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LIGHT-DEPENDENT QUENCHING OF CHLOROPHYLL FLUORESCENCE IN PEA CHLOROPLASTS INDUCED BY ADENOSINE 5′-TRIPHOSPHATE

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Summary

Addition of ATP to chloroplasts causes a reversible 25–30% decrease in chlorophyll fluorescence. This quenching is light-dependent, uncoupler insensitive but inhibited by DCMU and electron acceptors and has a half-time of 3 minutes. Electron donors to Photosystem I can not overcome the inhibitory effect of DCMU, suggesting that light activation depends on the reduced state of plastoquinone. Fluorescence emission spectra recorded at −196°C indicate that ATP treatment increases the amount of excitation energy transferred to Photosystem I. Examination of fluorescence induction curves indicate that ATP treatment decreases both the initial (F_0) and variable (F_v) fluorescence such that the ratio of F_v to the maximum (F_m) yield is unchanged. The initial sigmoidal phase of induction is slowed down by ATP treatment and is quenched 3-fold more than the exponential slow phase, the rate of which is unchanged. A plot of F_v against area above the induction curve was identical plus or minus ATP. Thus ATP treatment can alter quantal distribution between Photosystems II and I without altering Photosystem II-Photosystem II interaction. The effect of ATP strongly resembles in its properties the phosphorylation of the light-harvesting complex by a light activated, ATP-dependent protein kinase found in chloroplast membranes and could be the basis of physiological mechanisms which contribute to slow fluorescence quenching in vivo and regulate excitation energy distribution between Photosystem I and II. It is suggested that the sensor for this regulation is the redox state of plastoquinone.

Abbreviations: PS II, Photosystem II; PS I, Photosystem I; F_m, maximum fluorescence level when all Photosystem II traps are closed; F_0, minimum fluorescence level when all Photosystem II traps are open; F_v, $F_m - F_0$; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; Tricine, N-tris(hydroxymethyl)methylglycine; LHC, light-harvesting complex; Q, primary electron acceptor of Photosystem II.

Introduction

The membranes of plant chloroplasts are able to regulate the amount and distribution of absorbed radiation arriving at PS II and PS I [1—3]. This forms the basis of the State I/State II transitions first seen in algae [4] and has been studied by observing the slow quenching of chlorophyll fluorescence seen in the period (up to 5 min) following the onset of strong illumination (see, for example, Refs. 5—9). The kinetics of this process are complex and a number of different quenching processes are probably involved (for review see Ref. 10). In the past, great emphasis has been placed upon the study of uncoupler-sensitive, 'high-energy state' quenching [6—8,11,12], a process postulated to involve changes in concentrations of divalent cations at the thylakoid membrane surface [13,14]. This line of investigation has recently been confused by the observation that uncoupler sensitive quenching in chloroplasts does not alter the distribution of excitation between PS II and PS I [15]. In contrast, slow quenching in algae [4,16—18], whole leaves [18] and protoplasts (Horton, P. unpublished data) does result in enhanced excitation of PS I. It is perhaps worthwhile therefore to consider other biochemical processes which may contribute to the physiological process of slow fluorescence quenching. Recently, Bennett has observed phosphorylation of thylakoid membrane proteins [19] including the major chlorophyll protein, the light-harvesting complex [20]. This phosphorylation uses ATP as substrate and is catalysed by a light-activated protein kinase, light activation requiring photosynthetic electron flow but being uncoupler insensitive [21]. Bennett has speculated that phosphorylation of light-harvesting complex may influence its energy transferring properties [20,22,23]. In this paper, an effect of ATP on chlorophyll fluorescence is described which has characteristics identical to those required for protein phosphorylation, and which gives information as to how quantal distribution may be controlled. Some of the fluorescence data in this paper are identical to those recently reported by Bennett et al. [24].

