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THE YIELD OF CHLOROPHYLL *a* FLUORESCENCE AS A MEANS TO TEST THE VARIOUS DEEXCITATION MECHANISMS IN THE ANTENNA SYSTEM OF GREEN PLANTS

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With the aid of measurements of the fluorescence yield, the efficiency of the various deexcitation mechanisms of an exciton in the light-harvesting system has been determined. For this purpose, the fluorescence of dark-adapted as well as of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated and preilluminated leaves of *Zea mays* L. was excited by single ultrashort laser pulses of different energies. The experimental results have served for the fitting of solutions of rate equations, which describe the deexcitation by linear relaxation processes like fluorescence and radiationless transitions, by annihilation of excitons, and by traps both in the ground state and in an excited state. We have obtained the following results: a ratio of antenna chlorophyll molecules to Photosystem II traps of 600:1, an annihilation constant $\gamma = 2 \cdot 10^{-8}$ $cm^3 \cdot s^{-1}$, a mean trapping time of $\bar{t} = 0.5$ ns, a trapping probability for traps in the ground state of $2 \cdot 10^{-8}$ $cm^3 \cdot s^{-1}$, and $6 \cdot 10^{-9}$ $cm^3 \cdot s^{-1}$ for traps in an excited state.

Introduction

The fluorescence yield measured in vivo at room temperature provides a powerful means of investigating the primary photosynthetic processes in PS II. In particular, the fluorescence of variable yield although emitted mainly by the antenna system, reflects the state and kinetics of the traps (P-680), and the primary electron acceptors Q and donors Z [1,2]. After absorption of an exciton by a dark-adapted trap the basic reactions occur according to the following scheme [3]:

$$Z \cdot \text{P-680} \cdot Q \xrightarrow{h\nu} Z \cdot \text{P-680}^* \cdot Q \xrightarrow{<<0.5\ \text{ns}} Z \cdot \text{P-680}^+ \cdot Q^- \xrightarrow{\approx 25\ \text{ns}}$$

$$Z^+ \cdot \text{P-680} \cdot Q^- \xrightarrow{\approx 0.5\ \text{ms}} Z^+ \cdot \text{P-680} \cdot Q \qquad (1)$$

Abbreviations: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Chl, chlorophyll; PS, photosystem.

The time specified above for the photooxidation of P-680 is an upper limit [4]. However, it is reasonable to assume that the photoxidation occurs much more rapidly, because it proceeds in PS I in less than 200 ps [5].

The excited state $Z \cdot \text{P-680}^+ \cdot Q^-$ absorbs also excitons from the antenna system, so that a high concentration of the states $Z \cdot \text{P-680} \cdot Q$ as well as $Z \cdot \text{P-680}^+ \cdot Q^-$ results in a low fluorescence yield [6,7], whereas a high concentration of the state $Z^+ \cdot \text{P-680} \cdot Q^-$ causes a high yield.

The fluorescence lifetime changes with the yield. It is 0.5–0.8 ns in dark-adapted plants (cf. the review by Campillo and Shapiro [8]) and increases to about 2 ns when the PS II are mostly in the nonquenching state $Z^+ \cdot \text{P-680} \cdot Q^-$ [9]. This state is created in a high concentration by DCMU treatment and preillumination or also by intensive irradiation over a period longer than its above-specified lifetime of about 10 ns [10,11].

