

ELECTRIC FIELD MODULATION OF THE FLUORESCENCE FROM *RHODOBACTER SPHAEROIDES* REACTION CENTERS

David J. LOCKHART and Steven G. BOXER

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

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The fluorescence intensity from randomly oriented *Rhodobacter (Rb.) sphaeroides* photosynthetic reaction centers at 77 K increases in an applied electric field. This is ascribed to a net reduction in the rate of the initial electron transfer reaction which competes with fluorescence due to the effect of the field on the free energy of charge separation. Quantitative analysis of the fluorescence change in an electric field indicates that the difference in permanent dipole moment between the ground and initially formed excited state is greater than between the ground and emitting state.

1. Introduction

The nature of the excited singlet state which initiates charge separation in photosynthesis has been the subject of interest for many years. The elucidation of the structure of the reaction center (RC) from *R. viridis* [1,2] and *Rb. sphaeroides* [3-6] by X-ray crystallography provides detailed information on the ground state configuration of the reactive components. Of course, the excited states of the chromophores are the functionally relevant entities, and the electronic nature of these states is not well understood. Recently there have been several new approaches to this problem: subpicosecond transient absorption spectroscopy [7-9], photochemical hole-burning spectroscopy [10-13] and Stark effect spectroscopy [14-16].

Photochemical hole-burning experiments on both species of RC demonstrate that narrow holes cannot be burned in the absorption band of the dimeric primary electron donor, also known as the special pair or P [10-13]. Because the observed holewidths were on the order of 400 cm^{-1} even at 1.3 K, it has been suggested that a very fast decay process follows the initial excitation with a time constant of tens of fs, the timescale given by applying the uncertainty principle to the observed holewidth [10-13]. A possible ultrafast process is the decay of the initial dimer excited state into a charge-transfer state, possibly in-

volving charge separation within the dimer. This hypothesis predicts that the initial excitation moves within tens of fs to an excited state with a much larger dipole moment; as a result, the fluorescence from the RC (lifetime of several ps) would be primarily from this much more dipolar state.

Although we could not rule out an ultrafast process to explain the broad holes, we have favored the alternative hypothesis that a broad hole is observed because the initial excitation is to a state whose equilibrium nuclear geometry is substantially different from the ground state [10,11]. Possible causes for the displacement include movement of the monomers comprising the special pair upon excitation, as in excimer formation, or an excited state with a large dipole moment. The latter hypothesis is very different from that discussed above: it predicts that the *initial* excited state should have a large dipole moment, and that the dipole moment of the populated state will not increase substantially until an electron actually moves from the special pair to the primary electron acceptor, believed to be a monomeric bacteriopheophytin and often denoted I. The rate constant for the reaction ${}^1\text{PI} \rightarrow \text{P}^+ \cdot \text{I}^-$ is approximately $3 \times 10^{11} \text{ s}^{-1}$ at room temperature, and about $8 \times 10^{11} \text{ s}^{-1}$ at cryogenic temperatures [7-9]. In this scheme, the fluorescence would come from essentially the same electronic state that was formed upon excitation.

We have shown by Stark effect spectroscopy that the lowest energy electronic absorption band of the special pair (Q_y band) is due to a transition between states with a substantial difference in permanent dipole moment, $|\Delta\mu_A|$ [15,16]; nearly identical results are obtained for the special pair in *R. viridis* and *Rb. sphaeroides* RCs [16]. $|\Delta\mu_A|$ is the dipole moment difference between the ground state and excited state to which the transition occurs at the instant of photoexcitation. Although the forward electron transfer reaction, ${}^1P \rightarrow P^+I^-$ (see fig. 1) is very fast, there is some fluorescence from the special pair (quantum efficiency about 4×10^{-4} [17], consistent with the observed rate of charge separation [7-9]). At low temperatures, there is only prompt fluorescence because the back reaction from P^+I^- to regenerate the excited state of P is a thermally activated process. A measurement of the Stark effect on the emission at low temperature should give similar results to what was measured in absorption if the initially excited state does not undergo an ultrafast (tens of fs) charge separation reaction. Because the fluorescence monitors the excited state until the time it decays by electron transfer to I (i.e. up to several ps), any rapid increase in the dipole moment of the excited state would be reflected in a much larger Stark effect on the emission than absorption. We do not find this to be the case.

