Biochimica et Biophysica Acta, 974 (1989) 289–293
Elsevier

BBA 42985

Chlorophyll fluorescence from spinach leaves: resolution of non-photochemical quenching

M. Hodges, G. Cornic and J.-M. Briantais

Institut de Physiologie Végétale, C.N.R.S., Gif-sur-Yvette (France)

(Received 3 October 1988)

Key words: Chlorophyll fluorescence; High energy state; Non-photochemical quenching; Photoinhibition; Relaxation kinetics; State transition

Using saturating light pulses of 800 ms duration, room-temperature chlorophyll fluorescence quenching in intact spinach leaves has been examined upon illumination with different light quantities and qualities. The observed non-photochemical quenching was resolved into three different components by their relaxation times when the actinic light was switched off. A rapid relaxation phase (half-time, approx. 40 s) is considered to arise from a pH-gradient-induced phenomenon. A slower phase (half-time, approx. 7.6 min) which is affected by the presence of NaF and is predominant when the leaf is preilluminated with low-intensity blue light shows the expected characteristics of a State-II-to-State-I transition. The slowest relaxation component (half-time, approx. 40 min) is observed after a photoinhibitory pretreatment and is therefore associated with the photoinhibition-induced fluorescence quenching.

Introduction

At room temperature, chlorophyll fluorescence from light harvesting chlorophyll *a/b* complex (LHC) containing higher plants and algae mainly originates from the LHC complexes associated with Photosystem II (PS II) (LHC II) [1]. This emission is widely used to investigate the functioning of the photosynthetic apparatus. Upon illumination of dark-adapted material, the fluorescence emission undergoes a number of changes in intensity before reaching a steady-state level which has been termed the Kautsky effect [2]. It rapidly rises from an initial low level, where all of the primary quinonic electron acceptor of PS II (Q_A) is oxidised (F_0), via an intermediate level (F_i) to a peak before decaying to a steady-state level [3]. The various induction transients reflect the onset of the photosynthetic processes and the slow quenching has been divided into photochemical and non-photochemical quenching [4]. The photochemical changes reflect the redox state of Q_A of which fluorescence is not a linear indicator (see Ref. 5). The non-photochemical quenching has been assigned to a number of different mechanisms including (i) the 'high-energy state' (HES) which might reflect in some way a pH-gradient induced by photosynthetic electron transfer [6]; (ii) The State I-State II transition, brought about by the phosphorylation/dephosphorylation state of the LHC II, which alters the distribution of excitation energy arriving at the two photosystems [7]; (iii) the redox state of the plastoquinone pool which, when oxidised, can decrease the fluorescence emission by 20% [8]; (iv) photoinhibition [9]; and (v) the xanthophyll cycle, where the conversion of violaxanthin to zeaxanthin is associated with a decrease in the fluorescence emission [10].

This non-destructive technique for probing the photosynthetic reactions is often used to investigate modifications induced by environmental stresses and therefore a qualitative definition of the different non-photochemical quenching processes is essential. This paper characterises the relaxation of the non-photochemical chlorophyll fluorescence quenching exhibited by spinach leaves after various pretreatments.

Materials and Methods

Modulated chlorophyll fluorescence was measured using a commercially available apparatus (PAM chloro-

Abbreviations: F_0, initial chlorophyll fluorescence level; F_i, intermediate chlorophyll fluorescence level; F_M, maximal chlorophyll fluorescence level before quenching; F_{SP}, maximal chlorophyll fluorescence level at steady state; F_{RT}, maximal chlorophyll fluorescence level during the relaxation time t; HES, high-energy state; LHC II, light harvesting chlorophyll *a/b* complex of Photosystem II; PS II, Photosystem II; FQR, ferredoxin-quinone oxidoreductase.

