BBA 47592

THE FLUORESCENCE DECAY KINETICS OF IN VIVO CHLOROPHYLL MEASURED USING LOW INTENSITY EXCITATION

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Summary

We report fluorescence lifetimes for in vivo chlorophyll *a* using a time-correlated single-photon counting technique with tunable dye laser excitation. The fluorescence decay of dark-adapted chlorella is almost exponential with a lifetime of 490 ps, which is independent of excitation from 570 nm to 640 nm.

Chloroplasts show a two-component decay of 410 ps and approximately 1.4 ns, the proportion of long component depending upon the fluorescence state of the chloroplasts. The fluorescence lifetime of Photosystem I was determined to be 110 ps from measurements on fragments enriched in Photosystem I prepared from chloroplasts with digitonin.

Introduction

An accurate determination of the kinetic law governing the excited state decay of in vivo chlorophyll is of fundamental importance to the understanding of the excitation energy transfer process in photosynthesis [1]. Conventional single photon counting techniques have been used, but these lacked sufficient temporal resolution for accurate in vivo lifetime determinations [2—4]. The advent of mode-locked lasers and streak cameras renewed interest in these measurements [5]. However, it soon became apparent that the high power of the laser pulses could give rise to anomalies as a result of exciton annihilation [6,7]. Once these effects were recognised and the laser pulse intensities controlled, it was possible to obtain results which correlated well with those predicted by steady-state fluorescence yield measurements [8,9]. Although there

is general agreement between ourselves and other investigators on the gross values of the fluorescence lifetimes under various conditions [5], there is still much controversy over the details of the form of the decay kinetics, i.e. whether it is a single or a sum of exponentials or a time-dependent function. In this paper we describe measurements of the fluorescence decay of in vivo chlorophyll *a* by a single-photon counting technique using low power excitation from a tunable dye laser pumped by an argon ion laser.

Experimental

Picosecond-tunable pulses were obtained from a Rhodamine 6G dye laser synchronously pumped by a mode-locked argon ion laser (CR 12 Coherent Radiation Ltd.). The dye laser output pulses were determined to be less than 10 ps full width at half maximum by a zero background second harmonic generation auto-correlation technique over the wavelength range of 580—640 nm. For the photon counting measurements the pulse repetition rate was reduced from 75 MHz to 33 kHz using a Pockels cell between crossed polarisers. A contrast ratio of better than 500 : 1 between the transmitted and rejected pulses was achieved. The subsequent laser output was divided along two paths by a beamsplitter. One was attenuated and used to excite the sample while the other was incident upon a Texas Instruments TI XL 56 silicon avalanche photo-diode which provided the start signal for the time-to-amplitude converter. Fluorescence emitted at right angles to the excitation beam was detected through appropriate filters by a Mullard 56 TUVP photomultiplier tube. Temporal linearity of the photomultiplier tube was obtained by reducing the light-sensitive area of the photocathode to 3 mm diameter. Time calibrations were carried out by monitoring the excitation pulses through suitable optical delays. The laser power at the sample cell was measured using an Alphametrics photometer. Experiments were performed with incident laser intensities within the range 10^9—10^{11} photons/cm^2 per pulse.

The green alga *Chlorella pyrenoidosa* was cultured as described previously [10]. Pea (*Pisum cativum*) chloroplasts were isolated with the outer envelope intact and hypotonically shocked immediately before additions were made and the fluorescence measured [11]. Details of the media are given in the text. Sample suspensions were flowed at a rate of 1 l/min for dark-adapted samples through a 1 cm pathlength cell from a reservoir and had a concentration of approx. 5—8 μg chlorophyll/ml or A_{680nm} = 0.3—0.5, as measured using an integrating sphere. Photosystem II reaction centres of chloroplasts were closed by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to a final concentration of 10 μM and pre-illumination with 633 nm light from a 0.5 mW CW HeNe laser; the sample flow rate of the chloroplast suspension was also decreased. A purified Photosystem I preparation was obtained from pea chloroplasts by isolation of a stroma lamellae vesicle fraction using 0.2% digitonin, as previously described [12]. Stroma lamellae vesicle samples were not flowed. All measurements were carried out at room temperature, and the fluorescence emission was observed at wavelengths greater than 665 nm using a Schott RG 665 filter.

