4 April 1997

ELSEVIER

Chemical Physics Letters 268 (1997) 143–149

CHEMICAL PHYSICS LETTERS

Fluorescence excitation spectra of membrane-bound photosynthetic reaction centers of *Rhodobacter sphaeroides* in which the tyrosine M210 residue is replaced by tryptophan: evidence for a new pathway of charge separation

M.E. Van Brederode [a,*], M.R. Jones [b], R. Van Grondelle [a]

[a] *Department of Physics and Astronomy, Free University of Amsterdam, De Boelelaan 1081, 1081 HV Amsterdam, Netherlands*
[b] *Krebs Institute for Biomolecular Research and Robert Hill Institute for Photosynthesis, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2UH, UK*

Received 15 October 1996; in final form 28 January 1997

Abstract

The B_A and H_A absorption bands of membrane bound reaction centers of *Rhodobacter sphaeroides*, in which the tyrosine M210 residue has been replaced by tryptophan appear to be only weakly present in the fluorescence excitation spectrum of P^* emission. In contrast, these contributions are clearly observable in the $P^+Q_A^-$ excitation spectrum. Upon reduction of Q_A, the B_A and H_A bands in the excitation spectrum are largely recovered. These findings are discussed in terms of a model in which, following excitation of the B_A or H_A pigments a direct path exists for charge separation that does not involve the excited low-exciton state of P. Similar observations were made in two other mutants in which tyrosine M210 was replaced by leucine or phenylalanine.

1. Introduction

In photosynthesis, the conversion of solar energy into electrochemical energy that can be used by plants, algae and photosynthetic bacteria, occurs in membrane-bound pigment–protein complexes termed reaction centres (RCs). In photosynthetic bacteria such as *Rhodobacter (Rb) sphaeroides* it is generally assumed that this energy transduction involves the light-driven transfer of an electron from a pair of excitonically coupled bacteriochlorophyll molecules (primary donor: P) located on one side of the membrane to a molecule of ubiquinone located on the opposite side of the complex [1].

The protein of the RC is embedded in the bacterial cytoplasmic membrane and is composed of three subunits, H, L and M, that encase the cofactors that participate in the light-driven electron transfer reaction [2]. The L and M subunits each have 5 transmembrane α-helices and are related by an axis of approximately twofold symmetry. The primary electron donor, P, lies close to the periplasmic face of the protein. Two monomeric bacteriochlorophylls (B), two bacteriopheophytins (H) and two ubiquinones (Q) are arranged in two approximately symmetrical branches that span the membrane. Despite

* Corresponding author.

0009-2614/97/$17.00 Copyright © 1997 Elsevier Science B.V. All rights reserved
PII S0009-2614(97)00155-3

this symmetry, transmembrane electron transfer occurs almost exclusively along the branch of cofactors most closely associated with the L subunit (B_A, H_A, Q_A) [1,3].

It is a generally accepted that in photosynthetic RCs all of the light energy that is harvested by the antenna pigment-protein complexes is funnelled to P, creating the P^* state from which the primary photochemical electron transfer occurs. This view is based upon femtosecond transient absorption, spontaneous emission and stimulated emission experiments in which the P^* excited state was detected as an intermediate following light excitation [4,5]. In support of this, fluorescence excitation spectra of bacterial membranes containing both the reaction centre and the light-harvesting complexes have clearly shown the efficient conversion of the light energy absorbed by the antenna pigments into a charge separated state [6]. Since the major emission of core antennae is at 900 nm, the most likely intermediate in this process is P, which has an absorption maximum at approximately 865 nm and overlaps with the antenna absorption band. The monomeric bacteriochlorophyll (B) and bacteriopheophytin (H) pigments, with absorption maxima at 800 and 750 nm respectively, lie too high in energy to participate in this downhill energy transfer and trapping process. For isolated RCs, or in a photosynthetically competent antenna deficient strain [7], the widely accepted view is that light energy harvested by the accessory chlorins B_A, B_B, H_A, H_B is rapidly transferred downhill to the low exciton state of P (possibly involving the high exciton component), creating the state P^* that drives the electron transfer process [8,9].

