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Trapping Kinetics in Mutants of the Photosynthetic Purple Bacterium *Rhodobacter sphaeroides*: Influence of the Charge Separation Rate and Consequences for the Rate-Limiting Step in the Light-Harvesting Process†

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ABSTRACT: The primary light-harvesting processes, energy transfer in the light-harvesting antenna, and trapping of the excited states by reaction centers were studied in several mutant strains of the photosynthetic purple bacterium *Rhodobacter sphaeroides*. The mutants had reaction centers in which the rates of electron transfer were modified by site-directed mutations at the M210 position. Low-intensity pump–probe laser spectroscopy was used to monitor the absorbance transients in the Q_y region of the antenna pigments, and it was found that despite a wide variation in charge separation rates within the RC, produced by the alterations at Tyr M210, there was relatively little corresponding variation in the overall trapping rate. These effects of the mutations on the trapping kinetics demonstrate that the rate-limiting step of the overall light-harvesting process is the transfer of the excitations from the antenna to the reaction center.

In all photosynthetic organisms most of the utilized sunlight is initially absorbed by the so-called light-harvesting antenna (LHA). This absorption produces excited states of the LHA pigments which are transferred to and trapped by photosynthetic reaction centers (RC) where the excited-state energy is used to drive an electron-transfer process. In photosynthetic purple bacteria, e.g., *Rhodospirillum rubrum*, the trapping of LHA excitations takes place in about 50 ps (Sundström et al., 1986; Timpmann et al., 1991). The electron-transfer processes in the isolated (and membrane-bound) RC can be followed spectroscopically with femtosecond time resolution, and the kinetics can be related to the available crystal structure (Deisenhofer et al., 1985; Allen et al., 1986). However, the study of the excited-state dynamics of the *in vivo* RC, which is closely associated with antenna complexes, is much more difficult. This is due to the overlap of the absorption bands of the pigments in the antenna with those within the RC and, more importantly, to the fact that these measurements are limited to very low excitation densities. Under the conditions normally used to study the isolated RC, multiphoton processes (singlet–singlet annihilation) in the LHA would seriously interfere with the trapping process. One of the open questions about the trapping process concerns the transfer of excitations from the LHA to the RC. In the absence of a high-resolution structure of the LHA, the amount of LHA pigment directly transferring energy to the RC is unknown. Models have been presented which set this coordination number (z) at 4 (Pearlstein, 1982), 6 (Pullerits & Freiberg, 1992), and 1 (Pearlstein, 1992). Although the energy-transfer rates be-

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tween pigments in the LHA have been well studied (Bakker et al., 1983), the actual rates of the transfer of excitations from the LHA to the RC are unknown and are difficult to measure since the excited-state absorption difference spectra of the special pair and the LHA are rather similar. These transfer rates are of special importance since it appears that this final excitation transfer event could be the rate-limiting step, the bottleneck, of the trapping process (van Grondelle et al., 1988; Visscher et al., 1989; Timpmann et al., 1991; Valkunas et al., 1992; Otte et al., 1993). Recently, several papers have been published on the subject of trapping in purple bacteria (Bakker et al., 1983; Sundström et al., 1986; Deinum et al., 1989; Müller et al., 1993a,b). In these papers it was concluded, from both time-resolved and steady-state fluorescence measurements, that the rate-limiting step should be the charge separation rate.

To decide this issue, we have for the first time measured the excitation trapping kinetics in photosynthetic systems as a function of the rate of charge separation by the reaction center. In this work we have made use of a set of mutant strains of *Rhodobacter sphaeroides*, which have been mutated at the reaction center M210 position, to modify the rate of primary charge separation (Finkele et al., 1990; Nagarajan et al., 1990; Du et al., 1992; DiMagno et al., 1993; Hamm et al., 1993). In mutants in which the tyrosine at the M210 position has been replaced by a leucine (M210Leu) or phenylalanine (M210Phe), the rate of primary electron transfer is reduced by up to 6-fold (Finkele et al., 1990; Nagarajan et al., 1990). Moreover, mutants with changes at the M210 position in RC's from *Rb. sphaeroides* and *Rhodobacter capsulatus* (Tyr M208 in *Rb. capsulatus*) have recently been found to exhibit strongly multiphasic charge separation kinetics (Hamm et al., 1993; Du et al., 1992). To reduce the complexity of the LHA energy transfer processes, a genetic expression system was used in which the peripheral LHA complex, LHII, was absent (Jones et al., 1992a). As a result the mutant strains mimic the "simple" LHA of *Rs. rubrum*, which also lacks LHII. These mutants have the advantage that the reaction center has a known structure (Allen et al., 1986) and that the rate of primary charge separation can be modified by site-directed mutagenesis.