Results

The data were analysed over 3 orders of magnitude of decay with single and two exponential decay characterisitics by an iterative convolution technique using a gradient expansion algorithm. As a check on the instrument's performance the dye molecule Rose Bengal was measured using 580 nm excitation and the same emission filters as used with the photosynthetic systems. The fluorescence had an exponetial decay over three decades decrease in fluorescence intensity of 597 ps in methanol and of 122 ps in water, which compares well with previous measurements of 543 ps [13], 655 ps [14], 118 ps [13] and 95 ps [14], respectively. Table I summarises the results obtained for chlorophyll in vivo.

Chlorella. Dark-adapted *Chlorella* analysed with the assumption of a single exponential decay gave reasonably good fits, as judged by a chi-square criterion but the calculated best fit data revealed small systematic deviations from the actual data which indicated that the decay was probably non-exponential. This may be seen in Fig. 1, where the fluorescence decay is close to, but not quite exponential over a 1000-fold decrease in intensity. The fit to the data could be considerably improved using two exponential terms, although the lifetimes varied slightly between different experiments. The two lifetimes obtained for dark-adapted *Chlorella* were found to be in the ranges 270—350 ps and 530—650 ps with the long component accounting for between 38 and 27% of the initial intensity.

No effect upon the lifetimes was discerned when the excitation wavelength was varied within the range 580—640 nm. Similarly, variation of the incident laser intensity from 10^9 to 10^{11} photons/cm^2 per pulse did not affect the fluo-

TABLE I

CHARACTERISTICS OF THE FLUORESCENCE DECAY OF IN VIVO CHLOROPHYLL *a*

The fluorescence yields (ϕ_{calc}) were calculated from the expression

$$\phi_{calc} = \frac{1}{\tau_0 I_0} \int_0^\infty I(t)dt$$

where τ_0 is the natural lifetime (19.5 ns)[30] of in vitro chlorophyll *a*. The mean lifetimes (τ_{mean}) were calculated from the expression $\tau_{mean} = (\alpha_1\tau_1 + \alpha_2\tau_2)/(\alpha_1 + \alpha_2)$ where α_1 and α_2 are the ratios of the lifetimes of τ_1 and τ_2, respectively, and $(\alpha_1 + \alpha_2) = 1$

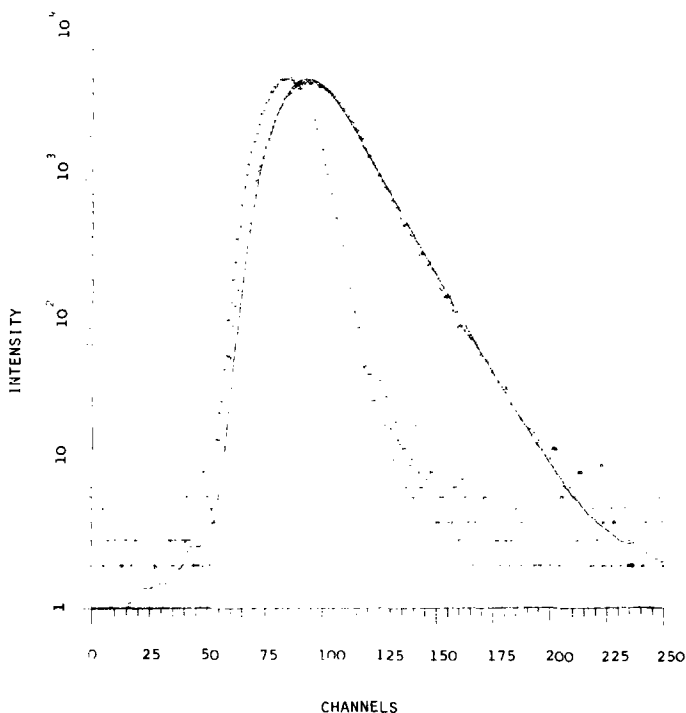| Sample	τ_1 (ps) *	τ_2 (ps) *	% $\frac{\tau_2}{\tau_1}$	ϕ_{calc}	τ_{mean} (ps)
Chlorella					
Dark adapted	492	—	—	0.025	—
Chloroplasts					
Dark adapted	413	1463	3.8	0.023	453
Light + DCMU	453	1328	9.9	0.028	540
Light + DCMU + Mg^{2+}	462	1342	36.6	0.040	784
Stroma lamellae vesicle fraction	113	1192	3—9	0.0058	—

* Estimated error, 5%.

Fig. 1. Time-resolved fluorescence emission of dark-adapted chlorella. Time scale = 32.9 ps/channel.

rescence decay. This gives us confidence that no excitation annihilation processes were occurring.

