Biochimica et Biophysica Acta, 681 (1982) 375–382
Elsevier Biomedical Press

BBA 41133

STUDY OF THE LONG-WAVELENGTH FLUORESCENCE BAND AT 920 nm OF ISOLATED REACTION CENTERS OF THE PHOTOSYNTHETIC BACTERIUM *RHODOPSEUDOMONAS SPAEROIDES* R-26 WITH FLUORESCENCE-DETECTED MAGNETIC RESONANCE IN ZERO FIELD

H.J. DEN BLANKEN, G.P. VAN DER ZWET and A.J. HOFF

Center for the Study of the Exited States of Molecules and Department of Biophysics, Huygens Laboratorium, State University of Leiden (The Netherlands)

(Received February 23rd, 1982)

Key words: Bacterial photosynthesis; Reaction center; Bacteriochlorophyll fluorescence; FDMR; (Rps. sphaeroides)

The triplet state of isolated reaction centers of *Rhodopseudomonas sphaeroides* R-26 has been studied by fluorescence-detected electron spin resonance in zero magnetic field (FDMR) at 4.2 K. The sign of the FDMR resonance monitored at the long-wavelength fluorescence band is positive (fluorescence increase); this confirms the earlier interpretation (Hoff, A.J. and Gorter de Vries, H. (1978) Biochim. Biophys. Acta 503, 94–106) that the negative sign of the FDMR resonance of the reaction center triplet state in whole bacterial cells is caused by resonant transfer of the singlet excitations from the antenna pigments to the trap. By monitoring the FDMR response as a function of the wavelength of fluorescence, we have recorded microwave-induced fluorescence spectra. In addition to the positive microwave-induced fluorescence band peaking at 935 nm, at 905 nm a negative band was found. The resonant microwave frequencies for these two bands, i.e., the values of the zero-field splitting parameters $|D|$ and $|E|$ of the triplet state being monitored, were different, those of the 905 nm microwave-induced fluorescence band being identical to the resonant microwave frequencies measured with absorption-detected zero-field resonance (Den Blanken, H.J., Van der Zwet, G.P. and Hoff, A.J. (1982) Chem. Phys. Lett. 85, 335–338), a technique that monitors the bulk properties of the sample. From this result and its negative sign, we tentatively attribute the 905 nm microwave-induced fluorescence band to a small (possibly less than 1%) fraction of antenna bacteriochlorophylls that are in close contact with the trap. The positive 935 nm microwave-induced fluorescence band with resonant microwave frequencies deviating from the bulk material is ascribed to a minority of primary donor bacteriochlorophyll dimers, which have a higher than normal fluorescence yield because of a somewhat slower charge-separation reaction. Is it likely that practically all long-wavelength fluorescence of isolated reaction centers stems from such impaired reaction centers.

Introduction

In reaction centers in the state PIX^- (P, primary donor; I, intermediate acceptor; and X, primary stable acceptor) the triplet state P^TIX is formed after optical excitation and charge separation as a result of the back reaction

$$P^+I^-X^- \rightarrow P^TIX^-$$

This triplet, first detected by electron spin resonance (ESR) by Dutton et al. [1] and later by fast optical absorption measurements [2], may be used

Abbreviations: ADMR, optical absorption-detected magnetic resonance; FDMR, fluorescence-detected magnetic resonance; ODMR, optically detected magnetic resonance; BChl; bacteriochlorophyll.

as a probe for the structure of the primary donor and its geometrical relationship with its accessory pigments in the reaction center, and with the antenna pigments outside the reaction center. One of the techniques employed for its study is fluorescence-detected magnetic resonance in zero magnetic field (FDMR) [3,4]. The FDMR spectrum was interpreted according to the model of Fig. 1 [5]: the reaction center in the triplet state forms photochemically a closed trap, because photochemistry occurs from the singlet, and not from the triplet excited state. Experimentally, it has been demonstrated that the triplet state does not form a deep excitation trap for antenna in the excited singlet state [6]. Thus, a decrease in P^T concentration caused by the application of microwaves resonant between the triplet sublevels in zero field corresponds to an increase in the concentration of quenching traps and hence to a decrease in antenna-fluorescence yield [5,6].

