Picosecond Fluorescence Studies of Photosystem II*

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Much effort has been expended in the measurement of the fluorescence decay kinetics of chlorophyll in vivo (for examples see papers cited in Harris et al., 1976). To the present time most techniques have lacked either the precision or the time resolution required for accurate determinations. Recent work using high-powered lasers has been hampered as a result of anomalous multiphoton effects (Campillo et al., 1976b; Porter et al., 1977). In the present paper we report measurements of Photosystem II fluorescence using a single-photon counting technique utilizing low-power excitation from a tunable dye laser. This method has the advantages of a much better time-resolution capability than conventional single-photon counting techniques (Wild et al., 1977; Spears et al., 1978) and of a higher sensitivity, enabling much lower incident light intensities to be used than those required for streak-camera or fast-shutter methods.

Experimental

The sample was excited by < 10 ps (FWHM) laser pulses, determined by a zero-background second-harmonic-generation autocorrelation technique, from a wavelength tunable (570–640 nm) Rhodamine 6G dye laser synchronously pumped by 90 ps (FWHM) pulses from a mode-locked argon ion laser (CR 12 Coherent Radiation). The pulse repetition rate of the dye laser was decreased from 75 MHz to 33 kHz by a Pockels cell between crossed polarizers. A contrast ratio of better than 500:1 between the transmitted and rejected pulses was achieved. Fluorescence was detected and time resolved by a time correlated single photon-counting technique. The time resolution of the instrument was limited by the transit time fluctuations of the photoelectrons in the photomultiplier tube, resulting in an apparent FWHM pulse of 700 ps for the excitation profile. Laser excitation intensities in the range of 10⁹ to 10¹¹ photons/cm² per pulse were used.

The alga Chlorella pyrenoidosa and pea (Pisum sativum) chloroplasts were prepared and used as described previously (Barber, 1968; Barber et al., 1978). Dark-adapted samples were flowed from a reservoir at 11/min through the illumination volume. Samples were also pre-illuminated by a 0.5 mW CW He/Ne laser, while the flow rate was decreased. For room-temperature experiments the fluorescence emission was monitored at wavelengths > 665 nm (Schott RG 665). Studies at 77 K were performed by immersing the sample in liquid N₂ in an optical Dewar. Fluorescence emission was observed at 685 nm (Balzer B40 685 nm interference filter).

Results and discussion

The data obtained from the fluorescence decays are summarized in Table 1. At the relatively low excitation intensities used, the dark adapted Chlorella have reasonably good exponential decays (see Fig. 1), but the calculated best fit to the data was obtained by assuming that the decay was composed of two exponential terms. The two components had lifetimes in the ranges of 270–350 and 530–650 ps with the longer decay consisting of between 38 and 27% of the total. The exact lifetimes varied from sample to sample. The decays could not be described by including a small component of 1–10 ns, which would be expected for closed traps (Porter et al., 1977). The lifetimes were not changed by either varying the excitation intensity from 10⁹ to 10¹¹ photons/cm² per pulse or by changing the excitation wavelength within the range of 570–640 nm.

The data for chloroplasts clearly show non-exponential behaviour. Under the four different experimental conditions, dark adapted (Fig. 1b), pre-illuminated in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea with (Fig. 3b) and without (Fig. 2b) Mg²⁺.

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Table 1. Characteristics of the fluorescence decay of Photosystem II

The fluorescence yields ($\phi_{\text{calc.}}$) were calculated from the expression:

$$\phi_{\text{calc.}} = \frac{1}{\tau_0} \int_0^\infty I(t) \, dt$$

where $\tau_0$ is the natural lifetime (19.5 ns) of chlorophyll a in vitro (Beddard et al., 1975). The mean lifetimes ($\tau_{\text{mean}}$) were calculated from the expression:

$$\tau_{\text{mean}} = \frac{\alpha_1 \tau_1 + \alpha_2 \tau_2}{\alpha_1 + \alpha_2}$$

where $\alpha_1$ and $\alpha_2$ are the ratios of the lifetimes of $\tau_1$ and $\tau_2$ respectively and $(\alpha_1 + \alpha_2) = 1$.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\tau_1$ (ps)</th>
<th>$\tau_2$ (ps)</th>
<th>$\tau_2/\tau_1$ (%)</th>
<th>$\phi_{\text{calc.}}$</th>
<th>$\tau_{\text{mean}}$ (ps)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorella</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark adapted</td>
<td>492</td>
<td>-</td>
<td>-</td>
<td>0.025</td>
<td>-</td>
</tr>
<tr>
<td>Chloroplasts</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dark adapted</td>
<td>413</td>
<td>1463</td>
<td>3.8</td>
<td>0.023</td>
<td>453</td>
</tr>
<tr>
<td>Light + 3-(3,4-dichloro-</td>
<td>453</td>
<td>1328</td>
<td>9.9</td>
<td>0.028</td>
<td>540</td>
</tr>
<tr>
<td>phenyl)-1,1-dimethylurea</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Light + 3-(3,4-dichloro-</td>
<td>462</td>
<td>1342</td>
<td>36.6</td>
<td>0.040</td>
<td>784</td>
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<tr>
<td>phenyl)-1,1-dimethylurea+Mg$^{2+}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>77 K (685 nm emission)</td>
<td>597</td>
<td>2244</td>
<td>15.8</td>
<td>0.044</td>
<td>857</td>
</tr>
</tbody>
</table>