Chloroplasts. The fluorescence decay for dark-adapted chloroplasts was fitted by a double exponential consisting of a major short component (413 ps) and a long component (1463 ps) comprising about 3.8% of the initial intensity (Table I). In low salt buffer upon pre-illumination and the addition of DCMU, the proportion of the long component increased to about 10%; the lifetime of the short component increased slightly. The addition of 5 mM Mg^{2+} caused a further increase in the proportion of the long component to about 37% of the total. The lifetime of the longer component under the three different conditions is the same within experimental error. The decay kinetics of dark-adapted chloroplasts are presented in Fig. 2.

Stroma lamellae vesicle fraction. Fig. 3 shows the time-resolved room temperature emission of the stroma lamellae vesicle Photosystem I (PS I) fraction. The fluorescence decay was again fitted by a double exponential, the major short component (113 ps) being attributed to the PS I emission. The proportion of long component (approximately 1.2 ns) varied for different preparations of the stroma lamellae vesicle fractions, being less the smaller the amount of Photosystem II (PS II) left in the preparation, determined from the 77 K emission spectrum. The fit to the tail of the emission curve is not the best possible since the complete decay curve was fitted by a double exponential, whereas it should have been fitted to a triple exponential decay (which unfortunately is not possible on our convolution programme). The shortest life-
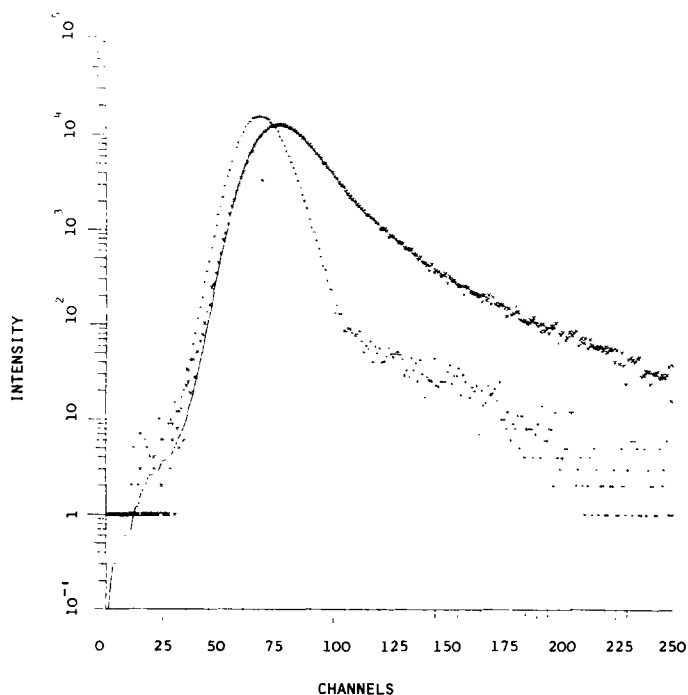
Fig. 2. Time-resolved fluorescence emission of dark-adapted chloroplasts. Time scale = 32.9 ps/channel. For the lifetime measurements the chloroplasts were diluted in water and then double strength low salt buffer was added. (Low salt buffer: 0.33 M Sorbitol/10 mM N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid, adjusted to pH 7.6 with tris(hydroxymethyl)aminomethane).
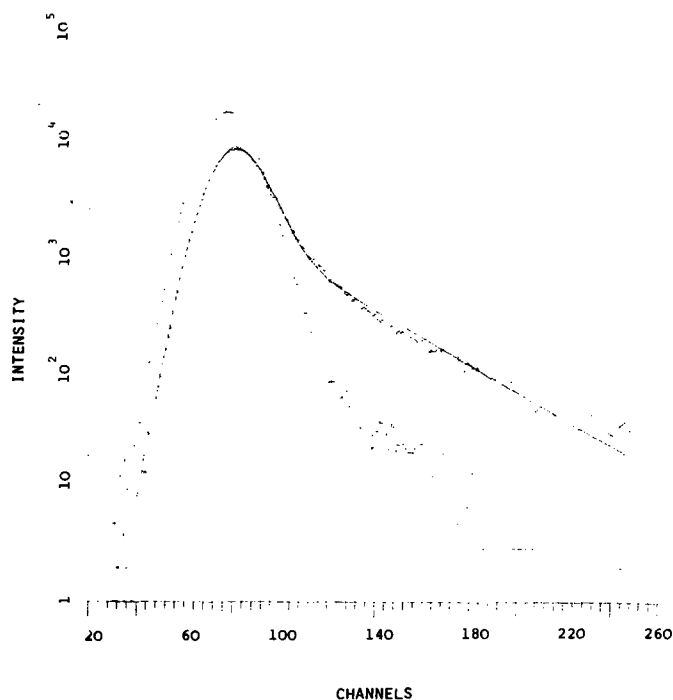