An alternative view is that the pigments in the active branch behave as a supermolecule [10], and from quantum mechanical calculations on the crystal structure it has been suggested that charge separated states that do not involve P could be energetically favoured [11–13]. It has been proposed that these states participate in a charge separation reaction, in which the excited RC first gives rise to the charge separated state $PB_A^+H_A^-$ which in turn develops to the state $P^+B_AH_A^-$.

The current debate about the role of P^* in electron transfer has focused on the mixing of low exciton states with either an internal charge transfer state or a charge transfer state involving the radical pair $P^+B_L^-$ [14–16]. Femtosecond experiments on the so-called heterodimer RCs of *Rb. sphaeroides* [14], which have a bacteriochlorophyll:bacteriopheophytin primary donor, and infrared experiments on wildtype (WT) RCs [17] have both provided evidence for the early participation of an internal charge transfer state in the primary charge separation. However, little is known about the participation of other charge transfer states of the RC in the charge separation process.

In this work we have examined whether the P^* state is an obligate intermediate in the light driven electron transfer in bacterial RCs, by investigating the influence of excitation wavelength on the relative efficiency of energy transfer to P, and of the formation of the charge separated state $P^+Q_A^-$. The experimental system consisted of a mutant reaction center (YM210W) expressed in an antenna-deficient strain of *Rb. sphaeroides*. It has been shown that as a result of this mutation the observed time constant associated with the charge separation from P^* increases from 4.5 to 72.5 ps at room temperature with Q_A in the neutral state [18], comparable with results obtained by others on detergent solubilised RCs [19,20]. At cryogenic temperatures with reduced Q_A the decay of P^* in membrane-bound YM210W RCs is 400/320 ps and the quantum yield of charge separation has decreased to 25/50% due to the long lifetime of P^* [21,22]. The long lifetime of P^* and the reduced yield of charge separation makes the YM210W mutant a good candidate for comparing fluorescence excitation of P^* emission spectra with action spectra for the formation of $P^+Q_A^-$, to determine whether the P^* state is a prerequisite for charge separation in *Rb. sphaeroides* RCs.

2. Materials and methods

2.1. Sample preparation and mutant construction

Mutant construction, protocols for the growth of *Rb. sphaeroides* RC-only strain and the preparation of membranes for spectroscopy have been described previously [7,23]. Aliquots of concentrated membranes were suspended in a 50 mM potassium phosphate 70% (v/v) glycerol buffer. All experiments

were performed in 1 cm cuvettes. The sample concentration was adjusted to have an OD at 807 nm of 0.22 for the fluorescence excitation experiments and 0.25 for the $P^+Q_A^-$ action spectrum. The reduction of Q_A for the fluorescence excitation spectra in Fig. 4 was performed with 25 mM sodium dithionite.

2.2. Fluorescence excitation spectra

Fluorescence excitation spectra were performed on a home-built spectrophotometer. The light source was a 150 W halogen lamp passed through a monochromator (Chromex 250 SM, the spectral resolution of the excitation monochromator was 3.2 nm). The fluorescence emission was collected perpendicular to the excitation and detected through a bandpass filter with a maximum emission at 930 nm (10 nm FWHM) or for the experiments in which only the spectral region between 500 and 550 nm was investigated through a 910 highpass filter. The detection of the fluorescence light was with a cooled S1 photomultiplier tube (Thorn EMI 9206B) using the single photon counting technique. The fluorescence excitation light was polarized at the magic angle (55°) relative to the horizontal polarization. The detection light was gathered unpolarized. The transmission of the bandpass filter was equal for horizontally and vertically polarized light. In this configuration isotropic fluorescence excitation spectra are recorded as can be calculated from the expression of the anisotropy r

$$r = \frac{F_{VV} - F_{VH}}{2F_{VH} + F_{VV}} \tag{1}$$

In this expression F stands for fluorescence emission and the first subscript indicates the polarization of the excitation light and the second the orientation of the detected fluorescence emission. The isotropic, F_{iso} spectrum equals the denominator in Eq. 1. Expressing F_{VV} and F_{VH} in F_{iso} and r gives:

