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VARIABLE CHLOROPHYLL *a* FLUORESCENCE FROM *P*-700 ENRICHED PHOTOSYSTEM I PARTICLES DEPENDENT ON THE REDOX STATE OF THE REACTION CENTRE

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Summary

1. Photosystem I particles enriched in *P*-700 prepared by Triton X-100 treatment of chloroplasts show a light-induced increase in fluorescence yield of more than 100% in the presence of dithionite but not in its absence.

2. Steady state light maintains the *P*-700, of these particles, in the oxidised state when ascorbate is present but in the presence of dithionite only a transient oxidation occurs.

3. EPR data show that, in these particles, the primary electron acceptor (X) is maintained in the reduced state by light at room temperature only when the dithionite is also present. In contrast, the secondary electron acceptors are reduced in the dark by dithionite.

4. Fluorescence emission and excitation spectra and fluorescence lifetime measurements for the constant and variable fluorescence indicate a heterogeneity of the chlorophyll in these particles.

5. It is concluded that the variable fluorescence comes from those chlorophylls which can transfer their energy to the reaction centre and that the states PX and P^+X are more effective quenchers of chlorophyll fluorescence than PX^-, where P is *P*-700.

Introduction

It is well known that the fluorescence yield of photosystem II (PS II) is dependent on the redox state of both Q [1] and *P*-680 [2]. In contrast to PS II the yield of Photosystem I (PS I) fluorescence at room temperature is very low and seems not to reflect changes in the redox state of the reaction

Abbreviations: PS I, Photosystem I; PS II, Photosystem II.

centre [3,4]. However the finding that $P\text{-}680^+$ is a quencher of PS II fluorescence has suggested that absence of PS I variable fluorescence may be due to the presence of $P\text{-}700^+$. That is by analogy with PS II one might not expect a maximum increase of PS I chlorophyll *a* fluorescence until both *P*-700 and the primary acceptor, X, are reduced.

There are recent reports that light induced variable PS I fluorescence yield changes can occur with particles enriched in this photosystem but the changes seen have been relatively small [5,6]. Only Ikegami [7] has shown considerable fluorescence yield changes by manipulating the redox state of the PS I reaction centre. He used ether extracted particles highly enriched in *P*-700 (*P*-700 : chlorophyll, approx. 1 : 8) and found four levels of fluorescence under different conditions which he attributed to the four possible redox states of *P*-700 and the primary electron acceptor. However, these particles show a low rate of light induced *P*-700 oxidation and no measurements of the redox state of the primary acceptor were made under the various conditions used.

In this paper we report variable fluorescence yield changes measured with PS I-enriched particles (*P*-700 : chlorophyll ratio, 1 : 30—50) which show a rapid rate of light induced *P*-700 oxidation and have EPR signals indicative of the primary electron acceptor, X, and the bound iron-sulphur proteins (centres A and B; ref. 8).

Methods

Particles enriched in PS I were isolated from washed broken spinach chloroplasts as described by Evans et al. [9] by Triton X-100 treatment. The only modification was that there was an additional wash of the hydroxyapatite column with 2 l 20 mM Tris · HCl buffer, pH 8.0 containing 0.2 M NaCl and 0.5% Triton X-100 to remove bulk chlorophyll. The preparations normally have a *P*-700 to bulk chlorophyll ratio of between 1 : 30 and 1 : 50 assuming a molar extinction coefficient for *P*-700 of 64 000 (see ref. 10). Chlorophyll was determined by the method of Arnon [11]. The particles were either used fresh or stored in liquid nitrogen. In all experiments the particles were suspended in 50 mM Tris · HCl buffer, pH 8.0. Sodium dithionite was made up in a vial gassed with oxygen-free nitrogen in 50 mM Tris · HCl buffer, pH 8.0.

Chlorophyll fluorescence was excited with blue light at $5 \cdot 10^4$ ergs $\cdot$ cm^{-2} $\cdot$ s^{-1} transmitted by a filter combination consisting of a Balzer Calflex C and 2 mm Schott BG18. Fluorescence was measured with an EMI 9558 photomultiplier screened by a Balzer 695 nm interference filter and a 2 mm Schott RG695 cut off filter. The chlorophyll concentration was 20 μg/ml. The fluorescence yield measurements were corrected for 10—20% reflected light which was estimated using a cuvette containing buffer but no particles.