It is desirable to test the above interpretation with FDMR experiments on isolated reaction centers without antenna-pigment complement. Then one expects that a decrease in the P^T concentration corresponds to an increase in the concentration of ground state P, and hence to an increase in reaction center fluorescence [7]. Thus, in going from the intact system to isolated reaction centers, one would expect the FDMR transitions to occur at the same frequencies (one is monitoring the same triplet state), but with reversed sign.

The experimental results corroborate only partly the above expectations. Monitoring the FDMR transitions of isolated reaction centers as a function of the detecting fluorescence wavelength in the reaction center long-wavelength fluorescence band [9], we find that the 920 nm fluorescence band in open reaction centers shows the expected increase in fluorescence, but in closed reaction centers a fluorescence band at 905–910 nm appears. Moreover, we measure distinctly different values of zero-field splitting parameters $|D|$ and $|E|$ for the 920 nm fluorescence band in open reaction centers and the new fluorescence band at 905 nm in closed reaction centers.

We checked the interpretation of Hoff and Gorter de Vries [5] that resonant microwaves cause a decrease in P^T concentration, by measuring the fluorescence response in intact cells and isolated reaction centers to an increase in P^T concentration resulting from illumination by an intense laser flash.

We conclude that:

(i) resonant microwaves cause a decrease in P^T concentration;

(ii) the 920 nm fluorescence band in open reaction centers belongs to a complex that contains a triplet state, and;

(iii) the new fluorescence band at 905 nm in closed reaction centers belongs to a complex with energy transfer to another complex containing a triplet state.

Our experimental results are discussed taking into account the results from optically detected magnetic resonance (ODMR) experiments monitoring the absorption instead of the fluorescence, which are presented in an accompanying paper [9]. The FDMR effect on the fluorescence of open reaction centers is due to a minority of reaction centers that fluoresce as a result of impaired charge separation. It is likely that almost all of the long-wavelength fluorescence of isolated reaction centers stems from this source, and that the bulk of intact reaction centers do not fluoresce, or only very slightly. The source of the FDMR signal observed at 905 nm is at present unclear; possibly a very small fraction of antenna BChl is responsible for the 905 nm fluorescence in closed reaction centers.

Materials and Methods

The reactions centers were prepared according to Feher and Okamura [10]. The reaction centers were passed twice over a DEAE column. The first time they were eluted with 0.12 M NaCl in TL buffer *, the reaction centers then had an A_{280}/A_{800} ratio of 1.26–1.30. In the second pass, they were eluted with an NaCl gradient of 0.04–0.2 M in TL buffer, the reaction centers then had an A_{280}/A_{800} ratio of 1.16. The spectra of the reaction centers were identical to published spectra [10]. As a conservative estimate we conclude that less than 10% of the reaction centers might still have an antenna-BChl complement.

Reduction of the iron-ubiquinone acceptor was

* TL buffer: 10 mM Tris-HCl, pH 8, 1 mM EDTA, 0.1% lauryldimethylamine *N*-oxide.

carried out under N_2 atmosphere by adding excess solid sodium dithionite at pH 8. After 2-fold dilution with ethylene glycol, the samples (A 0.3 in 3 mm at 375 nm, pH 8) were frozen slowly to 77 K.

FDMR experiments were carried out, as described by Van Dorp et al. [7] and Hoff [4], in a four-window liquid helium immersion cryostat at atmospheric or reduced pressure corresponding to a temperature of 4.2 and 1.2 K, respectively. The samples were excited continuously by broad banded light from a high-pressure 200 W Oriel mercury arc lamp, filtered by 10 cm water, Corning CS-7-51, Schott BG 38 and KG 3, resulting in a band width of 70 nm centered at 365 nm. The incident energy was approx. 500 mW/cm^2. The fluorescence was detected in a 90° configuration via KV 550, Schott RG 715 cutoff filters, a Bausch and Lomb (f/4.4, 740 nm blaze, 1200 g/mm) 0.5 m monochromator and an EMI 9684 B photomultiplier with S1 cathode cooled to −50°C. FDMR spectra were recorded using amplitude modulation of the microwaves (665 Hz, HP switch 33016 B) and lock-in detection (PAR 189 + PAR 5101). They were averaged 16 times (HP 5480 A CAT). The microwaves were generated by an HP 8690 B sweep oscillator with an 8699 B insert and amplified by a Varian solid-state amplifier VSP-7435-KL-496 to a level of 1 W. The frequency was measured by an HP 5246 L counter with an HP 5245 C frequency converter. No corrections were made for the spectral sensitivity of the detection setup (filters, monochromator, photomultiplier).

