Time-resolved measurements of fluorescence from reaction centres of *Rhodopseudomonas viridis* and the effect of menaquinone reduction

J.K.H. Hörber, W. Göbel, A. Ogrodnik, M.E. Michel-Beyerle[+] and R.J. Cogdell*

Institut für Physikalische und Theoretische Chemie, Technische Universität München, D-8046 Garching, FRG and
**Department of Botany, University of Glasgow, Glasgow G12 8QQ, Scotland*

The kinetics of the fluorescence emitted by the 'special pair' of bacteriochlorophyll *b* molecules in reaction centres from *Rhodopseudomonas viridis* was recorded in the near infrared, with a time resolution of 1 ns. In nonreduced reaction centres two decay components were resolved with lifetimes of <0.5 and 2.5 ns. Upon reduction of the menaquinone electron acceptor three decay components were detected with lifetimes of <0.5, 2.5 and 15 ns.

Bacterial photosynthesis   Primary process   Reaction center   Fluorescence

1. INTRODUCTION

Light absorbed by reaction centres of *Rhodopseudomonas viridis* is very rapidly transferred to two bacteriochlorophyll *b* molecules, which form the so called 'special pair' (P). This P is characterized by a long wavelength absorption band centred at 965 nm at room temperature, and it is this absorption band which is bleached when P is oxidized in the primary photochemical reaction. Prompt fluorescence from the P* state should decay in about 5 ps [1], since this is the time during which an electron is transferred from P* to one of the two reaction centre bacteriopheophytin *b* (Bpheo *b*) molecules. Subsequent electron transfer from Bpheo *b*⁻ to menaquinone proceeds within 230 ps [2], leading to the formation of the $P^+ MQ^-$ state, which recombines on a time scale of milliseconds.

In the presence of mild reducing agents such as ascorbate the bound *c*-type cytochrome is able to reduce P^+ with the characteristic time of 270 ns

[2]. If the reaction centre now receives another photon while it is in the $P MQ^-$ state, electron transfer from P can now only proceed as far as the Bpheo *b*. This prolongs the lifetime of the P^+ Bpheo *b*⁻ state (MQ is already reduced), allows charge recombination of the radical pair and is expected to give rise to a long-lived component of the fluorescence. This delayed fluorescence would then have the decay constant characteristic of the decay time of the radical pair, i.e. 15 ns [2]. Here we report on the fluorescence decay pattern of reaction centres from *Rps. viridis*, with a time resolution of 1 ns. The effect of the negative charge on the menaquinone is discussed in terms of its influence on the electron transport route that the electron takes upon leaving the excited special pair, P*.

2. MATERIALS AND METHODS

Reaction centres of *Rps. viridis* were prepared from LDAO solubilized membranes as described in [3], except that the deriphat (detergent; Henkel) stage was omitted. The purified reaction centres

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[+] To whom correspondence should be addressed

were then dialysed overnight against 20 mM Tris-HCl, pH 8.0, at 4°C and applied to a DES2, DEAE-cellulose column, which had been equilibrated in the same Tris buffer. The reaction centre band tightly binds to the top of the column. The column was now washed with 5–10 column volumes of 20 mM Tris-HCl, pH 8.0, with no added LDAO, with one column volume of buffer with 0.5 mM sodium dithionite and finally with two column volumes of buffer with 0.2% (v/v) Triton X-100. The reaction centres were then eluted from the column in the Triton-containing buffer by the addition of 300 mM NaCl. This procedure replaces the LDAO with Triton X-100 and both makes the reaction centres more stable and allows them to be subjected to reducing condition. During the fluorescence measurements the sample was maintained at 5°C. The time-resolved fluorescence measurements were carried out under two conditions: (i) the sample was used as prepared, with no further manipulation (untreated), (ii) the mild reductant sodium ascorbate was added to the sample to give a final concentration of 10 mM and a continuous light source (20 photons/s per RC) was focused onto the sample. This then, photochemically, puts the reaction centres into the P Bpheo b MQ^- state, since any oxidized P^+ is rapidly re-reduced by the ascorbate-reduced bound cytochrome.

The steady-state absorption and emission spectrum of reduced reaction centres are depicted in fig.1, together with the excitation and detection wavelengths of the time-resolved measurements. Pulse excitation was performed with a dye laser pumped by a Lambda M1000 nitrogen laser. The pulse width of the system was 2 ns at 590 nm, the wavelength used for all measurements to excite the reaction centres in the Q_x bands of the bacteriochlorophyll b molecules. The repetition rate was 10–20 Hz. No influence on the signal was observed by lowering the rate to 0.1 Hz. The excitation intensity (1 μJ/cm^2) used for all experiments corresponds to about 0.5% excited reaction centres within one pulse.