2. Experimental

Quinone-depleted *Rb. sphaeroides* RCs, R26 mutant, were prepared by standard methods (>99% quinone depleted as determined by kinetic spectroscopy) [18,19]. The RCs were embedded in poly(vinyl alcohol) films which were coated with semi-transparent Ni electrodes as described previously [15]. The optical density for the Q_y band of the special pair for various samples was between 0.06 and 0.26 at room temperature. Bacteriopheophytin a (BPheoa) was dissolved in toluene, and the solution was mixed with poly(methyl methacrylate) and cast into films as described above for the RCs.

The samples were immersed in liquid nitrogen and the fluorescence was detected from the front face. The excitation source was either the 752 nm line from a Kr-ion laser or broad-band light from a high-pressure Xe arc lamp which was filtered to pass 350-600 nm. The fluorescence was dispersed in a single monochromator (resolution 9 nm) and detected with a Si-avalanche photodiode. The electric field was generated with a power supply of local design (± 0 -5000 V, 0-500 Hz). The electric-field-modulated fluorescence was detected at the second harmonic of the field modulation frequency by a lock-in detector (the same phase angle was used for the BPheoa and the RC samples). High-quality fluorescence spectra in the absence of the applied field were generated by mechanically chopping the excitation light and using lock-in detection.

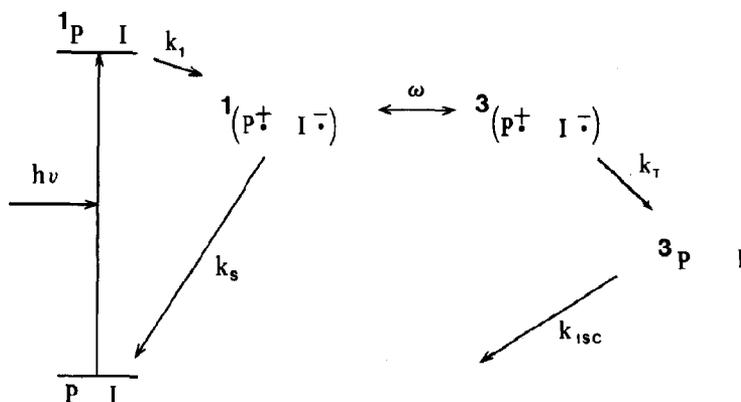


Fig. 1. Reaction scheme describing the initial events in bacterial photosynthesis when electron transfer to quinone is blocked. P is the primary electron donor or special pair and I is the initial electron acceptor.

3. Results

The fluorescence spectrum of Q-depleted *Rb. sphaeroides* RCs and the change in fluorescence in the presence of an applied electric field are shown in figs. 2A and 2B, respectively. The effect of an applied electric field on the Q_y region of the absorption spectrum for quinone-containing *Rb. sphaeroides* RCs has been presented earlier [16]. The absorption spectrum for the Q_y region of the special pair in Q-depleted *Rb. sphaeroides* RCs and the change in absorption in the presence of an applied electric field are shown in figs. 2C and 2D, respectively (the absorption feature on the blue side of the special pair band is the red side of the monomer bacteriochlorophyll band). The change in emission intensity versus electric field strength was found to be slightly subquadratic for RCs using either an ac or a dc field and quadratic for BPheoa (data not shown); the change in absorption was found to be quadratic in field

strength for both [16]. The fractional change in fluorescence was found to be independent of the exciting light intensity when the fluorescence signal was linear with excitation intensity (within 5%); essentially the same electric field effects were observed with broad-band and laser excitation. The effect of an applied electric field on the absorption and fluorescence spectra of pure monomeric BPheoa is shown in fig. 3, along with the absorption and fluorescence spectra in the absence of the field.

