Time-resolved measurements of fluorescence from reaction centres of *Rhodopseudomonas sphaeroides* R26.1

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The fluorescence decay pattern of the emission from the 'special pair' in reaction centres from *Rhodopseudomonas sphaeroides* R26.1 was recorded with a time resolution of 1 ns. The decay kinetics were monitored in photochemically 'open' reaction centres and in reaction centres 'closed' either by chemical reduction of the ubiquinone or by prior removal of the ubiquinone. As previously noted with reaction centres from *Rhodopseudomonas viridis* three fluorescence decay phases have been resolved. The origin of these phases is described in terms of a model which assumes that some of the initial charge separation in the reaction centre occurs in the 'M' branch as well as in the 'L' branch and that putting a negative charge on the quinone electrostatically inhibits the forward rate of the charge separation.

Bacterial photosynthesis *Primary process* *Reaction center* *Fluorescence*

1. INTRODUCTION

The elegant elucidation of the three-dimensional structure of the reaction centre from *Rhodopseudomonas viridis* by X-ray crystallography [1] has confirmed the generally accepted picture of the electron transfer events that occur in reaction centres of purple bacteria. An electron is transferred from the primary donor (the 'special pair' of bacteriochlorophyll molecules) to the first measurable acceptor, a monomeric bacteriopheophytin (Bpheo). This reaction is completed in 2–4 ps [2–4]. Over the next few hundred picoseconds the electron reaches the first stable electron acceptor, which in *Rps. sphaeroides* is ubiquinone [3]. The role of the monomeric bacteriochlorophyll, which in *Rps. viridis* has been shown to be located between the special pair and the Bpheo, is as yet unclear. This normal path of the electron transfer events takes place down the 'L' branch of the reaction centre.

One striking and unpredicted feature of the reaction centre structure that has come out of the X-ray analysis is the finding of a second, almost mirror image, chain of electron acceptors; the so called 'M' branch. The role of the M branch is still unresolved. Several years ago it was shown for reaction centres of *Rps. viridis* that both Bpheos could be photochemically reduced [5]. However the reduction of the second bacteriopheophytin was a much less favourable reaction. In our recent analysis of the decay kinetics of the fluorescence of reaction centers from *Rps. viridis* we proposed a model in which a small proportion of the initial charge separation from the special pair also involves the second Bpheo on the M branch. We have therefore extended our experimental observations to include reaction centres from *Rps. sphaeroides* in order to test whether a similar three-state branching model is still consistent with experimental findings.

2. MATERIALS AND METHODS

The reaction centres from *Rps. sphaeroides* R26.1 were prepared by the method of Clayton

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and Wang [6]. The LDAO used in the reaction centre preparation was exchanged for Triton X-100 as described [7].

Quinone-free reaction centres were prepared by treating reaction centres with 1 mM *o*-phenanthroline in the presence of 4% (v/v) LDAO at 25°C as described in [8]. After this procedure the extent of quinone removal was assayed spectrophotochemically by monitoring the photochemical bleaching of the bacteriochlorophyll at 865 nm.

During the measurement of the reaction centre fluorescence the sample was maintained at 5°C. The fluorescence emitted by the reaction centres was recorded with an 'untreated' sample with 'open' reaction centres. Then this same sample was reduced with sodium dithionite and the measurement repeated. These results from 'untreated' and 'reduced' reaction centres were compared with those obtained from the quinone-free ones.

The experimental set-up was as described in [7]. Pulse excitation was performed with a dye laser pumped by a Lambda M1000 nitrogen laser. The pulse width of the laser system was 2 ns at 590 nm used for the experiments to excite the reaction centre in the Q_x bands of the bacteriochlorophylls. The repetition rate was 20 Hz and the excitation intensity 1 μJ/cm^2, corresponding to 0.5% excited reaction centres within one pulse. Lowering the intensity by a factor of 10 had no influence on the decay pattern, and the fluorescence signal was linear with the excitation intensity. The detection wavelengths were selected by bandpass interference filters ($\Delta\lambda$ = 15 nm) at 860, 900, 925 and 950 nm, respectively. The rejection together with the Corning glass filter was 10^{-10} to 10^{-12} depending on the filter set used.

3. RESULTS

The time-resolved fluorescence decay patterns of reduced and untreated quinone-containing reaction centers are shown in fig.1A together with the shape of the exciting pulse. These decay patterns have been analysed by using the Marquardt algorithm [9] assuming that they follow a multi-exponential decay, and by deconvoluting the fluorescence signals with the exciting pulse shape.