Fluorescence excitation and emission spectra were measured using a Perkin Elmer MPF 4 spectrofluorimeter. The spectra were not corrected for the spectral sensitivity of the R446F S20 photomultiplier. The chlorophyll concentration was 2 μg/ml.

The absorption spectra and photooxidation of *P*-700 were determined with an Aminco DW2 spectrophotometer. The actinic light was the same as that described for the fluorescence measurements. The photomultiplier was pro-

tected by an RG695 cut off filter during the kinetic measurements and an RG630 cut off filter during the spectral measurements. The cuvette was placed 100 mm from the photomultiplier and measurements were made in the transmission mode to minimise the measurement of fluorescence from the particles. The chlorophyll concentration was 20 μg/ml.

EPR spectra were obtained using a Varian E4 spectrometer operated at X band frequencies (9.25 GHz). Samples were cooled to 9 K in an Oxford Instruments (Oxford, U.K.) liquid He cryostat. Samples were prepared in silica tubes (3 mm internal diameter) at a chlorophyll concentration of 1 mg/ml. Illumination at room temperature was with the same blue light system described for the fluorescence measurements. The light treated samples were illuminated at room temperature for the stated time and also during freezing in liquid nitrogen. The dark samples were protected from light during freezing and also during measurement of the EPR spectra.

Chlorophyll fluorescence lifetime measurements at room temperature were made using the picosecond laser and streak camera system previously described [12,13]. The sample was excited with a single 530 nm pulse (6 ps width at half maximum height) isolated by means of a Pockels cell from a train of pulses generated by a frequency doubled mode-locked neodymium glass laser oscillator. Fluorescence from the sample was passed through a Schott RG645 cut off filter and focused onto the slit of an S20 photocathode Imacon 600 streak camera (John Hadland (P.I.) Ltd.). A vidicon optical multichannel analyser (OMA 1205A and B, Princeton Applied Research) recorded the resulting streak trace in digital form which could then be displayed on an oscilloscope or transferred to punch tape for analysis. Streak speed used was 580 ps/50 OMA channels. For the light treatment, samples were illuminated for 5 min using the same blue light system described for the fluorescence measurements. The samples were then rapidly transferred to the laser apparatus and measurements were made within 30 s. Samples were contained in a 1 mm cuvette at a chlorophyll concentration of 400 μg/ml.

Results

Fig. 1 shows the fluorescence yield at 695 nm of PS I particles. Illumination in the absence of any added electron donor (trace a) causes an immediate rise to a steady level of fluorescence, for convenience designated F_A. Addition of dithionite in the light brings about a slow light dependent increase in fluorescence yield. Provided the particles are freshly prepared or have been stored at 77 K the increase in fluorescence yield is more than 100%, tending towards the level F_C. If the preparations are allowed to age at room temperature the ratio F_C to F_A steadily decreases.

Addition of reduced dichlorophenol indophenol did not alter the initial yield of fluorescence, F_A, but increased the rate of the dithionite induced fluorescence rise (not shown). After incubation of the particles with neutral red and dithionite in the dark for at least 5 min there was a rapid light-dependent rise in fluorescence yield to the level F_C complete in approx. 10 s (see Fig. 1, trace b). In this case, the initial level appears to be higher than the F_A level and is designated F_B in Fig. 1. Faster measurements using an oscillo-