The flash experiments to establish the fluorescence change corresponding to an increase in P^T concentration were performed at 77 K. Submicrosecond flashes were produced by a Lambda Physik excimer laser (EMG 102) and dye laser (FL 2000) combination (repetition rate 10 Hz, 385 nm, dye LC 3860). The fluorescence response was averaged 2^{13} times.

Results

In Fig. 2 we show the fluorescence spectrum of intact cells and isolated reaction centers in the state PIX^-. The isolated reaction centers fluoresce further to the red than the antenna-BChl component in intact cells, indicating that the fluorescence of the isolated reaction centers originates mainly, if not uniquely, from the reaction center pigments. In Fig. 3 we show the change in fluorescence, corresponding to a decrease in P^T concentration, effected by the application of microwaves resonant between two of the triplet substates. This type of

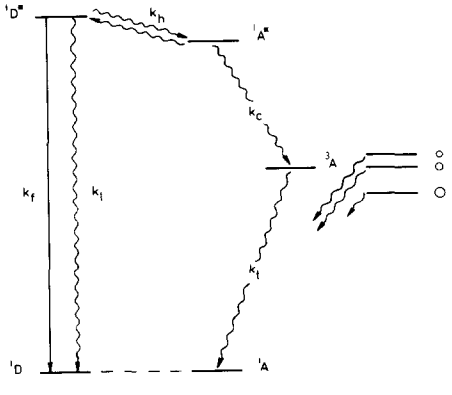

Fig. 1. Energy-level scheme of two interacting pigments: a donor (D) and an acceptor (A). Between D and A resonant singlet energy transfer is possible. In the triplet state the coupling between the pigments is much weaker than in the singlet excited state and the triplet is localized on the acceptor molecule. k_f, k_h, k_c, k_1 denote the rate of fluorescence, energy transfer, singlet-to-triplet conversion, and loss processes other then fluorescence or energy transfer, respectively. The relative population, and the decay rates of the three triplet sublevels of ^3A are indicated by open circles and arrows, respectively.

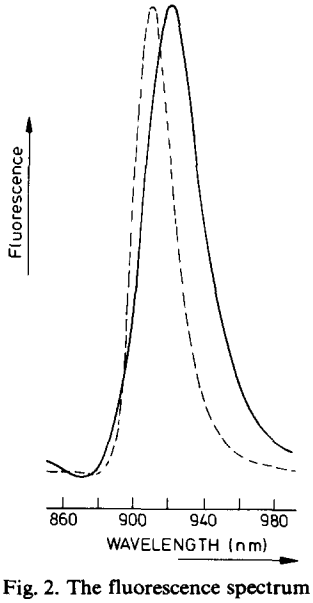

Fig. 2. The fluorescence spectrum of whole cells (— — —) and of isolated reaction centers (———) at 4.2 K. The optical resolution is 6 nm; the fluorescence was detected as described for the FDMR experiments in the text.

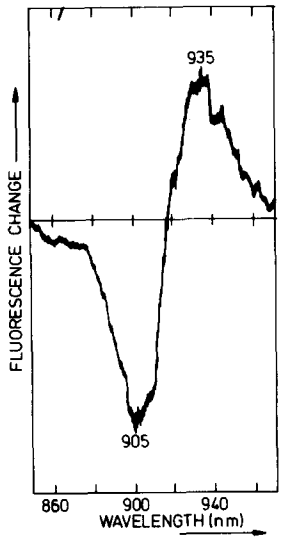

Fig. 3. The microwave-induced fluorescence spectrum of isolated reaction centers. The microwave frequency was 468 MHz; the optical resolution was 13 nm.

spectrum is called the microwave-induced fluorescence spectrum [11]. The change of sign in this type of spectrum proves that the microwave-induced fluorescence spectrum of isolated reaction centers is caused by more than one optical transition.

