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Phil. Trans. R. Soc. Lond. B 1989 **323**, 227-239
doi: 10.1098/rstb.1989.0006

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Phil. Trans. R. Soc. Lond. B **323**, 227–239 (1989)
Printed in Great Britain

The origin of chlorophyll fluorescence *in vivo* and its quenching by the photosystem II reaction centre

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Isolated chlorophyll *a*, in contrast to when it is dissolved in organic solvents, shows a lower and variable yield of fluorescence when bound to protein and embedded in the thylakoid membrane of photosynthetic organisms. There are two current theories that attempt to explain the origin of this variable yield of fluorescence. (i) It may be emitted directly from the photosystem II (PS II) antenna system and therefore in competition with photochemical trapping (prompt fluorescence). (ii) It may be derived from a recombination reaction between oxidized P_{680} and reduced pheophytin within the PS II reaction centre (delayed fluorescence).

We have isolated a PS II reaction centre complex that binds only four chlorophyll *a* molecules and can carry out primary charge separation. The complex contains no plastoquinone and therefore is devoid of the secondary electron acceptor Q_A. It does, however, contain two pheophytin *a* molecules, and one of these acts as a primary electron acceptor. The electron donor is P_{680}, which is either a monomeric or dimeric form of chlorophyll *a*. The isolated PS II reaction centre fluoresces at room temperature with a maximum at 683 nm, and the intensity of this emission is almost totally quenched when reduced pheophytin (bright light plus sodium dithionite) or oxidized P_{680} (bright light plus silicomolybdate) is photoaccumulated. The photo-induced quenching of chlorophyll fluorescence when sodium dithionite is present is also observed in intact PS II preparations containing plastoquinone Q_A. In the latter case Q_A is chemically reduced in the dark by dithionite. Bearing in mind the above two postulates for the origin of variable chlorophyll fluorescence it has been possible to investigate the relative quantum yields for the photoproduction of the $P_{680}Pheo^-$ state either in the absence (with isolated PS II reaction centres) or presence (with PS II-enriched membranes) of reduced Q_A. It has been shown that in the absence of Q_A^- the quantum efficiency for production of the $P_{680}Pheo^-$ is several orders of magnitude greater than when Q_A^- is present. This difference probably partly reflects the coulombic restraints on primary charge separation when Q_A is reduced and would suggest that under these conditions the PS II reaction centre is a less efficient trap. Such a conclusion is therefore consistent with postulate (i) that the increase in yield of chlorophyll fluorescence as Q_A becomes reduced is not due to a back reaction between P_{680}^+ and $Pheo^-$ but rather to a decrease in competition between emission and trapping. The results do emphasize however, that the $P_{680}Pheo^-$ and P_{680}^+Pheo states are quenchers of chlorophyll fluorescence.

In addition to the above, it has been noted that at 77 K fluorescence from the isolated PS II reaction centre reaches a maximum at 685 nm and does not have a peak at 695 nm. This observation appears to invalidate the postulate that the 695 nm emission is from the pheophytin of the PS II reaction centre.

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1. Introduction

When isolated and dissolved in an organic solvent such as ether, chlorophyll *a* has a relatively high fluorescence yield, ϕ_f, of about 30%. Almost all the remaining population of excited molecules de-excite to the ground state via intersystem crossing to the triplet state (Bowers & Porter 1967). As the calculated intrinsic lifetime, τ_i, for the lower excited singlet state of chlorophyll *a* is about 15 ns (this would be the lifetime if the only route for de-excitation is by fluorescence), then the measured or actual lifetime, τ_a, is about 5 ns under these conditions (Brody & Rabinowitch 1957). For chlorophyll *a in vivo* the maximum yield of fluorescence occurs when the reaction centres are closed and is usually termed the F_M level (in contrast to the F_0 level which is the minimum level when all the reaction centres are open). At the F_M level the measured fluorescence lifetime is in the region of 1.5 ns (Brody & Rabinowitch 1957) which corresponds to a fluorescence yield of 10%, assuming the relation $\phi_f = \tau_a/\tau_i$ can be applied. This calculation of the maximum *in vivo* yield contrasts with the yield estimated from steady-state measurements at room temperature, which relates the total amount of light absorbed, I_a, with the amount emitted as fluorescence, I_f (i.e. $\phi_f = I_f/I_a$). In this case the maximum value of ϕ_f is estimated to be approximately 3% (Latimer *et al.* 1956). The reason for this lack of correlation is that only about one third of the total chlorophyll *in vivo* is able to fluoresce with a significant yield, this being the chlorophyll *a* molecules associated with photosystem II (PS II). The emission maximum is at 685 nm. At liquid nitrogen temperature and below, however, additional emission bands appear with maxima at 695 nm and 735 nm. Although it is well accepted that the 735 nm emission is from chlorophyll *a* molecules associated with photosystem I (PS I) (Murata & Satoh 1987), the origin of the 695 nm fluorescence is less clear. Breton (1982) argued that this low-temperature emission came directly from pheophytin molecules within the PS II reaction centre. This hypothesis found considerable favour, so much so that it became the basis for assaying the presence of the PS II reaction centre (see, for example, Nakatani *et al.* 1984).