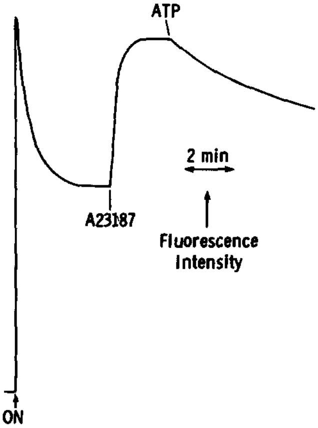
Materials and Methods

Chloroplasts were isolated from 12—16-day-old peas essentially by a method described previously [25] but incorporating the following modifications. The grinding medium contained 0.33 M glucose, 50 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 25 mM NaCl, 0.2% sodium iso-ascorbate and 0.1% bovine serum albumin, adjusted to pH 6.5 with NaOH. Initial purification was performed by squeezing the leaf homogenate through two layers of muslin followed by filtration through eight layers and cotton wool. After centrifugation, pellets were rinsed with 0.33 M glucose, 5 mM $MgCl_2$, 26 mM sorbitol, 4 mM Hepes, pH 7.6 and gently resuspended in 0.33 M sorbitol, 4 mM EDTA, 5 mM $MgCl_2$, 2 mM $MnCl_2$ and 100 mM Hepes, pH 7.6. The reaction medium for all experiments contained 0.1 M sucrose, 10 mM NaCl, 3 mM $MgCl_2$ and 10 mM Tricine, adjusted to pH 7.8 with NaOH. Chloroplasts were diluted to 15 μg chlorophyll/ml. Measurements of chlorophyll fluorescence were made at 90° to the excitation beam in a 1 cm × 1 cm thermostatically controlled and stirred cuvette, emission being defined by a Balzers 694 nm interference filter and measured by an EMI photomultiplier tube. Excitation was by means of a tungsten halogen lamp powered at 100 W and filtered through Corning 4-96 and 1-75 filters. The voltage developed across a load resistor was measured either by a strip chart recorder (for continuous light measurements) or, for induction curves, by a Datalab DL 406 Analyser equipped with a DL401B Sweep Timer set at 800 μs per address and a DL 403 Display. Induction was initiated by the opening of a Uniblitz electronic shutter with an opening time of 0.6 ms and the stirring was discontinued during measurements. Analysis of fluorescence induction curves was performed using programmes and routines provided on-line by the DL417 Function module. The analysis followed the approach of Melis and Homann [26] and consisted of: (1) normalization of F_v vs. t, (2) calculation and normalization of the area growth (A) above the induction curve vs. t, (3) computation of both the first-order rate constant (k_β) for the slow exponential induction phase and the proportion of area due to this phase (β_{max}), (4) plotting F_v vs. A and (5) substraction of an exponential curve, k_β from F_v, so enabling a similar analysis on just the fast sigmoidal part of the induction curve.

Fluorescence emission spectra at −196°C were recorded front face on samples of chloroplasts 1 mm thick. Excitation was defined by a 4-96 Corning filter and was chopped at a frequency of 520 Hz using a Rofin microchopper. Emission was detected through an Oriel monochromator with a resolution half-bandwidth of 2 nm and the photomultiplier output was measured using an EG and G Brookdeal 9503 Lockin Amplifier.

Results

Fig. 1 shows fluorescence emission at 21°C in freshly broken chloroplasts. Quenching of 50% of F_v occurred within 2 min. As expected, addition of an uncoupler reverses this quenching; in this experiment, the divalent cation



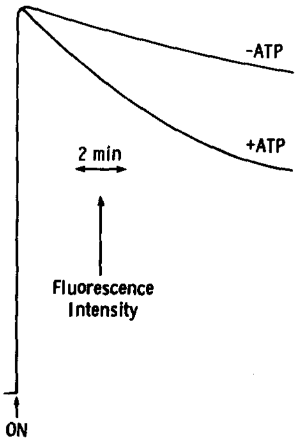


Fig. 1. Slow fluorescence quenching in chloroplasts. Fluorescence emission at 21°C was measured in a stirred cuvette as described in the text. Additions are Ionophore A23187 (1 μg/ml), and ATP (0.15 mM). Chlorophyll 10 μg/ml.

Fig. 2. Fluorescence quenching in uncoupled chloroplasts. Ionophore A 23187 (1 μg/ml) was added prior to illumination, as was ATP (0.15 mM) where indicated. Measurement and conditions as in Fig. 1.

