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Energy trapping and detrapping by wild type and mutant reaction centers of purple non-sulfur bacteria

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Abstract

Time-correlated single photon counting was used to study energy trapping and detrapping kinetics at 295 K in *Rhodobacter sphaeroides* chromatophore membranes containing mutant reaction centers. The mutant reaction centers were expressed in a background strain of *Rb. sphaeroides* which contained only B880 antenna complexes and no B800–850 antenna complexes. The excited state decay times in the isolated reaction centers from these strains were previously shown to vary by roughly 15-fold, from 3.4 to 52 ps, due to differences in the charge separation rates in the different mutants (Allen and Williams (1995) J Bioenerg Biomembr 27: 275–283). In this study, measurements were also performed on wild type *Rhodospirillum rubrum* and *Rb. sphaeroides* B880 antenna-only mutant chromatophores for comparison. The emission kinetics in membranes containing mutant reaction centers was complex. The experimental data were analyzed in terms of a kinetic model that involved fast excitation migration between antenna complexes followed by reversible energy transfer to the reaction center and charge separation. Three emission time constants were identified by fitting the data to a sum of exponential decay components. They were assigned to trapping/quenching of antenna excitations by the reaction center, recombination of the P^+H^- charge-separated state of the reaction center reforming an emitting state, and emission from uncoupled antenna pigment-protein complexes. The first varied from 60 to 160 ps, depending on the reaction center mutation; the second was 200–300 ps, and the third was about 700 ps. The observed weak linear dependence of the trapping time on the primary charge separation time, together with the known sub-picosecond exciton migration time within the antenna, supports the concept that it is energy transfer from the antenna to the reaction center, rather than charge separation, that limits the overall energy trapping time in wild type chromatophores. The component due to charge recombination reforming the excited state is minor in wild type membranes, but increases substantially in mutants due to the decreasing free energy gap between the states P^* and P^+H^-.

Abbreviations: PSU – photosynthetic unit; Bchl – bacteriochlorophyll; Bphe – bacteriopheophytin; P – reaction center primary electron donor; RC – reaction center; *Rb. – Rhodobacter*; *Rs. – Rhodospirillum*; EDTA – (ethylenediamine)tetraacetic acid; Tris – tris(hydroxymethyl)aminomethane

Introduction

The Emerson–Arnold photosynthetic unit (PSU) concept (Emerson and Arnold 1932) forms the basis of the modern understanding of the primary processes of photosynthesis. It may be defined as the statistical ensemble consisting of the reaction center (RC) and all the light-harvesting pigment molecules contributing excitation energy to the RC. The major experimental proof of the PSU concept was given by Duysens and collaborators (Duysens 1952; Vredenberg and Duysens 1963). According to the PSU concept, the primary steps of photosynthesis include: (i) absorption of a photon by antenna pigments; (ii) migration of antenna excitations (later called excitons) in the antenna ; (iii) transfer of excitons from the antenna to the RC; (iv) quenching of excitons in the RC by a light-driven oxidation reaction that takes place at a bacteriochlorophyll *a* (Bchl) dimer (P); (v) stabilization of the charge-separated state by a sequence of electron transfer steps within the RC.

The above scheme is intuitively clear and, as such, very useful. Yet, there are many questions on a detailed level that need to be clarified. Specifically, the knowledge of how antennas are coupled to the RC, both structurally and functionally, is still rather limited (for a review, see Freiberg 1995). The first clear experimental demonstration of an effective trapping of antenna excitations by the RC was given by Vredenberg and Duysens (1963) who observed an increase of the fluorescence yield when the RC became saturated by high light levels. (In some bacteria, fluorescence may be quenched more strongly when the primary donor, P, is oxidized (Deinum et al. 1991, 1992).) Twenty years later, it was demonstrated that the fluorescence yield, associated with the redox state of the RC, is correlated with the fluorescence lifetime (Sebban and Moya 1983; Freiberg et al. 1984). The average fluorescence lifetime of *Rhodospirillum (Rs.) rubrum* chromatophores changes from about 60 ps in the case of active RCs to about 250 ps upon closing the RC. The excited state lifetime of intact membranes is short compared to the lifetime of excitons in isolated antenna preparations which lack reaction centers (~700 ps at room temperature; Sebban et al. 1985; Bergström et al. 1988; Freiberg and Timpmann 1992). This suggests that the excited state lifetime is governed by quenching of excitons by the RC. However, the observed exciton lifetime is very long compared to the excited primary donor lifetime of isolated RCs without surrounding antenna (~3 ps; for reviews of reaction center primary photochemistry see Kirmaier and Holten 1987; Feher et al. 1989; Parson 1991; Martin and Vos 1992; Kirmaier and Holten 1993; Zinth and Kaiser 1993; Woodbury and Allen 1995), and is also long compared to the excitation migration time between neighboring antenna pigments (0.1 to 0.6 ps; Bakker et al. 1983; Visser et al. 1995; Bradforth et al. 1995; Valkunas et al. 1995).

Exciton kinetics in bacterial antenna systems have been investigated recently by a number of authors (Müller et al. 1993; Valkunas et al. 1992; Timpmann et al. 1993, 1995; Xiao et al. 1994; Bradford et al. 1995). At room temperature, direct trapping rate measurements are difficult because of the overlap between the thermally broadened spectral bands of antenna and RC. In many bacterial species, the light harvesting II complex, or B800–850 antenna, absorbs at both 800 and 850 nm and masks the reaction center bands in these regions. In species that possess only a single type of antenna complex (light harvesting complex I, or B880 antenna, which has only one transition in the near infrared at 880 nm), a more or less selective excitation of the RC and antenna pigments is possible, and this has been used to study the energy back-transfer from the RC to antenna (Wang and Clayton 1971). A rather limited (10–25%) back-transfer has been observed in different bacteria (Abdourakhmanov et al. 1989; Kleinherenbrink et al. 1992; Timpmann et al. 1993, 1995; Xiao et al. 1994). The low escape ratio implies that the PSU excited state decay is limited by a slow antenna-RC transfer rate (van Grondelle and Sundström 1988; Visscher et al. 1989; Somsen et al. 1996).