At room temperature the intensity of chlorophyll fluorescence varies considerably, responding primarily to limitations imposed on electron transport from PS II, which exert immediate influence on the degree of openness of the PS II reaction centres and establish a fluorescence level between F_0 and F_M. However, other fluorescence changes can also occur, usually with slower kinetics, that affect both F_0 and F_M. These changes are in response to a number of factors, including the establishment of a pH gradient across the membrane and alterations in the interaction between pigment systems. The precise molecular basis for the variation of fluorescence between the two extremes (i.e. F_0 and F_M) is a matter of controversy. On one hand it has been argued that it represents changes in various routes of de-excitation of the excited chlorophylls in the PS II light-harvesting system. As an appropriate basis for discussion of this possibility the standard equation for fluorescence quantum yield, expressed in its simplest form is

$$\phi_f = k_F/(k_F + k_H + k_{IC} + k_S + k_P[P]), \tag{1}$$

where k_F, k_H, k_{IC}, k_S and k_P are, respectively, the rate constants for de-excitation of chlorophyll via fluorescence, heat (vibrational relaxation), internal conversion to triplet (does not occur to any significance *in vivo*), transfer to non-fluorescing species such as PS I (spillover) and photochemistry. [P] is the fraction of open PS II reaction centres with the primary donor P_{680} reduced and primary quinone acceptor Q_A oxidized (P stands for $P_{680}Q_A$). When [P] = 1, the

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F_0 level is obtained, whereas when the reaction centre is closed (all Q_A is reduced and $[P] = 0$) the F_M level is achieved (Duysens & Sweers 1963). Because $\tau_i = 1/k_F$ and $\tau_a = 1/\Sigma_i k_i$ then equation (1) can be written in terms of the singlet lifetime

$$\phi_f = \tau_a/\tau_i. \tag{2}$$

If equation (1) correctly describes variable fluorescence, and assuming efficient energy transfer occurs between adjacent PSII units, then there should therefore be a linear relation between ϕ_f and τ_a on-going from F_0 to F_M. Such a relation was found by Briantais *et al.* (1973). With the advent of mode-locked lasers with picosecond time resolution a more complex picture has emerged. This very rapid technique has identified several phases in the decay but different laboratories using different deconvolution procedures have interpreted these phases in different ways. There is general agreement about the origin of the very short-lived lifetime (about 80 ps) as being derived from the chlorophylls of PSI. The problem arises with the medium and long lifetimes, which should correlate with variable fluorescence. Holzwarth & Moya and their colleagues have concluded that equation (1) is basically correct and only requires modification to take into account the heterogeneity of PSII, the so-called α and β centres (Holzwarth *et al.* 1985; Shatz & Holzwarth 1986; Moya *et al.* 1986). On the other hand, the analyses of Sauer and colleagues (Haehnel *et al.* 1982; Karukstis & Sauer 1983) do not agree with equation (1) but with a model that has been proposed by Klimov *et al.* (1977). The Klimov model argues that variable fluorescence is derived from a recombination reaction within the PSII reaction centre between P_{680}^+ and the primary acceptor pheophytin ($Pheo^-$). This back reaction is viewed as being a significant process and occurs when Q_A is fully reduced so that forward electron transfer to the quinone is not possible (Klimov & Krasnovsky 1981). Thus according to this model the variable emission is a very rapid form of 'delayed fluorescence' with an apparent lifetime of 1.5 ns governed by the rate of recombination between P_{680}^+ and $Pheo^-$. Klimov's main evidence for this hypothesis is that when the reaction centre is trapped in the state $P_{680}Pheo^-Q_A^-$ (e.g. in the presence of dithionite and strong light (Klimov *et al.* 1977), or under anaerobic conditions (Klimov *et al.* 1986)) the F_M level is significantly quenched. If the Klimov model is correct, and assuming that there is energy transfer between adjacent PSII units, then there will not be a correlation between ϕ_f and τ_a as predicted by equation (2). Indeed, Sauer and colleagues, using a three component analysis of the fluorescence decay, claim that from F_0 to F_M the main effect is an increase in the amplitude of the slowest components with nanosecond lifetimes (Haehnel *et al.* 1982; Karukstis & Sauer 1983).

Taking the results of Sauer and colleagues at face value, another explanation is possible that does not invoke the Klimov model, namely that PSII is composed of independent units with no or very little energy transfer between them. If this were the case then according to equation (2) there would be two distinct fluorescence lifetimes corresponding to an open and closed reaction centre. Thus interpretation of the relation between variable fluorescence yield and singlet lifetimes is complex and requires detailed mathematical modelling coupled with reliable experimental data (see Malkin 1974; Malkin *et al.* 1980). The inconsistencies and controversies which exist at present reflect the complexity and heterogeneity of the photosynthetic apparatus and the problems of instrumental response times and deconvolution procedures.