$$F_{VV} = F_{iso}\left(\tfrac{1}{3} + \tfrac{2}{3}r\right) \tag{2}$$

$$F_{VH} = F_{iso}\left(\tfrac{1}{3} - \tfrac{1}{3}r\right) \tag{3}$$

F_{HH} and F_{HV} have the same three-dimensional configuration as F_{VH} and therefore can be expressed in a formula equal to Eq. 3. Measuring in a configuration in which two times as much vertically polarized as horizontally polarized excitation light is used (55° relative to horizontal polarization), will thus give an isotropic spectrum. The fluorescence excitation spectra were corrected for the power spectrum of the excitation light.

2.3. $P^+Q_A^-$ excitation spectra

Reversible light induced $P^+Q_A^-$ absorption difference spectra were performed on an apparatus essentially as described in Ref. [24]. In brief, excitation light (λ = 749–930, 200–1000 $\mu W/cm^2$) was provided by a Ti:sapphire laser pumped by an Ar-ion laser. Probe light was provided by a 150 W halogen lamp in combination with a monochromator. Probe and excitation beams were at 90° and the probe beam was polarized at the magic angle relative to the vertically polarized excitation light. The probe light was modulated at 100 kHz by a photoelastic modulator placed between two polarizers. The excitation beam was chopped at 28 Hz. The transmission and the change in transmission due to the laser excitation were recorded using double lock-in detection of the transmission. The delta OD was calculated according to $c\Delta I/I$, where I is the probe light intensity and ΔI is the difference in intensity of the probe light between the transmission with and without the excitation light on. The relative yield of $P^+Q_A^-$ formation was estimated from the integral of the bleaching of the long wavelength P absorption band. Care was taken to ensure that the experiment was performed in a region were the signal was linear with respect to the excitation energy.

3. Results

The isotropic fluorescence excitation spectrum of the YM210W mutant at 77 K with Q_A in the neutral state is shown in Fig. 1. Surprisingly, the fluorescence excitation spectra shows dramatic deviations from the absorption spectrum in the Q_y region, with the B band (at 807 nm) and the H band (at 755 nm) not being present to the expected full extent. In the B

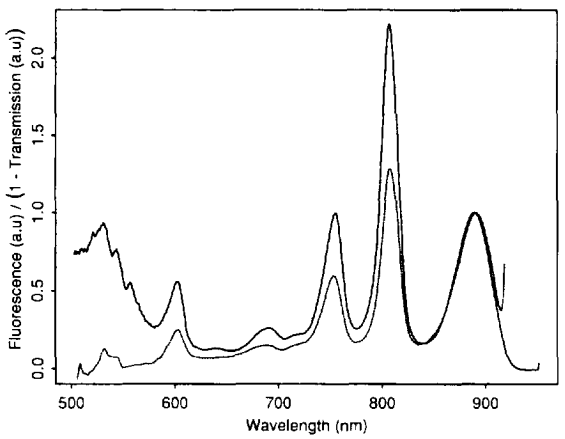

Fig. 1. 1 − Transmission (T) spectrum (solid line) and fluorescence excitation spectrum of RC-only membranes from the YM210W mutant of *Rb. sphaeroides* (dotted line), obtained at 77 K under conditions where Q_A was not pre-reduced. For the purpose of comparison the spectra are normalized on the lower exciton component of the Q_y transition of P. The intensity of the B (Q_y) band (in the excitation spectrum at 807.5 nm) is approximately 57% of that in the 1 − T spectrum and the maximum is shifted by approximately 1 nm to the red compared to the absorption spectrum. The intensity of the H(Q_y) band is reduced to 60% in the excitation spectrum and shows a blue shift of approximately 2 nm (752.5 versus 754.5 nm). At wavelengths below 650 nm direct comparison of the spectra is complicated due to the presence of the carotenoid (neurosporene) in the RC and excess carotenoid in the membrane.

band the peak of the excitation spectrum is slightly red-shifted to the absorption spectrum, whilst in the H band there is a similar blue shift. The simplest interpretation of this is that the pigments located in the active branch (B_A and H_A) [25–27] are missing in the excitation spectrum. Essentially the same features can be seen in the fluorescence excitation spectrum reported for detergent isolated YM210W RCs, although this was not noted by the authors [20].