Another mechanism of quenching is observed

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when the concentration of the excited antenna molecules of Chl *a* is so high that the interaction of the excitons becomes detectable. For example, possible interactions are of the form:

$$S_1 + S_1 \rightarrow S_0 + S_n \quad (2)$$

or

$$S_1 + T_1 \rightarrow S_0 + T_n \quad (3)$$

where S_0, S_1 and S_n are te ground, the first singlet and a higher singlet state, and T_1 and T_n analogous triplet states of Chl *a*. Above an excitation energy of 10^{14} photons/cm^2 per pulse, about every hundredth Chl *a* molecule is excited and a decrease in the fluorescence yield can be observed [8,12–14]. Reaction 2 is preferred when the exciting pulse is much shorter than the fluorescence lifetime of the dark-adapted sample. However, if the exciting pulse is longer than this fluorescence lifetime or if a train of short pulses is used, reaction 3 occurs [15,16]. Most of these quenching experiments have been performed with DCMU-treated samples in the state $Z^+ \cdot P\text{-}680 \cdot Q^-$. A comparison of the fluorescence of DCMU-treated samples with that of untreated ones excited by short pulses of different energy should provide a means of studying the various ways of deexcitation in the antenna system. To date there are three groups dealing with such measurements.

Monger et al. [17,18] have investigated the fluorescence of photosynthetic bacteria, excited by pulses of 15–20 ns length. First of all the dark-adapted sample shows an increase in fluorescence yield with growing pulse energy, reflecting a changed state of the traps. However, at high energies the yield decreases by annihilation as is usually the case. The samples in an environment of low redox potential, i.e., closed traps, have a constant high yield after weak and moderate excitation whereas the yield decreases with strong excitation. In either case the annihilation is due to the quenching effect of triplets of bacteriochlorophyll and of carotenoids.

Sonneveld et al. [19] have determined the fluorescence yield of PS II of dark-adapted *Chlorella* theoretically and experimentally. The plot of fluorescence yield vs. pulse energy has a maximum only at a moderate pulse energy when the exciting pulse is longer than about 25 ns. However, the maximum vanishes if the pulse is shorter. This has been explained by the authors by the assumption that the traps in state $Z \cdot P\text{-}680 \cdot Q$ as well as in state $Z \cdot P\text{-}680 \cdot Q^-$ quench excitons with equal probability from the antenna system, while the following state $Z^+ \cdot P\text{-}680 \cdot Q^-$ does not. Because of the disappearance of state $Z \cdot P\text{-}680^+ \cdot Q^-$ after its mean lifetime, only with longer pulses is it possible to observe a decrease in the concentration of this quencher.

These results are in disagreement with the findings of Leupold et al. [20] who investigated wheat leaves. Dark-adapted samples exhibited a maximum in the plot of fluorescence yield vs. pulse energy in about the same energy range as in the experiments by Sonneveld et al. [19], although excited by pulses as short as 2 ns.

In this paper this discrepancy is clarified by an experiment with ultrashort single pulses of about 8 ps duration. So the pulses are clearly shorter than the relaxation time of state $Z \cdot P\text{-}680^+ \cdot Q^-$.

Moreover, comparison of fluorescence yield curves of either dark-adapted or DCMU-treated samples together with a kinetic model proposed here provides deeper insight in the deexcitation of an excition in the light-harvesting system.

Theory

Some authors [21–23] formulated various mathematical descriptions of the fluoresence yield with respect to the energy of the exciting light, taking into account the change of the state of traps. Especially, Knox [24] investigated the action of traps on the concentration of excitons in the antenna system.

We neglect the inhomogeneous absorption spectra of chlorophyll and use, moreover, the approximation of the lake model, because a boundary of the exciton migration is of little importance [14]. Then we find for the concentration N of excitons in state S_1 in the antenna system the following non-linear rate equation:

$$\frac{dN}{dt} = -\Gamma N - \gamma N^2 - \kappa P N - \beta(P_0 - P)N \quad (4)$$

The concentration P of P-680 traps in the ground

state is governed by:

$$\frac{dP}{dt} = -\kappa PN \tag{5}$$

The initial conditions, realised by the irradiation of the sample by a pulse much shorter than the shortest relaxation time, are $N(t=0)=N_0$ and $P(t=0)=P_0$. Here, the depletion of the exciting pulse passing through the sample is neglected.