Correspondence: M. Hodges, (present address:) Laboratoire de Physiologie Végétale Moléculaire, Bât. 430, Université Paris Sud, 91405 Orsay Cedex, France.

phyll fluorescence measuring system, see Ref. 11). The leaf disc (10 cm^2) was placed in a Hansatech leaf oxygen electrode chamber and excited with non-actinic red light (3 $\mu E \cdot m^{-2} \cdot s^{-1}$; 660 nm), the fluorescence was detected between 710 and 760 nm. Actinic light was either white (150 $\mu E \cdot m^{-2} \cdot s^{-1}$) or modified with filters to give blue (Corning 4-96; 6 $\mu E \cdot m^{-2} \cdot s^{-1}$) or far-red (Balzer 706 nm interference filter; 1 $\mu E \cdot m^{-2} \cdot s^{-1}$). Photochemical and non-photochemical fluorescence quenching was followed by the light-doubling technique using a Schott KL1500 lamp which gave 800 ms pulses of white light at an intensity of 750 $\mu E \cdot m^{-2} \cdot s^{-1}$. Fluorescence relaxation was carried out either in the dark or in the presence of the far-red light source. All experiments were carried out at 25 °C in 3% CO_2 and 21% O_2.

The relaxation of the non-photochemical quenching was calculated by

$$\frac{F_{RT} - F_{SP}}{F_M - F_{SP}} = F_t$$

where

F_M = maximum fluorescence before quenching;
F_{SP} = maximum fluorescence at the steady state;
F_{RT} = maximum fluorescence during the relaxation at time t (see Fig. 1) and plotted as $\log(1 - F_t)$.

To study the effect of NaF on the relaxation kinetics, 10 cm^2 leaf discs were submerged in 100 mM NaF during 1 h. Photoinhibition was induced by illuminating attached whole leaves with white light (intensity, 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$) in 1% O_2 and in the absence of CO_2.

Results

Figure 1 shows an example of the fluorescence signal measured during the experiments described below and which contains certain information concerning the non-photochemical relaxation phases. Switching on the modulated red light, the initial fluorescence level (F_0) is obtained and the superposition of the saturating 800 ms light pulse increases the fluorescence emission to the maximal level (F_M). When the fluorescence has relaxed back to near F_0 the actinic light is turned on and during the slow changes in fluorescence, saturating flashes are superimposed to give the new F_m level (this gives rise to the spikes in Fig. 1). After the steady-state fluorescence level is reached the actinic light is turned off and the relaxation of the fluorescence quenching of F_M is monitored by giving saturating pulses. Finally, the modulated light is switched off so that the leaf is in the dark and the relaxation of the quenching is followed by turning on the modulated light and by giving a saturating pulse. It can be seen from Fig. 1 that both a fast and a slow relaxation component led to a quenching of the F_0 level. Furthermore, the slow phase could not fully

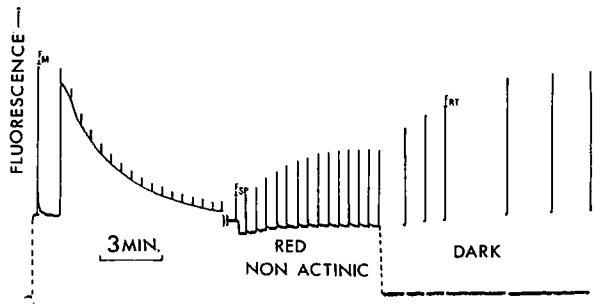

Fig. 1. An example of the chlorophyll fluorescence signal measured to characterise the non-photochemical quenching components seen in vivo in spinach leaves. The F_0 is attained after dark adaptation by turning on the modulated measuring light. The initial F_M is measured by the superposition of the saturating 800 ms light pulses. The F_m during the slow fluorescence changes induced by the addition of a non-modulated actinic light is monitored by the addition of the saturating pulse which gives rise to the spikes. The relaxation of the F_M quenching is monitored either in the presence of the measuring light (RED NON ACTINIC) or in the absence of all light sources (DARK).

relax in the presence of the weak non-actinic measuring light of the PAM fluorometer. It was also seen that the extent of the relaxation of the slow phase also depended upon the frequency of the 800 ms saturating light pulses. Even when pulses were given every 30 s a complete reversal of the slow component was not possible (data not shown). Therefore, after the almost complete relaxation of the fast phase (approx. 1 min), pulses were given every minute (or with larger intervals).

