CHANGES OF IN VIVO BACTERIOCHLOROPHYLL FLUORESCENCE YIELD IN *RHODOPSEUDOMONAS SPHAEROIDES* AT LOW TEMPERATURE AND LOW REDOX POTENTIAL

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1. Introduction

Recent work using picosecond lasers with reaction centres isolated from *Rhodopseudomonas sphaeroides* has indicated that, following a very short flash, a state P_F with a distinctive absorbance spectrum, is generated within a few picoseconds [1,2]. P_F may correspond to a complex of the oxidised reaction centre bacteriochlorophyll dimer with a reduced bacteriopheophytin molecule, i.e. state P^+BPheo^- [3,4]. Unless normal photochemistry is blocked this state decays with a half-time of about 100 ps to 250 ps, presumably with the concomitant reduction of the 'primary' acceptor, X (probably an iron-ubiquinone complex, see refs. [5,6]) giving state P^+X^-. If, however, X is already in the reduced state part of P_F decays, in the absence of carotenoids, into a state P_R, identifiable by its absorbance spectrum. At room temperature the quantum yield of this state is low (about 10%) but at 15°K is near 100% [7]. A triplet state of the reaction centre has been identified by ESR techniques [8,9] and P_R probably corresponds to this triplet state [4,5].

It seemed possible that such reaction centre states might give rise to changes in the fluorescence yield of bacteriochlorophyll. Fluorescence yield measurements on a slow time scale have established the existence of 'traps' for absorbed energy, corresponding to the reaction centre complex, in which a high fluorescence yield is associated with a closed trap, unable to undergo photochemistry.

Abbreviations: ESR, Electron Spin Resonance; MOPS, 3-(*N*-morpholino) propane sulphonic acid.

Vredenberg and Duysens [10] have suggested a relationship between the in vivo bacteriochlorophyll fluorescence yield and the redox state of the reaction centre bacteriochlorophyll:

$$\frac{1}{\Phi_F} = A + B\,[P]$$

where Φ_F is the fluorescence yield, [P] represents the concentration of P in the reduced state, and A and and B are positive constants, and provided experimental evidence in support of this; under the conditions of these experiments, the acceptor X was presumably largely in the oxidised state. Other authors have reported observations indicating that this relationship may not be valid under all conditions [11] and that the state of the primary acceptor, X, may also influence the fluorescence yield [11,12].

The work reported below examines changes in fluorescence yield and in the absorbance spectrum in whole cells of *Rps. sphaeroides* R26 on a rapid (microsecond) time scale, at low temperatures, with and without the primary acceptor, X, reduced.

2. Materials and methods

Cells of *Rps. sphaeroides* wild type were grown as previously described [13] and cells of *Rps. sphaeroides* R26 (kindly supplied by Dr G. Feher) anaerobically in modified Hutner medium. Before use they were resuspended in 50 mM MOPS, 50 mM

KCl, 1 M sucrose, at pH 7.0, and the resulting suspension mixed to 50% v/v with glycerol. This suspending medium gave samples free from cracking when frozen to 77°K. The final suspension of cells had an absorbance of 0.9 in 1 cm at 865 nm which corresponds to a concentration of 6 μM BChl as estimated from the absorption spectrum and the in vivo extinction coefficient in [14]. For samples under reducing conditions the cell suspension was reduced with excess sodium dithionite under an atmosphere of nitrogen. Preliminary experiments used 100 mM sodium dithionite but it was found that lower concentrations gave more reproducible samples on freezing.

Fluorescence was measured, during and after a Xenon flash as described previously [15] but using an S1 photomultiplier. A Schott RGN 9/2 filter excluded false light from the photomultiplier.

Absorbance changes were measured on a single beam spectrophotometer using an S20 photomultiplier, protected from stray actinic light by a Corning CS 4-96 filter. Actinic light was provided by a Xenon flash lamp ($t_{1/2}$ = 10 μs) from which light of wavelengths greater than 780 nm was selected by a cut off filter. Signals were averaged by a Datalab DL1025 to improve the signal to noise ratio. The whole system had a time constant of 4 μs.