Using low-intensity picosecond pump–probe spectroscopy, we were able to observe the effects of the modified rates of charge separation in these mutants on the trapping process. From the comparison of the trapping in the different mutants, it became clear that indeed the transfer from the antenna to the RC is a "slow" process. In addition, these measurements show that the dynamic equilibrium between the charge-separated state P^+I^- and the excited state $\{P, LHA\}^*$ is shifted in favor of the latter in the M210Leu and M210Phe mutants. Since this change in equilibrium position reflects the free energy difference, it might be possible to use these kinds of measurements as a method to monitor free energy changes in the RC arising from alterations to some of the residues important in primary electron transfer.

MATERIALS AND METHODS

The genetic system used for the expression of mutated reaction center genes is described in Jones et al. (1992b). Details of the construction of the mutations are described elsewhere (Jones et al., 1994). Mutated M subunit genes were introduced into the *Rb. sphaeroides* deletion strain DD13/G1 using plasmid pRKEH10. Details of this procedure are also described elsewhere (Jones et al., 1993, unpublished results).

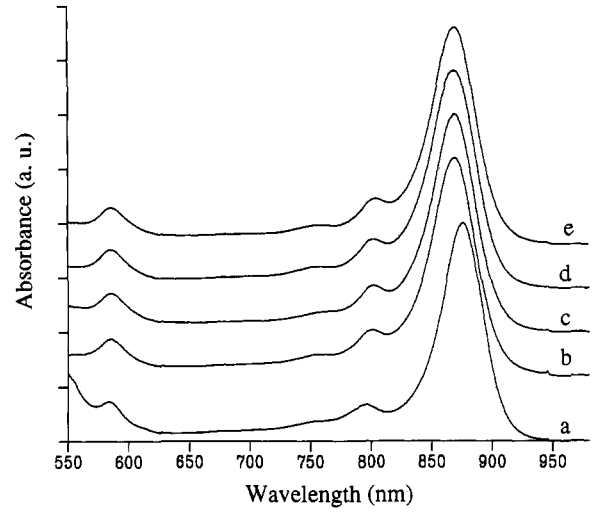

FIGURE 1: Room temperature absorption spectra of (a) *Rs. rubrum* membranes, (b) LHI–RC_{wt}, (c) LHI–$RC_{M210His}$, (d) LHI–$RC_{M210Phe}$, and (e) LHI–$RC_{M210Leu}$.

The mutant strains of *Rb. sphaeroides* were grown as described in Jones et al. (1992a); chromatophore membranes were prepared using a French press and were subsequently purified by sucrose-gradient centrifugation. The measurements were done in a 50 mM sodium phosphate buffer at pH 8.0. To maintain open (active) reaction centers, 1 mM phenazine methosulfate (Research Organics, Ltd.) and 40 mM sodium ascorbate were added to the sample. The picosecond absorption measurements were performed with a home-built instrument similar to that described in Sundström et al. (1986). Briefly, a pulse train of near-IR light pulses of 2–3 ps with a repetition rate of 1 MHz was obtained from a dual-jet cavity-dumped dye laser (Coherent-702), operating with Styryl-9 and IR140. The dye laser was synchronously pumped by a mode-locked frequency-doubled Nd:YAG laser (Coherent-Antares). The pulse train was split in a pump and a probe beam with a 2:1 intensity ratio. The pump beam was modulated with an acousto-optic modulator at 70 kHz. The path length of the probe beam could be varied with 0.1-μm accuracy with a 10-cm optical delay line. Measurements were performed in a cuvette rotating at 500 rpm, with a 13-cm diameter and a 2-mm light path. The pump and probe beams were attenuated and focused to a 100-μm diameter spot in the sample, resulting in an excitation density per pulse of 10^{13} photons/cm^2. The probe beam was detected with an S1-type photomultiplier connected to a lock-in amplifier. We checked for singlet–singlet annihilation and singlet–triplet annihilation by further lowering the pulse intensity and/or the repetition rate, which gave no change in the kinetics. The autocorrelation of the pulse was measured in a $LiIO_4$ frequency-doubling crystal at the position of the sample. Room temperature absorption and fluorescence spectra were recorded on a home-built absorption and fluorescence spectrophotometer (Kwa et al., 1993; Visschers et al., 1991).

