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A study of the regulation and function of energy-dependent quenching in pea chloroplasts

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In this study, the regulation and possible functions of energy-dependent quenching (quenching coefficient, *qE*), have been investigated through a series of experiments on isolated pea chloroplasts. The results presented here suggest that this type of quenching provides some protection against photoinhibition, but is of little or no importance in the regulation of the intrinsic yield of Photosystem II (Φ_p), which is, instead, controlled directly by the proton gradient. The relationship between *qE* and the transthylakoid proton gradient, as measured by 9-aminoacridine fluorescence quenching (*q9-AA*), showed an apparent redox-state dependency, this being reflected in higher *qE:q9-AA* ratios under oxidised conditions. A simple model is presented to explain these observations.

Introduction

A quenching of chlorophyll fluorescence associated with the energy state of the chloroplast was first reported by Murata and Sugahara [1]. This 'energy-dependent' quenching (defined quantitatively by the quenching coefficient, *qE*) has since been shown to be linearly related to the extent of the transthylakoid proton gradient [2]. Mechanistically, the formation of *qE* is thought to result from conformational changes within the light-harvesting pigment beds of Photosystem II [3]. These changes are assumed to be induced through protonation of the lumen surface, which occurs when Mg^{2+} is exchanged for protons across the thylakoid membrane during ΔpH formation [4].

The chlorophyll fluorescence signal has frequently been used as a 'probe' of the energy status of the chloroplast. However, an inhibition of *qE* formation by antimycin A, which can occur in the absence of any effect on ΔpH formation, has been reported [5]. From the nature of this inhibition, and what is known about antimycin A as an inhibitor, it was proposed that *qE* formation is co-regulated by the transthylakoid ΔpH and the redox state of an electron carrier involved in cyclic electron flow around PS II, or in a protonmotive 'Q or *b*' cycle. This proposal is supported by the identification of 'ferredoxin-quinone reductase' as the primary binding site for antimycin A in chloroplasts [6].

Abbreviations: PS II Photosystem II; *qQ*, photochemical quenching coefficient; Φ_s, quantum yield of Photosystem II; Φ_p, the Φ_s:*qQ* ratio; *qE*, 'energy-dependent' quenching coefficient; *qI*, quenching coefficient of photoinhibition; *q9-AA*, quenching coefficient of 9-aminoacridine; *qNP*, non-photochemical quenching coefficient; *qR*, coefficient for relative contribution of other non-photochemical quenching.

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A number of recent studies have produced evidence to suggest that the regulation of *qE* is closely related to the regulation of other processes within the chloroplast. For example, the quantum yield of PS II (Φ_s) has been shown to decline with increasing light intensity [7]. This reduction in quantum yield can be attributed, at least in part, to redox control of PS II (i.e., the reduction of Q_A). Weis et al. [8] have related the quantum yield of Photosystem II, to the capacity of the reducing side of Q_A for electron transport, by dividing Φ_s by the coefficient of photochemical quenching (*qQ*) to produce a metric for the 'intrinsic' quantum yield, i.e., the quantum yield of 'open' centres. In their experiments, they observed an inverse linear relationship between *qE* and the Φ_s:*qQ* ratio (Φ_p), determined over a range of irradiance levels. A decline in Φ_p with increasing light intensity has also been reported in barley protoplasts [9] and leaves [10]. Although these changes in ϕ_p also correlate with *qE*, some of these data indicate a deviation from the linear relationship, reported in Ref. 8. This may be partly due to methodological problems encountered in measuring oxygen-evolution rates at low light intensities, close to the compensation point.

Enhanced levels of 'state-transitional' quenching of fluorescence (quenching coefficient, *qT*) have been demonstrated under conditions where *qE* was inhibited both by uncoupling with nigericin, and, in the coupled state, with antimycin A [11]. This stimulation of *qT* was accompanied by increased levels of phosphorylation of the LHC-II and 9 kDa phosphoproteins within PS II. A stimulation of quenching associated with photoinhibition (quenching coefficient, *qI*), has also been observed, where *qE* formation was inhibited by uncoupling [12].

In this study, the regulation and possible function(s) of the mechanism underlying energy-dependent quenching, are investigated through a series of experiments on pea chloroplasts.