If the rate limiting step is the transfer from the antenna to the reaction center, then the rate of overall energy trapping in chromatophores will depend only weakly on the rate of initial electron transfer in the RC. One way to test this prediction is to systematically vary the rate of charge separation in the RC. Beekman et al. (1994) have done this using a series of RC mutants in *Rhodobacter (Rb.) sphaeroides* in which the charge separation time varies from roughly 3 to 20 ps in isolated reaction centers, and measured the antenna excited state decay by picosecond transient absorbance spectroscopy. They found that the overall rate of antenna excited state decay was only weakly dependent on the charge separation rate over this range. However, in this series of mutants there was not a wide enough range of charge separation rates to see a large effect on the overall antenna excited state decay rate (the maximum change observed was about 30%).

It would be informative to test these ideas using a larger number and range of charge separation times. A

series of reaction center mutants has been constructed in *Rb. sphaeroides* with charge-separation time constants varying from a few picoseconds to more than 50 ps (Allen and Williams 1995). These mutants remove or add hydrogen bonds between the dimer Bchl and nearby protein residues. In wild type *Rb. sphaeroides* RCs, there is only one hydrogen bond to a nearby amino acid (His at the L168 site). It is possible to remove this hydrogen bond by changing the His to a Phe (Murchison et al. 1993) and also to create new hydrogen bonds by introducing His residues at positions L131, M160 and M197 (Williams et al. 1992; reviewed by Allen and Williams 1995). The addition of each hydrogen bond to the dimer causes a 50–120 mV increase in the midpoint potential of P. This results in both a decreased driving force and a slower rate for the initial electron transfer. The decay times of the excited singlet state of P in the mutant RCs are up to 15 times longer than those from wild-type bacteria, as determined by transient absorbance measurements of isolated mutant RCs (Williams et al. 1992; Murchison et al. 1993; Woodbury et al. 1994; Peloquin et al. 1994; Woodbury et al. 1995). The bacteriopheophytin to quinone electron transfer rate in these mutants changes little, varying between 150 ps and 250 ps (Williams et al. 1992; Murchison et al. 1993; Woodbury et al. 1995).

In the series of mutants described above, there clearly is a relationship between the ability of the organism to grow photosynthetically and effects of the mutations on the reaction center rates and thermodynamics. In the highest potential mutant, LH(L131) + LH(M130) + FH(M197), the photosynthetic growth rate drops by nearly an order of magnitude (Lin et al. 1994). Thus, even though the dependence of the antenna excited state decay time on the charge separation rate in the reaction center is apparently weak (Beekman et al. 1994), the mutations do have a major effect on overall photosynthetic function. It would be difficult to ascribe the growth deficiency to the yield of charge separation within the reaction center since that is roughly 50% at physiological temperatures in reaction centers isolated from the most extreme mutant (Woodbury et al. 1995). One explanation lies in the possibility that the free energy of the overall charge separation process in the photosynthetic membranes is less favorable than that in isolated reaction centers, because the excited antenna lies below P* in free energy. The thermodynamic and kinetic parameters reported in the literature give estimates of the free energy increase between A* and P* of between 20 and 50 meV. In some of the reaction center mutants with a high P/P^+ midpoint potential, the free energy difference for charge separation between P* and $P^+H_A^-$ in isolated reaction centers approaches zero (Allen and Williams 1995). The small driving force in these mutants could substantially decrease the overall charge separation yield due to loss of efficient energy trapping by the reaction center. In fact, in the highest potential mutant, the overall reaction from A* to $P^+H_A^-$ may actually be slightly unfavorable and driven only by the irreversible conversion of $P^+H_A^-$ to $P^+Q_A^-$.

In this study, the effect of the RC mutations described above on the trapping rate of excitations in the antenna is investigated by picosecond-resolution, time-correlated single photon counting. This is performed by expressing the mutant RCs in a background strain of *Rb. sphaeroides* in which the B800–850 antenna genes have been deleted, in order to simplify the energy transfer process. For comparison, emission kinetics in the membranes of a *Rb. sphaeroides* B880 antenna-only mutant (lacking RCs and the B800–850 antenna) and of wild-type *Rs. rubrum* chromatophores are also described. A preliminary report of this work has been presented previously (Freiberg et al. 1995).

Experimental methods

Bacterial strains. Chromatophores isolated from eight strains of purple non-sulfur bacteria were used in this study. Seven of these were *Rhodobacter sphaeroides* mutants with changes in the RC and B880-containing *puf* operon and a deletion of the B800–850-encoding *puc* operon. The *Rb. sphaeroides* host strain, ΔBALM, is derived from the wild-type 2.4.1 strain and has chromosomal replacements of both the *puf* and *puc* operons (Williams and Taguchi 1995). The *puf* operon was expressed in this host strain from a plasmid. The strain with wild-type RC genes (WT-1A) contains the plasmid pRKENB (Paddock et al. 1989; Lin et al. 1994). Strains with the single RC mutations ((HF(L168), LH(M160) and LH(L131)) also contain plasmids that have been described previously (Williams et al. 1992; Murchison et al. 1993). The double and triple mutants were originally constructed in a plasmid that has a mutation in the *puf*A gene (Lin et al. 1994), so fragments containing these mutations were cloned into a plasmid with wild-type B880 antenna genes. A strain that lacks RCs was made by constructing a plasmid that has a deletion of the 172 bp

*Kpn*I-*Kpn*I fragment in the *puf*L gene. The *Rs. rubrum* strain used was generously provided by Dr. William Parson.

Bacterial growth and chromatophore isolation. Cultures of the *Rb. sphaeroides* strains were grown semi-aerobically in a rich medium as previously described (Paddock et al. 1989). After harvesting, the cells were resuspended in 10 mM Tris-HCl (pH 8) and sonicated (3 times, 3 minutes each). The cell extracts were incubated with DNase and then centrifuged at low speed (8000 rpm, 30 min, Sorvall GS3 rotor) to remove whole cells and debris. The membranes were isolated by high speed centrifugation (45 000 rpm, 2 h 15 min, Beckman Ti45 rotor) and resuspension of the pellet by homogenization in 15 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.1 M NaCl. The chromatophore preparations were stored at –75 °C in 15% (v/v) glycerol.