RESULTS

Figure 1 shows the absorption spectra of membranes from the mutants used in this study, together with the spectrum of *Rs. rubrum* membranes for comparison. The spectra compare well with the absorption spectrum of *Rs. rubrum*, apart from the obvious difference in the peak wavelength of the LHI near-IR absorption band. The mutants differ slightly with respect to the position of the 800-nm band of the reaction center; this has been observed before for similar mutations in

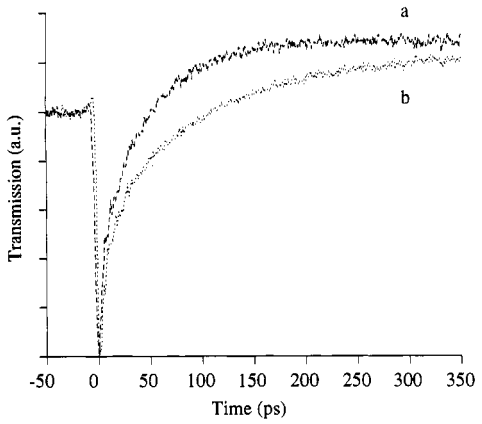

FIGURE 2: Single-wavelength pump–probe kinetics at 861 nm in the presence of 1 mM PMS and 40 mM ascorbate: (a) LHI–RC_{wt} and (b) LHI–$RC_{M210Phe}$. The kinetics were normalized at $t = 0$.

Rb. sphaeroides (Gray et al., 1990). The amount of antenna pigments per RC is the same in all mutants (21 ± 4) as judged from the relative intensity of the B875 antenna peak and the 800- and 750-nm RC bands.

Figure 2 shows two characteristic single-wavelength picosecond pump–probe traces recorded at 861 nm, which corresponds with excitation into the "blue" region of the Q_y band of the antenna. As a result the initially produced ensemble of excited states is far from the thermal equilibrium distribution (van Grondelle et al., 1987; Valkunas et al., 1991). The first process to occur is therefore an equilibration of the excitations, which takes place at a time scale of about 1 ps (Bakker et al., 1983; Valkunas et al., 1991, 1992; Visser et al., unpublished results). This initial (blue-shifted) ensemble of excited states has a net (sum of ground-state bleaching, excited-state absorption, and stimulated emission) excited-state difference spectrum which corresponds with a (weak) transmission increase at the wavelength of observation (also 861 nm). The initial transmission increase seen in the traces at $t = 0$, which disappears with pulse-limited kinetics in these experiments, could be this effect. Note that we cannot distinguish it from a possible coherent coupling signal. However, kinetics measured at the wavelength where, in the equilibrated antenna, the contributions from excited-state absorption, stimulated emission, and ground state bleaching cancel each other and where, therefore, the fast phase is more readily observed (data not shown) lead us to believe the initial feature actually is the above described equilibration phenomenon. The thermally equilibrated ensemble of excitations, on the other hand, has a large transmission decrease at this wavelength which is due to excited-state absorption and which gives rise to the strong negative signal in the traces after $t = 0$. In the case of the pseudo-wt the excited state of the antenna decays single exponentially (see Discussion) with a lifetime of approximately 50 ps and results in a net transmission increase, which is due to the formation of P^+. For two of the reaction center mutants the antenna excited-state decay is clearly nonexponential, and we have aproximated the decays (and both the pseudo-wt and LHI–$RC_{M210His}$) with two lifetimes. The results of the fits of the pump–probe traces are shown in Table 1.

Measurements performed on the LHI–$RC_{M210His}$ gave rise to traces which were almost indistinguishable from the LHI–RC_{wt}, whereas the LHI–$RC_{M210Phe}$ strongly resembled the LHI–$RC_{M210Leu}$ traces. For clarity, therefore, traces for the LHI–$RC_{M210His}$ and LHI–$RC_{M210Leu}$ mutants are not shown. It should be noted that the fitted steady-state level to which the traces decay (offset in Table 1) is identical for all mutants, indicating similar radical pair yields. The same conclusion was drawn from xenon flash induced P^+Q^- spectra (results not shown). We have also measured the relative fluorescence quantum yield of the mutants (with open reaction centers), normalized on the fluorescence yield from the wild-type mutant; the values were 0.9 ± 0.1 for the M210His mutant and 2.3 ± 0.1 for both the M210Phe and the M210Leu mutant. The relative fluorescence quantum yields compare well with the weighed average lifetimes of the RC–LHI mutants. We take this as evidence that there was no uncoupled antenna and that the pump–probe measurements were performed at annihilation-free conditions and with open reaction centers. A more complete spectroscopic characterization of the mutant strains used here will be presented elsewhere (Beekman et al., unpublished results).

