

On the efficiency of energy transfer and the different pathways of electron transfer in mutant reaction centers of *Rhodobacter sphaeroides*

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Abstract

The efficiency of energy transfer from the monomeric pigments to the primary donor was determined from 77 K steady-state fluorescence excitation spectra of three mutant reaction centers, YM210L, YM210F and LM160H / FM197H. For all three reaction centers this efficiency was not 100% and ranged between 55 and 70%. For the YM210L mutant it was shown using pump-probe spectroscopy with B band excitation at 798 nm that the excitations which are not transferred to P give rise to efficient charge separation. The results can be interpreted with a model in which excitation of the B absorbance band leads to direct formation of the radical pair state $B_A^+H_A^-$ in addition to energy transfer to P. It is also possible that some $P^+B_A^-$ is formed from B^* . In previous publications we have demonstrated the operation of such alternative pathways for transmembrane electron transfer in a YM210W mutant reaction center [van Brederode et al. (1996) *The Reaction center of Photosynthetic Bacteria*, pp 225–238; (1997a,b) *Chem Phys Lett* 268: 143–149; *Biochemistry* 36: 6855–6861]. The results presented here demonstrate that these alternative mechanisms are not peculiar to the YM210W reaction center.

Abbreviations: RC – reaction center; P – special pair of bacteriochlorophyll molecules; H_A – Bphea present in the active branch of the reaction center; H_B – Bphea present in the inactive branch of the reaction center; B_A – Bchl present in the active branch of the reaction center; B_B – Bchl present in the inactive branch of the reaction center; Q – quinone

Introduction

In the reaction center (RC) from *Rhodobacter sphaeroides*, excitation of any of the transitions of the monomeric pigments results in sub-ps downhill transfer of the excitation energy, creating the excited state of the special pair of bacteriochlorophyll molecules (P^*) (Breton et al. 1986; Stanley et al. 1996; Jonas et al. 1996). On the basis of steady state fluorescence excitation spectra at 77 K of a mutant which has very slow P^* driven electron transfer (YM210W), we recently concluded that the efficiency of energy

transfer in the bacterial RC is not always 100% (van Brederode et al. 1996, 1997a). In particular, the pigments in the active branch seemed to be less efficient in transferring energy to P. However, the action spectrum for the formation of $P^+Q_A^-$ showed that the excitations which did not give rise to P^* formation did result in efficient charge separation. We concluded from these experiments that primary charge separation in the bacterial RC can proceed without the involvement of P^* . This finding was confirmed by sub-ps pump-probe experiments using selective excitation of the P and B absorption bands (van Brederode et al.

1997b). Whereas P band excitation only resulted in very slow charge separation characterized by lifetimes of 80 and 500 ps, B band excitation at 799 nm resulted in the formation of the absorbance difference spectrum characteristic for $P^+H_A^-$ within a few picoseconds. The spectral shape and evolution of the early time-gated difference spectra were interpreted with a model in which the states $P^+B_A^-$ and $B_A^+H_A^-$ are formed from B_A^* , with the subsequent formation of the $P^+H_A^-$ state. These processes take place in parallel with energy transfer from B_A^* to P.

Prior to our experimental work, several calculations based on the crystal-structure indicated that alternative charge separation paths such as those proposed above are indeed possible (Fischer and Scherer 1987; Warshel et al. 1988; Creighton et al. 1988). These calculations mainly considered the possible formation of $B_A^+H_A^-$ as an alternative charge separated state. This $B_A^+H_A^-$ state could either be formed from B^* or from P^* , with in the latter case the requirement that a significant amount of H^* and/or B^* are mixed into the P^* state (Fischer and Scherer 1987; Warshel et al. 1988). Fischer and Scherer (1987) proposed that in *Rhodospseudomonas viridis* the radical pair $B_A^+H_A^-$ can be formed from B_A^* on a sub ps time-scale. In contrast the formation of $B_A^+H_A^-$ from P^* (with some B^* mixed in) would occur in 10 ps. This alternative process was suggested not to occur along the inactive branch, since the free energy of the $B_B^+H_B^-$ state was calculated to lie considerably above that of P^* . A lifetime of 0.7 ps was calculated for the reaction $B_A^+H_A^-$ to $P^+H_A^-$, whilst this process would proceed more than an order of magnitude slower among the pigments of the inactive branch. Free energy calculations based on the crystal structure of *Rhodobacter sphaeroides* have also indicated that the formation of $B_A^+H_A^-$ as an initial intermediate is not impossible (Creighton et al. 1988). The same calculations also favour the two step electron transfer model with $P^+B_A^-$ as the primary reaction product from P^* . We do not know of model calculations in which $P^+B_A^-$ is formed directly from B_A^* .