The chromatophore absorption spectra of the *Rb. sphaeroides* strains (not shown) have a single, predominant maximum in the near-infrared spectral region at 875 nm, due primarily to the B880 antenna, and minor peaks at 800 nm and 760 nm which are RC transitions. These spectra are very similar to the absorption spectra of the wild type *Rs. rubrum* chromatophores, with the exception of a small (5 nm) difference in the peak position of the B880 antenna transition (for a discussion of the spectral properties of *Rb. sphaeroides* in which the B800–850 antenna genes have been deleted, see Hunter 1995). Comparing the relative intensity of the near infrared RC transition at 800 nm to the B880 antenna transition near 880 nm in the various strains used, it appears that the number of antenna Bchl molecules per RC is essentially the same in the wild type and mutant *Rb. sphaeroides* strains as well as in *Rs. rubrum.* All the samples were suspended in a 100 mM Tris-HCl buffer (pH 8.0) giving a final optical density of 3 in a 1 cm cuvette at 875 nm. For the emission experiments, either a 0.15 cm pathlength cuvette was used with right angle detection or a 1 cm cell was used with front surface detection.

Time-correlated single photon counting. The time-correlated single photon counting system used to monitor the emission decay kinetics has been described previously (Gust et al. 1990; Taguchi et al. 1992; Peloquin et al. 1994). For these experiments, the sample was excited by pulses of about 10 ps duration from a cavity-dumped synchronously pumped rhodamine 6G or styryl-9 dye laser (Spectra Physics). The dye lasers were pumped by an Antares mode-locked Nd:YAG laser (Coherent). The emission photons were detected by an S1 type microchannel-plate photomultiplier tube (Hamamatsu). The overall instrument response function in different experiments was 50–70 ps (FWHM). The emission kinetics at 890 nm were recorded with the detection monochomator set to a 16 nm spectral bandwidth and were followed over a 10 ns time interval. Two different excitation wavelengths (at 590 nm into the Q_x absorption band of Bchl and at 860 nm into the Q_y bands of B880 antenna and the RC) that provide nearly homogeneous excitation of the PSU and several excitation pulse repetition rates (between 76 MHz and 380 kHz) were utilized, but essentially no differences were observed in the kinetic traces. The kinetics were analyzed by fitting to a sum of exponential decay terms

$$\sum_i A_i \exp\left(\frac{-t}{\tau_i}\right),$$

(where the preexponential amplitudes, A_i, and the lifetimes, τ_i, are fitting parameters), convoluted with the measured instrument response function (light scattered from the sample at the excitation wavelength).

All the measurements were performed at room temperature in order to minimize the possible effects of spectral inhomogeneity in the RC and antenna transitions (Freiberg and Timpmann 1992) as well as any effects of the small relative shifts in the Q_y transition energy of the RC special pair in the mutants (Allen and Williams 1995; Woodbury and Allen 1995; Mattioli 1995). Special care was taken to keep most of the RCs in a photoactive (open) state during the measurements. Emission saturation curves were recorded, as in Freiberg et al. (1984) and Borisov et al. (1985), ensuring that the measurements were performed in the linear, low light regime. In order to achieve acceptable count rates, either phenazine methosulfate (PMS) or terbutryn was added to the buffer and the sample was stirred rapidly. Under these conditions, excitation intensities of roughly 0.1 mW/cm^2 could be used without forming an appreciable steady-state $P^+Q_A^-$ population. Introduction of PMS allows an additional pathway for return of the electron to P^+ in the RC. Terbutryn blocks electron transfer to the secondary RC quinone. The proper concentrations of PMS and terbutryn were determined by directly measuring the RC $P^+Q_A^-$ lifetime in *Rs. rubrum* chromatophores. The differential absorbance around 870 nm recovered in these samples within 7 ms in the presence of 32 μM PMS and within 67 ms with 0.5 mM terbutryn. The results from the fluorescence decay measurements were the same

regardless of whether terbutryn or PMS was used to enhance the recovery rate of RCs after charge separation.

Results and discussion

Time-correlated single photon counting measurements were performed on seven strains of *Rb. sphaeroides* as well as on wild type *Rs. rubrum*. Each of the *Rb. sphaeroides* strains lacked the B800–850 antenna due to deletion of the *puc* operon, as described in the Experimental section above. Six of the *Rb. sphaeroides* strains also contained either point mutations or deletions in the L or M genes of the RC, resulting in a variety of charge separation lifetimes. At least three exponential decay components were required to fit the picosecond emission kinetics from chromatophores of each of the *Rb. sphaeroides* strains that contained functional RCs. In contrast, only two decay components were needed for either the wild type *Rs. rubrum* or the *Rb. sphaeroides* B880 antenna-only (no RC or B800–850 antenna) mutant strain. The decay time constants and amplitudes, averaged over several separate measurements, are collected in Table 1. The given error in the fitting parameters (± 10%) is an estimate based on a statistical evaluation of many repeated measurements performed with wild type samples. Note that processes, such as the P* to $P^+H_A^-$ electron transfer reaction in wild type reaction centers, which take place on time scales less than 10 ps, are not resolved due to the limited time resolution of this instrument.

Qualitatively, the two longest decay constants determined from the fitting, τ_2 and τ_3, have similar values in all RC mutants (about 250 ps and 700 ps, respectively). In contrast, the shorter decay constant, τ_1, varies roughly linearly with the previously measured P* decay time, τ_{rc}. An empirical formula for the τ_1 dependence on τ_{rc} determined by a linear fit to the points shown in Figure 1 is (in picoseconds): $\tau_1 \approx 56(\pm 3) + 2.0(\pm 0.1)\,\tau_{rc}$. The errors shown are statistical errors determined during the linear fit.