3. Results

Figure 1 shows the change in the fluorescence yield during the first 16 μs of a flash in cells under reduced (fig.1a) or non-reduced (fig.1b) conditions. In both cases it can be seen that there is a doubling of the fluorescence yield over the course of a 100% flash. Also shown is the fluorescence yield during a flash of lower intensity (7.9%) in which the yield undergoes little change. For technical reasons measurements made during the first microsecond of the flash are not reliable and this latter measurement therefore establishes a Φ_0 level. The initial fluorescence yields in figs.1a and 1b are not strictly comparable, probably as a result of differences in freezing. By giving such low intensity flashes at intervals after an initial exciting flash the decay of the increase in fluorescence yield may be monitored. It can be seen in fig.1 that, although in the case of cells with dithionite the fluorescence yield has dropped to about half its maximal value after 100 μs (the first point at which the fluorescence may be monitored using our apparatus), in the case of untreated cells the fluorescence is still at its maximal level after 1 ms. Figure 2 shows the decay kinetics in both types of sample. The fluorescence is plotted as a reciprocal plot to give the change in

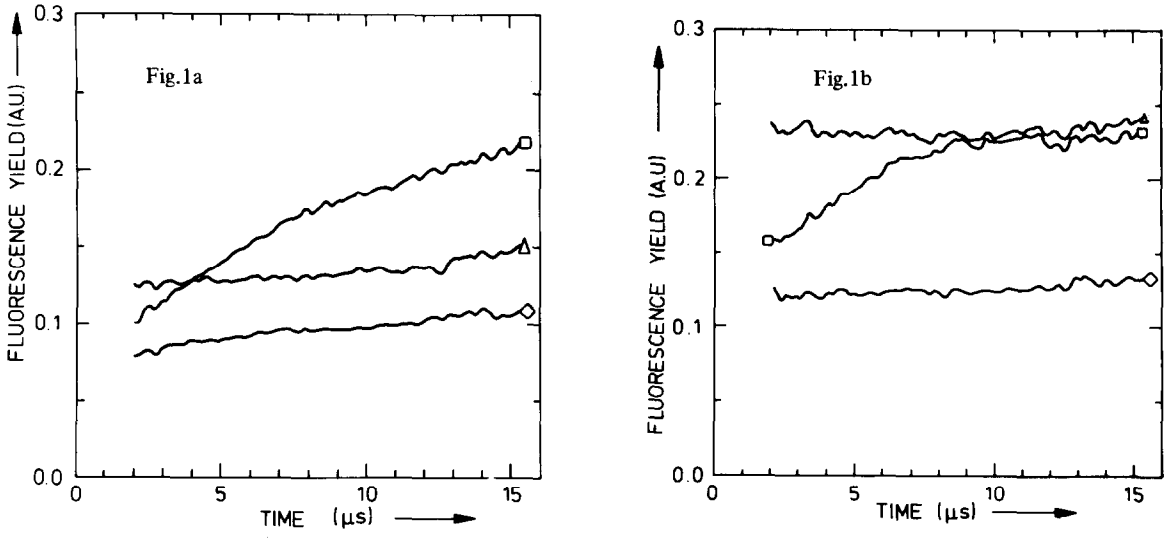

Fig.1. Rise kinetics of fluorescence yield during flash. (□) maximum intensity flash (100%), (◇) 7.9% flash with no previous actinic flash, (△) 7.9% flash 100 μs after 100% actinic flash, (a) sample reduced by 20 mM sodium dithionite, (b) unreduced sample.

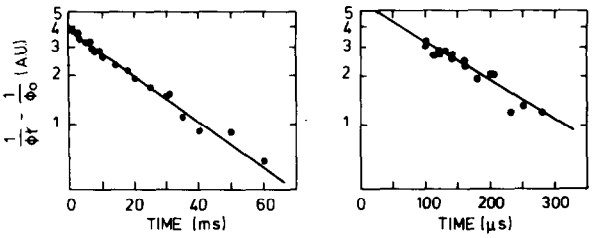

Fig.2. Decay kinetics of fluorescence yield after flash. The kinetics were followed after a 100% ($t_{1/2}$ = about 12 μs) actinic flash by monitoring the fluorescence yield with a low (7.9%) intensity ($t_{1/2}$ = about 16 μs) interrogating flash. The fluorescence yield is plotted as the difference between the reciprocals of the fluorescence yield with no actinic flash and the fluorescence yield at the given time after a 100% actinic flash which yields the decay of closed traps. Times are measured from the start of the actinic flash. The fluorescence yield was determined at 5 μs after the start of the interrogating flash. Left: unreduced sample. Right: reduced sample.

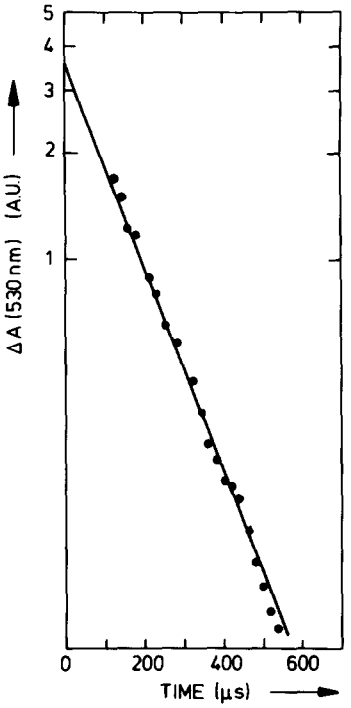

Fig.3. Decay kinetics of absorbance changes. Sample reduced with 10 mM sodium dithionite, decay kinetics of absorbance change at 530 nm.