To check the FDMR results, we have measured the change in fluorescence yield as a result of a submicrosecond laser flash, superimposed on continuous illumination by a mercury arc lamp. This type of spectrum we call the flash-induced fluorescence spectrum. The laser flash gives a transient increase in the triplet state concentration. Therefore, one can unambigiously determine whether with FDMR one is monitoring the fluorescence of a pigment that transfers energy to the complex on which the triplet is located or the fluorescence of the species on which the triplet is created. In the former case, the flash-induced fluorescence experiment yields an increase in fluorescence, in the latter case the fluorescence decreases. In agreement with the FDMR data, we find that at 77 K in intact cells, an increase in the fluorescence yield of antenna BChl corresponds to an increase in P^T concentration [6]. In contrast, in isolated reaction centers at 77 K an increase in P^T concentration yields a decrease in fluorescence at the red side of the 920 nm fluorescence band. At 4.2 K an increase in P^T concentration corresponds to an increase in fluorescence yield at the blue side of the 920 nm fluorescence band of isolated reaction centers. At 77 K the increase in the fluorescence at the blue side in isolated reaction centers was too small for a reliable measurement.

In Fig. 4 we show the FDMR transitions at the blue and the red side of the 920 nm fluorescence band in isolated reaction centers. It is seen that the

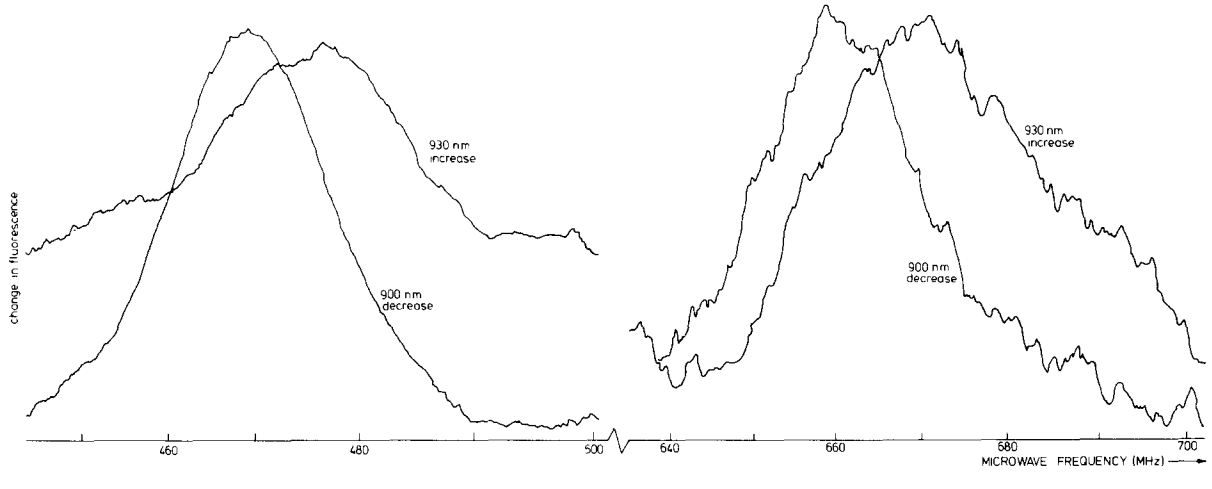

Fig. 4. The resonant microwave transitions between the triplet sublevels of isolated reaction centers monitoring the 900 and 930 nm fluorescence with an optical resolution of 13 nm. The 930 nm fluorescence shows an increase and the 900 nm fluorescence shows a decrease by the application of resonant microwaves; for ease of comparison the 900 nm band has been inverted.

TABLE I

FDMR AND ADMR FREQUENCIES OF WHOLE CELLS AND ISOLATED REACTION CENTERS OF *RPS. SPHAEROIDES* R-26

	ν_1 (MHz) (±1)	ν_2 (MHz) (±1)	$\lvert D\rvert$ ($\times 10^{-4}$) (cm^{-1}) (±0.4)	$\lvert E\rvert$ ($\times 10^{-4}$) (cm^{-1}) (±0.4)	Sign		
					Microwave-induced fluorescence, 4.2 K	77 K	Flash-induced fluorescence, 4.2 K
FDMR							
whole cells $\lambda = 910$ nm	468	653	186.8	30.8	−	+	+
isolated reaction centers $\lambda = 935$ nm	475	668	190.5	32.2	+	−	?
isolated reaction centers $\lambda = 905$ nm	469	661	188.3	32.0	−	≈0	+
ADMR							
isolated reaction centers $\lambda = 890$ nm	468	661	188.0	32.3			