4. Discussion

4.1. Stark effect spectroscopy

A detailed analysis of the Stark effect on the absorption band of a randomly oriented, immobilized sample has been discussed previously [15]. A band due to a transition between states with a difference

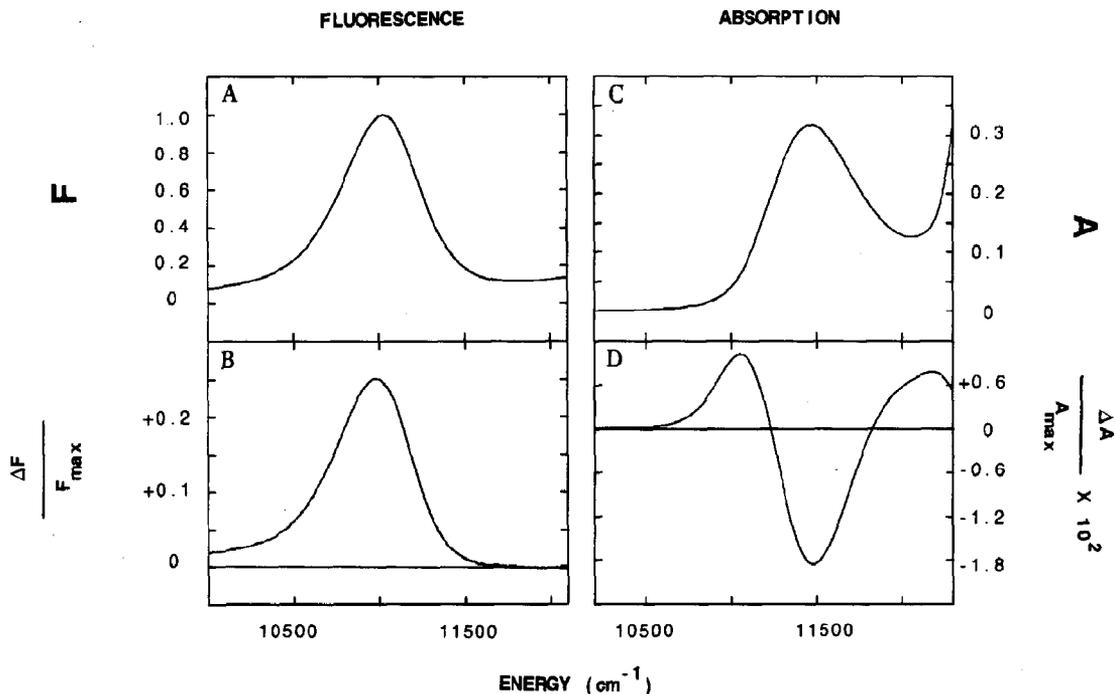


Fig. 2. (A) Fluorescence spectrum of *Rb. sphaeroides* RCs in zero applied field at 77 K; (B) the effect of an applied electric field on the fluorescence spectrum at 77 K ($F_{\text{ext}} = 8.9 \times 10^5$ V/cm); (C) absorption spectrum of the Q_y transition of the special pair in zero applied field at 77 K (the absorbance increase on the high energy side is due to the Q_y band of the bacteriochlorophyll monomers in the RC [16]); (D) the effect of an electric field on the absorption spectrum at 77 K ($F_{\text{ext}} = 8.9 \times 10^5$ V/cm). The same sample was used for all spectra. A_{max} and F_{max} are the values of the absorbance and fluorescence, respectively, at the peak of the bands.

