Biochimica et Biophysica Acta, 1057 (1991) 320–330
© 1991 Elsevier Science Publishers B.V. 0005-2728/91/$03.50
ADONIS 000527289100105H

BBABIO 43368

The relationship between zeaxanthin, energy-dependent quenching of chlorophyll fluorescence, and trans-thylakoid pH gradient in isolated chloroplasts

Graham Noctor [1], Deborah Rees [1], Andrew Young [2] and Peter Horton [1]

[1] Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield (U.K.)
and [2] School of Natural Sciences, Liverpool Polytechnic, Liverpool (U.K.)

(Received 19 July 1990)

Key words: Photosynthesis; Carotenoid; Zeaxanthin; Chlorophyll fluorescence; Non-photochemical quenching; pH gradient

The quantitative relationship between energy-dependent quenching of chlorophyll fluorescence (q_E) and trans-thylakoid pH difference (ΔpH, estimated with 9-aminoacridine) was compared in chloroplasts from leaves preilluminated at low oxygen in the absence of CO_2 with chloroplasts from leaves darkened under the same conditions. The extent of both phenomena was varied by changes in actinic light intensity. Chloroplasts from preilluminated leaves contained high levels of zeaxanthin (up to 15% of total carotenoid) and were capable of forming q_E at lower ΔpH values than chloroplasts from dark-adapted leaves, which lacked zeaxanthin. Infusion of dithiothreitol into leaves prior to preillumination prevented the light-induced formation of zeaxanthin; chloroplasts prepared from these leaves showed the same relationship between q_E and ΔpH observed in chloroplasts fom dark-adapted leaves. The rate of appearance and disappearance of the change in relationship between q_E and ΔpH upon leaf preillumination and darkening was closely matched by the kinetics of synthesis and degradation of zeaxanthin. The quantitative relationship between q_E and quenching of the dark-level of fluorescence (F_0) was very similar in chloroplasts from preilluminated or dark-adapted leaves, containing disparate amounts of zeaxanthin. In both sets of chloroplasts, q_E was inhibited by antimycin A. These latter observations suggest that the formation of q_E involves mechanistically similar features in chloroplasts containing or lacking zeaxanthin. Overall, the data are discussed in terms of action of zeaxanthin as a 'quenching amplifier', functioning physiologically to allow q_E formation in the absence of high ΔpH potentially prohibitive to high rates of CO_2 fixation.

Introduction

Excitation energy can be be dissipated at Photosystem II (PS II) in several ways, as is apparent from kinetics of formation and relaxation of non-photochemical quenching of room-temperature chlorophyll fluorescence (q_N) in leaves [1,2] and protoplasts [3]. Chief among these mechanisms under most conditions is q_N associated with formation of a trans-thylakoid pH difference, or ΔpH [3–6]. This phenomenon is referred to as q_E, and forms in the light and relaxes in the dark within 2–10 min [1–3]. It has been implicated both in protection of the PS II apparatus from excess light [7] and in dynamic control over the rate of electron transport through PS II [3,8–12]. The mechanistic details of q_E, however, remain obscure.

Abbreviations: DTT, dithiothreitol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid; Mes, (2-[*N*-morpholino]ethanesulphonic acid); PS I, Photosystem I; PS II, Photosystem II; Q_A, primary stable electron acceptor of PS II; F_0, F_m, chlorophyll fluorescence level when all PS II reaction centres are open, closed; q_0, quenching of F_0 fluorescence; q_N non-photochemical quenching of chlorophyll fluorescence; q_E, energy-dependent quenching of chlorophyll fluorescence; q9-aa, fractional quenching of 9-aminoacridine fluorescence; Chl, chlorophyll; Zea, zeaxanthin; Anth, antheraxanthin; Viol, violaxanthin; HPLC, high-performance liquid chromatography; ΔpH, difference in pH between the intrathylakoid space and the external medium; CF_1, catalytic portion of the chloroplast reversible protonmotive ATPase.

Correspondence: P. Horton, Robert Hill Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield, S10 2TN, U.K.

Following the discovery of a correlative link in leaves between the level of the xanthophyll zeaxanthin and the extent of a slowly-reversing component of q_N, it was suggested that zeaxanthin might act as a quencher of excited chlorophyll [13,14]. Zeaxanthin is formed by enzymatic de-epoxidation of violaxanthin, via the intermediate antheraxanthin; an epoxidase catalyses the reverse reaction [15,16]. The epoxidase shows maximum activity at neutral pH and is probably located on the stromal side of the thylakoid membrane [17]. The de-epoxidase has a low pH optimum (pH 5), suggesting location on the inside [18]. Illumination and the consequent drop in lumen pH might therefore be expected to promote zeaxanthin formation, with epoxidation prevalent in the dark [15,16].