Materials and Methods

All the experiments described in this study were carried out on chloroplasts isolated from young plants of *Pisum sativum* L. cv. Kelvedon Wonder. Seeds were dry sown in trays of vermiculite at a density of approx. 250 $g \cdot m^{-2}$. Plants were germinated under Growlux tubes (150 $\mu mol \cdot quanta \cdot m^{-2} \cdot s^{-1}$). The temperature during the 16 h photo-period was 18°C and the night temperature 16°C. Two days after first emergence, the young seedlings were placed under Wotan HQI-E lamps (metal halide and dysprosium additives), which provided 320 $\mu mol \cdot quanta \cdot m^{-2} \cdot s^{-1}$. The light and dark temperatures under these lamps were 22 and 16°C, respectively. The plants were irrigated continuously through the base of the trays which were stood on capillary matting.

12–14 day old plants were dark-adapted for 24 h, prior to harvesting. Intact pea chloroplasts were prepared according to the method of Cerovic and Plesnicar [13] and resuspended in a medium containing 330 mM sorbitol, 10 mM KCl, 1 mM EDTA, 50 mM Hepes buffer, 2.5 mM $MgCl_2$ and 20 $mg \cdot ml^{-1}$ titrated to bovine serum albumin, pH 7.9 with fresh KOH. Chloroplasts were osmotically 'swollen' by suspending them in assay medium which contained 100 mM sorbitol (30% isotonic), 10 mM KCl, 1 mM EDTA, and 50 mM Hepes. All experiments were carried out at 23°C and pH 7.9. The osmotic swelling of chloroplasts leads to the dissipation of many of the stromal components. The dissipation of stromal ATP was of particular importance in the titration of *qI* against *qE*, since ATP-dependent quenching (*qT*) [14] is difficult to distinguish from qI in chloroplasts. Swollen chloroplasts were used, in preference to broken chloroplasts, because they tend to show higher levels of energy-dependent quenching.

The simultaneous measurement of chlorophyll and 9-aminoacridine fluorescence signals and oxygen uptake was performed using apparatus similar to that described in Ref. 5; the only major difference being that a low intensity modulated actinic light (provided by a Walz fluorimeter) plus a non-modulated actinic light of varying light intensity, were used in place of a single modulated actinic light. Light-saturation pulses were delivered from a Volpi 250HL through a Uniblitz shutter. Another Volpi 250HL was used, either to provide additional non-modulated actinic light, or as a secondary light saturation pulse source at low levels of actinic light.

The F_0 level of chlorophyll fluorescence (and F_v/F_m ratio) were determined separately, with the modulated-light source reduced to a non-actinic level. A constant level of F_0 was assumed throughout individual runs. The coefficient of photochemical quenching (qQ) was calculated as $(\Delta F_v Q/F_v Q) \times 100$ (see Fig. 1). This gives a value of 100 where charge separation is 100% efficient and approaches 0 when Q_A is largely reduced. qE was calculated from $(\Delta F_v E/F_v E) \times 100$, after being reversed during a 1.5 min dark period, or by the addition of 50 μM DCMU. The term qR is used here to quantify non-photochemical quenching remaining after the reversal of qE and is given by $(\Delta F_v R/F_v R) \times 100$. Quenching of 9-aminoacridine fluorescence ($q9\text{-}AA$) is defined as the percentage quenching of the total scale.

Reagents were added through the top of the chamber using a micro-syringe. The volume of ethanol in the chamber was never more than 2% of the total. 9-aminoacridine was used at a concentration of 1 μM.

Results

The relationship between the quantum yield of Photosystem II and energy-dependent quenching

Measurement of Φ_p in leaves is complicated by respiratory O_2 uptake, occurring simultaneously with CO_2-dependent oxygen evolution. This is particularly true at low levels of irradiance. In the experiments described here, this complication is removed by the use of isolated chloroplasts. Another problem, which can be experienced with isolated chloroplasts which are fixing CO_2, is that oxygen evolution, at PS II may be partly offset by 'pseudo-cyclic' electron flow, reduction of oxygen occurring on the reducing side of PS I. This problem is overcome by using the 'Mehler reagent' methyl viologen as an electron acceptor. The lack of any Calvin-cycle activity in osmotically swollen chloroplasts ensures that the peroxidation reaction, catalysed by methyl viologen on the reducing side of P-700, is the only terminal electron acceptor. Thus, this system allows for the accurate assessment of rates of electron transport down to low levels of irradiance.