To detect the fluorescence signal a Varian 152A photomultiplier was used, operating at 190 K in an analogous mode. Its rise time was 0.6 ns and the InGaAsP-photocathode had a quantum efficiency of about 5% at 1060 nm. The photomultiplier was protected by a my-metal shield from magnetic
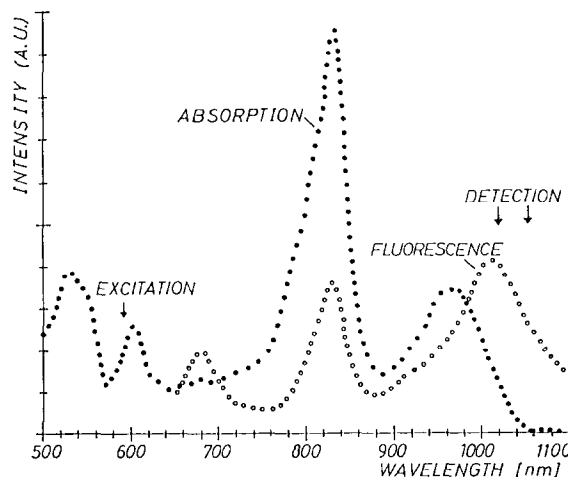

Fig.1. Absorption and emission spectra of a *Rps. viridis* reaction centre preparation (15 μM). The excitation source was a 450 W CW xenon lamp. The excitation wavelength selection was made by a monochromator together with Corning glass filters. Detection and excitation wavelength of the time-resolved measurements are marked by arrows.

fields and by Corning glass filters from stray light. In order to be independent of the sample concentration, a front surface geometry for excitation and detection was used (fig.2). To check the influence of small magnetic fields (<650 G) on the reaction centre fluorescence, the sample and its cooling system was placed between two Helmholtz coils. An optical wave guide was a practical way to obtain a wide detection angle together with sufficient distance between the photomultiplier and the magnetic field without too great a loss of signal. The detection wavelengths (1020 nm, 1060 nm) were selected by bandpass interference filters ($\Delta\lambda$ = 20 nm). The rejection was, together with Corning glass filters, 10^{-10} to 10^{-12} depending on the filter set used.

A Tektronix transient digitizer system (R7912) coupled to a PDP 11/34 computer was used to capture and analyse the signal of the photomultiplier. Because of the slow readout frequency of the transient digitizer, the system was only triggered if the excitation pulse had the proper intensity determined by two constant fraction triggers (CFT1000) and a fast ECL-logic. In this way a maximum rate of 3 measurements/s could be stored on a floppy-disk. The special way of digitizing a very fast analog signal with the transient digitizer system
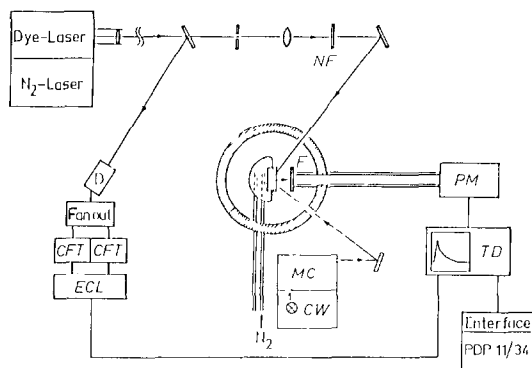
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Fig.2. Experimental set-up, for description see text.

can give rise to systematic errors. So if more decay patterns should be averaged, they had to be just of the same height to exclude additional errors. Only then the sum of all measurements can be corrected for writing errors of the electron beam on the target. For this purpose software was designed which determines the pulse shape from some hundreds of single measurements with respect to the pulse height and the upper and lower edge of the electron writing-beam on the silicon diode array. In this way the transient digitizer was used like a multichannel analyser of 512 horizontal time channels with an amplitude resolution of 8 bits each.

3. RESULTS

To compare the fluorescence from untreated and reduced reaction centres directly, we adopted the strategy of photochemical reduction of the menaquinone in situ (as described in section 2). Initially the fluorescence from untreated reaction centres was measured and the raw data stored. Then, ascorbate was added to the sample in the cuvette and CW light was turned on. It was possible over a 15 min period of illumination with the CW light to observe a gradual rise in the reaction centre fluorescence as the reaction centres were put into the P Bpheo b MQ$^-$ state. After about 15 min illumination equilibrium between unreduced and reduced reaction centres under these conditions was achieved. This equilibrium strongly favours reaction centres in the P Bpheo b MQ$^-$ state, since both processes, P$^+$ Bpheo b^- recombination and P$^+$ re-reduction by reduced cytochrome, are fast compared to the millisecond time scale of recombination of P$^+$ Bpheo b MQ$^-$. The fluorescence

decay measurements are now made on 'reduced' reaction centres characterized by a higher fluorescence quantum efficiency (fig.3). The reduced state of P Bpheo b MQ$^-$ slowly disappeared in the dark. About 25 min after the CW light was turned off the level of reaction centre fluorescence was back down to its initial level prior to the CW illumination.