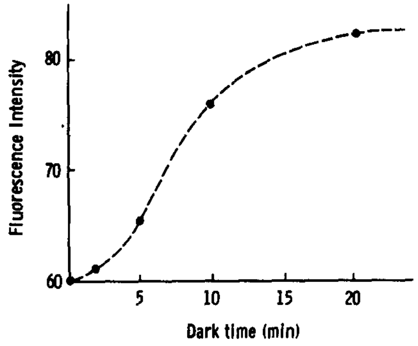
ionophore A23187 was used [12]. An identical effect was seen with nigericin, provided K^+ rather than Mg^{2+} was the predominant surface cation [27], and with gramicidin. After A23187 addition no further elevation of fluorescence occurred upon nigericin or gramicidin addition. If 0.15 mM ATP is added to these uncoupled chloroplasts, fluorescence quenching is observed (Fig. 1). In the experiment illustrated by Fig. 2 ionophore A23187 was added initially. Clearly, a significant fluorescence quenching is induced by ATP addition. The fluorescence decrease seen in the absence of ATP was irreversible, was enhanced at very light intensity and was probably a photodestructive effect. A plot of the difference between +ATP and —ATP demonstrated that quenching has a $t_{\frac{1}{2}}$ of approx. 3 min and a maximum extent of 25—30% of F_m. Addition of DCMU (5 μM) caused inhibition of the ATP-induced quenching, as did the addition of the electron acceptors methyl viologen and potassium ferricyanide (Table Ia). PS I-mediated electron transfer using ascorbate/diaminodurene in the presence of DCMU did not restore ATP-induced quenching. The fluorescence quenching was found to be reversible (Fig. 3); after 20 min in darkness over 90% of the quenched fluorescence was recovered. This represents almost completely recovery of ATP-induced quenching since, in this experiment, the minus ATP sample showed 7 units of irreversible quenching. Addition of 10

TABLE I

ATP-INDUCED FLUORESCENCE QUENCHING UNDER DIFFERENT INCUBATION TREATMENTS

Fluorescence quenching after 10 min incubation was measured as described in Fig. 2, and values represent the difference between fluorescence levels of plus ATP and minus ATP samples, expressed as a % of the 10 min fluorescence level minus ATP (i.e. $F_{(+ATP)} - F_{(-ATP)}/F_{(-ATP)}$. In (c) the designated light period was followed by dark incubation so that the total time from first turning on the light was always 10 min. In (d) all samples received 20 s light plus 10 min dark after ATP addition; the second time value given indicates the length of the interval between the end of the illumination and ATP addition. Both (c) and (d) contained 10 mM NaF. Concentrations: 5 μM DCMU; 0.1 mM methyl viologen; 0.5 mM potassium ferricyanide; 0.5 mM ascorbate; 0.4 mM diaminodurene (DAD); chloroplasts 10 μg chlorophyll per ml.

Treatment		ATP-induced ΔF (%)
a	10 min dark	0
	10 min light	27
	10 min light + DCMU	3
	10 min light + methyl viologen	0
	10 min light + ferricyanide	0
	10 min light + DCMU + ascorbate/DAD	7
b	10 min light	22
	10 min light + NaF	28
	10 min dark + NaF	0
c	0.2 s light	10
	2 s light	17
	20 s light	21
	5 min light	28
d	20 s, 0	20
	20 s, 1 min	19
	20 s, 2 min	15
	20 s, 5 min	7



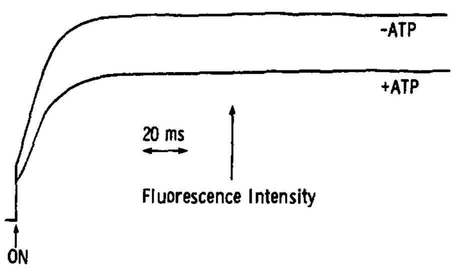


Fig. 3. Recovery of fluorescence level in darkness. Chloroplasts were exposed to 10 min light as in Fig. 2 and then incubated in darkness for various times before recording the fluorescence intensity. Units are arbitrary, with an inital F_m of 92. A minus ATP control decayed seven units with no dark recovery.

Fig. 4. Fluorescence inductions in the presence of DCMU. Chloroplasts were given 5 min illumination ±ATP as in Fig. 2, but at a chlorophyll concentration of 15 μg/ml and containing 10 mM NaF. After 7 min in darkness 5 μM DCMU was added. Induction curves were recorded 1 min after as described in the text.

mM sodium fluoride completely inhibited the dark recovery as well as enhancing the maximum ATP effect by 5—10% (Table Ib).