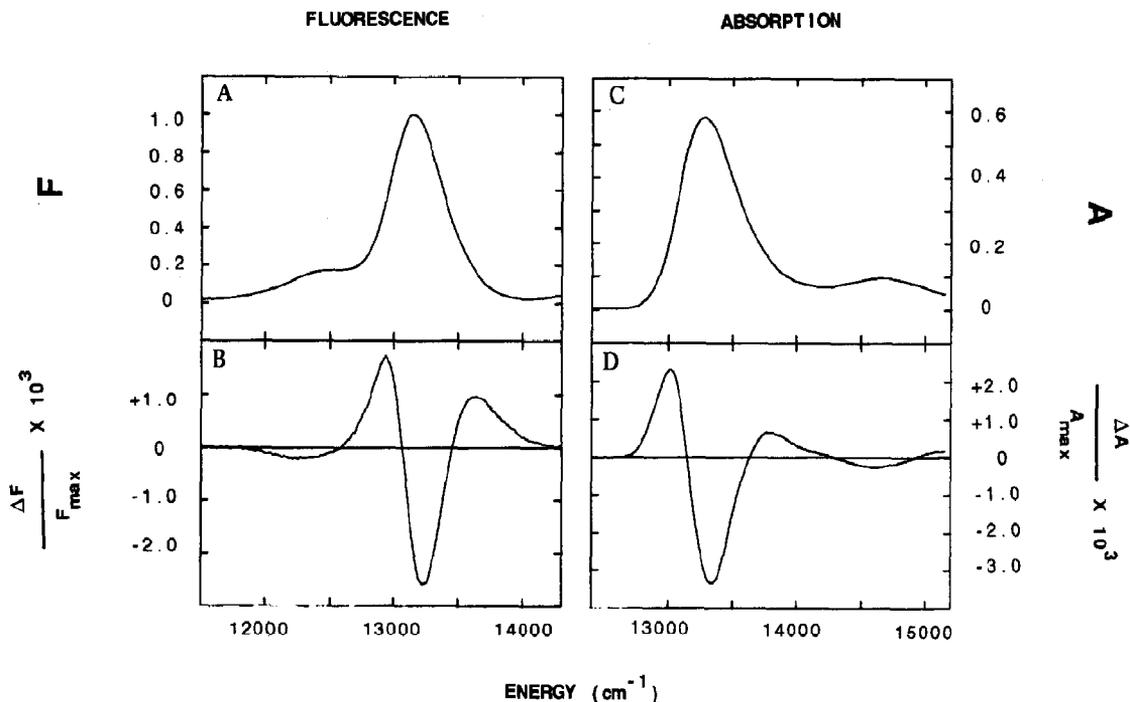


Fig. 3. (A) Fluorescence spectrum of bacteriopheophytin **a** in zero applied field at 77 K; (B) the effect of an applied electric field on the fluorescence spectrum at 77 K ($F_{\text{ext}}=9.2 \times 10^5$ V/cm); (C) absorption spectrum of the Q_y transition in zero applied field at 77 K; (D) the effect of an applied electric field on the absorption spectrum (data obtained with $F_{\text{ext}}=4.24 \times 10^5$ V/cm and scaled to $F_{\text{ext}}=9.2 \times 10^5$ V/cm to facilitate comparison with fluorescence; ΔA was found to be quadratic in applied field strength).

in permanent dipole moment, $|\Delta\mu|$, is broadened in the presence of an electric field. If the change in absorption, ΔA , is small, it has approximately the shape of the second derivative of the absorption spectrum; the magnitude of ΔA is proportional to the square of the applied field, $|\Delta\mu|^2$, and the second derivative of the absorption spectrum. The same principles apply to the Stark effect on the fluorescence spectrum [20–23]^{#1}. In order to distinguish the change in dipole moment for absorption and fluorescence, we use the notation $\Delta\mu_A$ and $\Delta\mu_F$, respectively. If polarized light is used to probe ΔA , the angle between the applied electric field and the electric vector of the probing light can be varied to determine the angle ζ between $\Delta\mu$ and the transition dipole moment for the transition of interest [15]. Similar methods can be