Fig. 2 shows the relaxation of the fluorescence quenching produced by either low-intensity blue light or high-intensity white light. The blue light treatment led to a total quenching of 30% and gave a single relaxation component with a half-time of 10.5 min. The white light

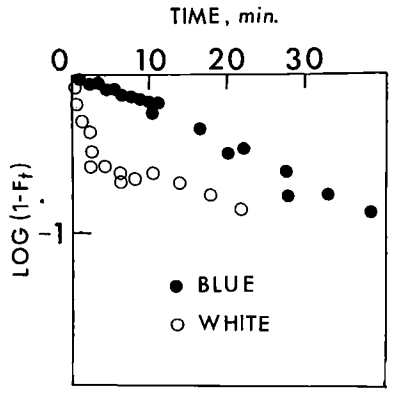

Fig. 2. The relaxation of the fluorescence quenching produced by low-intensity blue light (●) and high-intensity white light (○). The quenching characteristics were:

	Total quenching	$t_{1/2}$ (amplitude, %)
Blue	30%	10.5 min (100%)
White	60%	11.8 min (30%) and 46 s (72%)

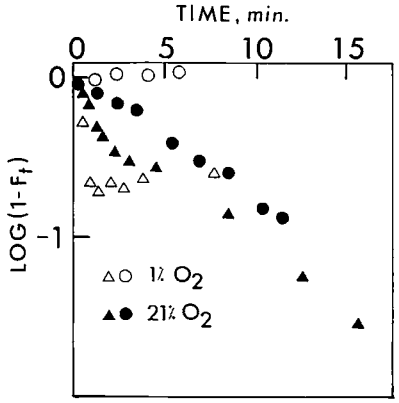

Fig. 3. The relaxation kinetics of fluorescence quenching achieved as in Fig. 1 followed at 3% CO_2 in either 1% O_2 (△, ○) or 21% O_2 (▲, ●). The quenching characteristics were:

	20% oxygen	1% oxygen
Blue (○)	4 min	−40 min
White (△)	4 min and 30 s	−40 min and 23 s

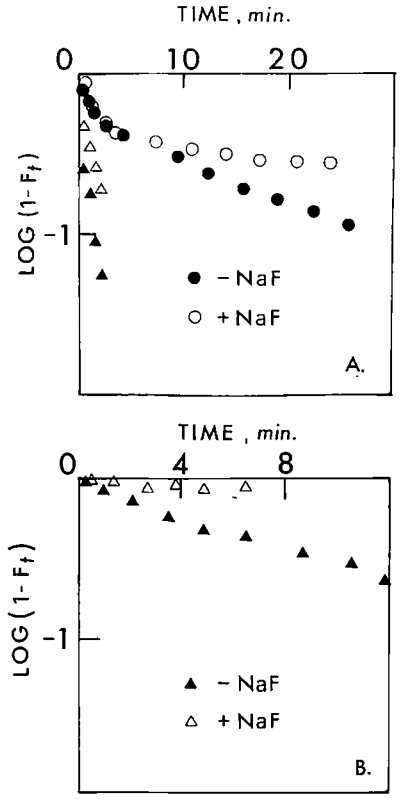

Fig. 4. The relaxation of fluorescence quenching carried out as in Fig. 1 with white (A) or blue (B) light in the presence (open symbols) or absence (closed symbols) of NaF. The triangles in (A) give the fast phase after the subtraction of the slower component. The quenching characteristics were:

	−NaF	+NaF
Blue	5.5 min	34 min
White	11 min and 44 s	37 min and 57 s

treatment produced a larger total quenching of 60% and two relaxation components with half-times (and relative amplitudes) of 46 s (72%) and 11.8 min (30%).