DISCUSSION

The major observation of this work is that the replacement of the tyrosine residue at the M210 position of the reaction center of *Rb. sphaeroides* by either a leucine or a phenylalanine affects the excited-state decay kinetics in an intact LHA–RC system in two ways: (1) the fast (40–50 ps) excited-state decay component, associated with the formation of the state P^+I^-Q, slows down by at most 30% and (2) the kinetics become distinctly biexponential with a second component in the 200–300-ps region, contributing about 40% of the amplitude. In the following we will give an explanation of these phenomena on the basis of a simple model and a semiquantitative estimate of the various parameters involved. In order to get a better understanding of the processes that cause the complicated trapping kinetics, we will first discuss the nature of the multiexponential signals in the case of the mutated reaction centers. First of all, we should note that even in the case of wild-type chromatophores there is a slow trapping component of about 250 ps with a low amplitude (0–10% of the initial amplitude) (Sundström et al., 1986; Müller et al., 1993a). This long lifetime, which is not observed in the decay of P* in isolated RC's, is caused by the addition of the antenna to the RC. Because LHA* is approximately isoenergetic with P*, we can look upon the addition of the LHA to the RC as a 13-fold increase of the degree of degeneracy of the excited state relative to the charge-separated state; this arises from changing from an excited state "delocalized" over two pigments, the dimer P, to an excited state delocalized over 24 LHA pigments plus 2 RC pigments. At room temperature this corresponds to a decrease in the free energy difference between the states P^+I^-Q and {P, LHA}* of 66 meV. At room temperature the thermal equilibrium between P^+I^-Q and {P, LHA}* lies >95% toward the state P^+I^- in the case of the wild-type species. Therefore, the excited-state decay of the antenna exhibits two lifetimes, a fast component of 50 ps, which can be thought of as the settling of the equilibrium between P^+I^-Q and {P, LHA}*, and a slow component of about 250 ps due to the decay of P^+I^-Q into P^+IQ^- which results in the decay of the remaining population of {P, LHA}*. This argument does not hold quantitatively since the two lifetimes, 50 and 250 ps, do not differ enough for them to be considered independently.

The RC mutations have a direct effect on the energy gap between the states P* and P^+I^-Q (Parson et al., 1990). From delayed fluorescence measurements, on detergent-isolated RC's, it is known that the energy gap changes from −120 meV (in wild-type RC's) (Taguchi et al., 1992) to −70 meV in the case of the M210Phe mutant (Volk et al., 1993). For isolated RC's with the M210Phe mutation this implies that after the

Table 1: Results Obtained from Fits to the Pump–Probe Data Collected at 861 nm[a]

	exp 1 (ps)	amp 1	exp 2 (ps)	amp 2	offset	
wt	45.7	–1			0.31	3.5[b,c] 2.7 (80%) and 12.1 (20%)[d] 2.3 (80%) and 7 (20%)[e]
YM210H	50.7	–0.98	250	–0.02	0.32	3.7[d]
YM210F	57.1	–0.64	250	–0.36	0.32	16,[b] 10.5[c] 5.4 (53%) and 40 (47%)[d] 6.1 (42%) and 26 (58%)[e]
YM210L	59.9	–0.63	250	–0.38	0.32	22,[b] 16[c,f]

[a] In all the fits we fixed the 250-ps component to fit the decay time of the secondary charge separation process in the RC. The offset is the amplitude of a nondecaying component; this compensates for the residual bleaching due to the formation of P^+IQ^-, which has an "infinite" lifetime on the time scale of our measurement. The last column lists the charge separation rates measured by several research groups on isolated reaction centers of both wild-type and the Tyr M210 mutants from *Rb. sphaeroides* and *Rb. capsulatus* (Tyr M208 in *Rb. capsulatus*). [b] From *Rb. sphaeroides*; Finkele et al., 1990. [c] From *Rb. sphaeroides*; Nagarajan et al., 1990. [d] From *Rb. capsulatus*; DiMango et al., 1992. [e] From *Rb. sphaeroides*; Hamm et al., 1993. [f] In the paper of Nagarajan et al. the tyrosine at M210 was mutated to an isoleucine residue instead of a leucine residue.