Fig. 2A shows Φ_p, determined from swollen pea chloroplasts over a range of irradiance values between 30 and 2500 μmol · quanta · $m^{-2} \cdot s^{-1}$. Chloroplasts were assayed in the presence of 0.1 mM methyl viologen ± 3 μM antimycin A. Steady-state values of qQ, $q9\text{-}AA$ and the rate of oxygen uptake were measured after 8 min irradiance. The most obvious feature of these results is a decline in Φ_p with increasing irradiance, particularly between 30 and 500 μmol · quanta · $m^{-2} \cdot s^{-1}$. In Fig. 2, parts B and C, the rate of oxygen uptake and the value of qQ are both slightly lower in the presence of antimycin A, over the range of irradiance levels used. From Fig. 2A, the slightly higher values of Φ_p, where antimycin A is present, indicate that the decrease in qQ, induced by antimycin A, is proportionally greater than the decrease in oxygen uptake.

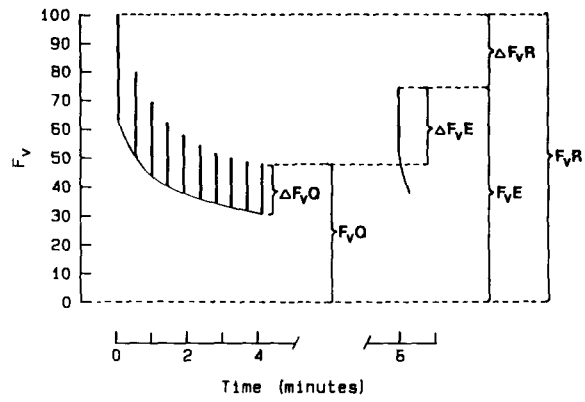

Fig. 1. Method used to calculate values for qQ, qE and qR. Terms are described in the text.

Fig. 3 shows Φ_p plotted against both $q9\text{-}AA$ (A) and qE (B), $\pm$antimycin A. These results show a clear correlation between Φ_p and $q9\text{-}AA$, whether antimycin A is present or not. Conversely, the relationship between Φ_p and qE is dramatically altered by the presence of antimycin A. This is particularly evident at the lowest values of Φ_p, where control values for qE are around 10-times higher than in the presence of antimycin A.

The data in Table I shows Φ_p, $q9\text{-}AA$ and qE calculated from chloroplasts in the coupled and uncoupled states, under conditions which were otherwise identical to those in the previous experiment. These results show that Φ_p is increased by uncoupling at both 64 μmol · quanta · $m^{-2} \cdot s^{-1}$ and 1080 μmol · quanta · $m^{-2} \cdot s^{-1}$, and in the presence and absence of 3 μM antimycin A. At