The ability of NaF to inhibit reversal of ATP induced quenching allowed measurement of the length of illumination necessary for this effect. It was found that 200 ms light could elicit 40% of the maximum ATP effect, observed after an ensuing 10 min dark period; 75% effectiveness resulted from 20 s of illumination (Table Ic). In a similar experiment it was found that ATP need not not be present during illumination; addition of ATP immediately following a 20 s illumination gave 20% quenching, compared to 21% with ATP added prior to illumination. The light-activated state decays with a half-time of 2—3 min as evidenced from adding ATP at varying times after the end of 20 s illumination (Table Id). Control experiments in which up to 20 mM $MgCl_2$ were added showed that ATP-induced quenching was not due to metal ion chelation by ATP. Also, EDTA added under the conditions of the above experiments causes no fluorescence quenching.

TABLE II

RELATIVE FLUORESCENCE INTENSITIES OF CHLOROPLASTS AT —196°C

Fluorescence emission at —196°C was recorded for samples treated as in Fig. 1 for (a) and Fig. 2 for (b). Samples were taken out by syringes and injected into a pre-cooled 1 mm thick cuvette. In (b) the mean values are expressed ±95% confidence limits using nine different samples for each.

Conditions		F 685/F 735
a	light	0.90 (0.89—0.91, n = 4)
	light + ionophore A 23187	0.98 (0.97—1.00, n = 4)
	dark	0.94 (0.93—0.95, n = 3)
b	light - ATP	0.95 ± 0.02
	light + ATP	0.65 ± 0.03

TABLE III

EFFECT OF ATP TREATMENT ON KINETIC PARAMETERS DERIVED FROM FLUORESCENCE INDUCTION CURVES

F_m and F_v (in arbitrary units) were determined from curves of the type shown in Fig. 6. Semi-logarithmic plots of the area growth above such curves enabled the proportion of area due to the slow β phase (β_{max}) and its rate constant k_β to be calculated. Subtraction of a curve with rate constant k_β allowed the absolute values for the amplitudes of $F_v(\alpha)$ and $F_v(\beta)$ to be obtained and examination of $F_v(\alpha)$ gave values for k_α, the initial rate constant determined from semi-logarithmic area plots. All data are the mean of eight replicates with 95% confidence limits given.

Parameter	—ATP	+ATP	% change
F_v/F_m	0.703 ± 0.001	0.701 ± 0.002	0
β_{max}	0.327 ± 0.003	0.350 ± 0.002	+7
$k_\beta(s^{-1})$	23.4 ± 0.1	23.5 ± 0.1	0
$k_\alpha(s^{-1})$	116 ± 3	98 ± 5	—16
F_m	105 ± 9	79 ± 6	—25
F_v	75 ± 6	55 ± 3	—26
$F_v(\alpha)$	69 ± 6	49 ± 3	—29
$F_v(\beta)$	6.1 ± 0.6	5.5 ± 0.6	—10

Fluorescence emission spectra at —196°C of ATP-quenched chloroplasts showed an alteration in emission at 695 nm relative to 735 nm, in comparison to controls (Table II). On the other hand, uncoupler sensitive quenching induced by the presence of the proton motive force as in Fig. 1 was accompanied by only a negligible change in the —196°C spectrum, confirming the recent finding of Briantais et al. [15]. This suggests that ATP-induced quenching is due to an increased fraction of absorbed energy being transferred to PS I. In this respect, the ATP effect resembles divalent cation depletion of membranes [1,6,8]. It was of interest, therefore, to investigate the effect of ATP on

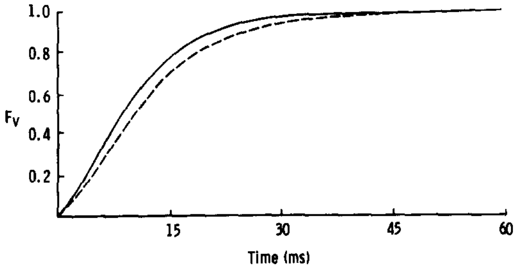


Fig. 5. The fast phase of fluorescence induction. $F_v(\alpha)$ from plus ATP (- - - - -) and minus ATP (———) induction curves are shown on an expanded time-scale and normalised. Data derived as described in Table III.