In this work we show that the possibility of alternative electron transfer pathways is not something specific to the YM210W mutant RC. We have examined the efficiency of energy transfer in two other YM210 mutants (YM210L and YM210F) and in a mutant which has two extra hydrogen bonds between the protein and the bacteriochlorophylls of P (LM160H/FM197H) and find that in all these RCs that the efficiency is less than 100%. For the YM210L RC time

gated absorbance difference spectra measured upon 798 nm excitation show that part of the primary charge separation takes place without the involvement of P^* .

Materials and methods

Construction of the YM210L and YM210F mutant strains of *Rb. sphaeroides* has been described in Beekman et al. (1996). The LM160H/FM197H double mutant was constructed by two sequential rounds of mismatch oligonucleotide-mediated site directed mutagenesis, using the procedures described by Beekman et al. (1996) and Vos et al. (1995). In all cases, the mutant RCs genes were expressed in a strain of *Rb. sphaeroides* that lacks the structural genes of the light-harvesting complexes giving a 'RC-only' (antenna deficient) strain (Jones et al. 1992). All strains were grown under semiaerobic conditions in the dark; the preparation of RC-only membranes for spectroscopic measurements was as described by Beekman et al. (1992).

The fluorescence excitation spectra at 77 K were measured essentially as described by van Brederode et al. (1997a), with the difference that the isotropic spectrum was calculated from the excitation spectrum with horizontally and vertically polarized excitation light. The excitation density used to measure the fluorescence excitation spectra was $< 150 \mu W / cm^2$.

Pump-probe measurements were performed as described by van Brederode et al. (1997b). Briefly, our excitation pulse was centered at 798 nm and had a width of ~ 10 nm. The excitation frequency of the laser was 30 Hz. The FWHM of the instrument response was ~ 350 fs.

Analysis of the data was performed as described by van Brederode et al. (1997b) with the addition that a correction for a wavelength dependence of time zero of the instrument response due to the group velocity dispersion was performed. To achieve this we analysed our transient CS_2 signal measured over the different wavelength windows simultaneously with a third order polynomial function to describe the variation of time zero at the different wavelengths. The polynomial function thus obtained was used to describe the time-zero variation for the pump-probe experiments. The time-gated spectra shown in Figure 2 have been corrected for group velocity dispersion and were reconstructed from the global analysis.

Results

Fluorescence excitation spectra

Figure 1A–C shows the 77 K fluorescence excitation spectra with Q_A in the neutral state for the YM210L, YM210F and LM160H / FM197H mutant RCs respectively, and compares the excitation spectrum (dashed) with the 1-transmission (1-T) spectrum (solid). For the YM210F and the LM160H / FM197H RCs the fluorescence excitation spectra with Q_A reduced are also shown (dotted). It can be concluded from the deviations between the 1-T spectra and the fluorescence excitation spectrum with Q_A in the neutral state that for all three RCs the efficiency of energy transfer from the monomeric pigments to P is not 100%. The amount of ‘missing’ fluorescence in the B and H bands of the fluorescence excitation spectrum is approximately 40% and 40%, respectively, for the YM210L RC, 40% and 30%, respectively, for the YM210F RC and 45% and 40%, respectively, for the LM160H / FM197H RC.