concentration of traps (see introduction) assuming energy transfer between photosynthetic units [10]. In cells with dithionite a half decay time of 110 μs and in cells without dithionite a half decay time of 18 ms were found (both values being an average of 9 separate experiments). It was found that the assumption of an inverse relationship between fluorescence yield and traps (and therefore of non-independent units) gave a better fit to the experimental data than the assumption of a linear relationship (assuming also that the decay is first order, as measured for the absorption changes). In addition analysis based on the latter assumption gave values for the decay half-times approximately 20% smaller than those given above.

Figures 3 and 4 show the kinetics and spectra of absorbance changes accompanying the fluorescence changes described above. In unreduced samples an absorbance change can be seen decaying monophasically with a half-time of 20 ms and showing a spectrum (fig.4) between 400 nm and 630 nm closely similar to that previously observed in isolated reaction centres under equivalent conditions and ascribed to P^+ [6]. In samples with dithionite an absorbance change decaying monophasically with a half-time of 110 μs was observed (fig.3). The spectrum of this change resembles the spectrum of P^R in isolated reaction centres [6] although some discrepancies are apparent. These discrepancies may be a result of the different samples, cells and reaction centres, used in the two measurements.

By using a lower concentration of sodium dithionite it was found possible to create a mixed population of reaction centres with reduced and with oxidised primary acceptors within the same samples. With such a sample it was possible to measure both the P^R and P^+ changes as a function of light intensity in the same sample. From fig.5 it can be seen that although the flash used was not fully saturating, the two changes showed a similar intensity dependence and that within the limits of accuracy of the measurement the ratio of the two components remained constant over the range of intensities employed (fig.5, inset).

In parallel experiments on fluorescence yield changes in reduced *Rps. sphaeroides* wild type at even lower temperatures (2°K), using a rotating disk interrupted He/Ne laser as an excitation source (switching time 25 μs), and increase in fluorescence yield with a half-time of 110 ± 10 μs has been measured. This

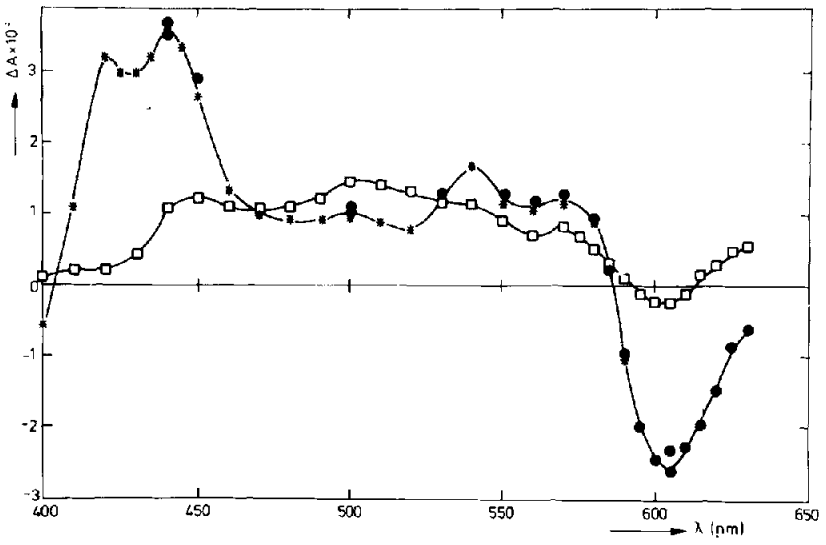

Fig.4. Spectra of absorbance changes. (□) Sample reduced with 10 mM sodium dithionite. Absorbance change between 100 μs and 800 μs after the flash. (*●) Unreduced sample. Absorbance change 100 μs after flash. Different symbols indicate two different samples.

increase in fluorescence is attributed to populating the triplet state. The rate of increase under weak excitation light conditions can be shown to be equal to the triplet decay rate, and correlates well with measurements of the triplet lifetime using ESR techniques in zero magnetic field under the same conditions [16]. This half-time was determined to be 74 μs, 81 μs and 330 μs for the triplet x, y and z levels, respectively, yielding an average half-time (which obtains at higher temperatures) of 104 μs ± 10%.