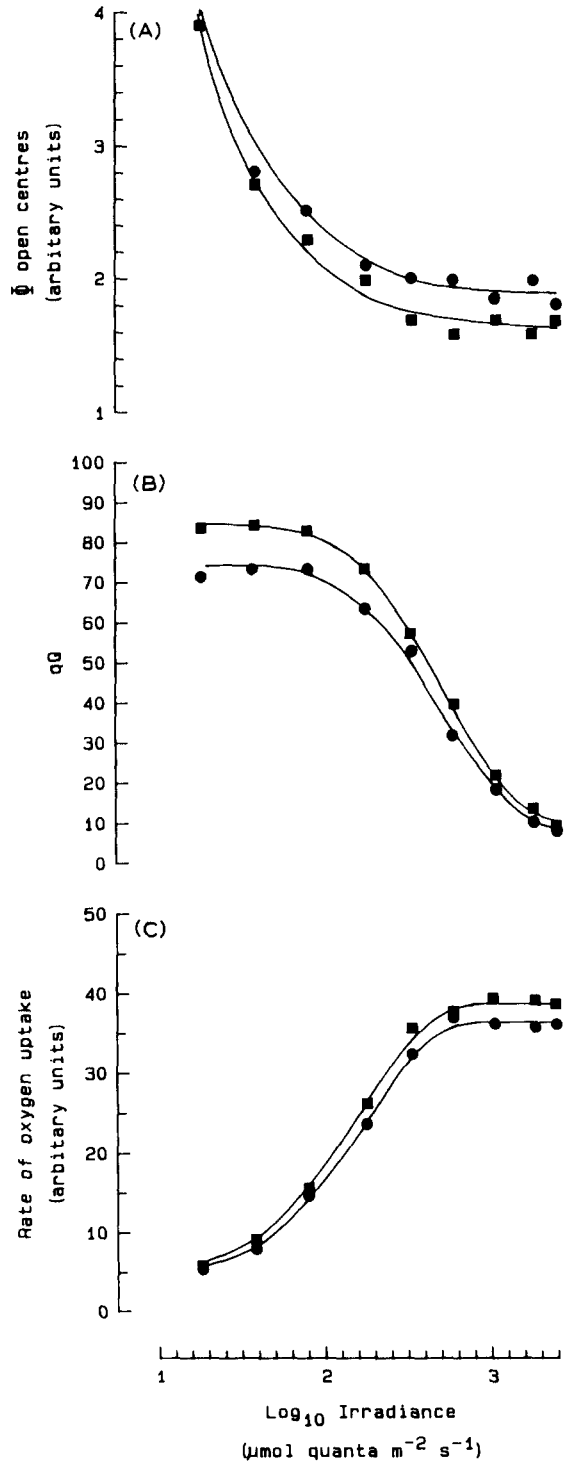

Fig. 2. Changes in the level of Φ_p (A), the level of qQ (B) and the rate of oxygen uptake (C), with increasing irradiance levels. ●, +3 μM antimycin A; ■, no antimycin A. See text for assay conditions.

low light, where *q9-AA* is low, this increase is around 25% of the coupled value where antimycin A is present and 50% of the coupled value in the absence of antimycin A. At high light, where *q9-AA* is appreciably higher, the respective values are 130% and 160%.

As in the previous experiment, the differences seen in Φ_p between treatments can be correlated with differences in *q9-AA*. One obvious exception to this is a 13% increase in Φ_p between low and high levels of irradiance in the uncoupled state. Again, Φ_p is enhanced in the presence of anti-

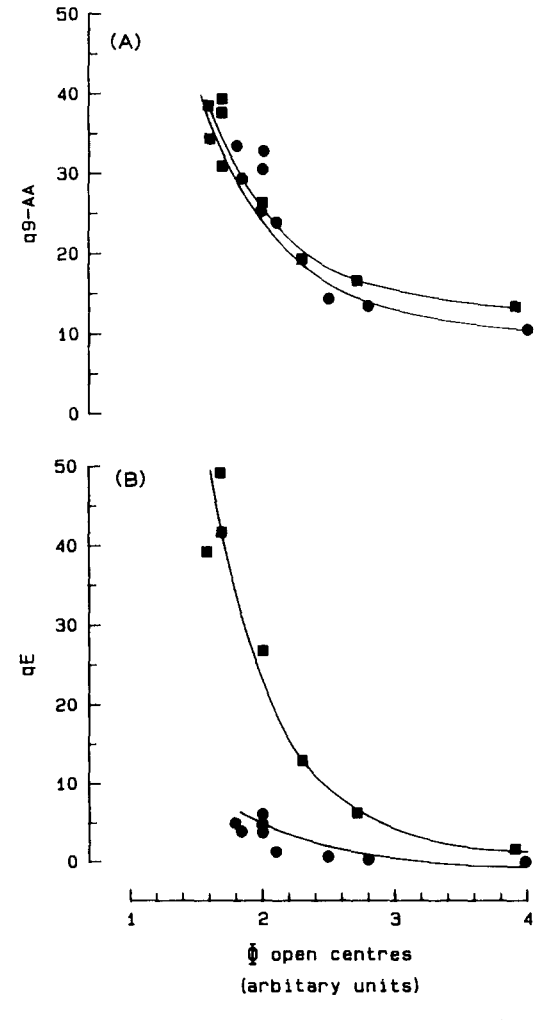

Fig. 3. Relationships between Φ_p and *q9-AA* (A) and between Φ_p and qE (B) over a range of irradiance levels between 30 and 2500 μmol·quanta·m^{-2}·s^{-1}. ●, +3 μM antimycin A; ■, − antimycin A. Details of assay conditions are given in text.

TABLE I

VALUES OF Φ_p, *qE* AND *q9-AA* IN SWOLLEN PEA CHLOROPLASTS IRRADIATED IN THE PRESENCE OF 0.1 mM METHYL VIOLOGEN AT 23° C AND pH 7.9

Values shown are the mean of five replicates. See text for conditions. Low light; 64 μmol·quanta·m^{-2}·s^{-1}; high light: 1080 μmol·quanta·m^{-2}·s^{-1}; chloroplasts were uncoupled using 1 μM nigericin; (+) and (−) refer to presence and absence of 3 μM antimycin A. Units for Φ_p are arbitrary. See Materials and Methods section for calculation of *qE* and *q9-AA*.

	coupled						uncoupled	
	(+)			(−)			(+)	(−)
	Φ_p	*q9-AA*	*qNP*	Φ_p	*q9-AA*	*qNP*	Φ_p	Φ_p
Low light	2.2	24	7	1.8	26	22	2.8	2.7
High light	1.4	46	23	1.2	51	56	3.2	3.1

mycin A. In the uncoupled state, this enhancement is very low, being around 5% of the control. In the coupled state, the addition of antimycin A has increased Φ_p by 15–20%.