The amplitude ratio of the shortest and the intermediate decay components, A_1/A_2, decreases as τ_{rc} increases, but clearly in a non-linear fashion (Table 1). The antenna-only mutant reveals a prevailing 750 ps decay component, similar to what has been seen previously both in antenna-only membranes (Hunter et al. 1990) and in isolated B880 antenna complexes (Freiberg and Timpmann 1992). This suggests that the 700 ps kinetics in the RC mutants is at least partly due to emission from antenna complexes that are not effectively coupled to the RC.

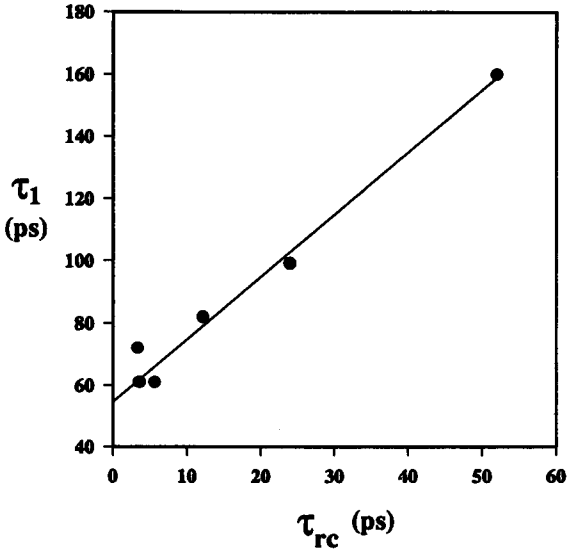

Figure 1. The shortest emission lifetime in *Rb. sphaeroides* mutant and wild type chromatophores, τ_1, as a function of the P* decay time in the isolated RCs, τ_{rc}. The values used in this plot are given in Table 1. The solid line is a linear fit through the points as described in the text.

In order to analyze the origin of the two shorter decay components in the RC mutants, the following four-level kinetic scheme (Källebring and Hansson 1991; Xiao et al. 1994) was applied:

$$A^* \underset{k_{-1}}{\overset{k_1}{\Leftrightarrow}} P^* \underset{k_{-2}}{\overset{k_2}{\Leftrightarrow}} P^+H_A^- \overset{k_3}{\Rightarrow} P^+Q_A^-.$$

This model takes into account the antenna exciton (A*) trapping by P (forming P*), quenching of excitons by the primary charge separation reaction, $P^*H \rightarrow P^+H_A^-$ (H_A is the Bphe on the A-branch of the RC), in the RC, and stabilization of the electron at the primary quinone acceptor site, Q_A. All processes, except the very last stabilization step, have been assumed to be reversible. In the above scheme, k_1 represents the total rate constant of energy transfer from the antenna to the RC (the sum over all possible pathways), k_{-1} is the total rate constant of energy transfer back to the antenna, k_2 and k_{-2} are the forward and the backward primary electron-transfer rate constants in the RC, respectively, and k_3 is the rate constant of electron transfer to the quinone. The model implicitly assumes that the excitation migration within the antenna is much faster than the transfer from the antenna to the RC (Somsen et al. 1996). It also ignores any decay paths to the

Table 1. Picosecond emission decay times (τ_i) and relative amplitudes (A_i) of mutant *Rb. sphaeroides* and wild type *Rs. rubrum* chromatophores[a]

Sample[b]	τ_1/A_1 (ps)(%)	τ_2/A_2 (ps)(%)	τ_3/A_3 (ps)/(%)	τ_{rc}[c] (ps)	D[d] (%)
WT-1A	72/86	280/7	710/7	3.4	6
HF(L168)	61/90	230/7	650/3	3.6	6
LH(M160)	61/78	240/14	700/7	5.7	9
LH(L131)	82/56	250/29	640/15	12.2	18
LH(M160) + LH(L131)	99/44	250/37	690/19	24	24
LH(L131) + LH(M160) + FH(M197)	160/25	400/50	780/25	52	41
B880 antenna-only mutant		230/13	750/87		
Rs. rubrum	55/99	330/1		5.1[e]	5

[a] All time constants and amplitudes have an error of ± 10% as described in the text.
[b] RC mutations are designated, for example, HF(L168), meaning that the histidine (H) residue has been replaced by the phenylalanine (F) residue at position 168 of the L subunit. WT-1A has wild type RC and B880 antenna genes. All *Rb. sphaeroides* strains contained a deletion in the chromosomal B800–850 antenna genes.
[c] P* decay times from Williams et al. (1992), Murchison et al. (1993), Peloquin et al. (1994), and Woodbury et al. (1995).
[d] The detrapping efficiency calculated using the simple kinetic model described in the text and Equation (1). As described in the text, these values are used for comparative purposes only and may not represent the actual values of D.
[e] This is the lifetime of P* in *Rs. rubrum* with quinones reduced (Wang et al. 1994). The lifetime under the conditions of the present measurement is probably shorter.

ground state from the intermediate excited and charge separated states. In order to apply this scheme to the different mutants studied here, one must also assume that the energy transfer between the RC and the antenna is unchanged by the RC mutations and that the rate of P* decay measured in isolated RCs from each mutant is the same as that in whole chromatophores.

An exact solution to the differential equations describing this kinetic system can be obtained by numerical methods, much as has been done previously (Källebring and Hansson 1991; Beekman et al. 1994). In this simulation, we searched for values of k_1 and k_{-1} which would account for the kinetic behavior of the entire set of mutants, using rate constants of electron transfer (k_2) and the standard free energy differences between P* and $P^+H_A^-$ previously determined for these mutants (Lin et al. 1994; Allen and Williams 1995; Woodbury et al. 1995; Bixon et al. 1995). Given the weak dependence of the overall trapping rate constant on the electron transfer rate constant in reaction centers (Table 1), k_1 must have a value very similar to the overall excitation trapping rate constant, roughly $(55\ \text{ps})^{-1}$, and this was apparent in the simulations. Unfortunately the value of k_{-1} in these simulations is much less well defined and depends strongly on the value used for the P* to $P^+H_A^-$ standard free energy difference, an energy that is seriously debated both in the wild type RCs and in the high P/P^+ potential mutants used in this study (Peloquin et al. 1994; Woodbury et al. 1995; Bixon et al. 1995). What can be said is that values for k_{-1} in the range of those previously published, $(8\ \text{ps})^{-1}$ to $(25\ \text{ps})^{-1}$ (Timpmann et al. 1993; Beekman et al. 1994; Xiao et al. 1994), are consistent with the data using standard free energy values for the P* to $P^+H_A^-$ reaction that are within the limits of values in the literature for the mutants in this study (Peloquin et al. 1994; Woodbury et al. 1995; Bixon et al. 1995). (The free energy values cited in these reports depend strongly on what model one uses to analyze transient optical data and vary by as much as 0.13 eV from model to model for any particular reaction center sample.) From k_{-1} and the charge separation rate constant in the reaction center, the detrapping ratio, D (the fraction of excitons on the RC that avoid quenching and are transferred back into the antenna), can be calculated as:

$$D = (k_{-1})/(k_{-1} + 1/\tau_{rc}). \quad (1)$$

Note that the definition of D varies between publications (e.g. Somsen et al. 1996). The value of D is uncertain for the same reason that k_{-1} is, but increases from 10–25% in the WT-1A *Rb. sphaeroides* membranes to over 50% in the triple mutant. Again, this is consistent with the values reported earlier (Timpmann et al. 1993; Beekman et al. 1994; Xiao et al. 1994).

Note that the values of D reported in Table 1 were based on an analysis using a simpler kinetic model described below and are given for the purpose of comparing mutants under a given set of assumptions rather than as necessarily the most likely values for D.

Numerical simulation as a method for analyzing data such as these has several shortcomings. First, for these data the model parameters are not well constrained, and it is very difficult to evaluate the error in parameters obtained in this way. Perhaps more importantly for the present report, it lends very little in the way of a conceptual model for understanding the source of the different kinetic components or the linear dependence of the overall early time excitation trapping rate on the rate of charge separation in the reaction center (Figure 1). An alternative approach is to derive a conceptually simple, but only approximate, solution to the kinetic system by assuming that k_2 is much greater than either k_{-2} or k_3. Under this assumption, one can consider the reversible reaction between A^* and P^* as being followed by a nearly irreversible decay of P^* forming $P^+H_A^-$. Then on a longer time scale, there is a competition between forward and backward electron transfer from $P^+H_A^-$ involving the rate constants k_3 and k_{-2}. Under this assumption, k_2 can be approximately identified with τ_{rc}, the observed lifetime of P^* in isolated RCs. Note that the observed lifetime of P^* is used, rather than the microscopic charge separation rate constant, since this includes all pathways of P^* decay. This is appropriate since for a nearly irreversible charge separation reaction, the antenna decay kinetics will only depend on the overall decay rate of P^*, independent of the pathway of the decay. Under the above assumptions, one can derive three approximate eigenvalues (observable decay rates) for the kinetic system:

$$\lambda_1 = k_1k_2/(k_1 + k_{-1} + k_2) \quad (2)$$

$$\lambda_2 = k_3 + k_{-1}k_{-2}/(k_2 + k_{-1}) \quad (3)$$

$$\lambda_3 = k_1 + k_{-1} + k_2 - k_1k_2/(k_1 + k_{-1} + k_2) \quad (4)$$

These equations result from a first-order binomial expansion of the solutions given in Xiao et al. (1994). Note that this is not a completely accurate representation of the kinetic system, but it lends conceptual insight into the expected behavior of the observed decays as a function of the rate constants in the model.

Equation (2) primarily describes the trapping and quenching processes. λ_1^{-1} can be identified with the fastest decay constant in the fit of the fluorescence decay and is linearly proportional to k_2^{-1} (the P^* decay time in the isolated RC, τ_{rc}): $\lambda_1^{-1} = k_1^{-1} + (1 + k_{-1}/k_1)k_2^{-1}$ or substituting in τ_1 for λ_1 and τ_{rc} for k_2^{-1}, one obtains:

$$\tau_1 = k_1^{-1} + (1 + k_{-1}/k_1)\tau_{rc} \quad (5)$$

which predicts the observed linear dependence of the fastest rate constant for antenna fluorescence decay on the charge separation rate. From comparison with the linear fit in Figure 1, it follows that $k_{-1}/k_1 = 1.0 \pm 0.1$, $k_1^{-1} = 56 \pm 3$ ps and therefore $k_{-1}^{-1} = 56 \pm 10$ ps. As can be seen from comparison to the numerical simulations, the simple model gives the expected value for k_1 but may underestimate k_{-1}. This is due to the fact that in the model the charge recombination reaction reforming P^* from $P^+H_A^-$ is essentially ignored as are all decay paths for the excited state other than trapping. Both of these assumptions become less and less valid as higher potential mutants are considered. In addition, for the mutants with the largest value of τ_{rc} , the observed rate constant does not simply reflect λ_1 because λ_3 probably contributes. For the mutants with the faster charge separation times or the wild type, λ_3 (which reflects the approach of the system to either steady-state or equilibrium with respect to the population of P^*) will have a value roughly equal to the P^* decay rate in isolated RCs. This is faster than the instrument used can accurately resolve and has little effect on the fitting. However, for mutants with 20 to 50 ps charge separation times, λ_3 and λ_1 will both be important, but probably not well resolved, and together determine the fastest observed decay rate. The combined result of all of these approximations is that as one goes from wild type to the higher potential mutants, the observed time constant, τ_1, is probably less and less representative of the calculated rate constant, λ_1. This may lead to an artificially small slope for the line in Figure 1 and therefore an underestimate of k_{-1}. In addition, it was shown by Szöcs and Barvik (1986) and Barvik (1993) that for sufficiently large quenching rates the antenna excitons effectively decouple from the RC and remain stored in the antenna avoiding transfer to the RC. As the effect is larger in the case of wild type and single mutant strains, it again diminishes to some extent the slope of the line in Figure 1.