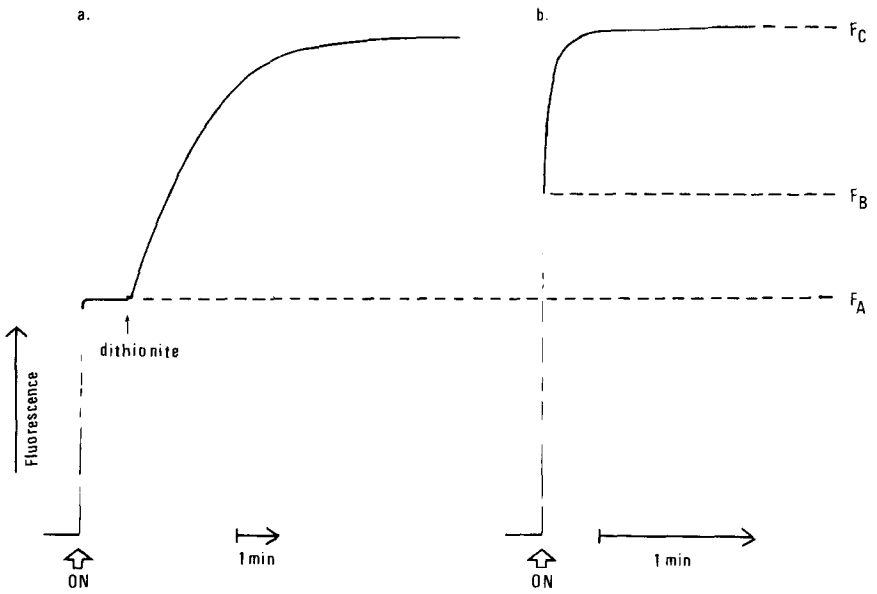


Fig. 1. Time course of fluorescence yield changes of PS I particles at room temperature. Trace a, no additions; trace b, plus 1 μM neutral red and 1.5 mM sodium dithionite. In trace a 1.5 mM sodium dithionite was injected as indicated.

scope did not detect the F_A level with this particular treatment as shown in Fig. 2a. When dithionite alone was present the initial fluorescence yield was again at the F_B level but the rise to F_C was approximately six times slower (see Fig. 2b).

In order to understand the significance of the above fluorescence changes we investigated the redox state of *P*-700 under the various treatments used. Fig. 3 shows the light minus dark difference spectrum of the absorption change in the 700 nm region of PS I particles in the presence of ascorbate. Maximum bleaching occurs at 697 nm with a shoulder at approx. 677 nm.

Fig. 4a shows the kinetics of *P*-700 photooxidation, which are very rapid, even though the actinic illumination intensity was somewhat lower than that used in the fluorescence yield experiments of Figs. 1 and 2. Reduction of *P*-700 in the dark was very slow unless an electron donor, in this case ascorbate, was added. Reduced dichlorophenol indophenol was a more efficient donor than ascorbate alone.

Fig. 4a also shows that addition of dithionite completely inhibited *P*-700 photooxidation except for a rapid transient. The transient only appeared on the first illumination cycle and, as Fig. 4b shows, incubation with dithionite in the dark for 10 min also inhibited the appearance of this transient. Dithionite also caused some irreversible bleaching at 697 nm. Neutral red had no additional effect on *P*-700 photooxidation than that seen with dithionite alone.

These results indicate that in the dark *P*-700 is in the reduced state, though complete reduction in the dark is slow unless an electron donor is present. In the absence of dithionite illumination fully oxidises *P*-700 but in its presence

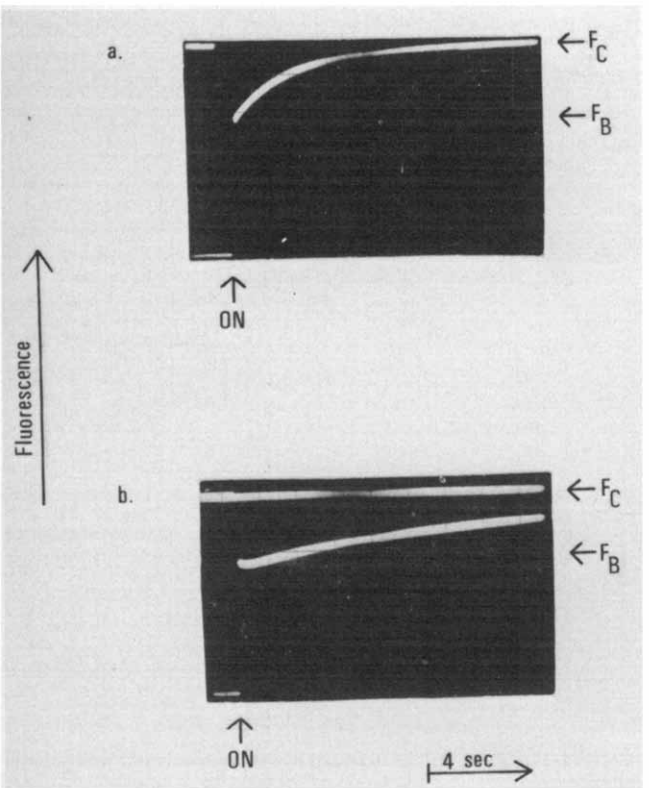


Fig. 2. Kinetics of fluorescence yield changes of PS I particles at room temperature preincubated in the dark for 10 min with (a) 1 μM neutral red and 1.5 mM sodium dithionite and (b) 1.5 mM sodium dithionite alone. F_C level was measured 2 min after the onset of illumination in each case.

this component essentially remains in the reduced state.

