Nonphotochemical Quenching of Chlorophyll Fluorescence in Higher Plant Leaves Studied by Delayed Fluorescence Decay Measurements

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Delayed chlorophyll fluorescence decay measurements on the second time scale were applied to investigate the state of photosystem II under different photosynthetic conditions. Leaves adapted to high and low light intensities were used to study the effects of nonphotochemical quenching (energy quenching) on the photosynthetic state. 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU)-treated leaves were used to characterize the photosynthetic state in the absence of a transthylakoid ΔpH, dithiothreitol (DTT)-treated leaves in the absence of the xanthophyll zeaxanthin. The fast decay components were the most affected by energy quenching as indicated by increased decay times. The slowest decay component was hardly affected, neither in amplitude nor in decay time. The measurements indicate a relaxation of energy quenching on the second time scale and the absence of damages in the electron transfer chain of PS II. The constant decay times of the DTT-treated leaf, comparable to those of the DCMU-treated leaf, indicate the obligatory role of zeaxanthin for most of the detected energy quenching.

Introduction

Light of the visible spectrum is absorbed by the antenna pigments of higher plant leaves. The electronic excitation, generated in the antenna complexes of photosystem II (PS II), is transferred to the reaction-centre P680 to initiate the charge separation. Pheophytin is the primary electron acceptor, transferring the electron via the primary quinone Q_A to the secondary quinone Q_B. Q_B^- remains in the binding pocket of the D1-protein until the second reduction by another electron. Q_B^{2-} is lost from the binding pocket, takes up two protons and becomes part of the plastoquinone-pool. An oxidized quinone Q_B binds in exchange at the D1-protein. The oxidized reaction centre $P680^+$ is reduced by an electron of the water splitting complex (for reviews see Govindjee and Coleman, 1993; Diner and Babcock, 1996).

Some of the charged molecules are rather stable. Q_A^- can be stabilized for seconds, Q_B^- for tens of seconds or sometimes up to hours. At the donor side of PS II, electrons are extracted from water molecules in five successive redox steps. Two of these so-called S-states, the S_2- and S_3-states, carry a positive charge and are stable for tens of seconds (Vass and Inoue, 1992; Inoue, 1996).

Thermally activated recombination of electrons of both quinones and positive charges of the S_2- and S_3-states leads to the re-excitation of the reaction centre P680. P680* can decay via fluorescence to the electronic ground state. The time course of this so-called delayed fluorescence can be followed on the microseconds- up to the minutes time scale, depending on the state of the electron transfer chain in PS II.

Two decay components were clearly characterized: the decay of the $S_2Q_A^-$ charge pair with a decay time of about 2 s and the decay of the $S_2Q_B^-$ and $S_3Q_B^-$ charge pairs with a decay time of about 50 s (Rutherford *et al.*, 1982; Demeter and Vass, 1984; Hideg and Demeter, 1985).

High light intensities are known to induce the deepoxidation of violaxanthin to zeaxanthin (xanthophyll-cycle). It is widely accepted that zeaxanthin is an integral part for photoprotection but functional only in connection with a transthylakoid ΔpH, giving rise to the so-called energy quenching state (qE). In the qE-state, part of the absorbed energy is dissipated by a nonradiative decay in the antenna

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complexes, observable by an increased quenching of chlorophyll fluorescence (Demmig-Adams *et al.*, 1996; Horton *et al.*, 1996; Gilmore, 1997).

Light intensities oversaturating the primary reactions induce photoinhibitory damages. PS II is affected at various sites as well as many other components of the electron transfer chain (Baker and Bowyer, 1994; Baker, 1996).

Delayed fluorescence makes it possible to investigate the state of PS II by the recombination of charge pairs. Vass *et al.* (1988) studied the effects of photoinhibition on the electron transfer activity in spinach thylakoids. They found no variations of the decay times, but a parallel decrease of all decay amplitudes with increasing time of photoinhibition.

We studied the effects of lower light intensities on the photosynthetic state of intact leaves. The applied intensity was high enough to induce the conversion of violaxanthin to zeaxanthin (xanthophyll-cycle), but insufficient to cause irreversible photoinhibitory effects.

Materials and Methods

Plants of *Pisum sativum* Lancet were grown on cultivation soil under natural light with a daily photoperiod of about 11 hours. All delayed fluorescence decay curves presented here were obtained in measurements on fully developed leaves and repeated twice. Measurements were also carried out on leaves of *Spinacia oleracea* Atlanta (two times), grown under the same conditions, giving the same results.