The quantum yield of fluorescence of the reaction centres in the untreated and MQ reduced states was determined by a photon-counting technique in comparison with a tungsten standard lamp [4]. The yield was 3 ($\pm$ 1) $\times$ 10^{-4} for untreated reaction centres and 8 ($\pm$ 3) $\times$ 10^{-4} for the MQ$^-$ reaction centres. These values are very similar to those measured from *Rps. sphaeroides* reaction centres [5].

After a complete measuring cycle the raw data are computed as described above. The resulting fluorescence decay patterns are identical at 1020 nm and 1060 nm within the resolution of the detection system. The pulse width of the excitation pulse was in all cases smaller than the fluorescence decay signals. The fluorescence decay patterns are analysed with the Marquardt algorithm [6], by assuming that they follow a multi-exponential decay law, and the fluorescence signal is convoluted with the exciting pulse shape.

$$F(t) = \int_0^t I(t - t')\, f(t')\mathrm{d}t'; \quad I(t) = \Sigma_i\, I_i\exp(-\,t/\tau_i)$$
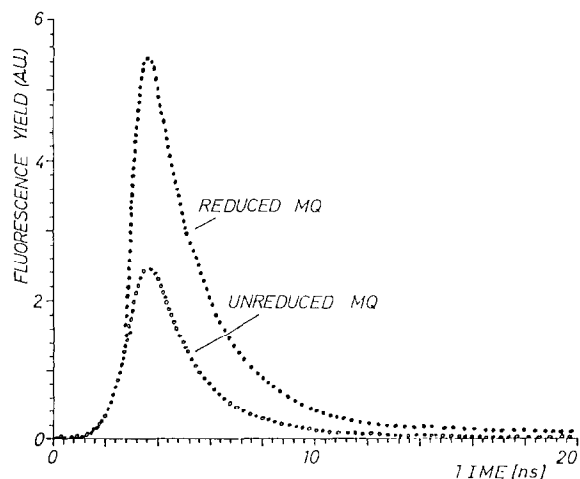

Fig.3. Decay pattern of the special pair fluorescence at 1020 nm, excited at 590 nm with a 2 ns laser pulse. (○) Recorded before reduction, (●) 15 min after the ascorbate was added and the CW light was turned on (see section 2).

Table 1

Measured fluorescence yields and lifetimes

n	Untreated reaction centres		Reduced reaction centres	
	y_n	τ_n (ns)	y_n	τ_n (ns)
1	1	<0.5	1.9 ± 0.3	<0.5
2	0.17 ± 0.3	2.5 ± 0.2	0.59 ± 0.5	2.5 ± 0.2
3	–	–	0.44 ± 0.5	15 ± 1

It turns out that even in the case of untreated reaction centres a biexponential fit is required, whereas for reduced reaction centres only a three-exponential fit can give satisfactory agreement. Since the time resolution of the detection system was limited by the transient digitizer to about 1 ns, the prompt fluorescence component just follows the exciting pulse. But if there would be a significant fluorescence component with a decay time between 0.5 ns and 1 ns, this would give rise to a detectable deviation from the excitation pulse shape in the maximum part of the measured fluorescence curve. Any fluorescence decaying faster than 0.5 ns, in this way, is attributed to the so called 'prompt fluorescence', and only the yield of this component can be derived. This amplitude rises upon reduction by a factor of 1.9. The slow component of fluorescence found unexpectedly in untreated reaction centres has a decay time of 2.5 ns and is within the obtainable resolution identical to the intermediate fluorescence component found in the reduced case. The amplitude of this component rises by a factor of 3.5 upon reduction. The slowest detected fluorescence component of 15 ns is only detectable in the case of reduced reaction centres. It can be increased by a magnetic field changing the time constant to 17 ns at 600 G. The experimental results are collected in table 1.