used in emission by varying the angle between the applied electric field and the polarization direction of an analyzing polarizer. When the electric field also affects the fluorescence quantum yield (as in the present case) by changing the free energy of the reactants and products of a competing electron transfer reaction, an analysis of the fluorescence polarization can be used to probe the *orientation* of the difference dipole moment between the reactant and product states (dominated in this case by the $P^+ \cdot I^-$ dipole moment, *vide infra*) relative to the fluorescence transition moment. Results and analysis of such experiments are presented elsewhere [24]. In this communication we treat the problem isotropically and semi-quantitatively.

The Stark effect on the Q_y region of the special pair absorption spectrum of quinone-depleted *Rb. sphaeroides* RCs (fig. 2D) provides a typical example of the expected second derivative lineshape. A quantitative analysis of $|\Delta\mu_A|$ at 77 K for the special pair Q_y transition gives the values $\zeta=38 \pm 2^\circ$ and

^{#1} For an effect due to a dipole moment difference, ΔA is proportional to $\nu d^2[A(\nu)/\nu]/d\nu^2$ [15]; ΔF is proportional to $\nu^3 d^2[F(\nu)/\nu^3]/d\nu^2$ [20–23], where $A(\nu)$ and $F(\nu)$ are the absorption and emission spectra, respectively.

$|\Delta\mu| = (7.0 \pm 0.5 \text{ D})/f$ [16], where f is the local field correction accounting for the difference between the applied field, F_{ext} , and the actual internal field, F_{int} , felt by the molecules under investigation. The value of f is uncertain, but it is likely to be about 1.2 [15]. The Stark effect spectra for both the absorption and the fluorescence of pure monomeric BPheoa in poly(methyl methacrylate) at 77 K (fig. 3) likewise show the expected second derivative lineshape. A quantitative analysis of the absorption gives $|\Delta\mu_{\text{A}}| = (2.6 \pm 0.14 \text{ D})/f$ and $\zeta = 9.5 \pm 2^\circ$ [16]. Assuming the same value of ζ for the fluorescence, we obtain $|\Delta\mu_{\text{F}}| = (1.9 \pm 0.2 \text{ D})/f$. As expected for a $\pi\pi^*$ transition in a rigid molecule, $|\Delta\mu_{\text{A}}|$ is approximately equal to $|\Delta\mu_{\text{F}}|$. The small difference may be due to minor polarization artefacts in emission or a different value of ζ ; a systematic study of a series of chromophores is in progress and should provide a more precise answer.

4.2. Upper limit on $|\Delta\mu_{\text{F}}|$ for the reaction center

Based on the results for the RC absorption Stark effect spectrum and pure BPheoa, it is striking that the effect of an electric field on the fluorescence spectrum of the RC (fig. 2B) shows very little evidence of a second derivative component; rather, it is very similar in lineshape to the fluorescence spectrum itself. Thus, it is not possible to directly extract the value of $|\Delta\mu_{\text{F}}|$ from the spectrum. We note at the outset of this analysis that the fluorescence quantum yield in the RC is very low and is consequently sensitive to contamination from RCs with degraded function. In the following we treat the observed fluorescence intensity in the 880–1000 nm region as being due to fully functional RCs; if some intensity were from non-functional RCs, this could decrease the observed value of $\Delta F/F_{\text{max}}$, and the values of $|\Delta\mu_{\text{F}}|$ calculated below would be too low. It is likely that the electric field modulated portion of the fluorescence is due to functional RCs and that the fluorescence is from intact dimers since the emission is too far to the red to be from monomeric BChl_a. Inspection of the shape of ΔF (fig. 2B) immediately suggests that $|\Delta\mu_{\text{F}}|$ is not large as there is no evidence for a second derivative component.