Though approximate, the simple kinetic model described above is useful not only in explaining the approximately linear relationship in Figure 1, but in obviating the origin of the longer decay component(s)

of the antenna excited state decay in chromatophores. Earlier studies by Freiberg et al. (1984) and Borisov et al. (1985) reported an almost single-exponential fluorescence decay at different excitation light intensities. In these studies the fluorescence decay rate simply slowed down as the RCs were oxidized due to increases in the actinic light level. However, later measurements suggested that the emission decay was strongly non-exponential at all excitation intensities, and that the origin of at least part of this kinetic complexity was in the ability of the system to undergo back-reactions reforming excited states from $P^+H_A^-$ (Woodbury and Bittersmann 1990). The same conclusion was theoretically reached by Källebring and Hansson (1991). The eigenvalue λ_2 in Equation (3) can be identified with the few hundred picosecond observed time constant, τ_2, in Table 1, and from Equation (3) one can see that this term is dominated by the kinetic rates surrounding $P^+H_A^-$. A $P^+H_A^-$ kinetic term only affects the fluorescence decay timecourse because reverse reactions lead to charge recombination of $P^+H_A^-$ reforming emitting states. Consistent with this notion, the τ_2 lifetime matches the decay time of $P^+H_A^-$ as the electron is transferred to Q_A. This also qualitatively explains the non-linear decrease of the amplitude ratio, A_1/A_2, with τ_{rc}. In a quasi-equilibrium limit,

$$A_1/A_2 \approx k_1k_2/(k_{-1}k_{-2}) = k_1(k_{-1})^{-1}\exp(\Delta G/kT) \quad (6)$$

where ΔG is the free energy gap between the states P^* and $P^+H_A^-$. In the series of mutants used in this study, the free energy gap becomes smaller and smaller as more hydrogen bonds are added and the ratio of A_1/A_2 decreases accordingly. Effectively biexponential kinetic decays for a similar model system were also deduced by Källebring and Hansson (1991).

In the work of Beekman et al. (1994), three *Rb. sphaeroides* RC mutants, different from those described here (mutations at the M210 position), were studied by picosecond transient absorption spectroscopy. A roughly linear lifetime dependence on τ_{rc} was observed in that case as well. However, the slope of the linear dependence was even smaller than that reported here. Also the measured excited state lifetimes (including the wild-type species) were shorter. (The decay time constant in membranes with wild type RCs was 46 ps and the slope of the dependence of the antenna excited state lifetime on τ_{rc} was two-fold smaller than that observed here.) One potential advantage of the measurements presented in this work is the larger range of charge separation times in the mutant RCs. This should result in a better evaluation of the dependence of energy trapping on the rate constant of electron transfer. In addition, the excitation intensities used for the fluorescence work reported here were lower than that in the previous transient absorbance measurements (Beekman et al. 1994), removing any effects from multiple photon absorption by the large antenna cross section.

Another difference between this set of RC mutations and those used by Beekman et al. (1994) is that much larger variations in the P/P^+ midpoint potential are seen in the mutants used in this report (as much as 260 mV above wild type). This means that the driving force for electron transfer from P^* to $P^+H_A^-$ in some of these mutants is very small (Lin et al. 1994). This puts a limit on the free energy gap between A^* and P^*. Clearly, this reaction cannot be unfavorable by more than a few tens of meV in order for significant quenching to occur at all in the triple mutant, LH(L131) + LH(M130) + FH(M197), since in this mutant P^* and $P^+H_A^-$ are apparently very close in free energy (Lin et al. 1994; Woodbury et al. 1995). This is consistent with values of k_{-1} on the lower end of the range (near $(25\ ps)^{-1}$) and thus also with low values of the free energy between A^* and P^* as reported in Xiao et al. (1994) and proposed theoretically by Novoderezhkin and Razjivin (1994).

Despite the differences between detailed values for rate constants, free energies and detrapping ratios in the literature, there is a qualitative agreement among most of the recent papers on the subject (Abdourakhmanov et al. 1989; Kleinherenbrink et al. 1992; Valkunas et al. 1992; Timpmann et al. 1993, 1995; Beekman et al. 1994; Xiao et al. 1994 and the present work) that the available data can be modeled in terms of rapid energy equilibration among the B880 antenna complexes followed by rate limiting incoherent energy transfer to the RC which is significantly slower than the rate of charge separation. The present work also verifies one of the predictions made by that model, that for large changes in the rate constant of charge separation in the RC (a factor of about 15), much smaller relative changes are seen in the overall trapping rate constant of excitons in the antenna (a factor of roughly 2.5). It was recently deduced (Pullerits et al. 1995) that excitons in the B850 peripheral antenna of *Rb. sphaeroides* extend over 4 ± 2 bacteriochlorophyll *a* molecules at room temperature. The exciton size in the core antenna is not yet determined, but in view of the presumed structural similarity between the two antenna complexes,

it is reasonable to assume some exciton delocalization also in the core antenna (Novoderezhkin and Razjivin 1994). This may influence the physical interpretation of the results (Barvik 1993). Our further investigations will focus on that issue.

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References

Abdourakhmanov IA, Danielius RV and Razjivin AP (1989) Efficiency of excitation trapping by reaction centers of complex B890 from *Chromatium minutissimum*. FEBS Lett 245: 47–50

Allen JP and Williams JC (1995) Relationship between the oxidation potential of the bacteriochlorophyll dimer and electron transfer in photosynthetic reaction centers. J Bioenerg Biomembr 27: 275–283

Bakker JGC, van Grondelle R and Den Hollander WTF (1983) Trapping, loss and annihilation of excitations in a photosynthetic system. II. Experiments with the purple bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas capsulata*. Biochim Biophys Acta 725: 508–518

Barvik I (1993) Computer modeling of coherence effects in excitation transfer in hexagonal PSU. Acta Phys Slov 43: 114–129

Beekman LMP, van Mourik F, Jones MR, Visser HM, Hunter CN and van Grondelle R (1994) Trapping 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. Biochemistry 33: 3143–3147

Bergström H, Westerhuis WHJ, Sundström V, van Grondelle R, Niederman RA and Gillbro T (1988) Energy transfer within the isolated B875 light-harvesting pigment-protein complex of *Rhodobacter sphaeroides* at 77 K studied by picosecond absorption spectroscopy. FEBS Lett 233: 12–16