transition frequencies are distinctly different; both deviate somewhat from the frequencies measured for FDMR transitions in whole cells (Table I). That there is a slight difference between isolated reaction centers and whole cells is not unexpected, in view of the sensitivity of the FDMR frequencies to the surrounding matrix and to slight changes in configuration of the P-890 dimer (the number refers to the absorption maximum of the longest-wavelength absorption band at cryogenic temperature). In Fig. 5 the microwave-induced fluorescence response at 460 and 475 MHz is shown. The changes in concentration of the singlet ground state of P-890 that are brought about by microwave irradiation of $P\text{-}890^T$ can also be measured by monitoring its absorbance at 890 nm instead of the fluorescence [12]. With this technique (called ADMR) one monitors the bulk properties of the sample. For comparison, Table I also shows the transition frequencies found with the ADMR technique. It is seen that the ADMR frequencies coincide with those of the FDMR measured at 905 nm.

During the experiments we observed a 5–10-fold increase in the quantum yield of the 920 nm fluorescence in isolated reaction centers over a period of 1–2 h. However, samples with different illumination history gave similar FDMR results.

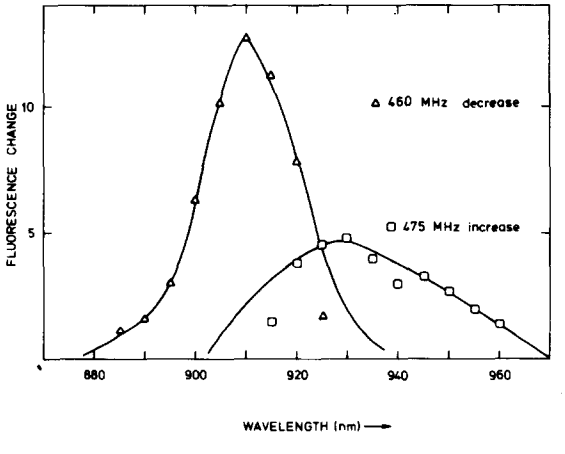

Fig. 5. The microwave-induced fluorescence spectra of isolated reaction centers with resonant microwaves at 460 or 475 MHz. Resonant microwaves at 475 MHz induce an increase and resonant microwaves at 460 MHz induce a decrease in fluorescence. For ease of comparison the band recorded at 460 MHz is inverted. The optical resolution is 13 nm.

Discussion

When interpreting our microwave-induced fluorescence, FDMR and ADMR spectra we have to accommodate three important features:

(i) the change of sign of the microwave-induced fluorescence spectrum across the long-wavelength fluorescence band, or more precisely, the fact that predominantly only part of the microwave-induced fluorescence spectrum responds to microwaves set at a particular frequency (460 MHz for the blue part and 475 MHz for the red part);

(ii) the red and blue parts of the microwave-induced fluorescence spectrum show different resonant FDMR frequencies, i.e., different values of the zero-field splitting parameters $|D|$ and $|E|$;

(iii) the transition frequencies of the blue part of the microwave-induced fluorescence spectrum coincide with the frequencies for the reaction center triplet as measured with ADMR.

Let us first concentrate on the '935 nm' FDMR. It gives rise to the only FDMR transition with a sign corresponding to that of our ADMR experiments, which measure the bulk properties of the sample. As we have not seen two distinct ADMR bands in the long-wavelength absorption region [9], it follows from the flash-induced fluorescence experiments that the 935 nm FDMR is due to a triplet located on P-890 itself and that no other FDMR band can be ascribed to the $P\text{-}890^T$ complex. Yet, why are the transition frequencies of the 935 nm FDMR different from those of the bulk ADMR? We think that this difference is caused by so-called site-dependent properties of the reaction centers. A 'site' is defined as an environment of the pigment with a particular set of $|D|$ and $|E|$ values, hence a particular set of microwave-transition frequencies. We have found that although most of the 890 nm absorption band shows ADMR transitions at 468/661 MHz, the ADMR frequencies and the width of the ADMR transitions change somewhat with detection wavelength (resolution 3 nm) in the red and blue regions of the 890 nm band. As we have evidence that at least part of the width of this band stems from pigments with a somewhat different 0-0 singlet-singlet transition (Ref. 13 and unpublished results), it is not surprising that the ADMR varies slightly with detection wavelength. If we assume that all P-890 sites fluo-