Dithiothreitol (DTT) blocks the formation of zeaxanthin (Bilger and Björkman, 1990) and was provided through the cut petiole. Leaves were set into a 3 mM DTT-solution and kept in darkness for a minimum of twelve hours to allow uptake and epoxidation of zeaxanthin to violaxanthin (Demmig-Adams *et al.*, 1990; Ruban *et al.*, 1991). Prior to the first delayed fluorescence decay measurements, the DTT-treated leaves were submitted to weak white light (about 20 W/m^2) for about 20 minutes to induce photosynthetic activity.

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) blocks the electron transfer after the primary quinone Q_A (Velthuys, 1981). Leaves were put into a 10^{-3} M solution for about 15 min, long enough to block all PS II centres.

Leaves were adapted to high light intensities by exposing them to an intensity of 300 W/m^2 (Schott KL1500 lamp). Minimal exposure time was one hour to allow most of the violaxanthin to be converted to zeaxanthin (Härtel *et al.*, 1996; Jahns and Miehe, 1996); the intensity was weak enough to avoid photoinhibitory effects.

Leaves were adapted to low light intensities by exposing them to 20 W/m^2 of white light. The leaves were submitted to this intensity for a minimum of two hours to allow epoxidation of zeaxanthin to violaxanthin. The reverse step of de-epoxidation was strongly reduced during that time period (Härtel *et al.*, 1996; Jahns and Miehe, 1996).

Delayed fluorescence decay measurements were carried out in a home-built single photon counting setup. The leaves were illuminated by well defined light intensities of a light emitting diode (selected LED, HP HLMP8103, with a red-shifted peak wavelength of 650 nm). The illuminated leaf area was strictly controlled by an iris diaphragm (diameter of 1.2 cm), positioned on the leaf surface. Each illumination time was 15 min to allow photosynthetic adaptation to the applied intensity. After the LED was switched off, a shutter in front of the photomultiplier (RCA C31034 02) was opened. The delay time between switching off the LED and opening the shutter was below one second. A long pass filter (RG665, Schott) restricted the spectral sensitivity to the wavelengths of chlorophyll fluorescence. Pulses of the photomultiplier, representing single photons, were amplified (EG& G Ortec VT110), discriminated (Ortec T101/N), and registered by a multi-channel analyser card (Nucleus) with an integration time of 0.4 s per channel.

Delayed fluorescence decay curves were analysed by iterative deconvolution techniques (Marquardt algorithm). The quality of the fits was checked by the reduced chi-square criterium χ^2_{red} and the random distribution of the weighted residuals.

Results

Delayed fluorescence decay measurements were carried out in dependence on the illumination intensity.

The first delayed fluorescence decay of the DCMU-treated leaf was measured after illuminating the leaf with 0.08 W/m^2, followed by the increased intensities of 0.6 W/m^2 and 9 W/m^2

(Fig. 1). The measurement with 0.08 W/m^2 was finally repeated. The calculated amplitudes α_i and decay times τ_i are listed in Table I. Two delayed fluorescence decay components were observed, both stable in decay time and relative amplitude at all applied intensities. The absolute amplitudes increased with increasing illumination intensities.The decay curves of the DCMU-treated leaf were first analysed with the expectation of one decay component. The reduced chi-square criterium gave χ^2_{red} above 3, the weighted residuals were significantly distorted. A biexponential decay gave much better results: all χ^2_{red} were between 1 and 1.1 with randomly distributed weighted residuals.

Measurements on the DTT-treated leaf were carried out following the same protocol. The corresponding delayed fluorescence decay curves are shown in Fig. 1, the decay components are listed in Table II. Both fast decay times are similar to those of the DCMU-treated leaf. Also a new decay component appears with a decay time of about 50 s. All decay times were unaffected by the applied intensities. The 50 s-component was stable in its amplitude at all applied intensities, while the amplitudes of both fast decay components increased with increasing illumination intensities.

A different response to the illumination intensities was seen in the measurements on the low light-adapted leaf (Fig. 1, Table III), despite the same measuring protocol as above. Both fast decay components increased in amplitude and in decay time with increasing light intensities. Only the slowest decay component was unaffected, both in amplitude and in decay time. The overall amplitude of the delayed chlorophyll fluorescence decay in this photosynthetic state was much lower compared with the DCMU- and the DTT-treated leaf.