Redox-state regulation of energy-dependent quenching

In this section, the relationship between *qE* and ΔpH, as defined by *q9-AA*, has been studied under conditions where the level of photochemical quenching (qQ) is varied by the addition of different subsaturating concentrations of methyl viologen. Although the redox state of ferredoxin-quinone reductase is not necessarily related to the redox state of Q_A, it seems reasonable to assume that the redox state of both carriers will be affected by the addition of methyl viologen, since the reduction of O_2, catalysed by methyl viologen, competes with ferredoxin for electrons from PS I. Therefore, any variation between *qQ* and the slope of the relationship between *qE* and *q9-AA* will be consistent with the regulation of *qE* formation by the redox state of ferredoxin-quinone reductase under these conditions.

Dark-adapted, swollen chloroplasts were irradiated with 1080 μmol · quanta · m^{-2} · s^{-1} red light. Once steady-state levels of *q9-AA* and *qE* were reached (after approx. 3 min irradiance), the transthylakoid proton gradient was gradually reversed by the addition of small aliquots (50–200 nM) of the ionophoric uncoupler, nigericin. Reversal of *q9-AA* was closely followed by a reversal of *qE*.

Although, under the conditions used here, most of the steady-state level of non-photochemical quenching (quenching coefficient, *qNP*) is energy-dependent, the progressive reduction of *qE* by uncoupling obviously increases the relative contribution of other non-photochemical quenching mechanisms, which are collectively defined by the quenching coefficient, *qR*. Clearly, *qR* is a potential source of error in the determination of the relationship between energy-dependent quenching and the transthylakoid proton gradient.

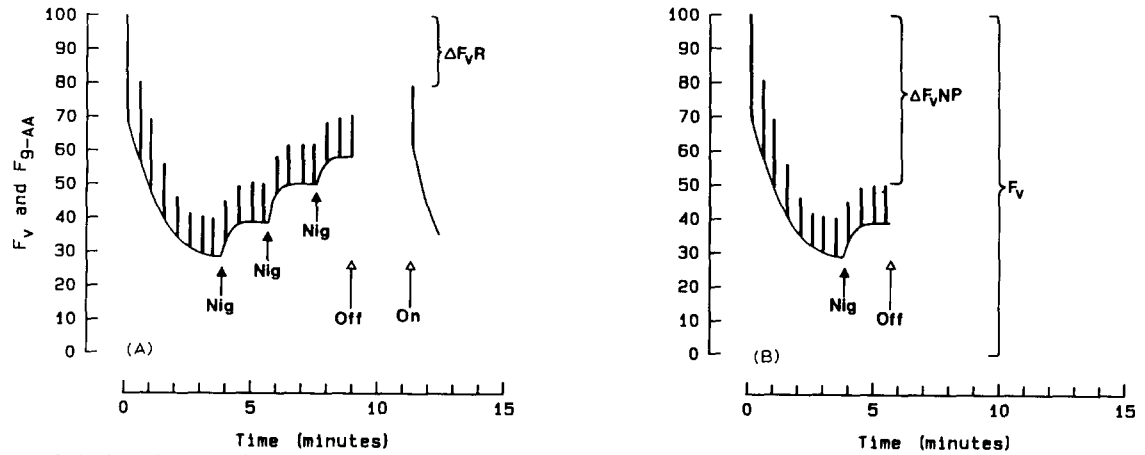

Fig. 4. Method used to calculate accurate values for *qE* at several points throughout a run. Nig, points at which small aliquots of nigericin were added. Off, light off; On, (saturating) light on. See text for details of calculation.