An insight into the fate of the excitations that are absorbed by B and H but not transferred to P comes from the action spectrum of $P^+Q_A^-$ formation (Fig. 2), which is similar to the absorption spectrum with the B and H bands being present to their normal extent. This experiment reveals that in the YM210W mutant there is efficient $P^+Q_A^-$ formation without involvement of the P^* state. Further support of this comes from the data in Fig. 3, which shows the fluorescence excitation spectrum of YM210W under conditions where Q_A is reduced, compared with the

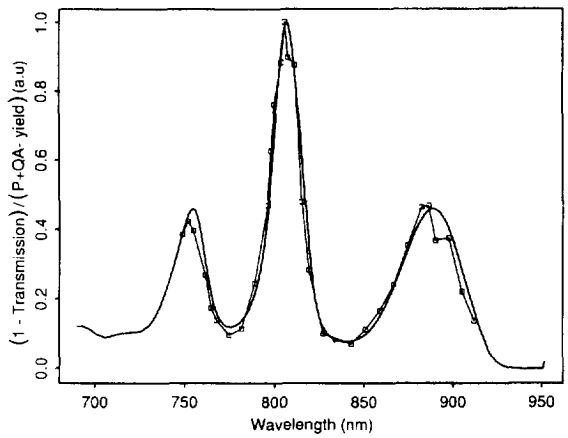

Fig. 2. 1 − Transmission spectrum (solid line) and action spectrum of the formation of the radical pair state $P^+Q_A^-$ (squares) in RC-only membranes of the YM210W mutant at 77 K. The spectra are normalized to the 806 nm band.

absorption spectrum and the fluorescence excitation spectrum with Q_A not pre-reduced. The effect of reducing Q_A is to approximately double the level of fluorescence emission, the extra emission being at-

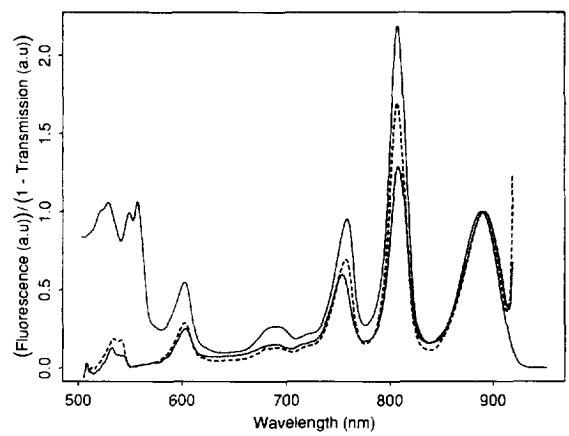

Fig. 3. 1 − Transmission spectrum (solid line) and fluorescence excitation spectrum (dashed) of RC only membranes of the YM210W mutant at 77 K under conditions in which Q_A is pre-reduced eliciting a 3 nm electrochromic red-shift of the H Q_y absorption band. For comparison the fluorescence excitation spectrum from Fig. 1, in which Q_A was not pre-reduced is shown (dotted). All spectra are normalized on the Q_Y transition of P. The intensities of the B and H bands in the fluorescence excitation spectrum with reduced Q_A have increased to 75 and 73% respectively. This increase is attributable to thermal repopulation of P^* from $P^+H_A^-$.