A variety of approaches of increasing sophistication can be taken to analyze the data in fig. 2B more

quantitatively. It can be shown that, in general, zeroth, first and second derivative components are expected when Stark effects due to $|\Delta\mu_{\text{A}}|$ and $|\Delta\mu_{\text{F}}|$ are combined with electric field effects on the quantum yield of a process which competes with fluorescence [25]. In order to be useful, this general analysis requires comprehensive data as a function of excitation and emission wavelength and polarization. Both the observed spectral shift and slightly sub-quadratic field dependence are potential consequences of these more general considerations under certain conditions; however, it will be some time before sufficient experimental information is available to warrant the application of this general analysis. For now, we consider simply the magnitude of $\Delta F/F_{\text{max}}$ and the lineshape.

In order to calculate $|\Delta\mu_{\text{F}}|$ from the observed spectra we deconvolve the Stark spectrum into a sum of zeroth, first and second derivative components of the observed fluorescence spectrum since $|\Delta\mu_{\text{F}}|^2$ is inversely proportional to the second derivative of the fluorescence. In order to obtain a fluorescence spectrum of sufficient quality to reliably calculate derivatives, the fluorescence spectrum in fig. 2A was fit to a sum of four Gaussians; this gave an excellent fit to the fluorescence for subsequent processing^{#2}. The observed Stark effect spectrum was then fit to a sum of the derivatives of the fluorescence spectrum fit. The best fit to the Stark effect spectrum was obtained by adding in very little of a second derivative component (the average value for independent data sets was nearly zero), i.e. an excellent fit can be obtained assuming $|\Delta\mu_{\text{F}}| = 0$. In order to determine if larger contributions from a second derivative component might still give a reasonable fit, we fixed the amplitude of the second derivative component, and allowed only the zeroth and first derivative components to vary to minimize the deviation between

^{#2} There is no physical significance to the Gaussian components used in the best fit. The second derivative of the fluorescence spectrum itself is too noisy to be useful. The special pair fluorescence spectrum is always contaminated on the blue side with some background fluorescence, presumably from degraded RCs or traces of residual antenna. The Stark effect on this background is negligible (fig. 2B), consequently we adopt the conservative approach of only comparing the observed and computed Stark effect spectrum for the red two-thirds of the special pair fluorescence band.

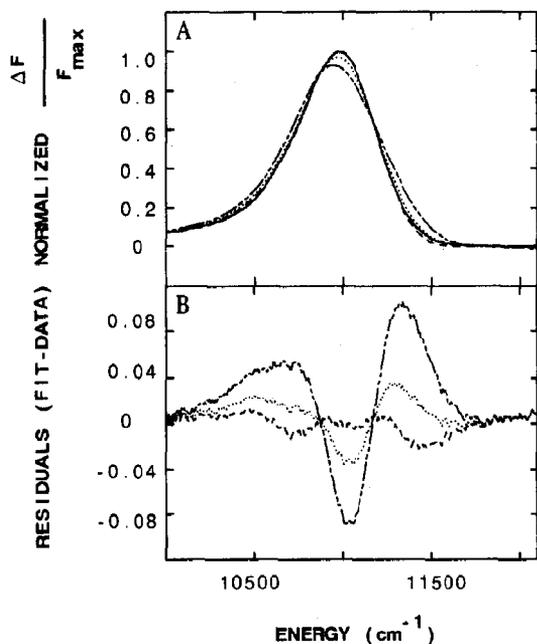


Fig. 4. (A) Quantitative analysis of the observed change in fluorescence in the presence of an electric field (—): best fit to a sum of zeroth, first, and second derivative components of the fluorescence spectrum (---), and best fits using fixed second derivative components of the fluorescence corresponding to $|\Delta\mu_F| \leq (4.7 \text{ D})/f$ (...) and $|\Delta\mu_F| \leq (8.7 \text{ D})/f$ (----). The indicated values of $|\Delta\mu_F|$ are the maxima consistent with the magnitude of the second derivative contribution; (B) residuals (fit minus data) of the fits in (A).

the data and the fit. This procedure is illustrated in fig. 4 for several fixed percentages of a second derivative component corresponding to the values of $|\Delta\mu_F|$ indicated in the figure caption. It is evident that only a rather small value of $|\Delta\mu_F|$ is compatible with the data; to be most conservative we find that $|\Delta\mu_F| < |\Delta\mu_A|$.