It must be concluded that, despite considerable effort by many laboratories, the precise mechanism which gives rise to variable chlorophyll fluorescence *in vivo*, including the processes of energy transfer, is still unclear. Such a lack of knowledge therefore makes it difficult to

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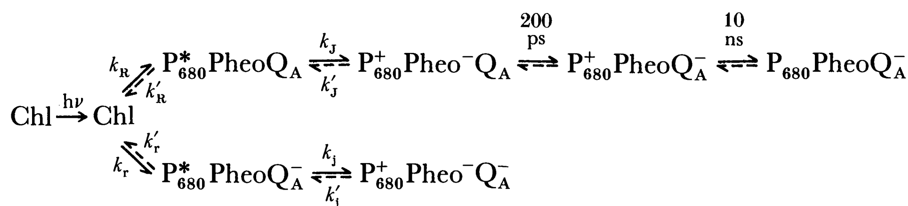
interpret the functional significance or understand the molecular mechanisms that underlie the various fluorescence quenching phenomena for the changes in F_0 and F_M observed in intact systems (Krause & Weis 1984). The recent isolation of the PS II reaction centre (Nanba & Satoh 1987; Barber *et al.* 1987) offers a new opportunity to investigate some important basic questions regarding the origin of variable fluorescence.



Scheme 1

These questions can be formulated in terms of the scheme of the initial photochemical events shown in scheme 1. If the rate constants k_R and k_r are smaller than k_J and k_j, then the rate of trapping of excitons by the reaction centre is limited by diffusion of excitons in the antenna pigment system. On the other hand, if they are larger then the processes are limited by the trapping act itself. In the latter case an exciton may visit a reaction centre and return to the antenna system several times without causing any photochemistry (k_R and $k_r > k_J$ and k_j). In both cases the yield of fluorescence would be expected to follow equation (1) (assuming energy transfer between PS II units). If k_J or $k_j > k_R$ or k_r then every excitation that reaches an open reaction centre (i.e. P_{680}Pheo state) has a high probability of causing a charge separation. If Q_A is oxidized then the electron is rapidly passed to it; hence the primary excitation results in stable photochemistry. However, if Q_A is reduced and P_{680} is oxidized, then the state $P^+_{680}Pheo^-Q^-_A$ would be created, and it is Klimov's proposal that the recombination of the primary radical pair gives rise to variable fluorescence. It seems quite reasonable to assume that k_R and k_r will have similar values, but can such an assumption be made for k_J and k_j? It seems likely that these two latter rate constants would be different, bearing in mind the electrostatic constraints that Q^-_A could place on the generation of the $Pheo^-$ state.

The isolation of the PS II reaction centre free of Q_A provides a chance to measure the quantum yield for the formation of the $P^+_{680}Pheo^-$ state in the absence of the electrostatic field generated by Q^-_A and to investigate the possible role of this radical pair in generating fluorescence.

2. Materials and methods

Thylakoid membranes have been isolated from seedlings of *Pisum sativum* (var. Feltham First) by using the method of Nakatani & Barber (1977) except that the medium used for the homogenization step was 50 mm KH_2PO_4, pH 7.5 (NaOH), 0.35 m KCl and 0.5 mm EDTA. The chloroplast pellet was obtained by centrifugation at 5000 *g* at 4 °C for 10 min and then resuspended in 6 mm $MgCl_2$ to rupture any intact plastids by osmotic shock. A PS II-enriched membrane fraction (Berthold, Babcock & Yocum: BBY) was prepared from these thylakoids by following the procedure of Berthold *et al.* (1981). Normally the BBYs were stored at 4 mg Chl ml^{-1} at −80 °C in the presence of glycerol.

To isolate the D1/D2/cytochrome b_{559} complex a PS II-enriched membrane sample of

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200 mg chlorophyll was thawed and washed, to deplete extrinsic membrane polypeptides, by dilution to 0.8 mg Chl ml^{-1} in 50 mM Tris, pH 9.0, incubation on ice in the dark for 10 min and then centrifugation at 40000 *g*, 4 °C for 20 min. The pellets were then resuspended in 50 mM Tris, pH 7.2 (200 ml) and 33 ml of 30% Triton X-100 to give a final chlorophyll concentration of 0.8 mg ml^{-1} and a Triton:chlorophyll ratio of 50:1 (by mass). A 60 min incubation with stirring, in the dark, on ice was followed by centrifugation at 100000 *g* for 60 min and application of the supernatant to a column (16 mm × 300 mm) of Fractogel TSK DEAE-650 (S) (Merck-BDH) maintained at 6 °C. Extensive washing at 0.4 ml min^{-1} was done with 350 ml of 30 mM NaCl in a running buffer of Triton X-100 (2 g l^{-1}), 50 mM Tris-Cl, pH 7.2. This resulted in the return of the absorbance (at 280 nm) of the eluant to the same level as for the running buffer itself and removed more than 98% of the chlorophyll applied. The material remaining on the column was eluted by a linear concentration gradient of 2 mM NaCl ml^{-1}. Appropriate 2 ml fractions centred around 100 mM NaCl were pooled, diluted fourfold in running buffer and loaded on a smaller column (9 mm × 100 mm) of the same DEAE Fractogel. After further extensive washing with 30 mM NaCl in running buffer (about 50 ml at 0.5 ml min^{-1}) and a linear NaCl gradient (5 mM ml^{-1}) the complex eluted as a sharp peak (detected 280 nm) at about 110 mM NaCl. The purity of the sample was checked by its room-temperature absorption spectrum, by its chlorophyll:cytochrome b_{559} ratio and by its profile on sodium dodecyl sulphate polyacrylamide gel electrophoresis. The isolated complex is particularly unstable at room temperature and in bright light (Chapman *et al.* 1988). Throughout its isolation and subsequent handling, therefore, it is necessary to keep it on ice and avoid exposure to unnecessary light. With this care the complex is stable and indeed can be stored at liquid-nitrogen temperatures without obvious detrimental consequences. In its purest form this complex contains four chlorophyll *a* molecules, two pheophytin, one β-carotene, one cytochrome b_{559} and at least one non-haem iron atom. It binds no manganese or plastoquinone. It therefore cannot photocatalyse water oxidation or the reduction of Q_A and Q_B.