Recently, quantitative correlations between estimated total dissipation and zeaxanthin levels have been obtained in leaves [19–21], indicative of a role for zeaxanthin in q_E as well as in the slowly reversing quenching mentioned above. Rapid formation of q_N (within 2 min), consistent with expected kinetics of ΔpH formation, occurred only in leaves containing high zeaxanthin levels prior to illumination [19]. In leaves initially lacking zeaxanthin quenching formed within 5–10 min, with kinetics similar to those of zeaxanthin synthesis [19,20]. Additional evidence that zeaxanthin is involved in q_E has come from experiments with dithiothreitol (DTT), an inhibitor of violaxanthin de-epoxidase [22]. In one study, pre-treatment of leaves with DTT in the dark prevented formation of zeaxanthin upon illumination and also prevented formation of that component of q_N (presumably q_E) which relaxes rapidly on darkening [23]. In other studies of similar nature, however, only partial inhibition of this component has been reported [24,25].

On the basis of these data, it was proposed that two requirements exist for q_E formation, one of which is the presence of zeaxanthin, the other being formation of ΔpH [19–21,23,25]. Thus, q_E could be controlled kinetically either by the rate of ΔpH formation or by the kinetics of zeaxanthin synthesis: upon illumination of leaves lacking zeaxanthin, the rate of formation of q_E could be limited by the rate of zeaxanthin synthesis, which is expected to be at least as slow as onset of ΔpH. Since a component of q_E could be formed in the absence of zeaxanthin, it was further suggested that q_E may proceed by two distinct mechanisms, a zeaxanthin-dependent one in the light-harvesting/antenna chlorophylls and a zeaxanthin-independent one in the PS II reaction centre [23,25].

It is difficult to explore satisfactorily the relationships between q_E, ΔpH and zeaxanthin content in leaves, since changes in q_E and changes in zeaxanthin may not be causally related but may still be coincident owing to the dependence of both upon ΔpH. ΔpH cannot be measured in leaves whereas in isolated chloroplasts one can use 9-aminoacridine (9-aa) fluorescence [26] which, though it may not reflect exactly the absolute magnitude of the trans-membrane pH difference [27], can be used as a semi-quantitative and independent indicator of ΔpH [28]. Using this technique and chlorophyll fluorescence simultaneously, one can titrate ΔpH against either uncouplers or light intensity. Since uncouplers and inhibitors may be used to quantify q_E in chloroplasts [5], it is possible to examine the quantitative relationship between q_E and quenching of 9-aa fluorescence [29,30]. Such titrations have previously shown that chloroplasts isolated from leaves pre-treated to contain high levels of zeaxanthin are capable of q_E formation at lower ΔpH values than chloroplasts prepared from dark-adapted leaves, containing negligible zeaxanthin [29]. Maximum attainable q_E values differed only slightly between the two sets of chloroplasts [29], which appears at odds with the proposal that the presence of zeaxanthin is an absolute requirement for q_E formation.

In this paper, we have extended our initial observations [29] to examine further the change in ΔpH-dependence of q_E and its relationship to zeaxanthin content. We have also compared the q_E-associated quenching of dark-level (F_0) fluorescence in chloroplasts pre-treated to contain, or lack, high levels of zeaxanthin. The data lend further support to the notion that the formation of zeaxanthin functions physiologically as a ‘quenching amplifier’, acting to allow greater dissipation at a given ΔpH. The physiological significance of such action is considered.

Materials and Methods

Spinach plants were grown in a greenhouse under supplemented light with a 12 h photoperiod. Plants were harvested after 4–6 weeks and were dark-adapted for 24 h prior to leaf treatment and chloroplast isolation. Leaves were cut at the petiole base and floated on water in a plastic tray covered with transparent cling-film at 25 °C with the cut petiole under water. The water was bubbled with a CO_2-free nitrogen/oxygen mixture (composition as indicated in the text) for 10 min prior to treatment of the leaves. Unless otherwise indicated, treatment consisted of either illumination at 200 μmol m^{-2} s^{-1} for 50 min or of darkening under the same conditions for 50 min. The appropriate nitrogen/oxygen mixture was passed over the water throughout the treatment. For experiments involving infusion of dithiothreitol (DTT), leaves were placed overnight upright in a beaker containing 1 mM DTT solution. Air was fanned across the leaf surface in order to increase transpiration rate and aid uptake of DTT. During preillumination of leaves on the following morning the leaves were floated on water containing DTT at 1 mM. Control leaves for DTT treatment were treated in identical fashion except that DTT was omitted.

Unless stated otherwise, chloroplast isolation was performed essentially as in Ref. 31 immediately following treatment. 80 g of deribbed leaves were ground in 400 ml of medium containing 330 mM sorbitol, 5 mM $MgCl_2$, 2 mM sodium ascorbate and 10 mM $Na_4P_2O_7$ (pH 6.5). Chloroplasts were washed in 330 mM sorbitol/10 mM Mes (pH 6.5) and resuspended in RS2 medium (330 mM sorbitol/1 mM EDTA/1 mM $MgCl_2$/50 mM Hepes (pH 7.6)) to a chlorophyll concentration of 1–2 mg ml^{-1}. To ensure a high degree of intactness, these chloroplasts were layered over 10 ml RS2 medium containing 50% dialysed Percoll and centrifuged at $3000 \times g$ for 5 min. Resulting chloroplasts were resuspended in RS2 at a chlorophyll concentration of 1.7 mg ml^{-1}. This procedure gave chloroplasts which were more than 80% intact according to the method of Lilley et al. [32].