For the YM210F and the LM160H/FM197H RCs, reduction of Q_A leads to a partial restoration of the missing bands in the fluorescence excitation spectrum, in accord with previous observations for the YM210W mutant (van Brederode et al. 1997a). As argued previously, this result is a phenomenon which is expected when the H and B band excitations which are not transferred to P do in fact give rise to charge separation. Reduction of Q_A results in increased thermal repopulation of P^* from the state $P^+H_A^-$, independent of the mechanism that forms $P^+H_A^-$, and so to a relative increase in fluorescence from P^* following excitation of B and H. Given the large free energy difference between P^* and $P^+H_A^-$ on the long timescale (Ogrodnik et al. 1988), this recombination fluorescence probably mainly arises from unrelaxed $P^+H_A^-$ radical pairs (Woodbury et al. 1995) or from the top of the energetically distributed $P^+H_A^-$ population (Ogrodnik et al. 1994).

Pump-probe spectroscopy

The fate of the missing excitations in the B band was investigated in the YM210L RC by performing pump-probe spectroscopy, in which the RCs were excited on the blue side of the B absorbance band at 798 nm where mainly B_A is thought to absorb. In Figure 2 time gated absorbance difference spectra at 800 fs, 2 ps, 6 ps and 10 ps (Figure 2A) and at 15 ps, 30 ps, 80 ps and 250 ps (Figure 2B) are shown. The 800 fs absorbance difference spectrum shows that at

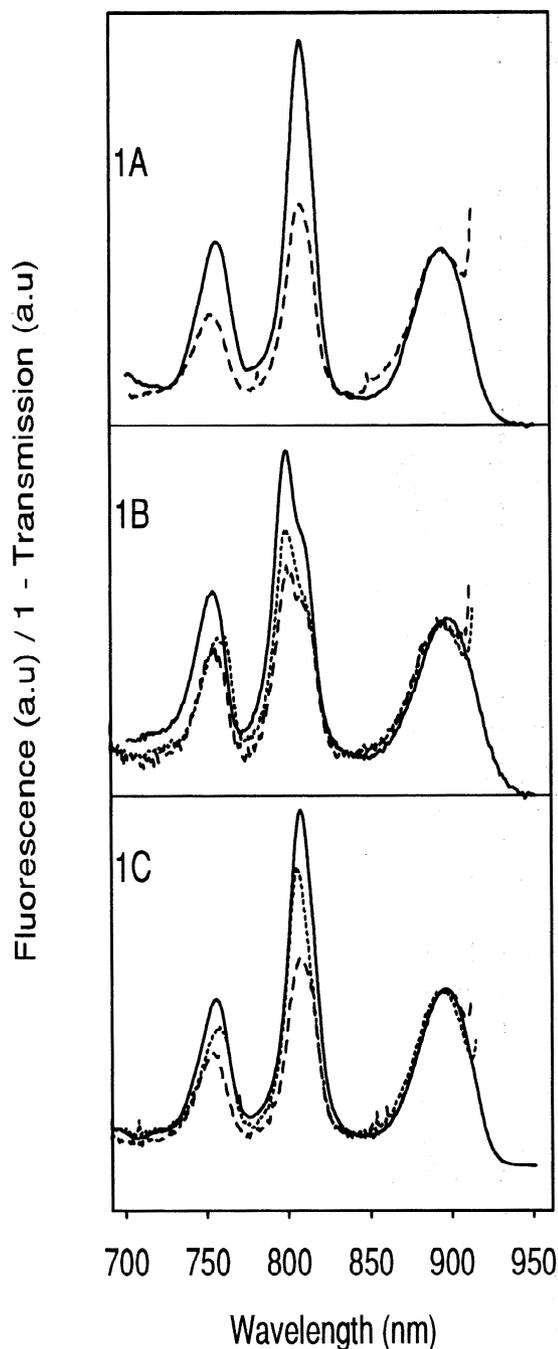


Figure 1. 77 K fluorescence excitation spectra of membrane bound mutant RCs from *Rb. sphaeroides* (1A, YM210L; 1B, YM210F; 1C, LM160H / FM197H). Solid line represents the 1-T spectrum obtained with Q_A in the neutral state, dashed line represents the fluorescence excitation spectrum with Q_A neutral, dotted line represents the fluorescence excitation spectrum with Q_A reduced. For all three RCs reducing of Q_A resulted in a small red-shift of the Bpheo Q_y absorbance band at 755 nm.