Assuming that the dipole moment of the ground state is the same in absorption and fluorescence, this result implies that *the dipole moment of the excited state does not increase in the time window sampled by the fluorescence*, i.e. several ps. The data are consistent with $|\Delta\mu_F|$ being considerably less than $|\Delta\mu_A|$. Possible mechanisms which might account for such a decrease include the following. (i) The mixing between pure charge-transfer states and exciton states may change as the excited states relax. (ii) The macrocycles may move more closely together as in excimer formation, or they may move parallel to each

other in a sliding motion decreasing the Mg-to-Mg separation. Either motion could reduce the degree of charge separation (changes of 0.2 Å correspond to changes of roughly 1 D) or change the nature of the state as in (i). (iii) The angle between the ground and excited state dipole moment could change due to any of the above thereby affecting the difference dipole moment.

4.3. Effect of an electric field on the quantum yield for charge separation

Although these experiments were designed to measure $|\Delta\mu_F|$, we have discovered an unexpected and substantial *increase* in the fluorescence yield in an applied electric field. In order to explain this novel effect we hypothesize that the field changes the energy of the initial intermediate, P^+I^- relative to ${}^1\text{P}$, thus affecting the rate of the electron transfer reaction which competes with fluorescence^{#3}. The center-to-center separation between P and I is about 17 Å [1], consequently the dipole moment of the P^+I^- state is expected to be about 80 D; as a result, the energy of this state will vary enormously in an applied field depending on the orientation of the dipole relative to the field (for an 80 D dipole aligned with the field, the energy decreases by 0.017 eV per 10^5 V/cm). A simple model for the dependence of the rate of electron transfer on free energy is the classical Marcus theory which predicts an inverted parabola for the dependence of $\ln k_{\text{et}}$ on ΔG^0 [27]. Since the ${}^1\text{P} \rightarrow \text{P}^+\text{I}^-$ reaction is believed to be activationless [9], the electron transfer rate is the maximum at zero field (the top of the parabola) and any change in ΔG^0 due to the applied electric field is expected to reduce k_{et} and increase the fluorescence. There is little question that the classical Marcus model is an oversimplification, however, *any* form of the $\ln k_{\text{et}}$ versus ΔG^0 curve which has a non-zero second derivative or is asymmetric over a suitable range about the zero field value will lead to a net change of the fluorescence yield for an isotropic sample in an applied electric field. Thus, although an analysis of the magnitude of the field effect requires a more precise model of the reaction dynamics (see below), this basic physical

^{#3} Fluorescence quenching by a possibly related mechanism in phthalocyanine films has been reported by Popovic [26].

picture can account for the results shown in fig. 2.

The effects of electric fields on the millisecond recombination kinetics in quinone-containing RCs ($P^+Q^- \rightarrow PQ$) have been measured for RCs oriented in lipid bilayers [28] and in Langmuir-Blodgett films [29-31]. Dutton and co-workers further showed that the quantum yield of P^+Q^- formation in these films is reduced by application of a field [31]; however, since this experiment could not separate the steps ${}^1PIQ \rightarrow P^+I^-Q \rightarrow P^+IQ^-$, it was not certain whether the electric field was affecting the first or second step or both. Our observation that the fluorescence quantum yield is increased by an electric field demonstrates that the first step can be affected ^{#4}.