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

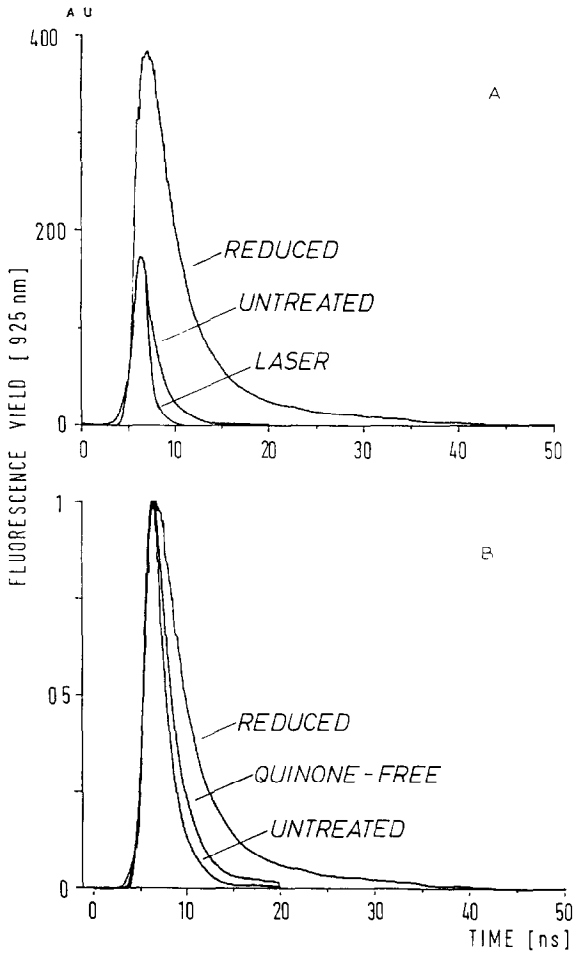

Fig.1. (A) Fluorescence decay patterns of untreated and reduced reaction centres of *Rps. sphaeroides* together with the exciting laser pulse. The difference in the amplitude shows the rising of the fluorescence signal after reduction of the quinone. The laser pulse is normalized with respect to the signal in the untreated case. (B) Fluorescence decay patterns of untreated, quinone-depleted and reduced reaction centres of *Rps. sphaeroides* normalized with respect to each other showing the change in width of the signal.

Just as was noted previously with reaction centres from *Rps. viridis*, the fluorescence signal emitted by reaction centres from *Rps. sphaeroides* is broadened with respect to the exciting pulse, even in the untreated case. Our analysis (see table 1) shows that this signal is composed of two components, a fast component (<0.5 ns) and a slower 2 ns component. The slower component has about 30% the intensity of the faster one.

Table 1

Measured fluorescence yields and lifetimes

State of the reaction centre	Relative yields			Lifetimes (ns)		
	y_1	y_2	y_3	τ_1	τ_2	τ_3
Untreated	1	0.25 ± 0.05	–	<0.05	2 ± 0.4	–
Reduced (600 G)	1.5 ± 0.05	0.70 ± 0.05	0.6 ± 0.05	<0.5	2 ± 0.2	11 ± 1 (13 ± 1)
Quinone-free (600 G)	1.2 ± 0.03	0.30 ± 0.03	0.2 ± 0.03	<0.5	2 ± 0.2	12 ± 1 (14 ± 1)

The decay times are an average from all measurements made under the same conditions. The variation given here is mainly from the variation between different samples. The results with a magnetic field of 600 G are shown in parentheses. The total fluorescence yield grows by a factor of 2.1 on reduction, on quinone-depletion by a factor of 1.4. The yields are all normalized with respect to the prompt fluorescence yield in the untreated case

Reduction of the reaction centres induces several changes in the fluorescence decay pattern. Most noticeable is a third, longer decay component, now seen with a decay time of about 10 ns. However, the intensity of the other two phases also increases (table 1). The intensity of the fast component increases by about 50% and the intensity of the 2 ns component rises by almost a factor of three. This decay pattern was seen with several different reaction centre preparations. With the quinone-free reaction centres the same three fluorescence decay phases were seen (fig.1B), but with different relative intensities (table 1).

The effect of an external magnetic field on the fluorescence decay pattern in the reduced and quinone-free reaction centres was investigated. In a field of 600 G only the slowest decay component was measurably influenced, showing a longer lifetime with the magnetic field (table 1).

Several measurements of the fluorescence decay pattern were made at different wavelength across the special pair emission band (fig.2). The decay kinetics were found to be independent of the detection wavelength within this band. Only in some samples, depending also on the age of the preparation, at the detection wavelength of 860 nm an admixture of a fluorescence component with a time constant of about 1 ns could be detected. Comparing absorption and emission spectra (fig.2) an admixture from a different emission band with its maximum at 800 nm is expected to contribute to the emission at 860 nm. The emission band at 800 nm is very sensitive to preparation and aging effects [10].