The first term on the right-hand side of Eqn. 4 describes all linear relaxations from state S_1 as spontaneous fluorescence k_{Fl}, radiationless transitions k_{NR} or intersystem crossing k_{ST}, i.e., $\Gamma = k_{Fl} + k_{NR} + k_{ST}$. Γ has a value of about $5 \cdot 10^8$ s^{-1} [9].

The second term γN^2 allows deexcitation by singlet-singlet annihilation. The theoretical computation of γ depends on the underlying mechanism of exciton transfer. The value provided by experiment is $\gamma = 5 \cdot 10^{-9}$ $cm^3 \cdot s^{-1}$ in the light-harvesting system [12]. Because we assume a very short exciting pulse, creation of triplet states in a high concentration and, therefore, also singlet-triplet exciton fusion, may be neglected [25].

The term κPN takes into account the fraction of excitons captured by traps in the ground state. The rate κP is the inverse of the mean trapping time $\bar{t}$ of an exciton captured by a trap. A theoretical determination of this time and of κ is impossible as long as the structure of the antenna system is not known exactly.

The last term of Eqn. 4 is proportional to the number of traps which have already absorbed an exciton. These traps are in state $Z \cdot P\text{-}680^* \cdot Q$ or $Z \cdot P\text{-}680^+ \cdot Q^-$. The latter is able to absorb a further exciton [4,5]. We assume that the transition time into this state is much shorter than the upper limit of 20 ns (cf. Introduction). Then the approximation is valid that all traps not in the ground state are in state $Z \cdot P\text{-}680^+ \cdot Q^-$. The constant β can be chosen independently of κ. Therefore, there is the possibility of taking into account that the ground and excited state of the traps have different trapping efficiencies.

The solution of Eqns. 4 and 5 provides $N = N(t)$ and the fluorescence yield is computed according to:

$$\phi = \frac{k_{Fl}}{N_0} \int_0^\infty N(t)\, dt \tag{6}$$

An analytical solution of the fluorescence yield can only be found for very small excitation densities, to wit in the form of the well known Stern-Volmer equation

$$\phi = \frac{k_{Fl}}{\Gamma + \kappa P_0} \tag{7}$$

Materials and Methods

A train of about 40 pulses at a wavelength of 1.06 μm, were generated in a passively mode-locked neodymium-glass laser. From the front of this pulse train a single pulse was selected by means of a Pockels cell. About 20% of the pulse energy was converted to light at 530 nm by second-harmonic generation employing a KH_2PO_4 crystal. The two pulses were separated in a polarizer. The pulse at 1.06 μm entered a two-photon fluorescence arrangement to measure its length. Only pulses with a duration of 8 ± 1 ps were considered in the experiments. The pulse at 530 nm was used to excite the fluorescence.

To achieve a nearly uniform radial intensity profile, the pulse diameter was expanded to 20 nm and subsequently truncated by an aperture of 6 mm diameter. The pulse energy was varied by calibrated nonsaturable filters. A mirror after the aperture directed a small part of the pulse energy to an energy-calibrated photomultiplier. The fluorescence of the sample was detected also by a photomultiplier, whereas cut-off filters blocked the remnant of the exciting pulse.

A complete description of the apparatus for picosecond studies has been given previously [26].

The fluorescence was measured on 10-day-old primary leaves of *Zea mays* L. We used whole leaves because they are more readily available and easier to handle than chloroplast suspensions. Half a charge of leaves was treated with DCMU for 24 h and preilluminated. The rest was dark adapted by being kept in the dark for at least 30 min. The interval between two single laser pulses was about 30 s, enough time to relax as shown by comparison of the first measurement to a later one. Before measurements the leaf was cut off. The fluorescence yield remained reproducible for many hours.