establishment of the dynamic equilibrium still more than 95% of the RC's will be in the charge-separated state P^+I^-, and consequently, transient absorption measurements will detect a "normal" amount of this state. However, in the LHI–$RC_{M210Phe}$ mutant the effect on the relative amounts of P^+I^-Q and {P, LHA}* is quite dramatic. The additional decrease of 66 meV due to the added antenna means that the free energy between the states P^+I^-Q and {P, LHA}* is close to 0. This fact explains the biphasic excited-state kinetics of the mutants. The initial fast settling of the dynamic equilibrium between P^+I^-Q and {P, LHA}* does not go beyond 60% P^+I^-Q. The further decay of the {P, LHA}* excited states is limited by the next step in the electron-transfer pathway: $P^+I^-Q \rightarrow P^+IQ^-$. As mentioned before, this argument does not directly yield the relative amplitudes of the fast and the slow processes; however, it is clear that the decreased energy gap results in a strong increase of the amplitude of the slow trapping lifetime.

The important thing to note from the fit of the kinetic traces is that the rate of the fast process is hardly affected by the modification made to the RC (45 ps for the wt vs 50, 57, and 60 ps for the M210His, M210Phe, and M210Leu mutants, respectively). Therefore, despite the decreased (by a factor of 3–6) rate of charge separation in the mutants (Finkele et al., 1990; Nagarajan et al., 1990), the settling of the equilibrium between P^+I^-Q and {P, LHA}* still occurs at a similar speed. This implies that the rate of charge separation cannot be the rate-limiting step. Since the migration processes internal to the LHA occur on a much faster time scale, they are of no importance. Therefore, the only candidate for the rate-limiting step is the transfer from the LHA to the RC (Valkunas et al., 1992).

For a quantitative analysis a full kinetic scheme has to be applied, including LHA*, P*, P^+I^-Q, and P^+IQ^- (see Figure 3), involving up to eight rate constants. We have not fitted the data to the model, but we used known values for the rate constants and the energy gaps, with only W_1 and W_2 as the adjustable parameters. We obtained good agreement between the model (Figure 3) and experiment for both the LHI–RC_{wt} and the LHI–$RC_{M210Phe}$ mutants using W_1^{-1} = 35 ps, W_2^{-1} = 8 ps, and k_2^{-1} = 200 ps for both mutants, and for the RC_{wt} γ^{-1} = 3.5 ps and $\Delta G_{P^*\_P^+I^-}$ = –120 meV and for $RC_{M210Phe}$ γ^{-1} = 15 ps and $\Delta G_{P^*\_P^+I^-}$ = –70 meV. In Figure 4 we have plotted the two generated LHA kinetics from the model and overlaid them on the experimental data. So the change in the charge separation rate and the energy gap between {LHA,P}* and P^+I^- can explain the results if we have a slow excitation energy transfer from the antenna to the RC. The published values for $\Delta G_{P^*\_P^+I^-}$ that we used were obtained from delayed fluorescence measurements on isolated reaction centers.

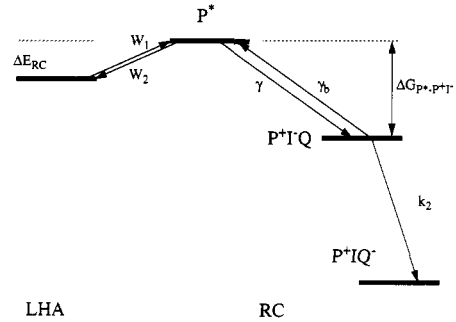

FIGURE 3: Energetic diagram of the model suggested for the description of the multiphasic trapping kinetics in the RC–LHA mutants with five rate constants. γ and γ_b are respectively the initial charge separation rate and charge recombination rate. The charge recombination rate is calculated using $\gamma/\gamma_b = \exp(-\Delta G_{P^*\_P^+I^-}/k_bT)$, in which k_b is the Bolzmann constant, T is the temperature (in kelvin), and $\Delta G_{P^*\_P^+I^-}$ is the free energy gap between the excited dimer P* and the initial charge-separated state. The rate k_2 is the secondary charge separation step in the reaction center (200 ps). W_1 and W_2 are the rate constants governing the transfer respectively from the antenna to the reaction center and back. ΔE_{RC} is the energy gap between the LHA and RC including the energy difference due to the degeneracy of the LHA; these rate constants are also balanced by thermal equilibrium.