Relative chlorophyll fluorescence yields and their time dependencies were measured by using a modulated fluorescence technique incorporating optical fibres manufactured by Heinz Walz (Effeltrich, F.R.G.). The reaction vessel was a well-stirred 1 cm diameter cuvette of a Hansatech oxygen electrode that was cooled to about 4 °C. The actinic light was provided by a quartz–iodine lamp, the intensity of which was varied with neutral-density filters; light was usually passed through a 3 mm Schott BG-18 filter and a yellow cut-off cellophane filter (515 nm, Lee Filters, Andover, U.K.). The transmitted light was green ('G' light), peaking at 525 nm with limits between 480 and 600 nm.

To estimate the intensity of absorbed light in the cylindrical cell the technique of potassium ferrioxalate actinometry was employed (Hatchard & Parker 1956). For this purpose we used different wavelengths of light suitable for actinometry (actinometric light, 'A'), such that the average light absorption by the actinometer solution with respect to 'A' matched the average light absorption of the sample with regard to the green 'G' light. The incident intensities of the A and G beams were then compared at the exit of the light guide by photoelectric measurements (Licor Model Li-185 radiometer). It was found that for the particular concentration of chlorophyll that we used routinely (*ca.* 7 μM) the average light absorption (weighted according to the filter transmission characteristics and light output data for quartz–iodine lamps versus wavelength) corresponded to the use of a standard actinometric potassium ferrioxolate solution of 0.15 M when measured at 484 nm with a narrow-band

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interference filter (Balzers). (The average absorption in a 1 cm path-length cell amounted to about 29%.) The absorbed intensity of G, I_G, is then given by $I_G = I_A(i_G/i_A)$, where I_A is the absorbed intensity of the A beam found directly by the actinometry and i_G and i_A are the corresponding photoelectric measurements. For the actinometric determinations we followed exactly the procedures outlined by Hatchard & Parker (1956).

Chlorophyll concentrations were assayed by the method of Arnon (1949) and cytochrome b_{559} was determined by recording the difference spectrum between the oxidized and reduced state (ferricyanide against dithionite) and applying an extinction coefficient of 15 mM^{-1}. Absorption spectra and light-induced redox changes were monitored with either a Perkin–Elmer 554 or 557 UV/Vis spectrophotometer (1 cm light path). Side illumination was provided by a quartz–iodine light source with an appropriate light guide and transmission filters (Calflex heat filter and 2 mm Schott RG 660 cut-off filter). The intensity of the actinic light at the cuvette surface was 114 μmol quanta m^{-2} s^{-1}. The photomultiplier was shielded by a 4 mm Schott BG18 broad-band filter. In some experiments the relative yield changes of modulated chlorophyll fluorescence were simultaneously measured with optical-density changes by placing the optical fibre of the Heinz Walz fluorimeter immediately above the 1 cm cuvette within the Perkin–Elmer 557 spectrophotometer. Emission spectra were measured with a Perkin–Elmer MPF 44A fluorimeter.

3. Results

Fluorescence emission

Figure 1 compares the emission spectra of the D1/D2/cytochrome b_{559} reaction-centre complex at room temperature and at 77 K. It can be seen that at room temperature the isolated reaction centre fluoresces with a maximum at 683 nm. At 77 K the emission peaks at about 685 nm and there is no additional peak at 695 nm. This latter observation contradicts the well-cited dogma that the low-temperature fluorescence at 695 nm emanates from pheophytin within the PSII reaction centre (Breton 1982). In fact it seems most probable that this low-temperature emission originates from the PSII chlorophyll-binding protein CP47 (Van Dorssen *et al.* 1987).

