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Evidence that the variable fluorescence in *Chlorella* is recombination luminescence

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The fluorescence lifetime of oxygen-forming photosynthetic systems as a function of closed traps has been studied by several groups using light and poisons (usually 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)) to fix the closed trap state during the experiment. These measurements have now been carried out using light alone, by means of pump and probe laser pulses and a very efficient fast photomultiplier-digitizing system. It is found that the absolute amplitude of fast fluorescence (mean τ, approx. 0.3 ns) remains constant until over half the traps are filled. The amplitude of the slow fluorescence ($\tau \approx 1.2$ ns) increases with pump energy, and its response is best fit with a lag or finite risetime of approx. 200 ps. This novel result is consistent with the hypothesis that the slow component of the fluorescence is actually recombination luminescence in the trap. Thus, the full trapping time, i.e., the time to form the P^+I^- state from an excitation in the O_2 photosystem, is relatively slow.

Introduction

Kautsky [1] first observed the increased yield of fluorescence on saturating photosynthetic systems with light some 50 years ago. This effect has served as a very useful indicator of the state of photosynthetic systems [2]. The use of pulsed lasers allowed the study of the temporal evolution of the change in fluorescence yield and its dependence on energy of the actinic pulse [3]. Considerable information concerning multi-excitation effects [4,5], the kinetics of these processes [6] and the optical cross-section of the photosynthetic units [7] have been obtained. The use of picosecond laser pulses and photon-counting techniques has allowed the kinetic measurements to be extended to the lifetime of the singlet excitation itself [6,8,9]. The main conclusions of these studies are that the higher yield fluorescence arises from a long-lived state whose amplitude increases with saturation of the system. A possible objection to these measurements is that they are all carried out on poisoned systems. The inhibitor (usually DCMU) may have other effects aside from that of 'closing traps' following pre-illumination. The rapid pulsing (at least 10 MHz) mode-locked lasers used in these measurements also provide sufficient excitation to the photosynthetic cells, even if flowing, that the truly dark-adapted state is difficult or impossible to measure [9].

The work presented here is based on a pump and probe method used previously in the measurements on changing fluorescence yield [3,10], and is free from the above problems. A variable intensity-actinic pulse is followed after a carefully determined interval by a weak test pulse which is used to determine the fluorescence lifetime. Thus, only light is used to prepare the photosynthetic

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; POPOP, 1,4-bis(2-(5-phenyloxazolyl))benzene; PPO, 2,5-diphenyloxazole; Chl, chlorophyll.

system in a state varying from completely dark-adapted to light-saturated. The fluorescence is measured by a fast microchannel plate photomultiplier and an ultrafast digitizer. Although the system is not as noise-free as is photon counting, it is simpler, adaptable and fast – about 30 s of measurement are sufficient to obtain useful data. Observations show that the slow fluorescence that increases with actinic light energy has a delay or rise time of 210 ± 40 ps. It is concluded that the charge recombination model for this fluorescence [11] accounts for this result.

Materials and Methods

The experiment consists in illuminating the algae with an actinic light pulse (approx. 5 ns) of energy from zero to maximal, followed after 30 μs by a short (approx. 0.3 ns) probe pulse. The fluorescence from this probe pulse is recorded via a microchannel plate photomultiplier (Hamamatsu R1294-01), an ultrafast digitizer (Tektronix 7912AD) and a computer (Hewlett Packard 9825A). Usually 128 pulses with and without the preilluminating pulse were averaged with interweaving of an equal number of baseline subtractions. The pulses were blocked as required by computer-controlled shutters. The actinic pulse was supplied by a N_2 laser (Molectron 1000) pumped dye laser (homemade) operated at 2 pps. The dye was Coumarin 440 (Molectron) in alcohol and emitted at 440 nm. An aperture was placed just before the teflon tubing to uniformly illuminate a 1 mm^2 area. The test pulse was generated by an atmospheric pressure N_2 laser (Photochemical Research Associates LN100A), an approx. 300 ps pulse at 337 nm. The delay between the pulses was determined by a home-made digital delay generator, and the jitter between the light pulses was negligible (20 ns) for the delay used (30 μs). This delay is chosen because in *Chlorella* at room temperature various quenchers have all decayed (τ ranges from 1 ns to 3 μs), but the opening of traps, i.e., reoxidation of the acceptor Q, has not appreciably occurred ($\tau \sim 600$ μs).