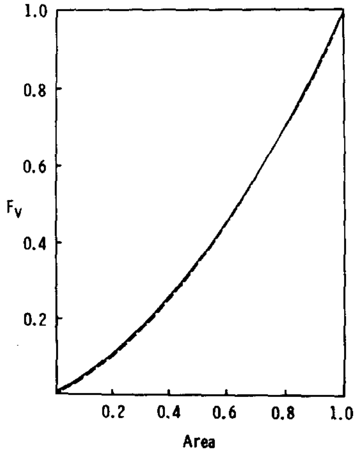


Fig. 6. Plots of variable fluorescence against area growth. Data obtained by plotting the normalized area growth against the normalized $F_v(\alpha)$ obtained from Fig. 5. - - - - - -, with ATP; ———, without ATP.

the kinetics of fluorescence induction, since depletion of divalent cations has marked effects on both the sigmoidicity [26,28—30] and the proportion of the slow phase of induction [26].

For this experiment chloroplasts were incubated for 5 min in the light with or without ATP to achieve near maximum ATP effect (Table Ic). In the presence of NaF, reversal is inhibited such that choroplasts could then be post-incubated for 7 min to allow complete re-oxidation of Q. Again, because of the stabilization of the ATP-induced state, DCMU could be safely added 1 min prior to recording the induction curve. Fig. 4 shows induction curves obtained in such an experiment. Quenching of both the F_m and F_0 fluorescence levels was observed after ATP treatment. The ratio F_v/F_m was, in fact, unchanged (Table III).

The fluorescence induction curve reflects the photoreduction of two types of PS II centres, α and β [26]. Analysis of the curves by a procedure similar to that described by Melis and Homann [26] revealed that ATP was preferentially affecting the faster α phase of Q reduction; thus $F_v(\alpha)$ was lowered almost 3-fold more than $F_v(\beta)$, giving rise to a small increase in the proportional area due to the slow phase, β_{max} (Table III). In addition, the rate constant (k_β) for reduction of Q_β is unchanged, whilst the initial rate constant (k_α) for the reduction of Q_α is decreased by 16% in ATP-treated chloroplasts.

The plot of $F_v(\alpha)$ vs. time in presence or absence of ATP obtained during this analysis is hown in Fig. 5 and clearly shows this decrease in rate of fluorescence induction. This curve, when transformed into a plot of F_v vs. area (Q^- concentration), can give information on the extent of PS II-PS II interaction, as recently demonstrated by Butler [31]. Fig. 6 reveals that PS II-PS II interaction, which determines the shape of this plot, is unchanged in ATP-treated chloroplasts. Finally, it should be added that the ATP effects on the induction curves require, as expected, pre-illumination, so that samples incubated in darkness for 12 min plus or minus ATP gave identical induction curves.

Discussion

Reversible non-photochemical quenching of chlorophyll fluorescence has previously been observed upon depletion of divalent cations from thylakoids (see, for example, Refs. 1,6,8,32) and in the presence of a transmembrane proton gradient [7,11—13,15]. The quenching induced by ATP addition, which occurs in uncoupled chloroplasts in the presence of divalent cations, is therefore unrelated to these two phenomena. It does resemble cation depletion in that ATP-treated samples have a lower PS II/PS I ratio of fluorescence emission at —196°C compared to untreated samples, just as cation depletion induces a similar change. In fact, the extent of ratio change (from nearly 1.0 to 0.65) is very similar in both treatments (Ref. 28 and unpublished data) despite the fact that cation depletion generally lowers the F_m by 50—60% and ATP causes only 25—30% quenching. It would seem that cation depletion might cause quenching by processes other than merely enhanced energy transfer to PS I.

However, important differences excist between cation depletion and ATP treatment. ATP causes no change in F_v/F_m and is hence quenching both F_0 and F_v to the same extent. Cation depletion lowers F_v/F_m, quenching F_v much more than F_0. Cation depletion also results in conversion of the sigmoidal induction curve to an exponential one and hence gives rise to a linear F_v vs. area plot. This is predicted by a photosystem model in which cations enhance coupling between light-harvesting complex and PS II and in which PS II interaction is mediated by energy transfer through light-harvesting complex [31,33, 34]. However, ATP-induced quenching is accompanied by a change in excitation distribution between PS I without a concomitant decrease in PS II interaction. It is suggested, therefore, that the PS II interaction which gives rise to sigmoidicity does not necessarily depend on energy transfer through light-harvesting complex. This would allow for a change in the association between light-harvesting complex and PS II or PS I to occur, so altering the emission spectrum at —196° C, without affecting PS II-PS II interaction. PS II interaction could involve energy transfer between PS II units [30-35] or two photon events in each reaction centre [36,37]. The fact that F_v/F_m is not modified is another argument in support of the hypothesis that ATP causes disconnection of light-harvesting complex from PS II with persistence of PS II interaction. In any quenching process, such as that described in current photosystem models [31, 33,34], F_m is necessarily more affected than F_0. (In this sense, the term 'ATP-induced quenching' is misleading and 'fluorescence lowering' would be more appropriate).

