Intermediate states between P* and Pf in bacterial reaction centers, as detected by the fluorescence kinetics

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The fluorescence lifetimes of the reaction centers isolated from the wild-type *Rhodopseudomonas sphaeroides* purple bacterium and those from the R26 mutant strain, lacking the carotenoid, were measured at low redox potential. In addition to the prompt fluorescence occurring directly from P* and the long delayed emission related to the radical pair state Pf, two other components are present. We suggest that they may come from intermediate states between P* and Pf, or reflect the stabilization of Pf itself.

Photosynthesis *Primary reaction* *Purple bacterium* *Phase fluorimetry* *Reaction center* *Fluorescence lifetime*

1. INTRODUCTION

During the last 10 years, the primary photochemical processes of bacterial photosynthesis have been investigated thanks to the progress both in the isolation and purification of the reaction centers, and in fast spectroscopy techniques. A scheme of the primary stages of the energy stabilization in reaction centers was proposed on the basis of absorption data. It is generally accepted that the excitation of the primary electron donor P870 (probably a dimer of bacteriochlorophylls) to its lowest excited singlet state P*, is followed, within 10 ps, by the transfer of an electron from P*, to a molecule of BPh (I) [1-12], giving rise to the radical pair state Pf (P^+I^-). It was suggested [9,11,13] that the electron transfer to I could occur via a molecule of bacteriochlorophyll, B800. However, this hypothesis was recently contested [14]. Authors in [15] suggested that in Pf, the electron could be localised either on BChl 800 or on the BPh molecule. The lifetime of Pf was determined to be about 200 ps [2,4,6,8,12,16,17] under normal conditions; this lifetime is lengthened to about 12 ns when electron transfer to the primary quinone Q is blocked by chemical reduction or by extraction of this acceptor [6, 18-21]. These data were obtained by fast absorption techniques. To understand better the processes leading to stabilization of the P* energy, fluorescence lifetime data from isolated reaction centers are still needed. Authors in [22] found a heterogeneous emission of the reaction centers, isolated from the R26 strain of *Rps. sphaeroides*. However, the excitation flash was too long to resolve the short (2 ns) component that they observed. We have measured here the lifetime of isolated reaction centers from wild-type *Rps. sphaeroides* and from the R26 strain lacking the carotenoid. We have employed a different technique from that in [22]. We used the phase shift method at very different modulation frequencies of light in order to resolve, accurately, lifetime components in a large range of time.

Abbreviations: LDAO, lauryldimethylamine oxide; BPh, bacteriopheophytin; BChl, bacteriochlorophyll

2. MATERIALS AND METHODS

Wild-type (strain Y) and R26 *Rps. sphaeroides* cells were grown anaerobically in the light in 'L, 17 μM Fe' medium [23]. Reaction centers were iso-

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lated with LDAO and purified as in [24], with an additional purification by DEAE-cellulose with a linear NaCl gradient. R26 reaction centers were isolated with LDAO, precipitated by 25% ammonium sulfate as in [25], and purified by DEAE-cellulose chromatography. Reaction center preparations were finally concentrated by ultracentrifugation, under N_2, on an Amincon PM 30 membrane in 10 mM Tris-HCl, 0.1 mM EDTA, 0.1% LDAO buffer (pH 8), and then dialysed against 10 mM Tris-HCl, 0.05% Triton X-100 buffer (pH 8). The reduction of reaction centers was accomplished, under anaerobic conditions, by addition of sodium dithionite (50 mM). Fluorescence experiments were performed either on fresh preparations, or on samples kept frozen in liquid N_2. No difference in the data was observed between these two preparations. The apparatus was roughly the same as in [26,27], except for the excitation light source. The modulated light source was a pulsed mode argon laser (model 164, Spectra Physics) using an acousto-optic modulator (model 365, Spectra Physics). The modulator driver was triggered by a TE 18 (Tekelec Airtronic) pulse generator. The frequencies employed in the experiments (7.25, 14.5, 29 and 58 MHz) were generated by oscillators; the corresponding alternative signals were measured through narrow electrical band pass filters. Fluorescence was detected at right angles to the excitation in a 2 × 2 mm cell. The absorbence of the samples was about 0.2 at 850 nm. The excitation energy (at 515 nm) was about 5 mW/cm^2. Fluorescence was isolated through a Schott RG830 glass filter. Calculations were carried out on an HP 9836 micro-computer.

3. RESULTS AND DISCUSSION

According to the theory of phase fluorimetry [28], one can easily calculate the average phase shift (τ_{pf}) or demodulation lifetimes (τ_{mf}) that are observed for a heterogeneous population of emitters, by using an excitation light modulated at frequency f:

$$\tau_{pf} = \left(\sum_i F_i \cos^2 \varphi_i \tau_i\right) \Big/ \left(\sum_i F_i \cos^2 \varphi_i\right)$$

$$\tau_{mf} = (1/2\pi f)(1/M^2 - 1)^{1/2}$$

$$\text{with } M^2 = \left(\sum_i F_i \cos^2 \varphi_i\right)^2 + \left(\sum_i F_i \sin \varphi_i \cos \varphi_i\right)^2$$

where F_i is the relative fluorescence intensity of component i, φ_i is the phase lag angle of component i at frequency f, and τ_i the intrinsic lifetime of component i.