4. DISCUSSION

The fast fluorescence decay component (<0.5 ns) undoubtedly represents the prompt fluorescence. It is therefore somewhat surprising that reduction of the ubiquinone results in an in-

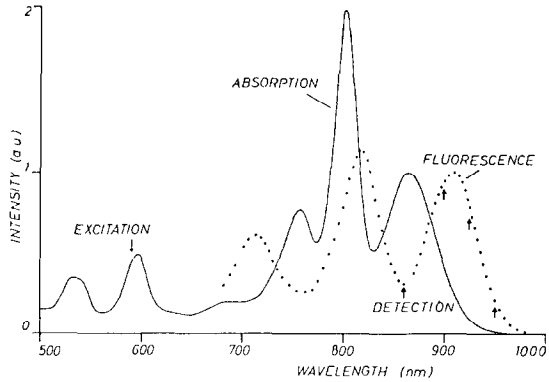

Fig.2. Absorption and emission spectra of a *Rps. sphaeroides* reaction centre preparation. The excitation source for the fluorescence measurement was a 450 W xenon lamp. The excitation wavelength of 590 nm was selected by a monochromator with additional Corning glass filters. Detection and excitation wavelength of time-resolved measurements are marked by arrows.

crease in the level of prompt fluorescence. However a similar increase in the intensity of prompt fluorescence was also noted in reaction centres from *Rps. viridis* upon reduction of the menaquinone. One simple and attractive explanation of this is that the presence of a negative charge on the quinone slows the forward rate of primary charge separation in the reaction centre by electrostatic interaction.

In reduced or quinone-depleted reaction centres, when the electron was prevented from moving from the Bpheo to the quinone a delayed component of fluorescence would be expected to arise from the recombination of the radical pair P^+ $Bpheo^-$. In absorption measurements radical pair lifetimes for quinone-depleted reaction centres of 13 ns have been observed [11–13]. The slowest fluorescence component clearly mirrors this radical pair decay. The decay however is magnetic field dependent. Hyperfine interaction induces singlet-triplet mixing in the initially singlet phased, radical pair state, thus opening a new recombination channel into a triplet state in addition to the singlet decay channel. Application of a magnetic field leads to Zeeman-splitting of the triplet states partially inhibiting singlet-triplet mixing and consequently changing the overall radical pair lifetime. Indeed an increase of the radical pair lifetime to 16.5 ns [12,13] in low magnetic fields has been observed in absorption measurements. Again this increase is mirrored in the fluorescence data, clearly indicating the radical pair state as the origin for this delayed component. Similarly in reduced reaction centres the magnetic field dependence of its lifetime marks the slowest fluorescence component to descend from radical pair states. In reaction centres blocked by reduction of the quinone a smaller triplet yield, resulting from radical pair recombination, when compared to quinone-depleted ones has been observed in absorption measurements [14,15]. This can be explained by a shorter lifetime of the radical pair in the reduced case as indeed is indicated by a speeding up of the associated, delayed fluorescence component. This is also reflected in absorption measurements yielding lifetimes of 7 ns and 9 ns at 0 G and 300 G, respectively [15].

If charge separation in the reaction centre took place exclusively in the L branch, then in photochemically open reaction centres only a single prompt fluorescence component would be expected. Furthermore in blocked reaction centres (by either quinone reduction or removal), only a single delayed component of fluorescence would be predicted. In all three cases however an additional delayed component of 2 ns lifetime was observed.

The origin of this intermediate 2 ns fluorescence decay component is not immediately obvious. However since it appears as a common feature in reaction centres from both *Rps. viridis* [7] and *Rps. sphaeroides* it is extremely unlikely to be an artefact. This view is also supported by the sensitivity of the intensity of this phase to the redox state of the quinone, which clearly links it to 'functioning' reaction centres.

It is possible to explain the origin of this 2 ns fluorescence decay phase if it is assumed that a proportion of the initial charge separation occurs in a proposed M branch as well as in the L branch, thus leading to an additional radical pair state on the M branch [16]. Though originating from radical pair recombination this fluorescence component is not expected to show a detectable magnetic field effect as the slow component does, because of its short lifetime. The basis of this type of three-state branched kinetic model (fig.3) has been presented elsewhere [17]. Within such a scheme the amplitudes and lifetimes of the three fluorescence components of reduced reaction centres yield only the relative rates in this case. With the rate R_L from absorption measurements [4] the absolute values for the rates (table 2) can be obtained. Assuming that the electrostatic effect of the excess charge at the quinone is mainly restricted to the L branch, the values R_M, r_M and γ_M obtained from reduced reaction centres must be identical to the values obtained for untreated reaction centres.