Recently, it has been shown that the radical pair relaxes on a time scale of 100–500 ps (Williams et al., 1992; Nagarajan et al., 1993) and consequently the free energy gap $\Delta G_{P^*\_P^+I^-}$ increases. Since the delayed fluorescence technique mainly monitors recombination luminescence from the relaxed radical pair state, we should consider the given values for $\Delta G_{P^*\_P^+I^-}$ as upper limits. Note, however, that the magnitude of the relaxation process is approximately the same for all the mutants including the wild type. Therefore, this effect (on its own) cannot account for the observed differences between the wild-type and modified reaction centers in our trapping experiments (using the unrelaxed energy gaps would mainly result in minor changes in the ratio between W_1 and W_2). Note that we also have not addressed the question of whether the primary charge separation is a one- or two-step process (DiMagno et al., 1993; Hamm et al., 1993) and whether this first step has a distribution of transfer rates (Du et al., 1992). However, since the trapping is only very weakly dependent on the rate of primary charge separation, this discussion is not very relevant for the main conclusions of this work.

From our measurements we saw great similarity of the LHI–RC_{His} mutant and the LHI–RC_{wt} species; this is consistent with the finding that in this mutant (1) the rate of charge separation is close to that of the wt (DiMagno et al., 1993) and (2) the redox potential of P^+/P is lower than in the wt

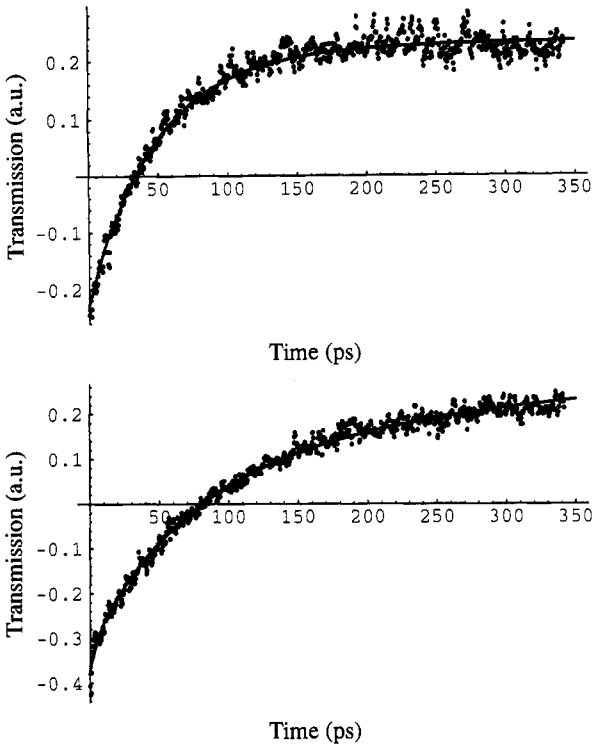

FIGURE 4: Generated kinetics obtained from the model together with the corresponding experimental results for (a, top) the wild-type and (b, bottom) M210Phe mutants, respectively. The parameters used in the model (Figure 3) are W_1^{-1} = 35 ps, W_2^{-1} = 8 ps, k_2^{-1} = 200 ps, $\gamma_b = \gamma \exp(-\Delta G_{P^{*}\_P^{+}I^{-}}/k_bT)$, and T = 300 K for both mutants. For the charge separation rate and energy gap the known values were used: γ^{-1} = 3.5 ps (Finkele et al., 1991) and $\Delta G_{P^{*}\_P^{+}I^{-}} = -120$ meV (Taguchi et al., 1992) in the case of the wild-type mutant and γ^{-1} = 15 ps (Finkele et al., 1991) and $\Delta G_{P^{*}\_P^{+}I^{-}} = -70$ meV (Volk et al., 1993) in the case of the M210Phe mutant.

(DiMagno et al., 1993; R. W. Visschers, personal communication), implying an increase of the free energy gap ($\Delta G_{P^{*}\_P^{+}I^{-}}$), resulting in an even further shift of the excited state {P, LHA}* vs radical pair ($P^{+}I^{-}Q$) equilibrium toward the radical pair state.

Our measurements clearly show that trapping of excitations from the LHA is limited by the transfer from the LHA to the RC. The energetics and kinetics of the first charge separation step seem to be designed to minimize back-transfer of excitations to the LHA; thus the forward rate of charge separation is only of minor importance (*in vivo*) and it is the rate of back-transfer that has to be minimized.

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