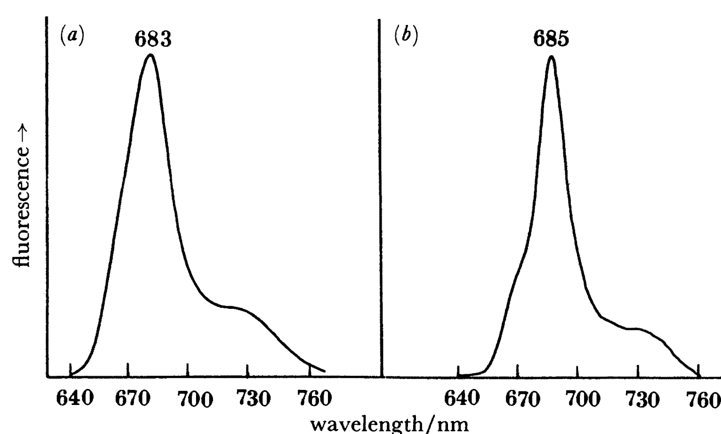

FIGURE 1. Fluorescence-emission spectra of PSII reaction centres: measured in 60 mM Tris-Cl buffer (pH 8.5) (*a*) at room temperature and (*b*) at 77 K. Excitation was at 420 ± 10 nm and emission was measured with a slit width of 2 nm.

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Fluorescence quenching

When the D1/D2/cytochrome b_{559} complex is exposed to bright actinic light in the presence of sodium dithionite, optical-density changes occur indicative of the photoaccumulation of reduced pheophytin (see figure 2). At pH 8.5 the photoreduced pheophytin is oxidized in the dark. Under similar conditions, the fluorescence from the isolated complex is reversibly quenched as shown in figure 3. With saturating white light this quenching can be as much as 80% to 90% of the total fluorescence. When measured simultaneously on one sample it can be shown that the two phenomena are kinetically coupled (see figure 4) and that the rates (see figure 5) and amplitudes of the signals were modified in the same way by the presence of low concentrations of methyl viologen. It seems that this redox mediator aided both the light-induced reduction and the dark oxidation of pheophytin within the reaction centre. With only sodium dithionite present the extent of the absorbance change indicated that one pheophytin molecule per cytochrome b_{559} can be fully reduced.

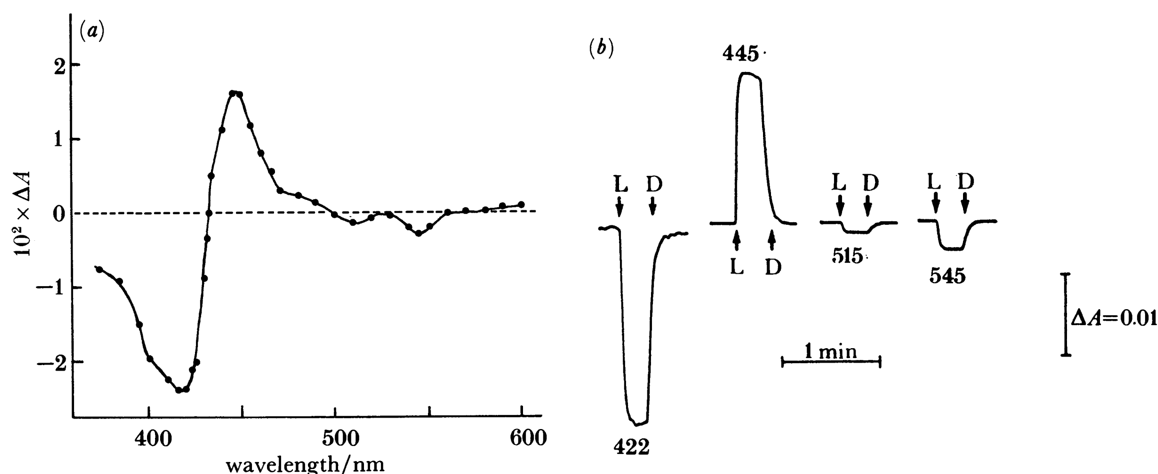

FIGURE 2. (*a*) Light–dark difference spectrum obtained in the presence of excess sodium dithionite (3 mg ml^{-1}) plus 1 μM methyl viologen. The PS II reaction-centre preparation was suspended in 60 mM Tris-HCl (pH 8.5) at 5 μg Chl ml^{-1} and measurements were made at 4 °C. (*b*) Light-induced absorption changes at four different wavelengths.

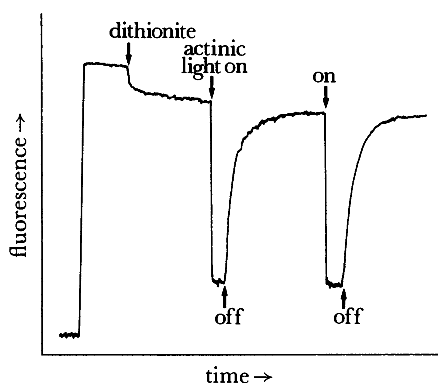

FIGURE 3. Light-induced quenching of chlorophyll fluorescence from PS II reaction centres measured under similar conditions to those given in figure 2. The fluorescence was excited by 1 μs pulses of light at 1.6 kHz from a light-emitting diode passing through a short-pass filter ($\lambda < 670$ nm). The fluorescence was detected at wavelengths greater than 700 nm. The actinic illumination was saturating white light.