The fluorescence was excited in the green

absorption minimum of the leaves at 530 nm. So only 48% of the exciting pulse was absorbed. Therefore, pulse depletion can be neglected to a good approximation. The concentration of excited antenna chlorophyll could not be determined in the usual way, as neither the absorption cross-sections at 530 nm nor the concentrations of the absorbing pigments were known. However, it is possible to determine the chlorophyll concentration per cm^2 of the leaf by measuring the absorption at 680 nm and using the here well known cross-section of $1.3 \cdot 10^{-16}$ cm^2 [27]. Now the exciton density can be evaluated with the help of this chlorophyll concentration per cm^2. To this end, energy transfer from the pigments absorbing at 530 nm to Chl *a* without losses has to be assumed. Then the relative exciton concentration is given by the ratio of the number of photons/cm^2 absorbed at 530 nm to the concentration of Chl *a* molecules altogether. The exciton concentration follows by using the familiar concentration of Chl *a* molecules in the chloroplasts of 0.1 M.

Eqns. 4 and 5 have been solved in two ways. An analytical approximation in the form of infinite series was calculated by a computer. Independently of that a solution was obtained by means of the Runge-Kutta method. The solutions obtained in both ways agree well. This and further tests have shown that the numerical error is clearly less than 10%. The error of the numerical integration of Eqn. 6 is also of this order.

The calculated fluorescence yield depends very sensitively on the set of parameters chosen. Therefore, evaluation of the parameters best fitting the experimental fluorescence data was possible with an error equal to or less than 30%, mainly caused by the uncertainty in the absolute values of the laser pulse energies.

Results and Interpretation

The dots in Fig. 1 represent the measured fluorescence yields ϕ, whereas the curves were obtained from solutions of Eqns. 4–6. The upper curve shows the well known decrease in ϕ with increasing excitation density due to exciton annihilation, when DCMU-treated and preilluminated leaves are applied. The lower curve depicts the yield of dark-adapted leaves.

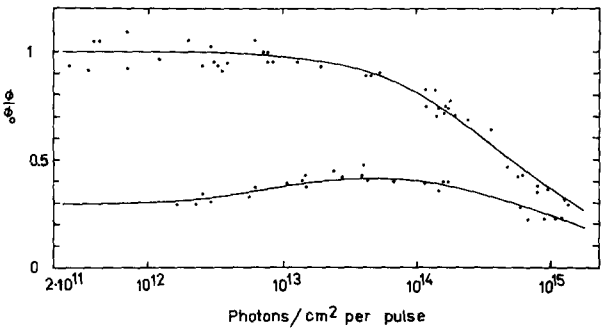

Fig. 1. Relative fluorescence yield vs. energy of the exciting pulse. Dots are experimental results, curves are the fitted theoretical solutions with the parameters specified in the text. Upper values: DCMU-treated and preilluminated leaves of *Zea mays* L. Lower values: dark-adapted samples. (The initial yield of DCMU-treated leaves ϕ_0 was used as the unit for the fluorescence yield of both curves.)

With the following set of parameters for the upper curve the values measured have been reproduced best: $\Gamma = 5 \cdot 10^8$ s^{-1}, $\gamma = 2 \cdot 10^{-9}$ $cm^3 \cdot s^{-1}$, $\kappa = \beta = 0$. Γ has been assumed according to its established value [9]. Simultaneously the necessary time scale is introduced through that in Eqns. 4 and 5. γ results from the fitting process and is in good accordance with experimental values of previous experiments [12,13].

The lower fluorescence yield of the dark-adapted leaves is best described by the parameters: $\Gamma = 5 \cdot 10^8$ s^{-1}, $\gamma = 2 \cdot 10^{-9}$ $cm^3 \cdot s^{-1}$, $\kappa = 2 \cdot 10^{-8}$ $cm^3 \cdot s^{-1}$, $\beta = 6 \cdot 10^{-9}$ $cm^3 \cdot s^{-1}$, $P_0 = 10^{17}$ cm^{-3}. Γ and γ have been chosen according to the first set. The three additional parameters can be uniquely determined, since they influence the fluorescence curve in a quite different way. Their error amounts to less than 30% (cf. Materials and Methods). In the following we will discuss these values.