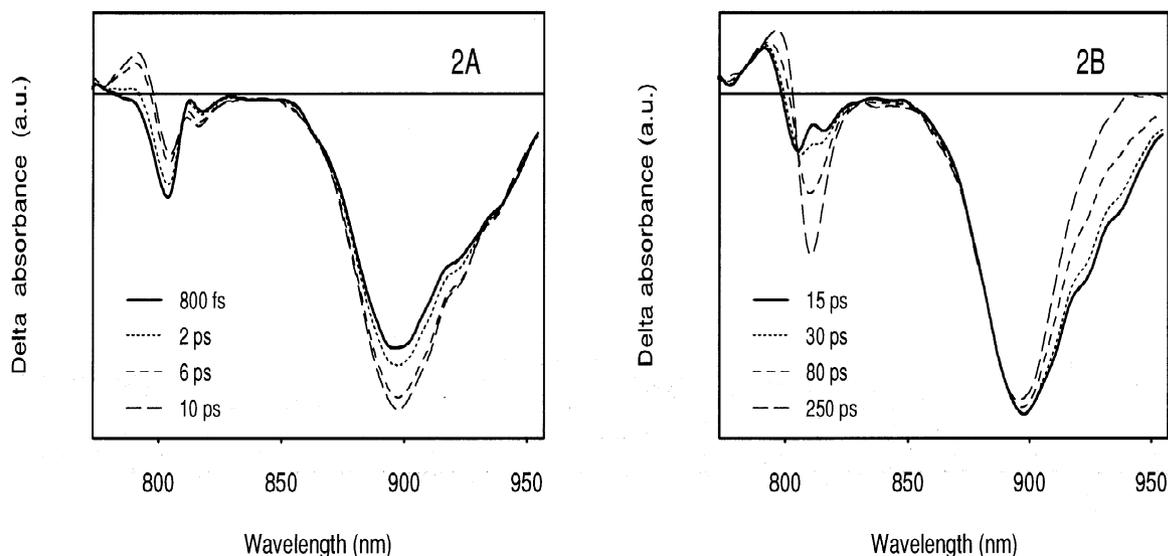


Figure 2. 77 K transient absorbance difference spectra of YM210L RCs obtained at 800 fs, 2 ps, 6 ps and 10 ps (A) and 15 ps, 30 ps, 80 ps and 250 ps (B) after 798 nm excitation.

a time when the ~ 180 fs energy transfer process is complete (see below), a strong bleach of the B absorbance band is present in addition to a bleach of the P groundstate absorbance band and the formation of P^* stimulated emission. This bleach of the B absorbance band is narrower and blue-shifted relative to the B absorbance band indicating that it is principally B_A that is bleached in this spectrum, probably due to the formation of B_A^+ or B_A^- . In the 2, 6 and 10 ps spectra a bandshift signal develops over the B absorbance band, which is characteristic for the formation of $P^+H_A^-$. That this $P^+H_A^-$ is not formed from P^* can be seen by examining the red side of the P^* stimulated emission region, where actually no change in the amount of stimulated emission is observed on this timescale. However, the development of the bandshift signal during the first 10 ps is accompanied by an extra bleach of the P groundstate absorbance band, which in our opinion reflects the reaction $B_A^+H_A^- \rightarrow P^+H_A^-$ (van Brederode et al. 1997b). The spectral evolution at later times (Figure 2B) shows the decay of P^* stimulated emission and further development of the bandshift signals in the B band region. From the fact that the P band bleaching signal does not diminish at longer time delays (Figure 2B) it follows that the yield of the final charge separated state $P^+Q_A^-$ is close to unity.