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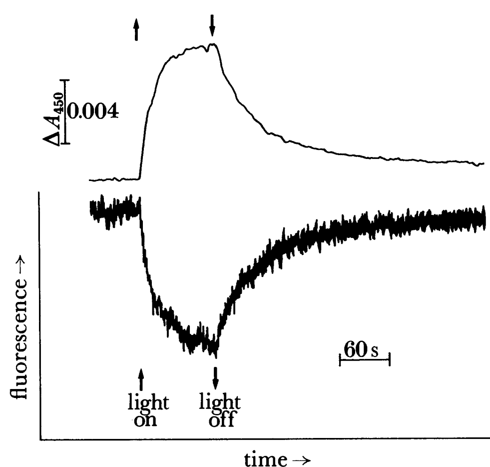
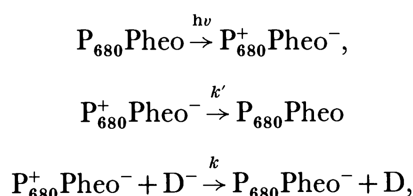


FIGURE 4. Simultaneous recording of the formation and decay of reduced pheophytin due to light on (upward arrows) and light off (downward arrows) measured as an absorption change at 450 nm and as chlorophyll fluorescence in a PS II reaction preparation treated with sodium dithionite and 0.3 μM methyl viologen. The reaction medium contained 5.2 μg Chl ml^{-1} in 60 mM Tris-Cl, pH 7.7 with 3 mg sodium dithionite ml^{-1}.

Quantum yields

The light-induced quenching of chlorophyll fluorescence in the presence of sodium dithionite has been observed by Klimov *et al.* (1977) for several different systems. In all cases dithionite serves the dual role of acting as an efficient electron donor to P^{+}_{680} and also as a chemical reductant of Q_A in the dark before experimentation. By comparing the fluorescence quenching in such systems with the quenching of fluorescence from the isolated PS II reaction centre, it should therefore be possible to investigate the effect of the presence of Q_A^- on the quantum efficiency for the production of the P^{+}_{680} $Pheo^-$ state.

The simplest reaction scheme can be written as



$$P_{680}Pheo \xrightarrow{h\nu} P^{+}_{680}Pheo^{-},$$

$$P^{+}_{680}Pheo^{-} \xrightarrow{k'} P_{680}Pheo$$

$$P^{+}_{680}Pheo^{-} + D^{-} \xrightarrow{k} P_{680}Pheo^{-} + D,$$

where D^- is the reduced donor to P^{+}_{680}.

The quantum yield for $Pheo^-$ accumulation, γ, would therefore be given by

$$\gamma = \phi_{PC}\, k[D^-]/(k' + k[D^-]), \qquad (3)$$

where ϕ_{PC} is the quantum yield of primary photochemistry and k is the bimolecular rate constant expressing the interaction of $P^{+}_{680}Pheo^{-}$ with the electron donor D^-.

When equal chlorophyll levels were placed in the measuring cuvette, with all other conditions identical, it was found that the rate of chlorophyll fluorescence quenching was considerably faster in the case of the isolated PS II reaction centres compared with thylakoid membranes or BBYs (see figure 6). This is not consistent with a constant quantum yield for the formation of $P_{680}Pheo^-$, because the isolated reaction centre would be expected to have a rate of fluorescence quenching 100 times less than the membrane system. This is because the latter

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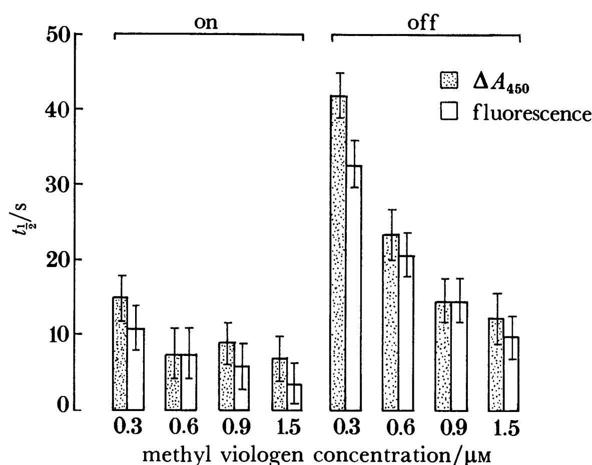

FIGURE 5. Comparison of the kinetics ($t_{\frac{1}{2}}$) for the formation (on) and decay (off) of the $Pheo^-$ absorption change (ΔA_{450}) and chlorophyll fluorescence quenching in a PS II reaction-centre preparation as a function of methyl viologen concentration. Error bars indicate limits of resolution of the $t_{\frac{1}{2}}$ measurements. Other conditions as for figure 4.