Fig. 3. Time-resolved fluorescence emission of the stroma lamellae vesicle fraction. Time scale = 31.9 ps/channel. The stroma lamellae vesicle fraction was resuspended in 50 mM Tris-HCl (pH 7.8)/2% (w/v) NaCl.

time component would then be due to PS I and the two residual longer components due to PS II. (see Discussion)

Discussion

Non-exponential decays of in vivo chlorophyll fluorescence. If the observed fluorescence decay is non-exponential, then its precise form contains information not accessible in a quantum yield determination. We wish to distinguish clearly between the different causes of non-exponentiality and suggest what kind of information may be obtained in the different cases.

Deviations from exponentiality can arise from two distinct causes: (a) an intrinsic time dependence in the emission probability. This form of non-exponentiality can give information on the mechanism of the energy transfer processes, (b) an inhomogeneity in the emitting species. Inhomogeneity may arise from trivial causes such as differences between individual chloroplasts or cells or may reflect different quenching probabilities in different physical regions of the same chloroplast or photosynthetic unit, and thus contain structural information. Additionally, non-exponential decays can be experimentally induced, for example from high intensity effects such as exciton-exciton annihilation [8,9] or stimulated emission [15], or from reabsorption [16]. These effects may also contain structural and dynamic information if properly understood [17].

The physical basis for an intrinsic time dependence for the quenching of excited chlorophylls is that some of the initially excited molecules may be very close to a trap and the excitation will reach the trap much more quickly than from initially excited chlorophylls far from the trap. This 'transient term' may be reasonably expected to affect only the initial portion of the fluorescence decay; at later times the probability of quenching will be time independent. This expectation is confirmed by recent calculations of Altmann et al. [18]. A transient term of the form predicted for this effect $[I(t) \alpha \exp -(at + bt^{0.5})]$ was not observed in the present experiments. Our data were always much better fitted by a single exponential (chlorella) or double exponential (chloroplasts) than by an equation of the above form. Such a transient term was reported with a Nd : glass/streak camera system at excitation intensities of 10^{13}—10^{14} photons/cm^2 per pulse [9,12]. At present, the time resolution of our apparatus is insufficient to definitely resolve this difference and we are working to improve our time resolution capability.

Non-exponential decays arising from structural heterogeneity may have several causes. (1) Contributions to the total emission from PS I as well as PS II. Although steady-state measurements of fluorescence have indicated that the 685 nm and 730 nm emissions be assigned to PS II and PS I respectively [19], attempts to separate spectrally the time-resolved emission have not been satisfactory. Sub-chloroplast particles enriched in PS I have been shown to emit mainly at 685 nm at room temperature [12]. Thus, spectrally resolved fluorescence decays may still contain contributions from both fluorescing photosystems. (2) Contributions from excitations near closed or open traps, i.e on the state of the primary donors and acceptors. Since the fluorescence yield of PS II is dependent upon the state of its reaction centre [20], further inhomo-

geneity may be introduced depending upon the ratio of open to closed traps which have different fluorescence lifetimes. (3) Heterogeneity in the light collection protein pigment complexes. The time scale of the experiment, i.e. the fluorescence decay time, is important in these cases. If there are a number of distinct sites (e.g. open and closed traps) and the excitation is able to visit them all during its lifetime, then the decay will be exponential; the sample will be homogeneous on this time scale.

Although it should be possible to distinguish the various cases, precise fluorescence decay curves are required, free, as far as possible, from any experimentally induced non-exponentiality. We have avoided annihilation or stimulated emission processes by using low incident light levels, typically 10^9 photons/ cm^2 per pulse which is at least four orders of magnitude less than the threshold for exciton annihilation processes [6]. Quenching by species built up by previous pulses was avoided by flowing the sample and by reducing the pulse repetition rate to 33 kHz. Normally, the contrast ratio of our electro-optic modulator was better than 500 : 1; this could be reduced to 200 : 1 without any discernible effect on the fluorescence decays. Finally, dilute samples were used to avoid reabsorption of fluorescence.

