusion derived from the models explored here is that the M-side  $P^* \rightarrow P^+H_M^-$  process in  $D_{LL}\text{-FY}_L\text{F}_M$  and the L-side  $P^* \rightarrow P^+H_L^-$  process in wild-type RCs both have a very weak temperature dependence.

As noted in the Introduction, the same is true of the M-side process  $P^* \rightarrow P^+\Phi_B^-$  in the H(L182)L mutant, wherein  $\Phi_B$  is a bacteriopheophytin that replaces  $B_M$  and  $P^+\Phi_B^-$  is below both  $P^*$  and  $P^+H_B^-$  in free energy.<sup>76</sup> This common weak temperature dependence holds for all of these processes initiated in  $P^*$  despite up to 20-fold differences in rate at a given temperature and presumably different underlying predominant mechanisms (e.g., a two-step process involving  $P^+B_L^-$  formation as an intermediate between  $P^*$  and  $P^+H_L^-$  on the L side of wild type compared to a one-step superexchange process that utilizes  $P^+B_M^-$  as a virtual intermediate for the formation of  $P^+H_M^-$  in  $D_{LL}\text{-FY}_L\text{F}_M$ ). The comparisons can be extended to include the  $P^+H_M^- \rightarrow P^+Q_A^-$  process in the wild-type RC, which has a rate constant of  $\sim(200 \text{ ps})^{-1}$  at 295 K and  $\sim(100 \text{ ps})^{-1}$  at 100 K and below.<sup>15</sup> Thus, the weak temperature dependence of electron transfer in the RC spans processes involving a nearly a 100-fold variation in rate constants, different degrees of electronic coupling, and different underlying mechanisms on the L and M branches.

Most descriptions of the rates of RC electron-transfer processes as a function of temperature are cast in terms of the Marcus framework with quantum corrections to incorporate vibrational modes of the cofactors and protein.<sup>117–120</sup> It is also typically assumes that any vibrational or structural relaxations resulting from excitation or previous charge separation steps are complete prior to the electron-transfer process of interest. If vibrational or structural relaxations are incomplete on the time scale of electron transfer, then the temperature (and free energy) dependence of a reaction's rate may be weaker than if the states are fully relaxed.<sup>29,106,121</sup>

Within the quantum-corrected Marcus formalism assuming equilibrated states, the reactions described above, including  $P^* \rightarrow P^+H_M^-$ , are in the so-called activationless regime in which the free energy change ( $\Delta\Delta G$ ) is comparable to the reorganization energy ( $\lambda$ ). A rather weak temperature dependence is also anticipated for electron transfer in the inverted region ( $\Delta\Delta G > \lambda$ ) when quantum effects are considered. Our results rule out the possibility that the process lies in the normal activated region ( $\Delta\Delta G < \lambda$ ) where the reaction should slow down substantially at low temperature. With the use of the values given in the Introduction and depicted in Figure 2, the free energy change for  $P^* \rightarrow P^+H_M^-$  in the  $D_{LL}\text{-FY}_L\text{F}_M$  is estimated to be on the order of 0.1 eV. This value is comparable to the reorganization energy estimated for electron transfer from  $P^*$  to the L-side cofactors in the wild-type RC.<sup>24,25,29</sup> Thus, the  $P^* \rightarrow P^+H_M^-$

process in  $D_{LL}\text{-FY}_L\text{F}_M$  would appear to be in the activationless regime, or nearly so.

In the activationless regime a 2-fold increase in rate with a reduction in temperature is consistent with the temperature dependence of the thermally weighted Franck–Condon factor. Alternatively, the 2-fold rate increase could derive from the temperature dependence of the electronic factor, which may arise from the protein shrinking slightly at low temperature and thereby bringing the cofactors closer together. One or both of these two factors can account for the 2-fold increase of rate of the M-side reaction  $P^* \rightarrow P^+H_M^-$  between 295 and 77 K.

## Conclusions

We have shown that M-side electron transfer to the acceptor  $H_M$  occurs in  $D_{LL}\text{-FY}_L\text{F}_M$  RCs at 77 K. The data and analysis are complex and require models to account for the pronounced nonuniformity of the  $P^*$  kinetics and the states produced by  $P^*$  decay. In particular, the application of the heterogeneous-RC model leads to the conclusion that about half of  $D_{LL}\text{-FY}_L\text{F}_M$  RCs are incapable of electron transfer to  $H_M$  at 77 K. However, regardless of the model used, one remarkable conclusion is the same: M-side electron transfer  $P^* \rightarrow P^+H_M^-$  is only weakly temperature-dependent, suggesting that, like the L-side process, the reaction is fundamentally activationless.

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