The algae were *Chlorella vulgaris,* grown in minimal media under fluorescent lights. They were used 'as is' after 3–6 days growth. The algae were flowed through 1 mm teflon tubing, which served to homogenize the light pulses, at a rate such that each pulse pair illuminated a fresh batch of dark-adapted algae. The fluorescence from the algae was isolated by a broad band interference filter: 680 ± 10 nm. When needed, this filter was replaced by a 337 nm filter to measure the form of the test pulse itself. A part of the test pulse was incident on a high speed photodiode which, through a suitable delay line, triggered the ultrafast digitizer. The time base of the digitizer was calibrated both by the use of the speed of light and by the use of the scintillators POPOP, $\tau = 1.13$ ns, and PPO, $\tau = 1.27$ ns, both in cyclohexane [12]. The calibration agreement was within 2%. The data were collected over 512 points at 20 ps (nominal) per point. The system linearity was checked directly by attenuators and also by showing that the scintillator fluorescence could be fitted to a single lifetime. Unlike our previous measurements [3,10] the photomultiplier was not gated. Thus, the actinic pulse had to be prevented from saturating the phototube. This is especially important for microchannel devices, since they have long recovery times (50 ms). The total pulsed current through the tube was kept 100-fold below saturation for the maximum actinic pulse energy by attenuating the fluorescence before the phototube. The energy of the test pulse was kept below 5% of this actinic maximum. Since the maximum actinic pulse did not quite saturate the system (80%), the test pulse was hitting only 4% of the traps. Tests with relatively inert systems (Chl solutions, dead algae) showed that the carry-over effects of the actinic pulse, i.e., any change of gain 30 μs after the 20-fold more energetic pulse, were small. A positive feature of the microchannel plate photomultiplier, in addition to its high time resolution, approx. 0.3 ns, is its insensitivity to wavelength time shifts. The use of a very short life-time red fluorescing dye (crystal violet, less than 2 ps [13]) as a wavelength converter showed that the time frame shift and increased lifetime were 40 ps. They will be neglected for this paper.

The data analysis was by reiterative convolution using the HP 9825A computer and all 512 points were used. The fast convolution method [14] was checked by exact calculation and found suitable. The least squares were weighted with a constant (unity) because the measured electrical signal has

an approximately constant noise, which was mainly low-frequency, limited by the amplifier band-width (600 MHz), and by ringing (approx. 1 GHz) from the phototube housing. The use of weighting factors appropriate to single-photon-counting (standard deviation equal to number of counts) or in between this and a constant, showed no significant change in errors. There was, however, the expected shift of the 'best' lifetimes and fraction thereof with these weigths. Given these observations, and the limited capacity of the small computer, I have limited the data fitting to two time constants. Unlike other fitting procedures, however, I did allow negative amplitudes when required (see Results). The result was shown to be unforced by analyzing data well fit by two positive components. If started with a negative amplitude, the program (slowly) wound its way back to the same positive amplitude and time constants as that with only positive amplitudes. All programs for data acquisition and analysis were home-made.

Results

The object of these experiments was to measure the change in lifetime of the 'prompt', i.e., less than 5 ns, fluorescence from *Chlorella* as a function of filling of the photosynthetic traps exclusively by light: no poisons. This is done by giving an actinic flash, waiting a specific time to allow all possible quenchers (triplets, Chl cations, etc.) to be removed, but not so long that the acceptor Q^- is reoxidized. For *Chlorella* at 300 K, the optimal time window is 15–30 μs [3,10].