We then investigated the redox state of the primary and secondary electron acceptors by EPR spectroscopy under the various treatments used. Four different samples were prepared at room temperature. In all cases the particles were incubated in the dark for 10 min in the presence of ascorbate either plus or minus dithionite and neutral red. The samples were then either frozen in liquid nitrogen in the dark or were illuminated at room temperature for 5 min before freezing in the light. With EPR it is possible to examine the redox state of *P*-700, X and the iron · sulphur centres, A and B (ref. 8). In Fig. 5 the instrument settings and temperature (9 K) were optimum for the $g = 1.76$ signal indicative of the reduced primary acceptor, X. Both the $g = 2.00$ signal indicative of $P\text{-}700^+$ and the complex signals centred at $g = 1.94$ indicative of the reduced iron · sulphur centres, A and B, were saturated to differing degrees.

Fig. 5 shows that in the dark plus ascorbate, X is in the oxidised state (no $g = 1.76$ signal, trace 1). Neither illumination in the presence of ascorbate (trace 2) nor addition of dithionite plus neutral red in the dark (trace 3) brings about any reduction of X although the iron-sulphur centres are reduced. Only on illumination in the presence of dithionite and neutral red (trace 4) is X reduced. Illumination at 9 K did not bring about any increase in the size of the

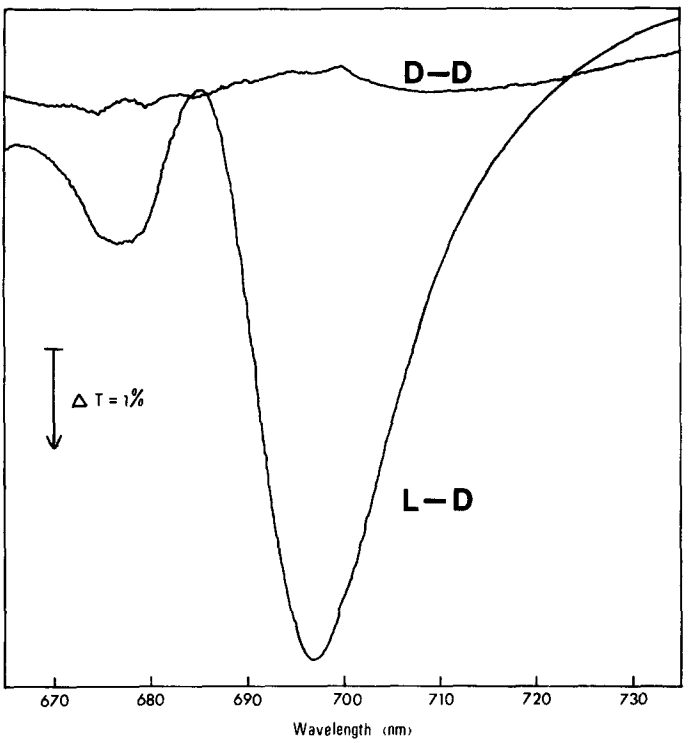


Fig. 3. Light minus dark difference absorption spectrum of PS I particles in the presence of 2 mM sodium ascorbate.