Bixon M, Jortner J and Michel-Beyerle M (1995) A kinetic analysis of the primary charge separation in bacterial photosynthesis. Energy gaps and static heterogeneity. Chem Phys 197: 389–404

Borisov AY, Freiberg A, Godik VI, Rebane K and Timpmann K (1985) Kinetics of picosecond bacteriochlorophyll luminescence *in vivo* as a function of the reaction center state. Biochim Biophys Acta 807: 221–229

Bradforth SE, Jimenez R, van Mourik F, van Grondelle R and Fleming GR (1995) Excitation transfer in the core light-harvesting complex (LH–1) of *Rhodobacter sphaeroides:* An ultrafast fluorescence depolarization and annihilation study. J Phys Chem 99: 16179–16191

Deinum G, Kramer H, Aartsma TJ, Kleinherenbrink FAM and Amesz J (1991) Fluorescence quenching in *Heliobacterium chlorum* by reaction centers in the charge separated state. Biochim Biophys Acta 1058: 339–344

Deinum G, Kleinherenbrink FAM, Aartsma TJ and Amesz J (1992) The fluorescence yield of *Rhodopseudomonas viridis* in relation to the redox state of the primary electron donor. Biochim Biophys Acta 1099: 81–84

Duysens LMN (1952) Transfer of excitation energy in photosynthesis. Thesis, State University of Utrecht

Emerson R and Arnold WA (1932) The photochemical reaction in photosynthesis. J Gen Physiol 16: 191–205

Feher G, Allen JP, Okamura MY and Rees DC (1989) Structure and function of bacterial photosynthetic reaction centres. Nature 339: 111–116

Freiberg A (1995) Coupling of antennas to reaction centers. In: Blankenship RE, Madigan MT and Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp 385–398. Kluwer Academic Publishers, Dordrecht

Freiberg A and Timpmann K (1992) Picosecond fluorescence spectroscopy of light-harvesting antenna complexes from *Rhodospirillum rubrum* in the 300–4 K temperature range. Comparison with the data on chromatophores. J Photochem Photobiol B: Biol 15: 151–158

Freiberg A, Godik VI and Timpmann K (1984) Excitation energy transfer in bacterial photosynthesis studied by picosecond laser spectrochronography. In: Sybesma C (ed) Advances in Photosynthesis Research, Vol 1, pp 45–48. Martinus Nijhoff/Dr. W. Junk Publishers, Hague

Freiberg A, Allen JP, Williams JC and Woodbury NW (1995) Trapping in mutant photosynthetic bacteria. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol 1, pp 863–866. Kluwer Academic Publishers, Dordrecht

Gust D, Moore TA, Luttrull DK, Seely GR, Bittersmann E, Bensasson RV, Rougee M, Land EJ, De Schryver FC and Van der Auweraer M (1990) Photophysical properties of 2-nitro-5,10,15,20-tetra-*p*-tolylporphyrins. Photochem Photobiol 51: 419–426

Hunter CN (1995) Genetic manipulation of the antenna complexes of purple bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp 473–501. Kluwer Academic Publishers, Dordrecht

Hunter CN, Bergström H, van Grondelle R and Sundström V (1990) Energy-transfer dynamics in three light-harvesting mutants of *Rhodobacter sphaeroides:* A picosecond spectroscopy study. Biochemistry 29: 3203–3207

Källebring B and Hansson Ö (1991) A theoretical study of the effect of charge recombination on the transfer and trapping of excitation energy in photosynthesis. Chem Phys 149: 361–372

Kirmaier C and Holten D (1987) Primary photochemistry of reaction centers from the photosynthetic purple bacteria. Photosynth Res 13: 225–260

Kirmaier C and Holten D (1993) Electron transfer and charge recombination reactions in wild-type and mutant bacterial reaction centers. In: Deisenhofer J and Norris JR (eds) The Photosynthetic Reaction Center, pp 49–70. Academic Press, San Diego

Kleinherenbrink FAM, Deinum G, Otte SCM, Hoff AJ and Amesz J (1992) Energy transfer from long-wavelength absorbing antenna bacteriochlorophylls to the reaction center. Biochim Biophys Acta 1099: 175–181

Lin X, Williams JC, Allen JP, and Mathis P (1994) Relationship between rate and free energy difference for electron transfer from cytochrome c_2 to the reaction center in *Rhodobacter sphaeroides*. Biochemistry 33: 13517–13523

Martin J-L and Vos MH (1992) Femtosecond biology. Ann Rev Biophys Biomol Struct 21: 199–222

Mattioli TA, Lin X, Allen JP and Williams JC (1995) Correlation between multiple hydrogen bonding and alteration of the oxidation potential of the bacteriochlorophyll dimer of reaction centers from *Rhodobacter sphaeroides*. Biochemistry 34: 6142–6152

Müller MG, Drews G and Holzwarth AR (1993) Excitation transfer and charge separation kinetics in purple bacteria. (1) Picosecond fluorescence of chromatophores from *Rhodobacter capsulatus* wild type. Biochim Biophys Acta 1142: 49–58

Murchison HA, Alden RG, Allen JP, Peloquin JM, Taguchi AKW, Woodbury NW and Williams JC (1993) Mutations designed to modify the environment of the primary electron donor of the reaction center from *Rhodobacter sphaeroides*: Phenylalanine to leucine at L167 and histidine to phenylalanine at L168. Biochemistry 32: 3498–3505

Novoderezhkin VI and Razjivin AP (1994) Exciton states of the antenna and energy trapping by the reaction center. Photosynth Res 42: 9–15

Paddock ML, Rongey SH, Feher G and Okamura MY (1989) Pathway of proton transfer in bacterial reaction centers: Replacement of glutamic acid 212 in the L subunit by glutamine inhibits quinone (secondary acceptor) turnover. Proc Natl Acad Sci USA 86: 6602–6606

Parson WW (1991) Reaction centers. In: Scheer H (ed) Chlorophylls, pp 1153–1180. CRC Press, Boca Raton