Chlorella and chloroplasts. Most of the early measurements of in vivo chlorophyll fluorescence decays utilising high power mode-locked solid-state lasers and optical shutter or streak camera detection gave anomalous decay times ($\leqslant$200 ps) for both *Chlorella* and chloroplasts, with various degrees of non-exponentiality in the decays [5]. The high incident laser intensities produced multiple excitations which then produced annihilation effects within the photosynthetic unit. When moderate (approx. 10^{13} photons/cm^2 per pulse) single-pulse excitation intensities were employed, lifetimes in the region of 450—650 ps [8,9] were observed, which compare well with the range of 350— 800 ps obtained by phase fluorimetric and standard photon-counting methods, (refs. 21—24 and also references cited in 25) and with values predicted from steady-state fluorescence yield determinations [26]. The gross values of the fluorescence lifetimes seem now to be accepted and the main point of conjecture is the form of the fluorescence decay.

Sauer and Brewington [3] have applied a similar technique to ours, but using a longer excitation pulse from a spark lamp. For dark-adapted *Chlorella* they obtained a fluorescence lifetime of 0.4 ns, in reasonable agreement with our result (Table I). At present, we cannot account for the slight non-exponentiality we observed in the fluorescence decay of *Chlorella*. It seems unlikely that the longer component of 530—650 ps is due to a proportion of the reaction centres being closed, since a lifetime of about 1.5 ns [9] would then be expected, and attempts to fit the data with decays longer than 1 ns proved unsuccessful. For dark-adapted spinach chloroplasts, Sauer and Brewington [3] obtained single exponential decays of 0.2—0.32 ns (depending upon the ionic medium) in contrast to our value of 413 ps (with a 3.8% component of 1463 ps) for pea chloroplasts. We do not know the reason for this discrepancy. For pre-illuminated chloroplasts with DCMU and NH_2OH they obtained [3] a biphasic decay of 0.48 ns (93%) and 2.0 ns (6.5%) which is in reasonable agreement with our result (Table I). We believe that our measurements of the dark-adapted state of chloroplasts indicate that some PS II reaction centres are

non-quenchers, i.e. effectively closed, on a statistical basis at any given time. This could possibly be due to some pre-illumination by scattered laser light (which seems unlikely since the effect was not observed in chlorella) or a result of damage to some of the chloroplasts during preparation. This explains the 3.8% of approx. 1.4 ns decay in the dark-adapted samples. Even upon addition of DCMU many PS II reaction centres remain open, presumably because of the low level of illumination, but the proportion of the long lifetime is now increased to approx. 10% (Table I) and addition of Mg^{2+} further increases the proportion of long component present to approx. 37%. The fluorescence yields and mean lifetimes are also consistent with this viewpoint. The weighted mean lifetimes calculated from our data can be compared to the lifetimes obtained by Moya et al. [27] who, using a phase fluorimetric technique and assuming a simple exponential decay, obtained a 4-fold increase in lifetime upon illumination, whereas our calculated mean lifetime increases by a factor of 1.7. The change in fluorescence yield also reflects this incomplete pre-illumination. Unfortunately, it was not possible to increase the level of light used for pre-illuminating the samples. 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 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 migration or trapping is the rate determining step. If an excitation was able to visit a number of traps, then an exponential decay varying continuously from approx. 400 ps to approx. 1400 ps is expected as more traps are closed. The model we envisage consists of light-harvesting pigments which can transfer their energy to many photosynthetic units. However, the photosynthetic units are located in 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 long to short components, which, as can be seen from Table I, 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 and Kitajima [28] 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 PS II fluorescence by PS I would be absent.

Stroma lamellae vesicle fraction. The PS I lifetime of 113 ps (±10%) is longer than most previous determinations (as cited by Searle et al. [12]) but is in agreement with the value of about 100 ps reported by Searle et al. [12]. We have assigned the minor long component (approx. 1.2 ns) to a residual amount of

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* Very recently Hipkins [31] independently reached similar conclusions from fluorescence-induction studies.

PS II still present in the preparation. Since the sample was not flowed, it was expected that the PS II reaction centres would be closed due to illumination by the laser, which resulted in this fluorescence lifetime being comparable to the long component seen in chloroplasts (Table I). On the basis of this lifetime (113 ps), and with the reasonable assumption that the natural radiative lifetime of chlorophyll is unchanged in vivo, we calculate a fluorescence yield of 0.0058. This is higher than the value of 0.003 measured by Boardman et al. [26], however, who also report that the fluorescence yield of PS II is a factor of 5 greater than that of PS I. The fluorescence yields of PS I and PS II reported by Brown [29] for different organisms also show a difference of a factor of 4—5 between the two Photosystems. The ratio of our calculated fluorescence yields for PS II and PS I is 3.9, which is in good agreement with the steady-state determinations.

Acknowledgements

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