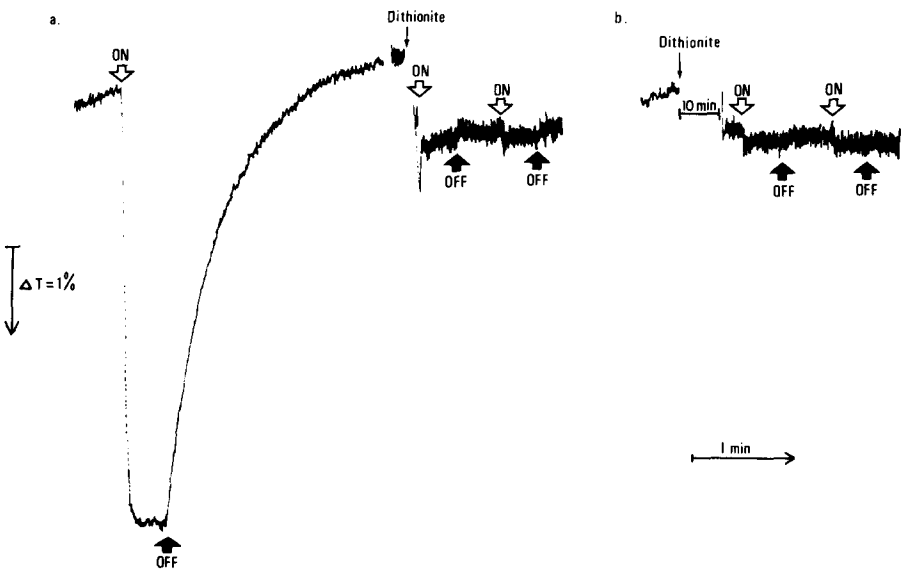


Fig. 4. Effect of sodium dithionite on the kinetics of photooxidation of *P*-700 in PS I particles. *P*-700 photooxidation was measured at 697 nm, reference wavelength 730 nm. 2 mM sodium ascorbate was present in both experiments and 1.5 mM sodium dithionite was added as indicated. In each experiment the recorder was operated on the rapid response mode after addition of sodium dithionite.

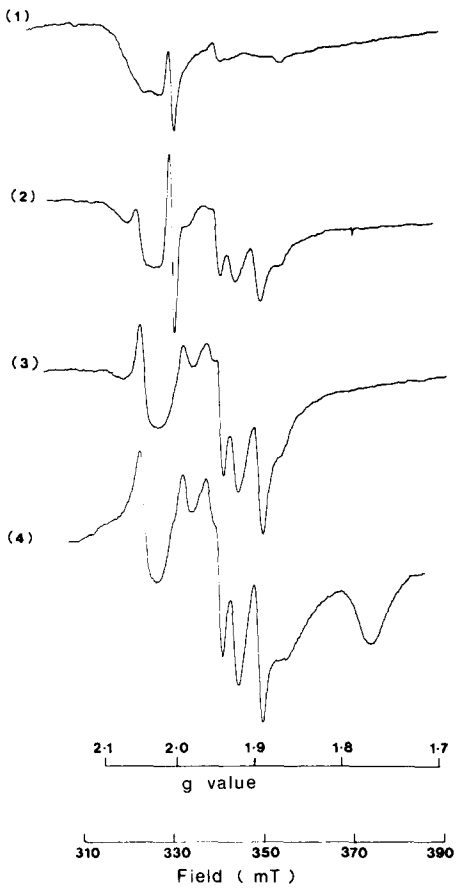


Fig. 5. Effect of light on the EPR spectra of PS I particles plus and minus sodium dithionite and neutral red. All samples were preincubated in the dark for 10 min. In traces 2 and 4 the samples were then illuminated for 5 min. All samples contained 5 mM sodium ascorbate and in traces 3 and 4 they contained 1.33 mM neutral red and 6.6 mM sodium dithionite. Modulation amplitude 1.6 m*T*, power 20 mW and scan rate 100 m*T*/min.

$g = 1.76$ signal nor did the $g = 2.00$ signal appear (not shown). Therefore under these conditions X must have already been fully reduced by the light dependent reduction at room temperature. It was found that the reduction of X was markedly slower in the presence of dithionite alone and that the relative rates of reduction plus and minus neutral red were essentially the same as those seen for the dithionite-induced variable fluorescence rise plus and minus neutral red (Figs. 1 and 2). These results indicate that X is in the oxidised state in the dark plus and minus dithionite and is reduced on illumination only if dithionite is present. The electron donor, neutral red, simply alters the rate not the extent of the X reduction.

We therefore conclude that the absorbance and EPR measurements show that the light-induced fluorescence changes are linked to changes in the redox state of *P*-700 and X.