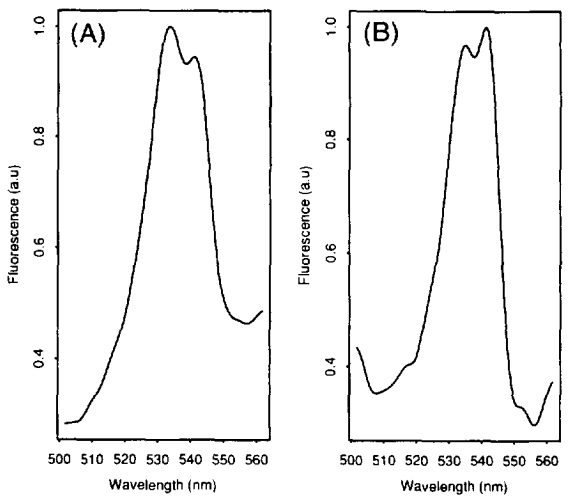

Fig. 4. (A) Fluorescence excitation spectrum in the H Q_x band of RC only YM210W membranes at 77 K without pre-reduction of Q_A. (B) Fluorescence excitation spectrum in the H Q_X band of RC only YM210W membranes at 77 K under conditions in which Q_A is reduced. Under conditions in which Q_A is not pre-reduced fluorescence is mainly observed upon excitation of H_B at 533 nm. The effect of reducing Q_A is to elicit an increase in intensity of the excitation spectrum at 541 nm, the location of a band in the absorption spectrum that is attributed to the H_A bacteriopheophytin.

tributable to thermal repopulation of P^* from the $P^+H_A^-$ state when forward electron transfer from $P^+H_A^-$ to $P^+Q_A^-$ is blocked by the chemical reduction of Q_A. As can be seen in Fig. 3 the H and B bands in the fluorescence excitation spectrum are partially restored in comparison to their extent in Fig. 2. This is what would be expected if excitation of the B_A and H_A pigments were translated into a charge separated state.

To confirm that it is really contributions from the B and H pigments in the active branch which are partially missing in the excitation spectrum, we measured excitation spectra in the Q_X region of the bacteriopheophytins with detection of the whole fluorescence emission of P^*. In this region the absorption of the active (H_A) and inactive (H_B) branch pigments is nicely separated. The fluorescence excitation spectrum in Fig. 4A is dominated by a contribution from H_B at 533 nm, whilst at 541 nm, where the contribution of H_A is expected, only a shoulder on the 533 nm band is observed. This contrasts the absorption spectrum of the YM210W mutant expressed in the R-26 carotenoid-less strain in which this relation is just the opposite with the absorption of H_A being more intense than the absorption of H_B [20]. In Fig. 4B it is strikingly shown that pre-reduction of Q_A leads to a partial restoration of the H_A Q_X band at 541 nm. Again, this indicates that the excitation of H_A is directly (without involvement of P^*) translated into a charge separated state, which with Q_A reduced gives rise to increased emission from P^* via thermal repopulation from the $P^+H_A^-$ state.

Similar experiments were performed with two other mutated bacterial RCs (YM210L and YM210F) with Q_A in the neutral state, yielding similar results to those reported here for YM210W at 77 K.

4. Discussion

In this Letter we have used *Rb. sphaeroides* RC carrying the mutation YM210W to show that the formation of the charge separated states $P^+Q_A^-$ and $P^+H_A^-$ can be established without the involvement of P^*. The absence of a major fraction (about 40%) of the H and B Q_y bands in the fluorescence excitation spectrum of P and their presence in the $P^+Q_A^-$ excitation spectrum can only be explained on the basis of this hypothesis. The rate of decay of the B_A/H_A excited state into a charge separated state (or a state leading to fast charge separation) must be of the same order of magnitude as the energy transfer rate from $B_{A,B}$ to P, which is about 100 fs [8,9], to explain the approximately 60% quantum yield of the alternative pathway(s). Earlier measurements on the formation of intradimer charge transfer states in RCs of purple bacteria have indicated rates of the same order of magnitude [14,17] and consequently are not impossible.