resce with equal yield, the FDMR transition frequencies should correspond to the ADMR transitions (as these reflect the bulk of the material). This is not so, the bulk does not show up in FDMR, hence the bulk of P-890 does not fluoresce or only very slightly. This agrees with the concept of fast charge separation with high quantum yield. Note that fluorescence with positive FDMR sign results from P-890 in open reaction centers, i.e., reaction centers in the state PIX^-. The pigment(s) on which the triplet is located in closed reaction centers, i.e., in the state P^TIX^-, do not fluoresce. It follows that the 935 nm fluorescence results from a minority of reaction centers with P-890 in an open state in which quenching is less effective, e.g., because of less efficient charge separation. That the reaction centers fluorescing at 935 nm may have a somewhat different pigment architecture is suggested by the observation that in contrast to the absorption spectrum, the fluorescence-excitation spectrum often shows a clearly split 800 nm band (data not shown).

Turning to the '905 nm' FDMR transition, we first note that the 905 nm fluorescence stems from closed reaction centers, i.e., from reaction centers in the state P^TIX. This follows directly from the sign of the FDMR effect and the flash-induced fluorescence experiments at 905 nm at 4.2 K. Secondly, this fluorescence is caused by reaction centers belonging to the bulk of the reaction center material, because the microwave-transition frequencies coincide with those measured by ADMR (for most of the 890 nm absorption band).

To explore possible explanations for the 905 nm microwave-induced fluorescence band, we consider Fig. 1, which represents the energy levels of a donor pigment ^1D interacting with an acceptor pigment ^1A. ^1A can be directly excited to its singlet excited state $^1A^*$, or it receives by energy transfer excitation from the excited singlet state $^1D^*$. From $^1A^*$, but not from $^1D^*$, charge separation can occur. At low temperatures ($T < 20$ K) charge recombination then leads quantitatively to a triplet state ^3A. $^1D^*$ in open reaction centers (i.e., no ^3A) does not fluoresce, or only very slightly, as $k_c \gg k_f$. However, when ^3A is generated, no energy transfer from $^1D^*$ to ^1A resulting in $^1A^*$ and ^1D is possible, and $^1D^*$ will fluoresce with high quantum yield. When $k_h \gg k_f$, the ratio of the quantum yield Y_c in closed centers to that in open centers, Y_o, is given by $Y_c/Y_o = 1 + \frac{k_c}{k_f + k_l} \gg 1$, where k_l represents decay channels of $^1D^*$ other then fluorescence or energy transfer. ^1A is regenerated by deactivation of ^3A, the rate of which is influenced by resonant microwaves. Hence, appreciable FDMR signals with negative sign can be obtained. The flash-induced fluorescence experiments provide a check on the sign of the FDMR effect, as a light flash always increases the triplet population. At 4.2 K (905 nm) and at 77 K (935 nm) the flash-induced fluorescence results corroborate our FDMR results. The failure in observing a flash-induced fluorescence effect on the 905 nm fluorescence at 77 K might be caused by a decrease in the lifetime of the $^1D^*$ level in closed reaction centers (which causes the 905 nm flash-induced fluorescence band at 4.2 K), because of an increase in the radiationless loss rate k_l.

Although our FDMR and flash-induced fluorescence results are conceptually well explained by the scheme of Fig. 1, it should be realised that Fig. 1 has been drawn for a photochemically active reaction center (open trap) and as such represents an oversimplification. For closed traps the energy-level scheme may change drastically. For example, when the singlet-singlet interaction between $^1D^*$ and $^1A^*$ is destroyed by triplet formation, then the new absorption spectrum will show the triplet-triplet absorption spectrum of ^3A and the appearance of the absorption spectrum of the monomeric pigment, whereas the exciton absorption bands of $^1D^*$ and $^1A^*$ disappear.

In the accompanying paper [9], we present data indicating that the triplet is localized (on an optical time scale) on one of the monomeric pigments D or A, i.e., the electronic interaction between ^1D and ^3A is weak, and that a monomer absorption band appears at 808 nm in the triplet absorption-difference spectrum. The negative sign of the 905 nm microwave-induced fluorescence band could, in principle, be explained by the assumption that this fluorescence stems from the monomeric BChl absorption at 808 nm. However, the difference between absorption and fluorescence peak wavelength would then amount to about 100 nm. A Stokes' shift of this magnitude is very unusual, so that we feel we can exclude the possibility that the

808 nm absorption band and the 905 nm fluorescence band belong to one optical transition.