Using the above equations, the characteristics (F and τ) of n elementary emitters are determined, in a unique way, by measuring T_p and T_m at n different frequencies. This problem was solved analytically in [29]. However, for more than 3 components, the problem becomes too complicated to be solved analytically. In this work, we have measured T_p and T_m at 4 light modulation frequencies: 7.25, 14.5, 29 and 58 MHz. These frequencies were chosen with the aim of measuring, with good precision, lifetimes in the range 500 ps–20 ns. The F_i and τ_i values were calculated by computation, by using the 'conjugate directions' method [30] of minimization of functions. The precision of our measurements ($\leqslant$10%) did not allow an analysis for more than 4 components (or 4 frequencies). Table 1 shows the results obtained for the fluorescence lifetimes of reduced reaction center preparations from the wild type and the R26 strain of *Rps. sphaeroides*. These data represent an average of 10 determinations, using 3 different samples of reaction center preparations. Data obtained from both strains give roughly the same decomposition (table 2) showing that the absence of carotenoid in the R26, does not greatly affect either the stages of the energy stabilization from P*, or the radiative deactivation of energy from Pf. The strong frequency dependence of the measured lifetimes (table 1), as

Table 1

Fluorescence lifetimes of reduced (50 mM $Na_2S_2O_4$) reaction centers from *Rps. sphaeroides*

Strain	T	f			
		7.25	14.5	29	58
Wild type	T_p	2.41	1.89	1.42	1.2
	T_m	5.8	4.61	3.05	2.05
R26	T_p	2.57	2.01	1.43	1.19
	T_m	5.9	4.86	3.24	2.17

τ_p and τ_m are the lifetimes obtained from measurement of the phase lag angle and from the modulation of the fluorescence signal, respectively. f is the modulation frequency (in MHz) of the exciting light. Buffer: 0.05% Triton X-100, 10 mM Tris–HCl (pH 8)

Table 2

Fitting of the measured lifetimes of table 1 on the basis of 3 or 4 components

	Four-component fit		Three-component fit	
	T(ns)	F(%)	T(ns)	F(%)
Wild type	0.04	11	0.04	11.5
	0.83	24.5	1.21	74.5
	1.45	50.5		
	12.95	14	12.8	14
S	0.015		0.017	
R26	0.04	11	0.04	11
	0.81	25	1.31	73.5
	1.51	48.5		
	12.3	16	12.35	15.5
S	0.013		0.019	

This decomposition was done by means of a function minimization program using the conjugate directions method. S, relative least square. Lifetimes (τ) are given in ns. F, relative weights of different components

well as the discrepancy between T_p and T_m values, point out a strong heterogeneity of the observed fluorescence emission. As indicated in table 2, the fitting of our results on the basis of 4 components, gives a smaller least square than with 3 components, and a much smaller one than with 2 components (not shown). The obtained decomposition shows the presence of a rapid (or direct) component in addition to the heterogeneous delayed emission (3 components). This very short component (40 ± 30 ps) is probably related to the direct emission from P* before any stabilization of excitation energy takes place. The obtained value is roughly in agreement with measurements of the electron transfer time between P and the BPh acceptor (≤10 ps) [1–12]. The small relative weight (11%) of this emission is consistent with the small energy loss required for a good efficiency of exciton capture in the reaction centers. The lifetime of the long component (12.3 or 12.9 ns) is very close to the lifetime value of Pf (12 ns), measured by absorption techniques [6,18–21]. This emission seems then to arise from charge recombination in Pf. The absence of components longer than 12 ns, in the delayed emission, suggests that the radiative deactivation from the Pf state occurs directly to the ground state rather than via reexcitation of P*. According to our results, most of such a back energy to P* would be deactivated by the high rate of the electron transfer to Pf, rather than directly, by fluorescence. In this case, a substantial lengthening (>12 ns) of the delayed emission would be observed, in contrast to our data. This result agrees with the hypothesis in [22], that only a small part of the decay of Pf occurs via P*. It must also be noted that the weight of this emission appears to be unexpectedly low (≃15%). According to the accepted scheme of the primary reactions in the reaction centers [15], one might have expected that most of the fluorescence from reduced centers would be emitted via, or from Pf. This is obviously not the case. The reason comes from the presence of two other components (0.83 and 1.5 ns)) in the delayed fluorescence. As for the component arising from charge recombination in Pf, these components are not observed when the acceptor quinone is not reduced. Thus, we suggest that these emissions are related to intermediate states whose lifetimes are lengthened when the electron transfer chain is blocked. It must be noted that on the basis of our results only, we cannot tell if these states emit fluorescence either by direct deactivation to the ground state, or from P* after a back transfer of their energy. If the latter hypothesis is right, then the effective lifetime of these states must be shorter than the measured fluorescence lifetimes, which are lengthened by several backward and forward transfers of the energy. These states, as in the case of Pf, must have a specific role in the stabilization of energy from P*. Our data are consistent with recent work of authors in [31] who suggested, on the basis of previous works [32–35], that electrostatic stabilization of the energy in the reaction centers could be provided by structural fluctuations of the proteins linked to the reaction center pigments, on the nanosecond time scale. The intermediate states that we observe could reflect different conformations of these proteins. However, we cannot exclude the possibility that these intermediate states belong to the Pf state itself. If this is the case, the lifetimes we observe here should only reflect the stabilization of Pf via substates.

Our results were analysed on the assumption that 3 components were present in the delayed part of the fluorescence. However, we cannot exclude the possibility that more than two short components (0.83 and 1.5 ns) may be present in this

emission. This would not greatly affect our conclusions. In that case, there could exist, instead of two, many states between P* and Pf associated to different conformations of the reaction center proteins. The energy deactivation from P* should then be more progressive, although irreversible. Unfortunately, no technique is actually accurate enough to resolve fluorescence emission composed of more than 3 or 4 elementary components. Further advances, in fast detector techniques, will help to resolve, more accurately, such complex emissions.

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