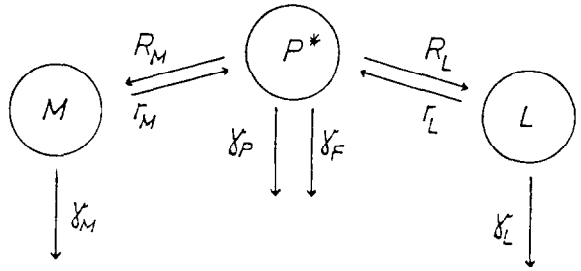

Fig.3. Three-state branched kinetic scheme for reaction centres, for details see text.

Table 2

Calculated rate constants

	Rates (ns^{-1})					
	R_M	r_M	γ_M	R_L	r_L	γ_L
Untreated	100 ± 20	0.6 ± 0.05	0.1 ± 0.02	(360)	–	(5)
Reduced	100 ± 20	0.6 ± 0.05	0.1 ± 0.02	200 ± 20	0.02 ± 0.002	0.09 ± 0.01
Quinone-free	100 ± 20	0.6 ± 0.05	0.1 ± 0.02	280 ± 20	0.01 ± 0.002	0.09 ± 0.01

From time-resolved fluorescence measurements only the relative rates can be evaluated and checked upon consistency under different preparative conditions. The absolute values are obtained with the forward rate to the bacteriopheophytin measured in absorbance [4]. Therefore this rate is noted in parentheses, like the transfer rate to the quinone in untreated reaction centres which is also taken from absorbance measurements for comparison. This rate affects none of the other rates, it only shows in relation to the back transfer rate in the reduced case that there is a negligible back transfer as expected for the high efficiency. This leaves the rate r_L undetermined

This gives a nontrivial consistency check for the model. The large value of γ_L measured in absorption [3] for untreated reaction centres implies a low intensity of the fluorescence component 3. Therefore recombination on the L branch competing with forward electron transfer to the quinone does not contribute in a measurable extent to delayed fluorescence. This is the reason why r_L in untreated reaction centres cannot be determined and only two fluorescence components are detected. A similar but even more critical consistency check of the model can be made by comparing the results of quinone-depleted reaction centres with the reduced case. The rates obtained independently for both cases with the three-state branching model fit together well within the accuracy of the data, showing also that changes in the L branch do not significantly affect the M branch. The changes in the amplitude relations of the different components in different preparations can be understood with the model just by changes of the forward and backward rates on the L branch listed in table 2.

Also from the forward and backward rates on the L and M branches the free energy difference between the fluorescent state P* and the two charge transfer states can be determined, yielding 0.23 ± 0.01 eV in the reduced, 0.26 ± 0.01 eV in the quinone-free case for the L and 0.13 ± 0.01 eV for the M state in both cases. These values are similar to those obtained for reaction centres of *Rps. viridis* in analogous measurements (0.25 eV for L, 0.13 eV for the M branch [17]). They differ significantly from other estimations [18] based on a two-state model giving smaller values for reaction centres of *Rps. sphaeroides* (0.15 eV).

For reduced reaction centres the forward rate on the L branch exceeds the one on the M branch by a factor of 2, whereas the recombination rate r_L is slower than the corresponding rate r_M on the L branch by a factor of 30, in quinone-depleted reaction centres by a factor of 60. For untreated reaction centres the branching ratio R_L/R_M is 3.6 favouring the L branch even more than in reduced reaction centres.

The phenomenology of multi-exponential decay has been reported [18,19] including an additional fourth fluorescence component between 0.5 and 1 ns. In the frame of our measurements there was no significance for such a component. It should become detectable, like the 1 ns component at 860 nm, if its intensity becomes comparable to the 2 ns component. Such a delayed fluorescence with a time constant of some hundreds of picoseconds also would not affect the use of the branched kinetic model, so with such a short time constant it may be produced by an intermediate state anywhere in the system [17]. To analyse such additional states further experiments are needed to obtain enough information to evaluate the four-state model coming up in this case.

At the moment the three-state branching scheme is able to explain the main features seen in time-resolved fluorescence measurements, without any further assumptions. It also shows how reduction or depletion of the ubiquinone influences the elec-

tron transfer in the reaction centre by changing only the forward and backward rate in the L branch. The greater change in the case of reduction may be due to the electrostatic effect of the additional charge in the reaction centre. These findings are very similar to those reported earlier on *Rps. viridis* [7], and the model developed in this case fits also for *Rps. sphaeroides* reaction centres.

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