The spectral characteristics of light-induced fluorescence changes are shown

in Figs. 6 and 7. Fig. 6 shows the room temperature fluorescence emission spectra for the two states, F_A and F_C. In the absence of additions there is a peak at 684 nm with a tail beyond 700 nm (trace a). On addition of dithionite and neutral red there is a general increase in fluorescence yield but also the peak wavelength changes from 684 to 688 nm (trace b). Fig. 6 also shows the emission spectrum of this variable fluorescence (trace b, trace a) where it can be seen that there is a peak at about 690 nm with a tail above 700 nm.

Fig. 7 shows the fluorescence excitation spectra, in the red region, of 730 nm fluorescence emission in the two states, F_A and F_C. The photomultiplier was protected by a 715 nm cut off filter. In the absence of additions there is a small 630 nm and a large 657 peak (trace a). Addition of dithionite and neutral red in the light causes an increase in both peaks (trace b). However the difference spectrum of the two states (trace b, trace a) shows that there is a greater increase at longer wavelengths as the peak is now at 685 nm instead of 675 nm.

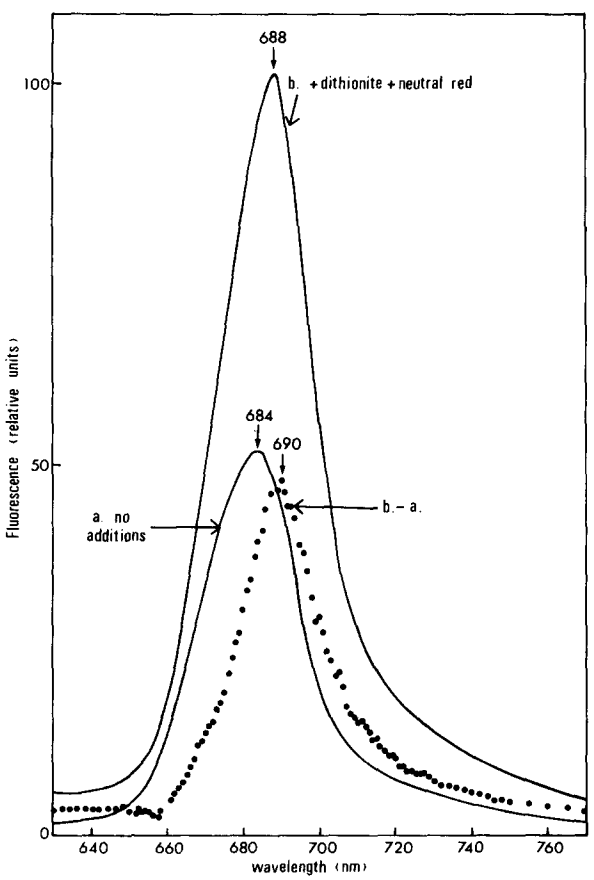
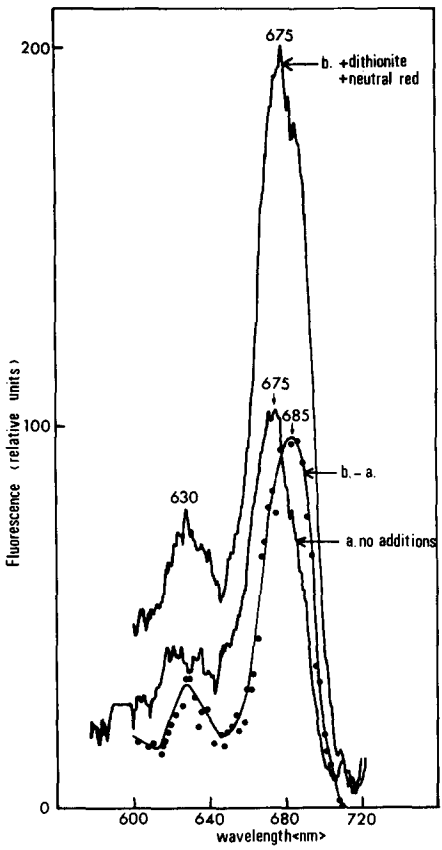


Fig. 6. Fluorescence emission spectra of PS I particles at room temperature excited at 430 nm. Trace a, no additions; trace b, plus 1.5 mM sodium dithionite and 1 μm neutral red. Dotted curve is trace b minus trace a. Emission curves were determined after all light-induced changes were complete. Emission slit width 3 nm, excitation slit width 10 nm.