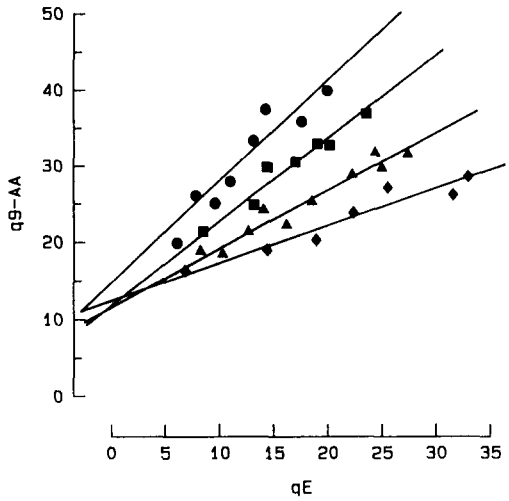

Fig. 5. Relationship between *qE* and *q9-AA* in swollen chloroplasts assayed in the presence of different subsaturating concentrations of methyl viologen (●, 0; ■, 25 nM; ▲, 100 nM; ◆, 250 nM). *q9-AA* and *qE* were progressively reversed from steady-state levels by the addition of small aliquots of nigericin. The range of values of *qQ* during each titration, and the assay conditions, are given in the text.

To overcome this problem, *qE* was 'isolated' from the other non-photochemical quenching processes by first determining the kinetics of *qR* formation. At the end of each run, *q9-AA* and *qE* were dark-reversed over 1.5 min. After this dark period, photochemical quenching was reversed by a saturating light pulse. In a separate experiment, *qR* was found to form linearly with time under the conditions used here (results not shown). Therefore, a value of $\Delta F_v R$ at any point along the run could be calculated from the value of $\Phi F_v R$ determined at the end of the run by simply multiplying the latter by T'/T, where T' is the point in time at which $\Delta F_v R$ is to be calculated, and T is the time at the end of the run (Fig. 4A). An accurate value for *qE* could then be determined at any point by subtracting $\Delta F_v R$ from $\Delta F_v NP$ and $F_v NP$ to give $\Delta F_v E$ and $F_v E$, respectively (see Fig. 4B).

In Fig. 5, *qE* is plotted against *9q-AA* at methyl viologen concentrations of 0, 50 nM, 100 nM and 250 nM. The range of values for *qQ* during each run were 9–11.5, 13–16, 20–22 and 29.5–32, respectively. It is clearly evident from these results that the slope of the relationship between *qE* and *q9-AA* is not constant over the range of methyl viologen concentrations used, being approximately three times as high at 250 nM compared to the control (zero methyl viologen). In contrast, the 'threshold' of *q9-AA* for *qE* formation appears to be fairly constant.

The redox-state dependency of the relationship between *qE* and *q9-AA* was further investigated by determining values for *qQ*, *qE* and *q9-AA* after 12 min irradiation at 1080 μmol · quanta · m^{-2} · s^{-1}, over a range of concentrations of methyl viologen between 0 and 1 μM. Assay conditions

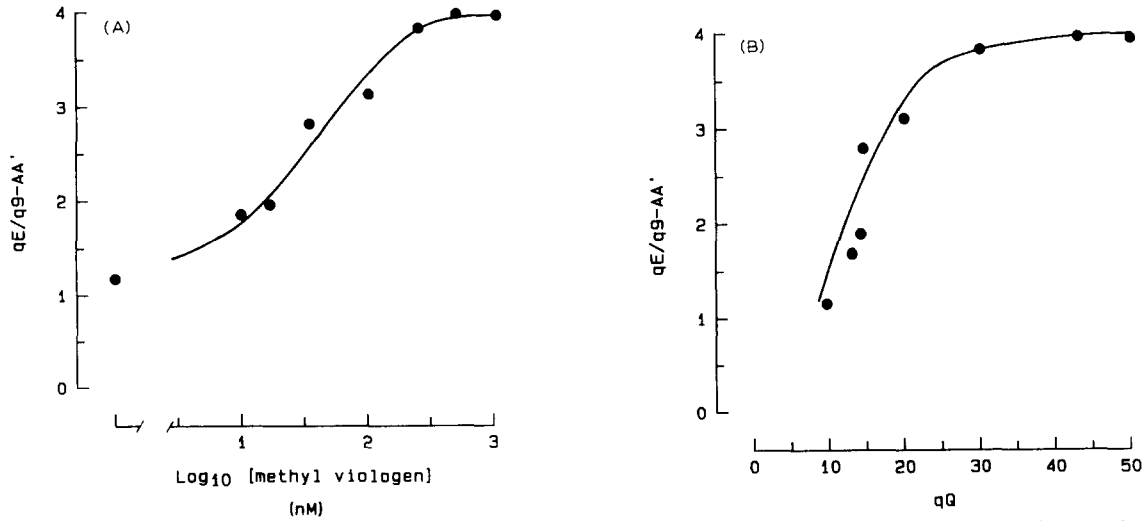

Fig. 6. Steady-state levels of *qE/q9-AA'* over a range of methyl viologen concentrations between 0 and 1 μM (A) and range of values for *qQ* (B). See text for details.