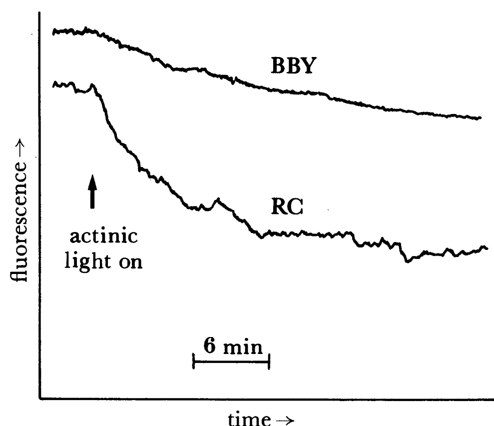

FIGURE 6. The kinetics of chlorophyll fluorescence quenching owing to weak, non-saturating, actinic light measured on PS II-enriched membranes (BBY) and isolated PS II reaction centres (RC). Both measurements were made under identical conditions with 7 μg Chl ml^{-1} present. Other conditions as in figure 4.

has a large antenna array. Hence it seems that the quantum yield in the reaction centres is approximately three orders of magnitude higher than in thylakoids or BBY particles. To calculate the quantum yields a version of the 'light titration' method (Malkin & Kok 1966) was employed. If it is assumed that as $Pheo^-$ accumulates the reaction centres become 'closed' to further photochemistry, then at the first approximation the following first-order rate equation in [Pheo] can be written.

$$\mathrm{d}[\mathrm{Pheo}^-]/\mathrm{d}t = \gamma I[\mathrm{Pheo}]/[\mathrm{Pheo}]_{\mathrm{T}}, \tag{4}$$

where I is the intensity of the absorbed light and $[\mathrm{Pheo}]_{\mathrm{T}}$ is the total concentration of reactive pheophytin ($[\mathrm{Pheo}]+[\mathrm{Pheo}^-]$). Therefore the first-order rate constant is equal to $\gamma I/[\mathrm{Pheo}]_{\mathrm{T}}$ and by measuring half-times ($t_{\frac{1}{2}}$) for the light-induced quenching it is possible to calculate γ by using equation (5),

$$\gamma = \ln 2[\mathrm{Pheo}]_{\mathrm{T}}/It_{\frac{1}{2}}. \tag{5}$$

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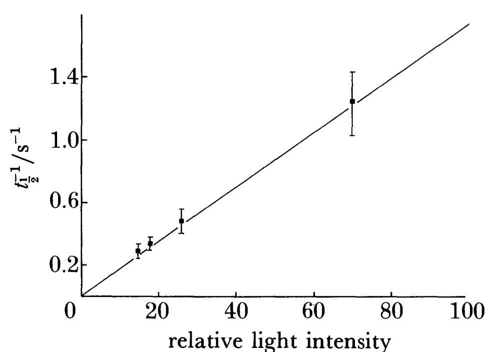

FIGURE 7. A linear relation between the intensity of actinic light and relative rate of chlorophyll fluorescence quenching (expressed as the reciprocal of the half-times). To cover a large intensity range white light was used. Other conditions as given for figure 6.

TABLE 1. QUANTUM YIELD FOR PHEOPHYTIN REDUCTION AS JUDGED BY THE RATE OF CHLOROPHYLL FLUORESCENCE QUENCHING

(Calculations were based on 400 Chl per reaction centre for thylakoids, 200 Chl per reaction centre for BBYs and 4 Chl per reaction centre for PS II.)

sample	light intensity µmol quanta s^{-1}	relative quantum yield	Q_A^-
thylakoids	30	$2 \pm 0.3 \times 10^{-5}$	+
BBY	30	$3 \pm 0.5 \times 10^{-5}$	+
PS II reaction centre	30	$1 \pm 0.2 \times 10^{-2}$	−
PS II reaction centre	6.1	$1.1 \pm 0.2 \times 10^{-2}$	−

For this calculation the reaction centre concentration has been crudely assumed to be associated with 400, 200 and 4 chlorophyll molecules for thylakoids, BBY particles and PS II reaction centres, respectively. As the quantum yields differ by an order of magnitude there is no need to be more accurate at this stage. Calculated values of quantum yields are given in table 1. As figure 7 shows, with white light (in this case to increase the light intensity range) the rate of fluorescence quenching, expressed as the reciprocal of half-time, was linear with light intensity, implying that there was no effect of a dark limiting step.

4. DISCUSSION

Based on fluorescence polarization studies, Breton (1982) proposed that the fluorescence emission at 77 K that peaks at 695 nm originates from the pheophytin molecule, which acts as the primary electron acceptor in PS II. Clearly, the data in figure 1 prove this hypothesis to be incorrect and indicate that this low-temperature fluorescence is emitted from another chlorophyll-binding protein of PS II, probably CP47 (van Dorssen *et al.* 1987). Indeed, this conclusion would be in line with the assumptions made by Butler (1978) in formulating his bipartite and tripartite models for describing the kinetics of chlorophyll fluorescence *in vivo*.

The photoquenching of fluorescence from the chlorophylls of the isolated reaction centre when sodium dithionite is present seems to be the same phenomenon observed by Klimov *et al.* (1977) with thylakoid membranes and PS II-enriched particles. Although not presented, we have also shown that the fluorescence of the isolated reaction centre is quenched by bright light

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when silicomolybdate is present (see Barber *et al.* 1987). This compound acts as an electron acceptor, allowing the P^{+}_{680}Pheo state to photoaccumulate. Previous studies with intact membranes have also indicated that P^{+}_{680} acts as a quencher of chlorophyll fluorescence (Butler 1973). Taken at face value, both observations indirectly support the Klimov model that all or a part of variable fluorescence originates from the back-reaction between P^{+}_{680} and $Pheo^{-}$. Pertinent to this theory is the efficiency at which this radical pair can be formed when Q_A is reduced and the chlorophyll fluorescence intensity is at its maximum, i.e. is the PS II reaction 'open' when in the $P_{680}PheoQ^{-}_{A}$ state? If it is not (i.e. a low efficiency of trapping), then there is no reason to evoke the Klimov model. If it is (i.e. high efficiency of trapping), then the recombination mechanism must be postulated to account for the high fluorescent state.