4. Discussion

It is clear from the results on fluorescence yield changes discussed above that at 77°K a state with a high fluorescence yield becomes populated on illumination both of cells which are under reducing conditions (where the centres exist in the dark as PX^-) and of untreated cells. This is in contrast to room temperature measurements which indicate that in reduced samples the fluorescence yield remains high, i.e. at the maximal

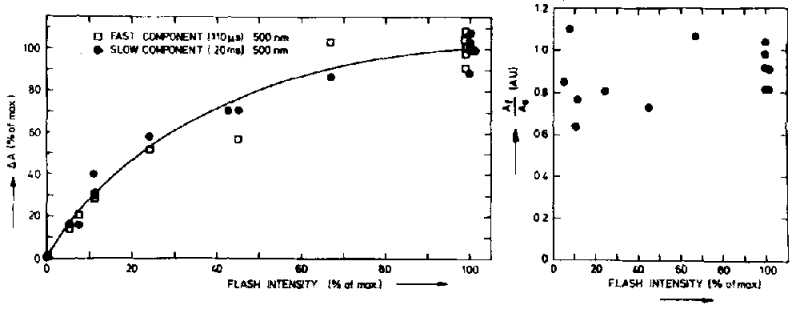

Fig.5. Intensity dependence of absorbance changes. The sample was reduced with 300 μM sodium dithionite giving a mixture of a slow and fast kinetic change as measured at 500 nm. Inset: Ratio of the fast and slow absorbance change as a function of intensity.

level attained in the light, throughout the flash (N. G. Holmes, unpublished observation) in keeping with earlier reports on a slower time scale [17,18].

The decay half-time for the fluorescence yield under reducing conditions must be considered approximate since, because of the tail of the actinic flash, the fluorescence yield cannot be monitored, using our apparatus, within the first 100 μs after the actinic flash. Comparison of these kinetics with the kinetics of absorbance changes seen under similar conditions, together with the spectra of those changes, allows identification of the high fluorescence states as P^+ in centres not reduced before the flash, and as P^T in reduced centres.

Figure 6 shows a scheme based on that of Parson and Cogdell [5] which can explain the above observations. These authors suggest, from a consideration of the quantum yields of photochemistry and of fluorescence in isolated reaction centres, that P_F may decay partly via a non-radiative pathway (other than via P_R. However, evidence from fluorescence studies at room temperature suggests that the fluorescence yield with centres blocked by the reduction of the acceptor is no less than that with centres blocked by the presence of state P^+, indicating that the turnover of state P_F does not, in this situation, diminish the probability of re-emission of a quantum (see above). We are currently investigating fluorescence yields under a variety of conditions in an attempt to resolve this apparent anomaly.

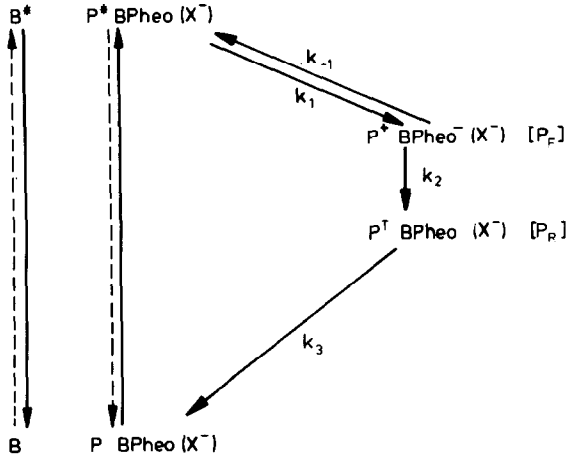

Fig.6. Schematic representation of reaction centres changes (after Parson and Cogdell [5]). (B), bulk bacteriochlorophyll, (P), reaction centre dimer, (BPheo) bacteriopheophytin, (X) primary acceptor, (P^T) triplet.

Therefore, according to this scheme, at room temperature, with the primary acceptor reduced, little of state P_R is generated and state P_F decays via the excited singlet state, causing a high fluorescence yield, whereas at 77°K, P_F is converted to P_R with a high efficiency and only whilst the reaction centre exists in state P_R is the fluorescence yield high. This is in accord with the temperature dependencies of the quantum yield for P_R formation in isolated reaction centres reported by Parson and co-workers [7]. These results also demonstrate that the decay rate of P_R is similar in intact cells to that reported by these workers in isolated reaction centres.

An alternative possibility, as discussed in [5], is that the increase in fluorescence yield on reduction of X may be due to a change in either or both of k_1 and k_{-1} (due possibly to electrostatic repulsion by the charge on X) and the P_F may then decay by a non-radiative mechanism. Such a scheme is also compatible with our results and can explain them in an essentially similar way to the first scheme. However, recent work [19] suggests that at lower temperatures P moves nearer to X; this would be expected to increase the electrostatic effect, and therefore it would, if the electrostatic effect were the only effect present, further increase the fluorescence yield of the reaction centres in state PX^- at low temperature, in contrast to our observations.

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