The features of the ATP effect seen in this paper are identical to those described in recent work by Bennett on the phosphorylation of light-harvesting complex [19—24]. These features are: (1) they are both light activated in a DCMU sensitive but uncoupler insensitive process, (2) the half-time for quenching and phosphorylation is 2—3 min, (3) the half-time for dephosphorylation and recovery of fluorescence is 5—10 min, (4) both require 0.1—0.5 mM ATP for maximum effect, (5) dephosphorylation and fluorescence recovery are inhibited by NaF at 5—10 mM.

Barber [38] had recently hypothesised that modulation of the surface charge on membrane proteins such as light-harvesting complex may be important in controlling excitation distribution. Covalent binding of a phosphate group to the exposed segment of light-harvesting complex, which is the site of phosphophosphorylation [23] and an important factor in controlling membrane stacking [39] would undoubtedly increase its surface negative charge and this could be basis of the proposed detachment of PS II from light-harvested complex. It is also of interest to note that the β centres, which are proposed to have less light-harvesting complex associated with them are much less affected than the α centres. A similarity differential effect of cation depletion on α and β centres was seen by Melis and Homann [26].

Bennett has suggested that light-induced reduction of ferredoxin is the basis of the requirement for electron transport for activation of the protein kinase that catalyses light-harvesting complex phosphorylation [21]. However, the fact that electron donation to PS I by ascorbate/diaminodurene can not cause light activation suggests that a segment of the electron transport pathway closer to PS II is involved. The activation of protein kinase by NADPH + ferredoxin [21] is consistent with Q or plastoquinone being involved, since it has been shown that addition of NADPH causes plastoquinone [40] and Q [41] reduction in ferrdoxin-dependent reactions. Inhibition of activation by DCMU suggests therefore that it is the reduction of plastoquinone that is necessary.

A model for regulation of excitation distribution during photosynthesis based on ATP-induced phosphorylation can be readily devised. Thus over excitation of PS II would cause plastoquinone reduction and activation of protein kinase. Subsequent phosphorylation would tend to redress the imbalance by increasing the rate of PS I excitation and plastoquinone oxidation. The effect may also be involved in promoting cyclic electron flow under conditions when depletion of endogenous NADP would cause inhibition of non-cyclic flow. Enhancement of cyclic electron transport may result from the redox poising effect of regulating the redox state of the plastoquinone pool as well as from a direct stimulation of its rate because of an increased fraction of energy being absorbed by PS I. The extra ATP synthesised would possibly have the effect of overcoming metabolic imbalances that prevent renewal of NADP from NADPH. For example, inhibition of 3-phosphoglycerate kinase by a high ADP/ATP ratio prevents re-oxidation of NADPH by glyceraldehyde phosphate dehydrogenase [42]. This kind of event may be particularly important during the induction period of photosynthesis [43] during which modification of exciton distribution can be observed in isolated protoplasts (Horton, P., unpublished data). The fact that light-harvesting complex phosphorylation occurs in leaves [21] strongly suggests that this process contributes in part to the slow quenching observed in vivo and accounts completely for the changes in exciton distribution. In this model it is the redox state of plastoquinone (which will respond to factors such as light intensity, acceptor concentration, adenylate levels and the relative rates of PS II:PS I excitation) that forms the basis for the control of photosynthesis at the level of light harvesting. Experiments are currently in progress to test this hypothesis.

Recent work in our laboratory [44] has shown that ATP-induced quenching can be elicited in darkness if plastoquinone is reduced potentiometrically, so considerably strengthening the above argument for the key role of plastoquinone. In addition, in collaboration with Dr. J. Allen, we have demonstrated directly that phosphorylation of light-harvesting complex accompanies our light-induced fluorescence decrease.

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