If the slow fluorescence is caused by recombination luminescence on re-exciting a closed trap by the test pulse [11], but the fast fluorescence comes from excitations in the antenna (possibly near the trap), one expects the amplitude of the fast fluorescence to be approximately constant. The reproducibility and stability of the measuring system allowed both lifetimes and relative fluorescence amplitudes to be measured in a succession of experiments. The reproducibility of the system was tested by repeating the measurement using only the probe pulse on *Chlorella* four times, spread over 6 h during a given run. Typical results were (see Table I): an absolute time drift of ± 20 ps, $\tau_1 = 0.30 \pm 0.04$ ns, $X_1 = 0.95 \pm 0.01$ and $\tau_2 = 1.20 \pm 0.13$ ns, where X_1 is the amplitude fraction having τ_1 time constant and the errors are root-mean-square. The observed very low time jitter is crucial to our results. The above data are in agreement with more highly time-resolved measurements [8,9]. The relevant data are presented in Table I. As in the previous measurements, the normalized amplitude of the fast component (X_1, column 5) is found to decrease as the traps are closed. However, the observed fluorescence intensity simultaneously increases (column 6). If the amplitude of the fast component of the fluorescence is multiplied by the measured fluorescence intensity (relative to that of the test pulse alone) the result (A_1, column 8) is in fact constant up to at least 50% saturation (D, row 4). An error of $\pm 2\%$ is caused by variation of the test pulse amplitude, and of $\pm 1\%$ by the curve fitting procedure. The identification of the amplitude of the fast component with the maximum amplitude of observed fluorescence is an approximation. It is actually determined by the relative ratio of the fast and slow lifetimes to the convolution time of the light pulse length and response time of the measurement system, and will be discussed in a subsequent publication with further data. The yield of the fast component at the highest achievable actinic pulse energy is definitely lower than unity (0.8, column 8, row E, Table I) and may reflect this approximation. Our previous measurements on total fluorescence yield showed quenching in the multi hit region; average hits, more than 3 [15]. This quenching was a complex function of time, but always totally recovered by approx. 15 μs. Further measurements with more intense actinic pulses are required. The limited power of our 10-year-old laser did not allow full saturation to be achieved. The increase in time constant (τ_1) between dark-adapted and slightly illuminated cells may be real, as it is seen in flowing cells preilluminated by steady light before entering the measuring cell. These effects will be considered elsewhere. For the present, the conclusion is that the fast fluorescence is approximately constant over an appreciable fraction (> 0.2, < 0.8) of trap filling by light alone.

It then follows that simple subtraction of the fluorescence of the test pulse alone (F_0) from that following an actinic flash, should give the time-

TABLE I

LIFETIMES AND AMPLITUDES OF FLUORESCENCE FROM PUMP-PROBE EXCITATION OF CHLORELLA AS A FUNCTION OF ACTINIC PULSE ENERGY

Time-base shift (column 3) is relative to that of test pulse alone. T_1 and T_2 are the fast and slow lifetimes and X_1 the fractional amplitude of the fast component obtained by curve fitting. Errors are as quoted in the text. Column 7 is the measured peak fluorescence intensity relative to that of the test pulse alone. A_1, column 8, is the true amplitude of the fast component, the product of columns 6 and 7. ϕ, column 9, is the integral of the measured fluorescence intensity, i.e., the total fluorescence yield. The maximum, saturated yield is estimated to be 3.2.

Expt.	Excitation energy	Time shift (ps)	T_1 ±0.04 (ns)	T_2 ±0.15 (ns)	X_1 ±0.01	Fluorescence intensity	A_1	ϕ
A	0	0	0.30	1.19	0.95	1	0.95	1
B	0.0125	−20	0.39	1.53	0.96	1.04	1.00	1.0
C	0.06	−20	0.45	1.27	0.90	1.14	1.03	1.3
D	0.25	+20	0.46	1.30	0.68	1.44	0.98	1.9
E	1	−20	0.28	1.43	0.48	1.73	0.83	2.7