The delayed fluorescence decay curves of the high light-adapted leaf were obtained after changing the succession of the applied intensities. The highest illumination intensity of 9 W/m^2 was used in the first measurement to maintain the high light-adapted state. The intensity was decreased in the following measurements to 0.6 W/m^2 and 0.08 W/m^2 (Fig. 1). The measurement with an intensity of 9 W/m^2 was finally repeated. Only the slowest decay component was stable, in amplitude as well as in decay time. Both fast decay times decreased with decreasing illumination intensity. Only the amplitude of the fastest decay component decreased systematically with decreasing intensities (Table IV).

A significant increase of the amplitudes of both fast decay components was seen in the final measurement with 9.0 W/m^2 (Table IV) compared to the first measurement with this intensity.

Discussion

We observed two decay components in our delayed fluorescence decay measurements on the DCMU-treated leaf. The decay times were 0.9 s and 2.7 s and the amplitude ratio 85% / 15%, constant at all illumination intensities.

Rutherford and Inoue (1984) measured a decay time between 1 and 2 s, Hideg and Demeter (1985) measured 3 s in the DCMU-treated state. They attributed this component to the recombination of the $S_2Q_A^-$ charge pair. Vass *et al.* (1988) investigated the effects of photoinhibition on spinach thylakoids and found no changes in the delayed fluorescence

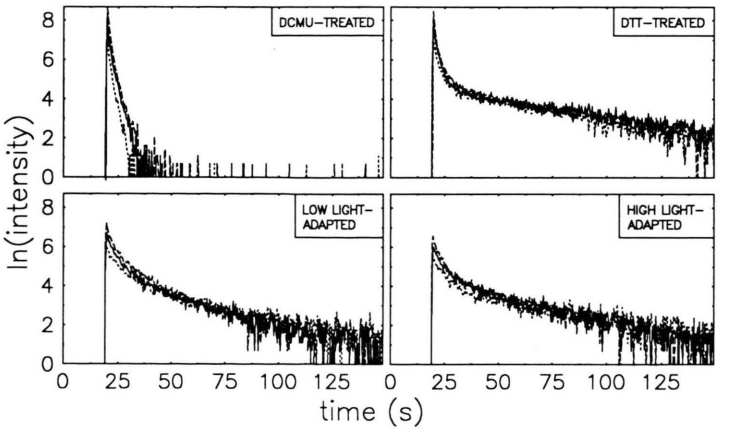

Fig. 1. Delayed fluorescence decay curves of the four investigated photosynthetic states. Applied intensities were from the bottom to the top 0.08 W/m^2, 0.6 W/m^2, and 9 W/m^2.

Table I. Decay components of the delayed fluorescence of the DCMU-treated leaf; comparison of the amplitudes (α_i) and decay times (τ_i) under the different illumination intensities.

Intensity [W/m²]	α_1 abs.	(rel.)	α_2 abs.	(rel.)	α_3 abs.	(rel.)	τ_1 (s)	τ_2 (s)	τ_3 (s)
0.08	1575	(86%)	257	(14%)	–		1.0	2.7	–
0.6	3652	(84%)	688	(16%)	–		0.9	2.8	–
9.0	7203	(87%)	1037	(13%)	–		0.8	2.7	–
0.08	1646	(82%)	373	(18%)	–		0.9	2.9	–

Table II. Decay components of the delayed fluorescence of the DTT-treated leaf.

Intensity [W/m²]	α_1 abs.	(rel.)	α_2 abs.	(rel.)	α_3 abs.	(rel.)	τ_1 (s)	τ_2 (s)	τ_3 (s)
0.08	837	(53%)	638	(41%)	91	(5.8%)	0.9	2.6	50
0.6	2573	(71%)	971	(27%)	98	(2.7%)	0.9	2.6	51
9.0	6320	(84%)	1115	(15%)	99	(1.3%)	0.7	2.9	49
0.08	901	(54%)	662	(40%)	95	(5.7%)	0.8	2.7	49

Table III. Decay components of the delayed fluorescence of the low light-adapted leaf.