At the moment we do not know whether or not the same process also occurs in WT RCs. In at least two other mutants of *Rb. sphaeroides* RCs (YM210L and YM210F) we have observed a similar absence of the B_A, H_A bands in the fluorescence excitation spectrum with neutral Q_A, and in both mutants these bands were partly restored upon the reduction of Q_A. For WT RCs fluorescence excitation spectra have been recorded, which were interpreted to show efficient energy transfer for the major part of the RCs

and may suggest that the new pathway does not contribute significantly to charge separation in WT RCs. A small fraction of RCs exhibiting slow charge separation, had a relatively blue shifted excitation spectrum in both the B and P bands and this was correlated with a possible slow superexchange mediated electron transfer from energetically high-lying states of P^* [28,29]. So far, we see no obvious connection between the blue-shifted minor fraction of WT RCs of *Rb. sphaeroides* and the new path of charge separation from B_A, H_A proposed by us to occur in YM210W and other mutants. Time resolved experiments are necessary to reveal the importance of this new pathway in mutant and WT RCs. Furthermore, we wish to stress that in WT RCs a large fraction of the fluorescence arises from slow components in the fluorescence decay and these probably are strongly mixed with fluorescence that originates from the dynamic equilibrium between $P^+H_A^-$ and P^* [30,31]. In that case both pathways will contribute equally to that fraction of the observed fluorescence.

The observation that charge separation in bacterial RCs can be driven from the singlet excited states of the H_A and/or B_A pigments without the involvement of P^* is intriguing because it indicates that there is at least one alternative route for charge separation in addition to the heavily studied reaction driven by P^*. In this context, it should be noted that a route of electron transfer to the $P^+H_A^-$ state involving the conversion of $[PB_AH_A]^*$ into $B_A^+H_A^-$ and then hole transfer from B_L^+ to P has been proposed [11–13]. Our findings may also have implications for energy transduction in photosystem II RCs (PSII). Despite clear indications of a strong structural similarity between PSII and bacterial RCs [32,33], it seems unlikely that the primary donor in PSII (P680) is a dimer of chlorophyll molecules. Rather, it may be that a description based on a multimer of (weakly) coupled pigments is more appropriate for PSII. Calculations for the PSII RC suggest that the two lowest states in the RC are, on average, localized on the pigments of either the active branch or the inactive branch [34]. The first state may be identified as "P680", while the second state is the so-called "trap" state, responsible for the low-temperature fluorescence of PSII RCs [35]. In this view charge separation is always driven from an excited state that involves both the monomeric chlorophyll and the pheophytin in the active branch. In this context, it would appear that our findings on bacterial RCs provide a precedent for charge separation through a mechanism that does not involve the excited state of a dimer.

Acknowledgements

This research is supported by the Dutch Foundation for Fundamental Research (NWO) through the foundation for Life Sciences (SLW) and EC contract CT930278. MRJ is a Biotechnology and Biological Sciences Research council Senior Research Fellow.

References

[1] N.W. Woodbury and J.P. Allen, in: Anoxygenic Photosynthetic Bacteria, eds. R.E. Blankenship and C.E. Bauer (Kluwer Academic Publishers, Dordrecht, 1995) p. 527.
[2] J.P. Allen, G. Feher, T.O. Yeates, H. Komiya, and D.C. Rees, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 5730.
[3] B.A. Heller, D. Holten, and C. Kirmaier, Science 269 (1995) 940.
[4] J. Breton, J.L. Martin, G.R. Fleming, and J.-C. Lambry, Biochemistry 27 (1988) 8276.
[5] M. Du, S. Rosenthal, X. Xie, T.J. DiMagno, M. Schmidt, D.K. Hanson, M. Schiffer, J.R. Norris and G.R. Fleming, Proc. Natl. Acad. Sci. USA 89 (1992) 8517.
[6] L.N.M. Duysens, Thesis, State University of Utrecht (1953).
[7] M.R. Jones, R.W. Visschers, R. Van Grondelle and C.N. Hunter, Biochemistry 31 (1992) 4458.
[8] R.J. Stanley, B. King and S.G. Boxer, J. Phys. Chem. 100 (1996) 12052.
[9] D.M. Jonas, M.J. Lang, Y. Nagasawa, T. Joo and G.R. Fleming, J. Phys. Chem. (1996) 12660.
[10] M. Bixon, J. Jortner, M.E. Michel-Beyerle and A. Ogrodnik, Biochim. Biophys. Acta 977 (1989) 273.
[11] S.F. Fischer, and P.O.J. Scherer, Chem. Phys. 115 (1987) 151.
[12] A. Warshel, S. Creighton and W.W. Parson, J. Phys. Chem. 92 (1988) 2696.
[13] P.O.J. Scherer and S.F. Fischer, Chem. Phys. 131 (1989) 115.
[14] L.M. McDowell, C. Kirmaier and D. Holten, Biochim. Biophys. Acta, 1020 (1990) 239.
[15] K. Wynne, G. Haran, G.D. Reid, C.C. Moser, P.L. Dutton and R.M. Hochstrasser, J. Phys. Chem. 100 (1996) 5140.
[16] G. Haran, K. Wynne, C.C. Moser, P.L. Dutton and R.M. Hochstrasser, J. Phys. Chem. 100 (1996) 5562.
[17] P. Hamm, and W.J. Zinth, Phys. Chem. 99 (1995) 13537.