The work presented here shows that there is a change in the quantum efficiency of pheophytin photoreduction by three orders of magnitude between PS II reaction centres free of Q_A and the PS II system with Q^{-}_{A} present. This large change could be due to three possible factors: a decrease in k', an increase in k or an increase in ϕ_{PC}. From flash spectroscopy it seems that the recombination rate constant decreases from about $5\times10^{8}\ s^{-1}$ in thylakoids (see Holzwarth 1987) to about $3\times10^{7}\ s^{-1}$ in isolated PS II reaction centres (Danielius *et al.* 1987; Takahashi *et al.* 1987). This would result in only a factor-of-ten difference between the quantum yields, assuming $k \ll k'$, and would be much less if $k \approx k'$ or $k > k'$. It is also hard to imagine changes in orders of magnitude in the interaction of the external electron donor (D^{-}) and P^{+}_{680} There is, however, a dependency on the concentration of methyl viologen (see figure 5), which seems to mediate the redox coupling between the reaction centre components and the sodium dithionite. Under the conditions of these experiments it seemed that this dependency was saturated at about 1.0 μM, although there could have been some limitations imposed by the response time of the instrument used. From chemical rate theory for solutions the maximum bimolecular rate constant k in an aqueous medium in the case of diffusion limitation is in the range 10^{9}–$10^{11}\ M^{-1}\ s^{-1}$. Multiplication by the methyl viologen concentration gives k in the range of 10^{3}–$10^{5}\ s^{-1}$, which clearly is much less than k'. It therefore seems reasonable to conclude that the interaction of the electron donor D^{-} and P^{+}_{680} is likely to be limited by other factors, such as binding.

From the above considerations, it is most likely that the large difference in quantum yield for the photoreduction of $Pheo^{-}$ between the isolated reaction centre and the intact PS II unit is a significant change in ϕ_{PC}. Presumably this change is due simply to the absence of Q_A in the isolated reaction centre, and when present the Q^{-}_{A} state exerts an electrostatic constraint on primary charge separation. This latter possibility finds support from the picosecond fluorescence measurements of Holzwarth (1987) in isolated thylakoids and flash absorption studies on PS II particles by Schlodder & Brettel (1988), both of which ascribe a smaller quantum yield of $P^{+}_{680}Pheo^{-}$ formation in the presence of Q^{-}_{A}, compared with conditions when Q_A can act as an electron acceptor.

Our observations, coupled with the fact that there seems to be a lack of correlation between the measured chlorophyll lifetime and the time of radical-pair recombination (Schlodder & Brettel 1988), places serious doubts on the Klimov model (but see Mimura *et al.* 1988). What can definitely be concluded is that $Pheo^{-}$ and also P^{+}_{680} are very effective quenchers of chlorophyll fluorescence, which indeed can be explained by the existence of low-lying excited state levels as indicated by the absorption spectra presented by Fujita *et al.* (1978). Thus it is possible that some of the various fluorescence-quenching phenomena observed in intact tissue

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are due to the establishment of these quenching species within the PS II reaction centre and are not a consequence of quenching processes within the antenna arrays.

We thank our colleagues who have made this work possible, D. J. Chapman, K. Gounaris, J. De Felice and K. Davis. We also acknowledge financial support from the Agricultural and Food Research Council and the Science and Engineering Research Council.

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Discussion

U. Schreiber (*Department of Botany, Universität Würzburg, F.R.G.*). For the particular type of PS II reaction-centre preparation that Professor Barber has isolated, he has shown convincingly that variable fluorescence does not originate from recombination. However, I noticed that in his preparation variable fluorescence was rather low. The ratio F_M/F_0 was about three, whereas it is up to six in intact leaves. I therefore still see the possibility that a substantial part of variable fluorescence *in vivo* may represent recombination luminescence.

J. Barber. Firstly I should emphasize that there is no variable fluorescence in the normal sense from the isolated PS II reaction centre, and therefore the concept of the F_M/F_0 ratio is meaningless. Moreover, I did not totally dismiss the Klimov recombination hypothesis. What has been found is that the states $P_{680}Pheo^-$ and $P^+_{680}Pheo$ quench the chlorophyll fluorescence emitted from the isolated PS II complex. This is consistent with the Klimov hypothesis but does not prove it. In fact, our quantum-yield studies suggest that the Klimov model is open to debate. My guess is that the recombination process does contribute to variable fluorescence but is not a major portion of it. The quenching $P_{680}Pheo^-$ and $P^+_{680}Pheo$ is probably photochemical in nature and therefore in competition with fluorescence.