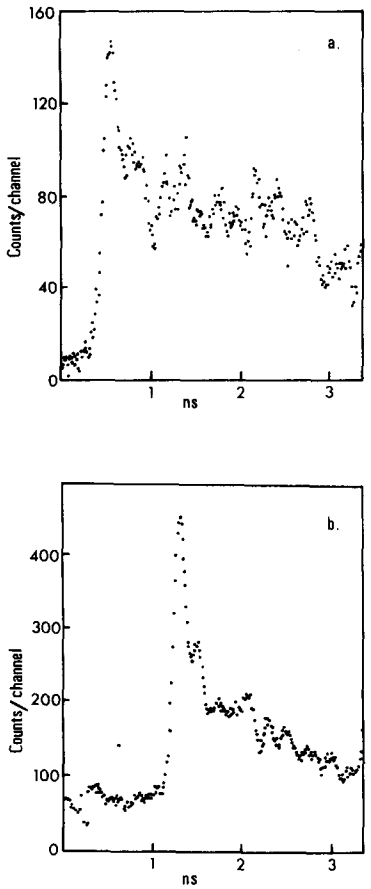


Fig. 7. Fluorescence excitation of PS I particles at room temperature emitting at 730 nm. Trace a, no additions; trace b, plus 1.5 mM sodium dithionite and 1 μM neutral red. Dotted curve is trace b minus trace a. Excitation slit width 5 nm, emission slit width 10 nm.

Fig. 8. Fluorescence decay of PS I particles at room temperature. a, dark-treated no additions; b, light-treated plus 10 mM sodium dithionite. The photon density of the excitation pulse was not more than $2.5 \cdot 10^{15}$ photons $\cdot$ cm^{-2}.

We have also examined the lifetime of fluorescence from these PS I particles under two different conditions either dark treated in the absence of dithionite (*P*-700 reduced but X oxidised) or light treated in the presence of dithionite (both *P*-700 and X reduced). In the former case the signal is dominated by a component with a long lifetime ($\tau_{1/e}$ = 4.2 ns) although there does also seem to be a shorter component, see Fig. 8a. In the latter case the fast component becomes more obvious and there is an apparent shortening of the overall lifetime (see Fig. 8b). However, if the 4.2 ns component (see Fig. 8a) is assumed to be due to chlorophyll not associated with functional antenna chlorophylls of *P*-700 then its subtraction from the decay shown in Fig. 8b should give an estimate of the lifetime of the fast component. When this is done a $\tau_{1/e}$ of approx. 500 ps is obtained.

Discussion

Our results clearly show that changes in chlorophyll fluorescence yield from PS I particles occur on manipulating the redox state of their reaction centres. In this discussion we will consider the following states: PXA, P^+XA^-, PXA^-, $P^+X^-A^-$ and PX^-A^- where P is P-700, X is the primary electron acceptor, A represents secondary electron acceptors (the iron-sulphur centres A and B, ref. 8).

In the absence of added reducing agents and electron acceptors, light converts PXA to P^+XA^- and since this is not accompanied by any significant fluorescence yield changes from the light harvesting chlorophylls, it can be assumed that both states are fluorescence quenchers and that the F_A level corresponds to P^+XA^-. When dithionite or dithionite plus neutral red are present in the dark the iron-sulphur proteins are reduced (see Fig. 5) and illumination coupled with efficient electron donation to P^+ drives the following reactions:

$$PXA^- \overset{\text{light}}{\rightleftharpoons} P^+X^-A^- \overset{\text{light}}{\underset{e^-}{\rightarrow}} PX^-A^-$$