Fig. 3 shows the relaxation of the non-photochemical fluorescence quenching induced by either the blue or white light treatments (in 21% O_2) in the presence of either 1% O_2 or 21% O_2. In this experiment, at 21% O_2, the blue light gave rise to a component with a half-time of 4 min while the white light preillumination produced a slow (4 min) and a fast (30 s) component. However, in the presence of only 1% O_2 the slow component did not relax (for both white and blue light treatments) and only a fast relaxation component (23 s) was seen with the white-light-treated leaves.

The relaxation kinetics and the sensitivity to O_2 levels of the slow component would be expected for a relaxation from State II to State I in which the plastoquinone pool must become oxidised to inactivate the LHC II kinase [12]. Fig. 4 shows the effect of treating the leaves with a solution of NaF (a phosphatase inhibitor which inhibits the dephosphorylation of the LHC II and therefore modifies a State II–State I transition). Fig. 4A shows the relaxation observed after a quenching induced by white light. It can be seen that in the presence of NaF that the slow relaxation phase was slowed down. In the absence of NaF the two components had half-times of 11 min and 44 s while in the presence of NaF the fast phase was unaffected (57 s) but the slow phase had a half-time of 37 min. Fig. 4B shows the same type of experiment as in Fig. 4A but for leaves preilluminated with blue light. Again it is seen that the presence of NaF slows down the relaxation of the slow phase (from 5.5 min to 34 min). It was observed that a similar incubation in the presence of 100 mM NaCl did not modify the relaxation characteristics (not shown).

Photoinhibition was carried out as described in Materials and Methods. This severe treatment led to a 20–40% inhibition of light-saturated CO_2 fixation. Fig.

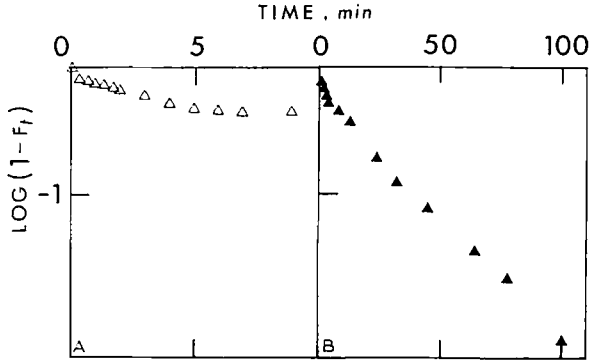

Fig. 5. The relaxation of fluorescence quenching after a photoinhibitory pretreatment. The data are given on a fast timescale (0–10 min) (A) and on an extended timescale (0–100 min) (B). The $t_{1/2}$ (and amplitudes, %) of the three phases were: 35 s (6%), 8 min (26%) and 40 min (68%). The total fluorescence quenching was 54%.

5A gives the relaxation of the observed non-photochemical fluorescence quenching on a 'fast' time-scale (0–10 min) while Fig. 5B shows the slower relaxation phases on an extended time-scale (0–100 min). An analysis of these curves shows the presence of a third component after the photoinhibitory treatment. The total fluorescence quenching was 54% and the half-times (and relative amplitudes) of the three phases were 35 s (6%), 8 min (26%) and 40 min (68%). It was observed that the slowest phase required the presence of O_2 for relaxation, it was inhibited in the dark and stimulated by the presence of weak far-red light (data not shown).

Discussion

The non-photochemical chlorophyll fluorescence quenching has been resolved into three components in spinach leaves which are summarised in Table I. The conclusions are in close agreement with the recent report by Horton and Hague [13] concerning the fluorescence quenching associated with barley protoplasts.