Intensity [W/m²]	α_1 abs.	(rel.)	α_2 abs.	(rel.)	α_3 abs.	(rel.)	τ_1 (s)	τ_2 (s)	τ_3 (s)
0.08	236	(40%)	279	(48%)	74	(12%)	0.7	5.4	41
0.6	582	(62%)	300	(32%)	60	(6.1%)	1.9	7.7	42
9.0	990	(68%)	397	(27%)	64	(4.4%)	1.8	8.7	41
0.08	240	(43%)	252	(45%)	68	(12%)	0.8	5.2	41

Table IV. Decay components of the delayed fluorescence of the high light-adapted leaf.

Intensity [W/m²]	α_1 abs.	(rel.)	α_2 abs.	(rel.)	α_3 abs.	(rel.)	τ_1 (s)	τ_2 (s)	τ_3 (s)
9.0	531	(70%)	161	(21%)	66	(8.7%)	2.2	7.8	42
0.6	241	(46%)	216	(41%)	65	(12%)	1.5	6.1	42
0.08	143	(40%)	159	(44%)	59	(16%)	0.9	4.3	45
9.0	699	(71%)	222	(23%)	64	(6.6%)	2.1	8.8	43

decay times compared to the normal state. Addition of DCMU to these thylakoids revealed two decay components with lifetimes of about 0.5 s and 2.7 s and an amplitude ratio in favour of the fast decay component, comparable to our results. They attributed the fast decay to the recombination of Q_A^- with an unspecified donor in PS II and the decay of 2.7 s to the recombination of the $S_2Q_A^-$ charge pair.

Other groups investigated the delayed fluorescence decay on the microsecond to millisecond time scale and found a number of decay times, most of them attributed to the recombination of the $P680^+Q_A^-$ charge pair (reviewed by Amesz and van Gorkom, 1978). So we conclude that the fastest decay component is to be considered as an average of all decay components on the subsecond time scale.

We also measured an increase of the overall amplitude of the delayed fluorescence decay with increasing illumination intensities, probably due to more produced charge pairs. By reproducing the first measurement it was shown that photoinhibitory damages were absent.

In comparison with the DCMU-treated leaf, an additional decay component of 50 s was observed in the delayed fluorescence decay of the DTT-treated leaf (Table II). This decay component was attributed to the recombination of the $S_2Q_B^-$ and $S_3Q_B^-$ charge pairs (Demeter and Vass, 1984; Hideg and Demeter, 1985). The 50 s component remained unchanged in amplitude as well as in decay time at all illumination intensities. Both fast decay times were stable and the expected increase in amplitudes with increasing illumination intensities was observed, too. But the ratio of the amplitudes of both fast decay components varied with the illumination intensities, probably due to the possible electron transfer out of PS II.

Reviewing the state of the high light- and the low light-adapted leaves at the different illumination intensities, only two conditions were clearly defined: the high light-adapted state in the first measurement with the highest illumination intensity and the low light-adapted state in the first measurement with the lowest illumination intensity. Under the other conditions changes in the amount of zeaxanthin can be expected due to the different illumination intensities of the LED within the experiments. This was confirmed by the final measurement on the high light-adapted leaf with the intensity of 9 W/m². The amplitudes of both fast decay components increased significantly, probably due to a decreased amount of zeaxanthin 50 min after the adaption to high light intensities.

The overall amplitude of both light-adapted states was decreased in comparison with the DTT-treated leaf. Moreover, the overall amplitude of the high light-adapted leaf was smaller than the amplitude of the low light-adapted leaf. This must be the result of different extents of energy quenching, probably due to different amounts of zeaxanthin.

Both fast decay times were affected by energy quenching, too. An increase was observed in the light-adapted states with increasing illumination intensities. Energy quenching must be the reason for

this effect because of the unaffected decay times in the DCMU- and DTT-treated leaf. Energy quenching is maximal after switching off the LED and relaxes in the following seconds. The delayed fluorescence decay is affected by this time-dependent quenching, that results in the apparently longer decay times. But both fast decay times, calculated by the numeric analysis, do not represent the true recombination times any more.

The unchanged decay times and amplitudes of the recombinating $S_2Q_B^-$ and $S_3Q_B^-$-pairs indicate that the electron transfer in PS II was unaffected by the high light treatment and by the illumination with the varying LED intensities. Since zeaxanthin can only be found in the antenna complexes (Yamamoto and Bassi, 1996) and in view of the decreased overall amplitude and the changes in both fast decay times in the light adapted states, the measurements support the current view that energy quenching is a reversible step of nonradiative energy dissipation in the antenna complexes in the presence of zeaxanthin and a ΔpH.

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