That is, the state PX^-A^- accumulates and the trap no longer acts as such an efficient quencher so that the bulk chlorophyll fluorescence rises to the maximum yield F_C. Support for the accumulation of the PX^-A^- state under these conditions comes from the absorbance and EPR studies presented above and from the recent work of Sauer et al. [14]. We found no evidence for the existence of high levels of the state $P^+X^-A^-$ (see Fig. 4). According to Sauer et al. [14] under these conditions the back reaction between P^+ and X^- is very fast. The transient P-700 photooxidation seen in trace a of Fig. 4 probably reflects those iron-sulphur centres which had not been reduced in the dark by dithionite. After a brief illumination period complete reduction would have occurred explaining why subsequent light on/off cycles produced no further P-700 oxidation transients. Reduction of the iron-sulphur centres in the dark by dithionite is known to be a relatively slow process and this is shown in Fig. 5 where the $g = 1.94$ signal is smaller in the dark treated sample than in the illuminated sample. As there is a rapid back reaction between X^- and P^+ it is likely that the F_B level seen initially on illumination in the presence of dithionite reflects the PXA^- state. It seems therefore, that P^+XA^- (F_A level) is a more effective quencher of chlorophyll fluorescence than PXA^- (F_B level) in agreement with the conclusions of Ikegami [7]. Ikegami used PS I preparations obtained by extraction of digitonin particles with ether, which had a very high P-700 : chlorophyll ratio (approx. 1 : 8). With these particles he identified similar fluorescence changes to those reported in this paper. He also found an additional fluorescence level between F_B and F_C which he attributed to a state corresponding to $P^+X^-A^-$ in our notation.

The fluorescence excitation and emission spectra presented in this paper suggest that there is more than one form of chlorophyll *a* present in these particles. The emission spectrum for the F_C level shows a longer wavelength peak than that for the F_A level as emphasised in the difference spectrum. The excitation spectra also indicate heterogeneity although energy transfer

does not occur between a proportion of the shorter and longer wavelength absorbing forms of chlorophyll. The existence of at least two forms of chlorophyll *a* in these preparations is very clear from the lifetime measurements. The decay component with a $\tau_{1/e}$ of approx. 4 ns almost certainly represents Triton X-100-solubilized chlorophyll or chlorophyll molecules weakly associated with the PS I particles. The short component having $\tau_{1/e}$ of approx. 500 ps seen when fluorescence is at the F_C level is attributed to light harvesting chlorophyll molecules tightly coupled to *P*-700. The lifetime of these chlorophylls for the dark treated system (*P*XA) is almost certainly less than 100 ps as suggested by previous measurements on PS I enriched particles [12,15]. Because of the contamination of the sample with long lived fluorescence species and the streak speeds used in the measurements, a component of less than 100 ps would not be resolvable. That the signals in Fig. 8a and 8b can be attributed to a low and high fluorescence state respectively has been shown by carrying out simultaneous quantum yield measurements using a reference pulse (unpublished data). The presence of detergent-solubilized chlorophyll in this type of particle preparation is not uncommon [16]. The effect of its presence is to give a background level of invariable fluorescence and also to under estimate the extent of the variable fluorescence.

In PS I particles prepared by digitonin treatment fluorescence emission at 77 K occurs at 735 nm while in Triton X-100-prepared particles there is a greater proportion of fluorescence emission below 700 nm than above [3,12, 16]. The fluorescence properties of digitonin particles are similar to those of PS I in the intact system. The Triton X-100 prepared particles used in this present work had a 715 nm emission peak at 77 K but no peak at 735 nm. As yet, PS I particles prepared by digitonin treatment have not been shown to have variable fluorescence changes dependent on the redox state of *P*-700 [2,4].

In conclusion it appears that light harvesting chlorophyll *a* molecules closely associated with *P*-700 can show variable fluorescence yield changes which are dependent on the redox state of the reaction centre. There is no evidence that the redox state of A affects the fluorescence yield although it is impossible to maintain the state X^- unless the secondary acceptors are also reduced. The highest yield is obtained when the state PX^-A^- is achieved. Observations of the variable fluorescence, which has its emission peak at approx. 690 nm in these particles, is hampered by the presence of Triton X-100-solubilized chlorophylls or chlorophylls loosely attached to the particles with a decreased efficiency of energy transfer to *P*-700. For this reason variable fluorescence yield changes are more likely to be observed with Triton X-100 preparations having low *P*-700 to chlorophyll ratios. How these observations can be related to digitonin or french press particles, which show fluorescence emission characteristics more similar to intact organisms, is not clear. Variable fluorescence has not been seen with these preparations but as far as we know, no measurements have been made under conditions where X is known to have been maintained in a reduced state.

Acknowledgements

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