Concentration of traps. The concentration P_0 of traps in the dark-adapted chloroplasts corresponds to $1/6 \cdot 10^{-3}$ M. Hence, because we assumed a chlorophyll concentration of 0.1 M we have a ratio of one trap per 600 antenna chlorophyll molecules. This is in remarkable agreement with the well known number of 300 antenna molecules, taking into consideration that P_0 is only the density of PS II traps, as the state of PS I traps does not influence the fluorescence yield, and the total concentration of PS I and PS II traps is twice P_0.

Mean trapping time. The mean trapping time $\bar{t}$ amounts to $(\kappa P_0)^{-1} = 0.5$ ns, and corresponds to the value for the fluorescence lifetime in dark-adapted plants given in the Introduction. This once more provides evidence that in dark-adapted samples the lifetime of the fluorescence is determined by the trapping time when the exciting pulse is weak and short. Moreover, it corresponds exactly to the theoretically obtained value given by Porter [28].

Competition between trapping and annihilation. Since κ is 10-times greater than γ, the energy flow into the traps at an exciton density of one or a few excitons per trap will take precedence over annihilation.

Efficiency of DCMU treatment. The experimental results are reproduced by the upper curve in Fig. 1 only with the values $\kappa = \beta = 0$. This confirms that the essential part of the traps after DCMU treatment and preillumination is neither in the ground state nor in state $Z \cdot P\text{-}68^+ \cdot Q^-$, but as expected in state $Z^+ \cdot P\text{-}680 \cdot Q^-$.

Absorption from excited traps. The ratio $\kappa/\beta = 3$ implies that the energy flow into traps in the ground state is 3-times greater than that into traps in state $Z \cdot P\text{-}680^+ \cdot Q^-$. Because there is no evidence that energy transfer through the antenna system depends on the state of the traps, we have no option but to assume that the probability of exciton capture by traps in state $Z \cdot P\text{-}680^+ \cdot Q^-$ is 3-times less than that of traps in the ground state. This seems understandable, as the probability of energy transfer depends on the quantum-mechanical state of the acceptor.

If the transition $Z \cdot P\text{-}680^* \cdot Q \rightarrow Z \cdot P\text{-}680^+ \cdot Q^-$ were no faster than the mean deviation of the trapping time $\bar{t}$, this ratio would depend on the pulse length. We do not know the mean deviation of $\bar{t}$, yet one can see that during this interval a considerable part of the traps obviously has been excited, since $\beta = 0$ does not give a fit to the data. So the charge separation in PS II seems to proceed in a time much shorter than 20 ns. The possibility that the second absorption starts with the given transition probability from state $Z \cdot P\text{-}680^* \cdot Q$ is unlikely, however, it cannot be ruled out.

Concluding Remarks

Detailed information can be obtained about the distribution of excitons in the antenna system to the different pathways of deexcitation by a comparative analysis of the plots of fluorescence yield vs. excitation energy of dark-adapted as well as DCMU-treated and preilluminated samples.

The course of the lower curve in Fig. 1 can be interpreted in the following way. For weak excitation the fluorescence yield is constant and described by the Stern-Volmer equation (Eqn. 7). With growing excitation density, depopulation of the ground state of the traps becomes detectable and the yield increases, because the excited traps have a 3-fold lower trapping efficiency. Exciton annihilation prevents a further increment and finally causes a rapid decrease in the yield, so that the upper and lower curves nearly coincide.

Both the explanations for the increase and decrease in yield do not agree with the arguments given by Sonneveld et al. [19]. However, a slight maximum of the yield is still to see in the figure of their paper even when the fluorescence is excited by the shorter pulses. This can be explained possibly by the arguments discussed above.

To test our results the fluorescence lifetime should be determined when the concentration of traps in state $Z \cdot P\text{-}680^+ \cdot Q^-$ is as high as possible. It should increase from 0.5 ns to about 1 ns because of the lower trapping efficiency of this state in comparison with the ground state.

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