The rapid phase (half-time of 40 s) can be associated with the so-called 'high-energy state' quenching. Such a mechanism would be expected to be insensitive to NaF and O_2 levels. However, it is still not known how a pH-gradient modifies the PS II fluorescence emission. Indeed, recently it has been shown that the addition of antimycin can inhibit the fluorescence quenching without altering the pH-gradient (as measured by 9-aminoacridine) [14]. It has been proposed that this type of quenching is regulated by a redox component of the electron-transfer chain, perhaps via the ferredoxin-quinone oxidoreductase (FQR) [15].

The '7.6 min' phase (see Table I) has the characteristics associated with the State I–State II adaptation. Firstly, the half-time for the relaxation of the fluorescence quenching is in close agreement with data already available in the literature (see, for example, Ref. 16). This mechanism would be expected to lead to an F_0 decrease (Fig. 1) as it alters the antenna size of the PS II. The inhibition of the relaxation by the addition of NaF infers that the origin of the relaxation is the dephosphorylation of the LHC II and hence a State-II-to-State-I transition. Furthermore, the sensitivity of the relaxation to low O_2 levels, where the reoxidation of the plastoquinone would be slowed down, would be expected for such a mechanism which requires the oxidation of the plastoquinone to inactivate the LHC II kinase. Finally, the incomplete reversal of the relaxation in the presence of the red measuring light is also in close agreement with the assignment of this relaxation phase to the reversal of the State transition.

The third component was seen only after a photoinhibitory treatment and is therefore assigned to the well-known but poorly understood photoinhibition-induced fluorescence decrease. The requirement of a low-intensity illumination for the reversion from the quenched to the non-quenched state is in agreement with previous reports concerning the recovery from photoinhibition [17,18]. The origin of the photoinhibition-induced fluorescence decrease is still not known. Recently, we have shown that the presence of chloramphenicol does not inhibit the relaxation of the F_M level although the F_0 is still affected [19]. This infers that protein synthesis encoded by the chloroplast genome is not necessary. The sensitivity to O_2 suggests that this type of quenching could also be controlled by a redox component and this might also explain the low-intensity far-red light requirement. It is possible that it is associated with the xanthophyll cycle as suggested in Ref. 20.

It is interesting to note that the photoinhibitory treatment led to a decrease in the amplitude of the fast relaxation component (see also Ref. 20) while not altering the 'middle' ($t_{1/2} = 7.6$ min) phase. This could be due to the fact that photoinhibition perturbs the mechanism which brings about the HES state quenching or that the photoinhibition-induced quencher is more effective and outcompetes the HES-induced quencher. Contrary to certain reports which show that photoinhibition leads to a decrease in LHC II phosphorylation [21,22] photoinhibition does not, under our conditions, modify the ability to carry out a state transition. This is perhaps not surprising because it has been shown that only a certain pool of phosphorylated LHC II gives rise to the associated fluorescence changes [23]. Unlike for the HES, the photoinhibition does not alter the state transition-induced fluorescence quenching. This might be expected if the State transition is carried out before the photoinhibition takes place. The phosphorylated LHC II which becomes dissociated from the PS II during the State transition would not undergo the structural modifications induced by the high light treatment and therefore when dephosphorylated and reassociated with the PS II would not be in a quenched state. This is indeed seen from a comparison of the data in Fig. 2 (white light) and Fig. 5 (photoinhibition) where the 'middle' phase produced a quenching of 18% and 14%, respectively.

TABLE I

The characteristics of the three non-photochemical chlorophyll fluorescence quenching components observed in vivo in spinach leaves

n.d., not determined.

phase	$t_{1/2}$	Inhibition			Origin
		1% O_2	NaF	red light	
1	40 ±15 s	no	no	no	high-energy state
2	7.6± 2.2 min	yes	yes	yes	State I–State II
3	39.5± 3.5 min	yes	n.d.	n.d.	Photoinhibition

Acknowledgements

M.H. wishes to thank the Royal Society (London, U.K.) for financial support through a Pickering Research Fellowship and the EPA Cephalosporin Fund. This work was also partly funded by the C.N.R.S. (France).

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