4. DISCUSSION

In general terms the fluorescence decay pattern described above for reaction centres from Rps. viridis is rather similar to that observed with reaction centres from Rps. sphaeroides [7–9]. For both types of reaction centres reduction of the quinone leads to a long-lived fluorescence component which reflects the repopulation of the excited

singlet state, P*, through recombination of P^+ Bpheo b^-. This is supported by equal time constants in fluorescence and transient absorption measurements [2] detecting the radical pair, and also by the corresponding magnetic field effect on this component in both cases. However, there are some detailed differences which extend our understanding of the primary processes in photosynthesis. The prompt fluorescence emitted by reaction centres from Rps. viridis rises by a factor of 1.9 upon reduction of the menaquinone. In similar experiments carried out with reaction centres of Rps. sphaeroides the relative increase of the prompt fluorescence was only 1.3 upon reduction
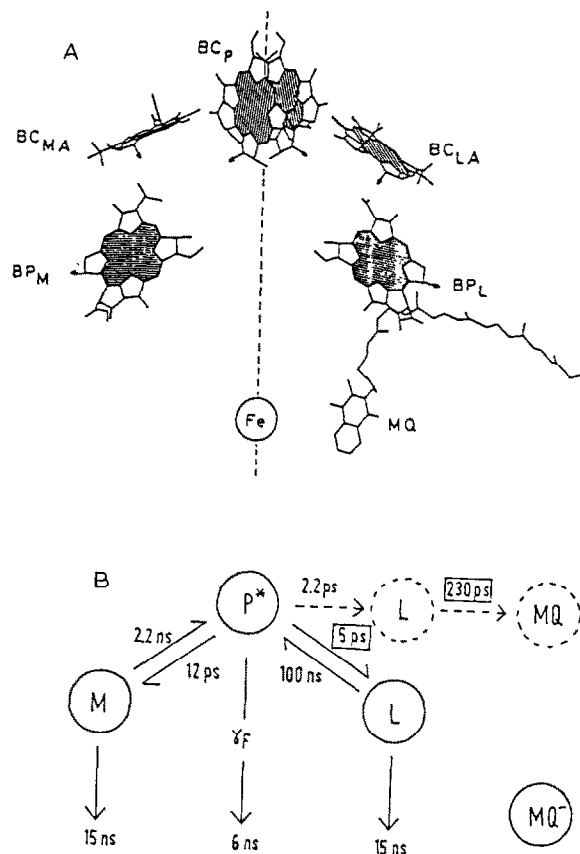

Fig.4. (A) Structure of the prosthetic groups in Rps. viridis reaction centres with two almost symmetrical branches (M and L) emerging from the 'special pair' molecules (BCP). (B) Three-state kinetic scheme where P* denotes the excited state of the BCP, and L, M two charge transfer states (i.e. BPL, BPM) responsible for the two delayed fluorescence components.

of the ubiquinone [9]. The increase in the level of prompt fluorescence upon menaquinone reduction can be understood if the negative charge on the quinone acted electrostatically to reduce the rate of the electron transfer from P* to Bpheo b. This prediction should be experimentally testable in the picosecond time domain.

The most striking result presented above was the characterization of an additional delayed fluorescence decay component between the prompt (<0.5 ns) and the slower component (15 ns). This intermediate component (2.5 ns) is observed in both untreated and menaquinone reduced reaction centres. But its yield is enhanced (× 3.5) by the menaquinone reduction. Such an intermediate component is also found in fluorescence decay patterns of *Rps. sphaeroides* [7–9], so it seems unlikely to be a preparative artefact. Also its identical spectral behaviour compared to the other fluorescence components, and especially its sensitivity upon photoreduction of the quinone make it very likely to be a characteristic feature of a reaction centre of purple bacteria and therefore it may provide further understanding of the primary processes together with the structure shown in fig.4A. This structure with its two almost symmetrical branches ('L' and 'M') of pigments which emerge from the special pair (BCp) leads us to the assumption that the first steps of charge separation may occur in either of the two branches X (= L,M) consisting of a monomeric accessory bacteriochlorophyll b (BC$_{XA}$) and a bacteriopheophytin b (BP$_X$) molecule [10]. In the reaction centres used only the menaquinone on the L branch was present, the ubiquinone on the M branch was lost during preparation. The resulting model system involves three states, one fluorescent state (P*) corresponding to the BC$_P$ and two non-fluorescent states M and L accounting for the participation of the pigments in either of the two branches. The coupling scheme of the model is shown in fig.4B together with the time constants corresponding to the transfer rates of charge separation and recombination and irreversible processes of nonradiative and radiative decay. The calculated parameters of the model given in fig.4B show the kinetic requirements which have to be met for the M branch to participate in the first steps of charge separation. It also clearly indicates that the predominant pathway is along the L branch.

Detailed analysis [11] showed that after 10 ps in only about 15% of the reaction centres the M branch should be involved. This might be the reason why the participation of the M branch pheophytin in picosecond absorption measurements was not evident. Since reduction of the quinone increases the probability of the participation of the M branch, we propose such measurements on reduced reaction centres in order to test the role of the M branch pheophytin.

ACKNOWLEDGEMENTS

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