were identical to those in the previous experiment. The threshold of *q9-AA* for *qE* formation, taken from the previous experiment, was subtracted from the original value of *q9-AA* to give *q9-AA'*. The *qE:q9-AA'* ratio then gives the slope of the relationship between *qE* and *q9-AA*. In Fig. 6, *qE:q9-AA'* is plotted against methyl viologen concentration and *qQ*. As in the previous experiment, *qE:q9-AA'* increases by approx. 200% between 0 and 250 nM methyl viologen (Fig. 6A). At higher concentrations, only a very small increase is seen.

In Fig. 6B, the increase in *qE:q-9AA'* seen with increasing concentrations of methyl viologen translates to a fairly linear relationship over a range of values for *qQ* between 10 and 30. At values for *qQ* above 30, this stimulation of *qE* by methyl viologen appears to be saturated.

Regulation of photoinhibition and energy-dependent quenching

In Ref. 5 the inhibitor antimycin A was found to inhibit the formation of energy-dependent quenching without reversing any *qE* which had formed before its addition. This feature of antimycin A has been exploited here, to regulate the level of *qE*. 2.5 μM antimycin A was added at different points through an induction curve in dark-adapted swollen pea chloroplasts, irradiated for 12 min with 2500 $\mu mol \cdot quanta \cdot m^{-2} \cdot s^{-1}$ red light, in the presence of 0.1 mM methyl viologen. Since antimycin A does not inhibit *q9-AA*, this technique allows for the calculation of *qR* formation under conditions where only the level of *qE* is varied. *qQ* and *qE* were reversed by the addition of 50 μM DCMU. Since swollen chloroplasts tend to loose their endogenous ATP to the reaction medium, leading to an inhibition of *qT* formation, any difference in the level of *qR* seen between treatments can be attributed to *qI*, since *qP* will be at a constant, maximum level in the presence of saturating DCMU. The results of the above titration are shown in Fig. 7. In the uncoupled state, under conditions which were otherwise identical to those used above, the addition of 2.5 μM antimycin A did not induce any increase in the level of *qI* (results not shown).

One possible reason for the increase in irreversible quenching, seen when *qE* is inhibited with antimycin A, is that molecules of chlorophyll are

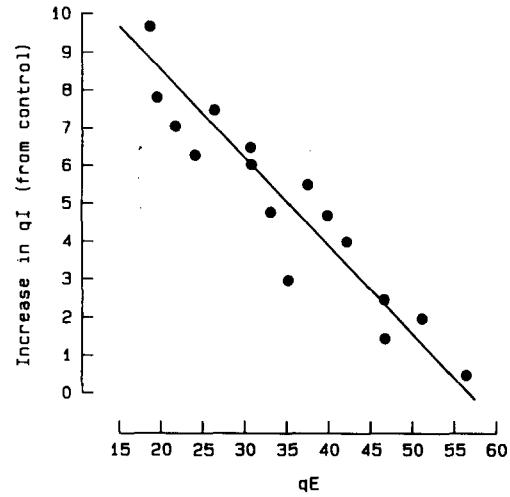

Fig. 7. Relationship between *qR* and *qE* under conditions where the level of *qE* is regulated by the addition of antimycin A at different points throughout the induction phase. See text for details.

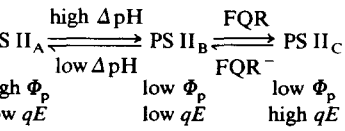

Fig. 8. Tentative model describing the formation of 'low-efficiency' and quenched states of PS II. See text for details. FQR, ferredoxin-quinone reductase.

photo-bleached. This possibility was investigated by measuring the chlorophyll concentration after a 12 min irradiance. Despite large differences in the level of irreversible quenching, no significant difference in the chlorophyll concentration was seen, ± antimycin A, or between irradiated samples and dark controls.