Finally, it is useful to see whether the *magnitude* of the increase in fluorescence in an applied electric field can be modeled by simple electron transfer theories. We model the situation simply as follows: (i) assume that the effect on the fluorescence intensity in an applied field is caused by a change in the rate of the initial electron transfer step due to a change in the energy of the P^+I^- state (we neglect the change in the energy of the 1P state); (ii) treat the P^+I^- dipole moment as a full electronic charge separated by 17 Å; (iii) assume that the electric field dependence of the rate of the activationless ${}^1PI \rightarrow P^+I^-$ reaction can be described by a simple Marcus theory expression with the reorganization energy equal to 0.26 eV, which is the standard free energy change in zero field for the initial reaction in quinone-depleted RCs [32]; (iv) assume that there are no other radiationless decay processes from the 1P state.

For an isotropic sample at 77 K and $F_{int} = 3 \times 10^5$ V/cm (we assume that $f=1.2$), the fluorescence intensity is calculated to increase by 14%, which is substantially larger than the change we observe (about

3%) ^{#5}. As mentioned above, it is very unlikely that the simple Marcus expression, in which the rate versus free energy curve is symmetric about $\Delta G^0 = -0.26$ eV, is applicable to the RC problem. Rather, a better model (though still oversimplified) would be to assume that the rate is independent of driving force for more negative values of ΔG^0 than the zero field value: in this case the fluorescence is expected to increase by about 7% for our experimental conditions, closer to the experimentally observed increase. A much more refined analysis of the electric field effect on fluorescence and the charge separation rate will be possible when data become available using polarized light and fully oriented RCs.

In conclusion, we have shown that the total fluorescence from *Rb. sphaeroides* RCs increases in an applied electric field. The magnitude of the increase obscures the direct observation of the second derivative component which is needed to obtain $|\Delta\mu_F|$. Nonetheless, it is possible to place an upper limit on $|\Delta\mu_F|$ by estimating the largest magnitude of the second derivative of fluorescence component which is consistent with the observed Stark effect spectrum. This analysis demonstrates that $|\Delta\mu_F|$ could be very small (close to zero), but could (in a most conservative analysis) be as large as $|\Delta\mu_A|$. Thus, the dipole moment of the excited state of the special pair does not increase rapidly, as it would if a charge transfer state were populated within tens of fs following photoexcitation. The fluorescence increase can be explained by the effect of an electric field on the energy difference between the 1P and P^+I^- states and thus on the rate of the electron transfer reaction which competes with the fluorescence. It should be noted that the fluorescence change in fig. 2B is detected with

^{#4} The ground state population under constant illumination could also be affected by an electric field, if the triplet state decay rate depends on field (the triplet state is formed with a very high quantum yield in quinone-depleted RCs at 77 K; quinone-depleted RCs were used for these experiments to avoid saturation). The fact that $\Delta F/F$ is independent of light intensity within the experimental error rules out the possibility that the effect in fig. 2B is due to a field effect on the ground state population.

^{#5} For the purpose of analyzing the magnitude of the fluorescence increase, results obtained at lower fields are used. In this regime the effect can more safely be treated perturbatively (i.e. the energy but not the nature of the states is changed). If the fluorescence quantum yield is proportional to $k_f/(k_i+k_f)$, where k_f is the rate of fluorescence (approximately 2×10^8 s⁻¹) and k_i (fig. 1) is the initial electron transfer rate constant of about 8×10^{11} s⁻¹ at 77 K, then the observed increase of about 25% in the fluorescence quantum yield for an applied dc field of 8.9×10^5 V/cm corresponds to an average increase in the 1P lifetime of about 0.2 ps. For an isotropic sample in an electric field there is a distribution of rate constants with the rate constant for a given subset of the population depending on the orientation of the RCs relative to the applied field.

excellent sensitivity; it would be possible to quantitatively measure changes at least one order of magnitude smaller, corresponding to reductions of the *average* charge separation time constant of hundredths of ps (see footnote 5). Thus, this cw method is exquisitely sensitive to changes in rate constants which would be extremely difficult to detect by transient methods and promises to be generally useful for studying electron transfer in model systems. Such studies will be reported shortly.

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