Peloquin JM, Williams JC, Lin X, Alden RG, Taguchi AKW, Allen JP and Woodbury NW (1994) Time-dependent thermodynamics during early electron transfer in reaction centers from *Rhodobacter sphaeroides*. Biochemistry 33: 8089–8100

Pullerits T, Chachisvilis M and Sundström V (1995) Exciton dynamics and delocalization length in B850 molecules of LH2 of *Rhodobacter sphaeroides*. In: Mathis P (ed) Photosynthesis: From Light to Biosphere, Vol 1, pp 107–110. Kluwer Academic Publishers, Dordrecht

Sebban P and Moya I (1983) Fluorescence lifetime spectra of in vivo bacteriochlorophyll at room temperature. Biochim Biophys Acta 722: 436–442

Sebban P, Robert B and Jolchine G (1985) Isolation and spectroscopic characterization of the B875 antenna complex of a mutant of *Rhodopseudomonas sphaeroides*. Photochem Photobiol 42: 573–578

Somsen OJG, Valkunas L and van Grondelle R (1996) A perturbed two-level model for exciton trapping in small photosynthetic systems. Biophys J 70: 669–683

Szöcs V and Barvik I (1986) Description of excitation transfer and damping in the primary processes of bio-(chemical) systems. J Theor Biol 122: 179–186

Taguchi AKW, Stocker JW, Alden RG, Causgrove TP, Peloquin JM, Boxer SG and Woodbury NW (1992) Biochemical characterization and electron-transfer reactions of *sym1*, a *Rhodobacter capsulatus* reaction center symmetry mutant which affects the initial electron donor. Biochemistry 31: 10345–10355

Timpmann K, Zhang FG, Freiberg A and Sundström V (1993) Detrapping of excitation energy from the reaction centre in the photosynthetic purple bacterium *Rhodospirillium rubrum*. Biochim Biophys Acta 1183: 185–193

Timpmann K, Freiberg A and Sundström V (1995) Energy trapping and detrapping in photosynthetic bacteria *Rhodopseudomonas viridis*. Chem Phys 194: 275–283

Valkunas L, van Mourik F and van Grondelle R (1992) On the role of spectral and spatial antenna inhomogeneity in the process of excitation energy trapping in photosynthesis. J Photochem Photobiol B: Biol 15: 159–170

Valkunas L, Trinkunas G, Liuolia V and van Grondelle R (1995) Nonlinear annihilation of excitations in photosynthetic systems. Biophys J 69: 1117–1129

van Grondelle R and Sundström V (1988) Excitation energy transfer in photosynthesis. In: Scheer H and Schneider S (eds) Photosynthetic Light-Harvesting Systems: Organization and Function, pp 403–438. Walter de Gruyter, Berlin, New York

Visscher KJ, Bergstrom H, Sundstrom V, Hunter CN and van Grondelle R (1989) Temperature dependence of energy transfer from the long wavelength antenna BChl-896 to the reaction center in *Rhodospirillum rubrum, Rhodobacter sphaeroides* (w.t. and M21 mutant) from 77 to 177 K, studied by picosecond absorption spectroscopy. Photosynth Res 22: 211–217

Visser HM, Somsen OJG, van Mourik F, Lin S, van Stokkum IHM and van Grondelle R (1995) Direct observation of sub-picosecond equilibration of excitation energy in the light-harvesting antenna of *Rhodospirillum rubrum*. Biophys J 69: 1083–1099

Vredenberg WJ and Duysens LMN (1963) Transfer of energy from bacteriochlorophyll to a reaction centre during bacterial photosynthesis. Nature 4865: 355–357

Wang RT and Clayton RK (1971) The absolute yield of bacteriochlorophyll fluorescence *in vivo*. Photochem Photobiol 13: 215–224

Wang S, Lin S, Lin X, Woodbury NW and Allen JP (1994) Comparative study of reaction centers from purple photosynthetic bateria: Isolation and optical spectroscopy. Photosynth Res 42: 203–215

Williams JC and Taguchi AKW (1995) Genetic manipulation of purple photosynthetic bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp 1029–1065. Kluwer Academic Publishers, Dordrecht

Williams JC, Alden RG, Murchison HA, Peloquin JM, Woodbury NW and Allen JP (1992) Effects of mutations near the bacteriochlorophylls in reaction centers from *Rhodobacter sphaeroides*. Biochemistry 31: 11029–11037

Woodbury NW and Allen JP (1995) The pathway, kinetics and thermodynamics of electron transfer in wild-type and mutant reaction centers of purple nonsulfur bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds) Anoxygenic Photosynthetic Bacteria, pp 527–557. Kluwer Academic Publishers, Dordrecht

Woodbury N and Bittersmann E (1990) Time-resolved measurements of fluorescence from the photosynthetic membranes of *Rhodobacter capsulatus* and *Rhodospirillum rubrum*. In: Baltscheffsky M (ed) Current Research in Photosynthesis, Vol 2, pp 165–168. Kluwer Academic Publishers, Dordrecht

Woodbury NW, Peloquin JM, Alden RG, Lin X, Lin S, Taguchi AKT, Williams JC and Allen JP (1994) Relationship between thermodynamics and mechanism during photoinduced charge separation in reaction centers from *Rhodobacter sphaeroides*. Biochemistry 33: 8101–8112

Woodbury NW, Lin S, Lin X, Peloquin JM, Taguchi AKT, Williams JC and Allen JP (1995) The role of reaction center excited state evolution during charge separation in a *Rb. sphaeroides* mutant with an initial electron donor midpoint potential 260 mV above wild type. Chem Phys 197: 405–421

Xiao W, Lin S, Taguchi AKW and Woodbury NW (1994) Femtosecond pump-probe analyses of energy and electron transfer in photosynthetic membranes of *Rhodobacter capsulatus*. Biochemistry 33: 8313–8322

Zinth W and Kaiser W (1993) Time-resolved spectroscopy of the primary electron transfer in reaction centers of *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis*. In: Deisenhofer J and Norris JR (eds) The Photosynthetic Reaction Center, pp 71–88. Academic Press, San Diego