Arguments based on an energy-level scheme like that of Fig. 1, but now applied to contaminating B-890-antenna pigments, can also explain the observation of a negative FDMR effect at the 905 nm fluorescence, provided energy transfer between this 'impurity' and P-890 is fast compared to charge separation. This possibility is difficult to accept because: (i) the optical characteristics of our reaction center preparation conform to the purest reaction center preparation described in the literature [10]; (ii) a second passage over a DEAE column did not affect the 905 nm FDMR. This treatment reduced the A_{280}/A_{800} ratio from 1.26–1.30 to 1.16 ± 0.03. The latter value is somewhat lower than the literature valule [10], possibly because of loss of secondary quinone acceptors which absorb in the ultraviolet; (iii) samples of different batches of reaction center preparation did not show variation in the 905 nm FDMR. However, to exclude the possibility that no contamination of antenna protein is present in sufficient quantity to give rise to 905 nm fluorescence in closed traps, one would have to determine protein impurity to better than a few tenths of a percent of reaction center protein, which is extremely difficult to do with present assay techniques.

In conclusion, we wish to emphasize that the relative amplitude of the FDMR effects is very strongly influenced by the ratio between the fluorescence yield with all traps closed (F_{max}) and that with all traps open (F_o) (see Eqn. 5 of Ref. 5). This means that the fluorescence intensity at 905 nm of reaction centers with reduced primary acceptor under conditions of illumination where the majority of reaction centers are open (i.e., in the state PIX^-) may well be low compared to that resulting from reaction centers with impaired electron transport. For ease of reference we have labeled the latter fluorescence the 935 nm fluorescence, based on the positive microwave-induced fluorescence peak in Fig. 3. This peak, however, is shifted somewhat to the red because of masking by the negative microwave-induced fluorescence through at 905 nm. In Fig. 5 one sees that the true peak wavelength of the positive microwave-induced fluorescence effect is close to that of the fluorescence band (920 nm, Fig. 2). This means that this band may well result almost completely from a minority of reaction centers with less than optimal rate of charge separation. Therefore, it would seem to be hazardous to draw conclusions on the primary processes in the intact reaction centers from experiments utilizing this fluorescence.

Acknowledgements

We thank Dr. J. Schmidt for his advice and encouragement. Dré de Wit cultured the bacteria and Lenneke Nan expertly prepared the reaction centers. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial assistance from the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

1 Dutton, P.L., Leigh, J.S. and Seibert, M. (1971) Biochem. Biophys. Res. Commun. 46, 406–413
2 Parson, W.W., Clayton, R.K. and Cogdell, R.J. (1975) Biochim. Biophys. Acta 387, 265–278
3 Clarke, R.H., Connors, R.E., Norris, J.R. and Thurnauer, M.C. (1975) J. Am. Chem. Soc. 97, 7178–7179
4 Hoff, A.J. (1976) Biochim. Biophys. Acta 440, 765–771
5 Hoff, A.J. and Gorter de Vries, H. (1978) Biochim. Biophys. Acta 503, 94–106
6 Holmes, N.G., Van Grondelle, R., Hoff, A.J. and Duysens, L.N.M. (1976) FEBS Lett. 70, 185–190
7 Van Dorp, W.G., Schaafsma, T.J., Soma, M. and Van der Waals, J.H. (1973) Chem. Phys. Lett. 21, 221–225
8 Clayton, R.K. (1977) Special Issue of Plant Cell Physiol. 87–96
9 Den Blanken, H.J. and Hoff, A.J. (1982) Biochim. Biophys. Acta 681, 365–374
10 Feher, G. and Okamura, M.Y. (1978) in The Photosynthetic Bacteria (Clayton, R.K. and Sistrom, W.R., eds), Ch. 19, Plenum Press, New York
11 Hoff, A.J. and Gorter de Vries, H. (1981) Isr. J. Chem. 21, 277–282
12 Den Blanken, H.J., Van der Zwet, G.P. and Hoff, A.J. (1982) Chem. Phys. Lett. 85, 335–338
13 Platenkamp, R.J., Den Blanken, H.J. and Hoff, A.J. (1980) Chem. Phys. Lett. 76, 35–41