[18] L.M.P. Beekman, I.H.M. Van Stokkum, R. Monshouwer, A.J. Rijnders, P. McGlynn, R.W. Visschers, M.R. Jones and R. Van Grondelle, J. Phys. Chem. 100 (1996) 7256.
[19] V. Nagarajan, W.W. Parson, D. Davis and C.C. Schenck, Biochemistry 32 (1993) 12324.
[20] S. Shochat, T. Arlt, C. Francke, P. Gast, P.I. Van Noort, S.C.M. Otte, H.P.M. Schelvis, S. Schmidt, E. Vijgenboom, J. Vrieze, W. Zinth and A.J. Hoff, Photosynth. Res. 50 (1994) 55.
[21] M.E. Van Brederode, L.M.P. Beekman, D. Kuciauskas, M.R. Jones, I.H.M. Van Stokkum and R. Van Grondelle, in: The Reaction Center of Photosynthetic Bacteria, ed. M.E. Michel-Beyerle (Springer, Berlin-Heidelberg, 1996) p. 225.
[22] M.H. Vos, M.R. Jones, J. Breton, J.-C. Lambry and J.L. Martin. Biochemistry 35 (1996) 2687.
[23] M.R. Jones, M. Heer-Dawson, T.A. Mattioli, C.N. Hunter and B. Robert, FEBS Lett. 339 (1994) 18.
[24] S.L.S. Kwa, S. Völker, N.T. Tilly, R. Van Grondelle and J.P. Dekker. Photochem. Photobiol. 59(2) (1994) 219.
[25] C. Kirmaier and D. Holten. Photosynth. Res. 13 (1987) 225.
[26] R. Van Grondelle, J.C. Romijn and N.G. Holmes, FEBS Lett. 72 (1976) 187.
[27] B. Robert, M. Lutz and D.M. Tiede FEBS Lett. 183 (1985) 326.
[28] G. Hartwich, M. Friese, H. Scheer, A. Ogrodnik, M.E. Michel Beyerle. Chem. Phys. 197 (1995) 423.
[29] G. Hartwich, H. Lossau, A. Ogrodnik and M.E. Michel-Beyerle, in: The Reaction Center of Photosynthetic Bacteria, ed. M.E. Michel-Beyerle (Springer, Berlin–Heidelberg, 1996) p. 199.
[30] M.G. Müller, K. Griebenow and A.R. Holzwarth, Chem. Phys. Lett. 199 (1992) 465.
[31] A.R. Holzwarth and M.G. Müller. Biochemistry 35 (1996) 11820.
[32] H. Michel and J. Deisenhofer, Biochemistry 27 (1988) 1.
[33] B. Svensson, I. Vass, E. Cedergren and S. Styring, EMBO J. 9 (1990) 2051.
[34] J.R. Durrant, D.R. Klug, S.L.S. Kwa, R. Van Grondelle. G. Porter and J.P. Dekker, Proc. Natl. Acad. Sci. USA 92 (1995) 4798.
[35] M.L. Groot, E.J.G. Peterman, P.J.M. Van Kan, I.H.M. Van Stokkum, J.P. Dekker and R. Van Grondelle, Biophys. J. 67 (1994) 318.