Discussion

It has been suggested that the decline by Φ_p, observed in leaves at high light intensity, is due to an increase in the thermal dissipation of energy, which is reflected by an increase in *qE* [8]. A model has been proposed in which there is a ΔpH-dependent conversion of PS II from a state in which the yields of both photochemistry and fluorescence are high, to a quenched state of low efficiency [15]. Although the data presented here are consistent with the notion of a ΔpH-depen-

dent regulation of PS II efficiency, a clear correlation between Φ_p and *qE* does not, in itself, infer a direct effect of energy-dependent quenching on Φ_p. In Fig. 2A, the slightly higher values for Φ_p at any given irradiance level, in the presence of antimycin A, could be explained by an inhibition of thermal deactivation within the antenna chlorophylls, reflected in much lower values for *qE*. However, the large difference in the relationship between Φ_p and *qE*, seen ± antimycin A in Fig. 3B, clearly shows that such a route for energy dissipation could not account for the large difference in Φ_p seen between low and high levels of irradiance. Conversely, the relationship between Φ_p and *q9-AA* in Fig. 3A is barely affected by the presence of antimycin A. These data indicate a primary role for the transthylakoid proton gradient in controlling the photochemical efficiency of PS II; qE may be a secondary consequence of the ΔpH effect, or merely a parallel event associated with ΔpH formation. Further support for this view is provided by a negligible increase in Φ_p between low and high irradiance in uncoupled chloroplasts, compared to a large decrease seen in coupled chloroplasts (Table I).

The correlation seen between Φ_p and *q9-AA* could be explained by a cycling of electrons around PS II, which is stimulated by low lumen pH. Such a cycle could involve a transfer of electrons between Q_B and P-680 through cytochrome *b*-559 [16,17]. Regulation of this cycle could come from a direct effect of lumen pH on electron donation to P-680 or through a reduction of the plastoquinone pool, induced by photosynthetic control.

Whilst energy-dependent quenching would appear to be of little or no importance in the regulation of Φ_p, the inverse linear relationship seen between *qE* and *qI* in Fig. 7 suggests that the mechanism responsible for this type of quenching offers some 'protection' against photoinhibition. Krause and Laasch have suggested that the level of *qI* is determined by the dynamic adjustment of thermal energy dissipation, as indicated by *qE*, to the energy requirement of photosynthetic carbon assimilation [7]. This proposal is compatible with the results presented here, and with recent work which suggests that qI can result primarily from increased thermal deactivation within antennae chlorophylls [18], due to light-induced zeaxanthin formation [19].

As yet, there is no evidence to suggest that energy-dependent quenching can occur in vivo, in the absence of a transthylakoid ΔpH. However, the recent studies with antimycin A [5] and methyl viologen (this report), clearly suggest that *qE* is also regulated through the redox-state of a component of the electron-transport chain. Although the results presented in Figs. 3 and 4 are compatible with the regulation of the *qE:q9-AA* ratio by the redox state of Q_A, the differential effect of antimycin A and *qQ* and *qE* described in Ref. 5 cannot be explained in this way. Alternatively, both the redox-state dependency of the *qE:q9-AA'* ratio and the inhibition of *qE* by antimycin A, are compatible with the concept of *qE* being regulated through the redox-state of FQR.

The tentative model presented in Fig. 8 is a summary of the results presented here. In this model, PS II_A is the dark-adapted or uncoupled state of PS II. The establishment of a transthylakoid ΔpH induces formation of PS II_B and a consequent reduction in Φ_p. Formation of PS II_C can only occur in the presence of oxidised ferredoxin-quinone reductase. Both the 'B-state' and the 'C-state' provide protection against photoinhibition.

The inverse relationship seen between *qE* and *qT* in Ref. 11 can be explained by the regulation of both quenching mechanisms through the redox state of ferredoxin-quinone reductase or cytochrome *b*-563. In this case, the conditions which support formation of the 'quenched' PS II_C would also inhibit protein phosphorylation. It is feasible that the synthesis of zeaxanthin, associated with photoinhibition [19], is also regulated by the redox state of ferredoxin-quinone reductase.

One of the main problems with the attribution of *qE* regulation to the redox state of ferredoxin-quinone reductase is the spacial separation between PS II, in the stacked regions of thylakoid membranes, and ferredoxin-quinone reductase, which is thought to be close to the cytochrome *bf* complex in the stroma-exposed regions. One possible explanation lies with the concept of 'PS II heterogeneity' (see Ref. 20 for a review). From this, it could be proposed that PS II_A is equivalent to PS II_α-centres within the stacked regions, and

that the 'low-efficiency' and 'quenched' centres (PS II_B and PS II_C, respectively) are analogous to PS II_β-centres, within the stroma-exposed regions.

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