Fig. 1. Time-resolved fluorescence emission of (a) dark-adapted Chlorella and (b) dark-adapted chloroplasts

In (b) for the lifetime measurements the chloroplasts were diluted into water and then double strength low-salt buffer [0.33 M-sorbitol/10 mM-N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid, adjusted to pH 7.6 with Tris(hydroxymethyl)amino-methane] added, and at 77 K, the fluorescence decay is well described as a sum of two exponentials. Dark-adapted chloroplasts had a short component of 413 ps and a minor long component of $\approx 1.4$ ns accounting for 3.8% of the initial intensity. In the presence of 10 $\mu$M-3-(3,4-dichlorophenyl)-1,1-dimethylurea and pre-illumination, the value of the short
component increases slightly to 453 ps and the proportion of long component increases to 10%. However, the fluorescence yield and mean lifetime still indicate that the pre-illumination was of insufficient intensity to close all the traps since the mean lifetimes are comparable to the lifetimes quoted by Moya et al. (1977). Addition of 5 mM-Mg$^{2+}$, which it has been proposed either decreases spillover of energy from Photosystem II to Photosystem I or alters the partitioning of absorbed light between the two systems (see Barber, 1976, for a recent review), further increases the proportion of long component to 37%. At liquid N$_2$ temperature the lifetimes of the two components are 597 and 2244 ps with the initial intensity of the longer component accounting for 15.8% of the total. The calculated mean lifetime of 857 ps is in very good agreement with the exponential lifetime of about 900 ps for the 690 nm emission of spinach chloroplasts measured by Campillo et al. (1977).

Most of the recent measurements of chlorophyll fluorescence in vivo have used relatively high-powered laser pulses (e.g. the second-harmonic output of a mode-locked Nd$^{3+}$ laser) with streak-camera or fast-shutter detection (see Govindjee, 1978, for a recent review). This has caused the formation of high enough concentrations of excited states, both singlets and triplets, to produce annihilation effects within the photosynthetic unit and has complicated the data obtained with these lasers. Our measurements greatly decrease the possibility of annihilation processes caused either by each pulse or passed over from one pulse to the next in the train by the use of relatively low incident light intensities. Our data are in fair agreement with data taken at moderate laser intensities ($10^{13}$–$10^{14}$ photons/cm$^2$ per pulse) using single laser pulses (Porter et al., 1977; Barber et al., 1978; Searle et al., 1977). This confirms that the threshold for the annihilation processes is $\approx 10^{13}$ photons/cm$^2$ per pulse as measured by Campillo et al. (1976a).

Barber et al. (1978) and Porter et al. (1977) have detected a transient decaying part in the fluorescence at short times. The fluorescence initially decays as $\exp(-at^{0.5})$ and then exponentially at longer times. This behaviour is to be expected for energy migration among the chlorophylls (Altmann et al., 1978). We have not been able to detect this effect, but our poorer time resolution and the uncertainties present when convoluting with decays other than exponentials may be the reason for this.

In the dark-adapted state, we believe that some reaction centres are effectively closed on a statistical basis and hence the presence of 3.8% of the $\approx 1.4$ ns component in the decay of the chloroplasts. The main effect of pre-illumination appears to be to increase the ratio of the long to the short components. We propose that the shorter lifetime is due

Fig. 2. Time-resolved fluorescence emission of pre-illuminated chloroplasts with (a) 10 µm- or (b) with 10 µm-3-(3,4-dichlorophenyl)-1,1-dimethylurea with 5 mM-Mg$^{2+}$

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to quenching of the exciton within the photosynthetic unit by an open reaction centre and the longer component is the result of excitation in a photosynthetic unit with a closed trap. We believe that our data are consistent with an isolated unit model, irrespective of whether energy migration or trapping is the rate-determining step. The model we envisage consists of light-harvesting pigments which can transfer their energy to many photosynthetic units. However, the photosynthetic units are located on potential-energy wells with the traps at the minima. Thus once an exciton moves within the vicinity of a trap it cannot return to the light-harvesting pigments, and thus to another photosynthetic unit. The proportion of closed traps would be reflected in the initial intensity ratio of the long to the short components, which as can be seen from Table 1 increases in proportion to the calculated fluorescence yield.

The effect of Mg$^{2+}$ is also to cause an increase in the ratio of long to short components. This supports the idea that cation-induced changes in fluorescence yield reflect changes in the partitioning of absorbed light between the two photosystems in agreement with the conclusions of Butler & Kitajima (1975), since if spillover was predominant then one would expect a lengthening of the value of the lifetime of the short component on addition of Mg$^{2+}$, which we have not observed, because quenching of photosystem I fluorescence by Photosystem II would be absent.

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Barber, J. (1968) *Biochim. Biophys. Acta* 150, 618–625

Studies of Photosystem II Centres by Fluorescence and Spectrophotometric Measurements*

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It has been shown that at least two phases are observed in the fluorescence rise measured in algae or in isolated chloroplasts in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea or at low temperature (Joliot et al., 1973; Melis & Homann, 1975, 1976). Different interpretations, which are not mutually exclusive, have been proposed. (a) Two

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