course of the slow or variable fluorescence (F_v). The resulting curves (Fig. 1) show a definite lag in the rise of F_v, relative to F_0. For a measurement limited by system response, in the limit of lifetime much longer than the excitation pulse width, the inflection point of the observed normalized fluorescence will be at the peak of the excitation pulse. This is clearly so, since the observed curve is then simply the integral of the excitation pulse. Since the observed variable fluorescence curves c, d and e have an inflection later than the peak of excitation, they must contain a lag or finite rise aside from the observed lifetime of the fluorescence. There is no lag between the excitation pulse (P) and F_0 beyond the apparent shift caused by the finite time constants. The maximum of the excitation, pulse a, is at channel 66, while the infections of F_v, curves c, d, e, is at channel 71–72. At 22 ps per channel, the delay is 110–132 ps. This is a minimum value, since the fluorescence decay time is not infinitely longer than that of the pulse response time. As a check, the inflection point of F_0, curve b, is at channel 55, well before the peak of the excitation pulse (a), and in turn the inflection of F_v is 110–132 ps before the peak of F_0 at channel 77. This conclusion can be quantified in two ways. First one can fit the excitation pulse, P, to the variable fluorescence allowing a time base shift to optimize the fit. Second, one can fit the same data with a negative amplitude exponential, which assigns a finite risetime to F_v. The results of the calculations are presented in Table II. The first method produces a lag of 210 ± 35 ps. The second results in a risetime of 180 ± 30 ps. Note that the amplitudes of the positive and negative compo-

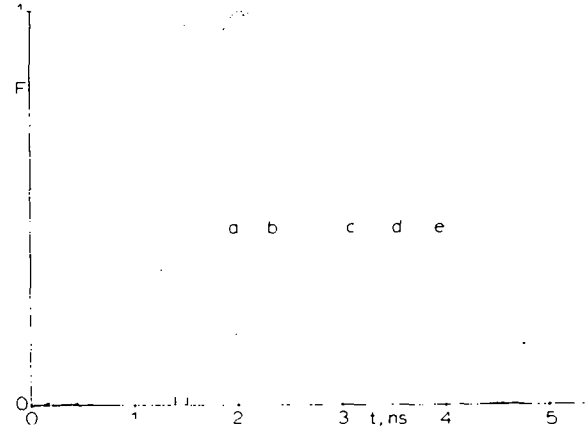

Fig. 1. Time lag in the variable fluorescence yield of *Chlorella*. The curves are: (a) the exciting pulse at 440 nm; (b) the fluorescence of *Chlorella* at 680 nm at zero pre-excitation energy, (c), (d), (e) the fluorescence of *Chlorella* after subtraction of F_0, i.e., curve (b), following increasing energy of excitation, rows (C), (D), (E) of Table I. A lag is required, since the inflection point of curves (c), (d), (e) has gone beyond the maximum of the exciting pulse a. (The bars on the ordinate indicate the maximum of the excitation pulse and the inflection point of F_v.) The data are averages of 128 sweeps, and have been normalized to unity. The oscillations in curve (a) are caused by ringing in the phototube housing. The bumps in curves (c), (d), (e) are for the moment attributed to noise.

TABLE II

THE FIT OF THE VARIABLE FLUORESCENCE IN CHLORELLA BY (1) A LAG AND (2) A FINITE RISETIME

(1) Data of F_v fit by convolution with P, with a variable lag and one lifetime. Two lifetimes did not significantly change the error. (2) Same data fit with two exponentials, but one allowed to have negative amplitude. Column 1 identifies the particular data in Table I.

Curve	Lag [1] (ps)	T (ns)	T_1 [2] (ns)	T_2 (ns)	X_1
B	200	1.30	0.21	0.98	−0.55
C	260	1.14	0.16	0.94	−0.62
D	200	1.30	0.15	1.17	−0.58
E	180	1.68	0.20	1.40	−0.53
Mean ± s.d.	210 ± 35	1.36 ± 0.23	0.18 ± 0.03	1.12 ± 0.21	−0.57 ± 0.04

nents are almost equal, as they will be for the simplest case of an intermediate in a linear system:

$$F_1 * F_2 = \int_{\tau=0}^{t} e^{-k_1\tau} e^{-k_2(t-\tau)} d\tau \propto e^{-k_2 t} - e^{-k_1 t} \qquad (1)$$

where k is the reciprocal of the lifetime of the state, and the subscripts 1 and 2 refer to the short and long lifetime states, the latter formed from the former.

Clearly, including more time constants in the analysis will not change the argument, it will only change the numbers somewhat. We expect that including a third, negative amplitude exponential in the analysis of the original data (Table I) would improve the fit. This must await further work. It is possible that analysis of the already published data [9,16] with a negative amplitude exponential would produce an equally good fit as that found with only positive amplitudes. The problem of least squares analysis of data by a series of exponentials is well known to be poorly poised [17–19]. Numerical analysis can only yield a best fit to data. The solution of the problem requires the judicious experiment.