Discussion

The fluorescence excitation spectra of the YM210L, YM210F and LM160H / FM197H RCs, show that the efficiency of energy transfer from the monomeric pigments to P at 77 K is between 55 and 70% in these mutants. Pump-probe measurements at 77 K of the YM210L RC with excitation of the B absorbance band at 798 nm show that the excitations which are not transferred to P are efficiently used to perform charge separation. The characteristics of this alternative charge separation process are a bleach of the B absorbance band that persists after the relaxation of B^* , and the formation of the $P^+H_A^-$ bandshift signal accompanied by a further bleaching of the P groundstate absorbance spectrum in several picoseconds. The delayed bleaching of the P absorbance band strongly indicates that the reaction pathway $B^* \rightarrow B_A^+H_A^- \rightarrow P^+H_A^-$ takes place in this mutant (van Brederode et al. 1997b). Global analysis of the data (not shown) revealed a lifetime of less than 200 fs for the relaxation of B^* and a lifetime of 4.3 ps for the delayed P band bleaching and the early $P^+H_A^-$ bandshift formation. Decay of P^* and further development of the bandshift over the B band was associated with a time constant of 100 ps. Earlier pump-probe experiments of detergent isolated RCs in which the YM210 was replaced by isoleucine (YM210I) with excitation (at 605 nm) of the Q_x absorbance bands of both P and B also resolved

a 5 ps component in the P band region in addition to a slower decay of P* (Nagarajan et al. 1993). These authors interpreted this 5 ps component as a separate relaxation in which the stimulated emission shifted to shorter wavelengths. However, the global analysis by these authors showed that an extra ingrowth of P absorbance bleaching signal could also for a large part contribute to this component. The fact that also changes are observed in the stimulated emission region in the experiments of Nagarajan et al., whereas in our experiments there seems to be no change in this region on this time-scale, can be explained by the fact that some fraction of P is excited directly with Q_x band excitation at 605 nm. It has been reported by several groups that a strong evolution of the P* excited state spectrum can occur following direct excitation of P (Vos et al. 1995; Woodbury et al. 1996; van Brederode et al. 1997b). In this respect we can therefore conclude that our results are not in contrast with previous non-selective pump-probe experiments on YM210 mutants.

It was not possible to judge whether the pathway $B^* \rightarrow P^+B_A^- \rightarrow P^+H_A^-$ which was demonstrated to be operating in the YM210W RC (van Brederode et al. 1997b) is involved as a branching reaction, since we did not make a direct comparison with an 880 nm excitation experiment from which we could determine the ratio between the amount of P absorbance bleaching and formation of P* stimulated emission. Comparison with the YM210W experiments described by van Brederode et al. (1997b) shows that the amount of stimulated emission of the YM210L is comparable to that of the YM210W mutant after B band excitation. We therefore conclude that probably a significant fraction of the B_A excitations forms P⁺B_A⁻. However, due to an uncertainty in the precise scaling of the data recorded in the P-band and B-band in the experiments reported here, we can not make an estimation about the relative amounts for both pathways.

To close, the experiments presented in this report demonstrate the operation of an alternative pathway for transmembrane charge separation in a number of mutant *Rb. sphaeroides* RCs. This shows that the alternative pathways demonstrated in previous reports are not a peculiarity of the YM210W RC, but rather are a feature of the RC that can be revealed when the principal route of P* driven electron transfer is slowed down. The first step in the alternative pathway occurs on a sub-picosecond timescale, and it is very likely that this reaction proceeds from a vibrationally-unrelaxed excited state, which would mean that it is not pos-

sible to describe the reaction within the framework of non-adiabatic electron transfer theory. Our principal conclusion from this work, therefore, is that the process of charge separation catalysed by the bacterial RC is perhaps even more complex than originally thought.

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