Discussion

A lag or risetime of 150–200 ps is observed in the increased fluorescence yield following photoexcitation of *Chlorella*. It is observed on subtraction of the constant fluorescence to obtain the variable fluorescence. It is observed either as a lag or via convolution of the response, allowing negative component exponentials. This finite rise-time of the increased fluorescence yield demonstrates an intermediate step between excitation and emission of the increased fluorescence yield. The simplest interpretation of this data is that there is a mean time of approx. 200 ps for the excitation to become trapped. The emission then arises by recombination luminescence in the trap, as first proposed by Klimov [11]. A corollary of this result is that although the amplitude of the long-lived fluorescence, F_v, may track the closing of traps, there is no necessary relation between this factor and the lifetime of the fluorescence. This is clearly so if the recombination luminescence is emitted directly by the trap. If the lifetime changes with closing of traps, then the relation between yield of fluorescence and the fraction closed factor may not be linear.

A simple explanation for the missing of this finite rise-time in previous data is that the fast fluorescence decay-time and the finite rise-time are too close to be easily resolved. The curve-fitting procedure will then fit the difference of the amplitudes of this time component, and the fit will result in a decrease of the amplitude of the fast component, just as is observed.

The observation that the rise-time of the variable fluorescence, 200 ps $= 1/k_1$ of Eqn. 1, is smaller than the mean fluorescence lifetime of F_0, 300 ps, suggests that the trapping is reversible, i.e., the probability of escape from an open trap is finite. The statistics of escape from open and closed traps has been analyzed [20]. As the probability of escape, suitably defined, becomes equal for open and closed traps, the effect of closing traps on observables such as oxygen evolution or

fluorescence yields becomes invisible. This is because, although more time may be spent in the antenna, the final result is independent of traps being opened or closed: simple Poisson statistics will be observed. The effect of escape will be observed on the fluorescence lifetime, since this directly tracks the mean time spent in the electronic excited state. A more detailed measurement of the lag or rise-time may allow the measure of the change of lag or rise-time with closing of traps.

The state that we have prepared by the combination of actinic flash and specific delay is Z^+PIQ^- where Z is primary donor, P is chlorophyll in the trap of Photosystem II (P-680), I is the primary acceptor, and Q is the stabilized acceptor. Thus the test pulse forms the state $Z^+P^+I^-Q^-$, in amount related to the energy of the actinic pulse, and with a time lag given by both the mean excitation trapping time τ_T and the electron transfer-time τ_{PI}: $Z^+P^*IQ^- \rightarrow Z^+P^+I^-Q^-$. It may be objected that the life-time of the observed fluorescent state, approx. 1.5 ns, is less than that observed for the P^+I^- state, 4.3 ns (for a review, see Ref. 21). However, those measurements were made on reduced material, i.e., the state is $ZP^+I^-Q^-$. The presence of Z^+ may well reduce the life-time by a factor of 3–4 because of the local electric field. The slowly increased fluorescence yield has a small (approx. 600 cm^{-1}) activation energy, shown by its 4-fold decrease in amplitude between 300°C and 70°C [21]. The life-time actually decreases by 20%. This small activation energy is consistent with the P^+I^- recombination, as pointed out by Klimov and Krasnovskii [21].

This view that the increased fluorescence yield with the 1.5 ns lifetime originates from charge recombination at the reaction center has another interesting consequence. Since the trap has a definite orientation in the thylakoid, an electric field across the thylakoid will affect this recombination. An electric field across the thylakoids has been shown to affect the fluorescence yield [22]. The observed effect seems to be remarkably unidirectional as regards electric field. It would be of great interest to measure the fluorescence lifetimes in the presence of such applied electric fields. One can interpret the data of Meiburg et al. [22] as supporting the mechanism of energy transfervia electron transfer proposed previously [23]. For this mechanism, a large field will effectively 'pin' the dipolar excitation. This will slow movement to the trap and increase the probability of loss by fluorescence. With the present measurement system it may be possible to measure such effects directly.

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