All chloroplast preparations were used at a chlorophyll concentration of 35 μg ml^{-1}. Experiments using intact chloroplasts were carried out in RS2. For experiments using broken chloroplasts, chloroplasts were osmotically shocked by incubation for 30 s in 30 mM $MgCl_2$/0.5 mM EDTA/10 mM Hepes (pH 7.6), after which sorbitol and Hepes were added to a final concentration of 330 mM and 55 mM, respectively. Final $MgCl_2$ concentration was 15 mM, which is the optimal concentration for q_E formation in these broken spinach chloroplasts [33]. In addition, all assays, whether with broken or intact chloroplasts, contained 0.1 mM methyl viologen, 0.1 mM NaN_3 and 1 μM 9-aminoacridine. 9-Aminoacridine fluorescence and chlorophyll fluorescence were assayed using equipment described in Ref. 30 at a temperature of 20°C. 1 min after addition of osmoticum and buffer, the Walz fluorimeter measuring beam was turned on. 30 s later a saturating flash or pulse was given to determine F_m prior to actinic illumination, and after a further 30 s red actinic light was applied. Application of saturating pulses was begun after 30 s actinic light and continued at 1 min intervals until 4.5 min actinic ilumination (see Fig. 1a). 1 min later, unless DCMU was added as in Fig. 1a, the actinic light was switched off. From 10 s before darkening to 2 s after, low intensity far-red illumination was applied to oxidise PS II acceptors and so ensure rapid return to a true F_0 level upon darkening (F_0'). Chloroplasts were given 5 min in the dark to allow relaxation of ΔpH-dependent non-photochemical fluorescence quenching (q_E): after 5 min, a saturating pulse was given to determine maximum fluorescence yield in the absence of q_E (F_m''). q_E was calculated as follows; $F_m' - F_0' = F' v_m$ (maximum variable fluorescence in the light). $F_m'' - F_0'' = F'' v_m$ (maximum variable fluorescence yield after 5 min recovery following actinic illumination). $q_E = (F'' v_m - F' v_m)/F'' v_m$. Coefficient for 9-aminoacridine fluorescence quenching (q9-aa) was taken as the fractional decrease in signal after 4.5 min actinic illumination. This experimental procedure was repeated at various actinic light intensities in order to explore the relationship between q_E and q9-aa. Where used, the concentrations of DCMU and nigericin were 50 μM and 2 μM, respectively.

Chloroplast samples containing 40 μg chlorophyll were taken at the beginning and end of the experiment and frozen in liquid N_2 prior to extraction of pigments for HPLC analysis as in Ref. 34. Carotenoids were quantified using a diode-array detector: absorbances at peak wavelengths were integrated and concentrations calculated using a common extinction coefficient of 2500 mol^{-1} cm^{-1}. Fractional content of pigments were calculated from data expressed on a mol : mol basis.

Results

In all experiments described below, differences in zeaxanthin content of chloroplasts were obtained by pre-treatment of leaves prior to chloroplast isolation. In chloroplasts, de-epoxidation of violaxanthin requires ascorbate [15,16]. Ascorbate was omitted during the experiment in order to prevent the formation of zeaxanthin in the chloroplasts. Comparison of pigment content of chloroplast samples taken prior to and after actinic illumination confirmed that no zeaxanthin was formed in the chloroplasts under actinic illumination (not shown).

Chlorophyll and 9-aminoacridine fluorescence traces for chloroplasts from darkened leaves ('dark' chloroplasts, left) or leaves preilluminated at 200 μmol m^{-2} s^{-1} at 2% O_2 in the absence of CO_2 ('light' chloroplasts, right) leaves are shown in Fig. 1a. The initial F_m induced by a saturating flash prior to actinic illumination was significantly lower in light chloroplasts, consistent with the idea that the higher zeaxanthin content of these chloroplasts is associated with long-lived or 'irreversible' quenching [13,14]. In this experiment, the actinic light intensity was saturating for ΔpH and q_E formation in both sets of chloroplasts. After 5.5 min illumination, DCMU was added. A biphasic rise in chlorophyll fluorescence is observed. The rapid phase corresponds to removal of photochemical quenching as DCMU binds to the PS II reaction centre and inhibits Q_A reoxidation, while the slower phase reflects the relaxation of q_E as ΔpH disappears due to the consequent inhibition of electron transport [5]. There is a clear difference in kinetics of q_E relaxation between the two sets of chloroplasts. In dark chloroplasts, q_E disappears with similar kinetics to ΔpH (1.5–2 min for total recovery of chlorophyll and 9-aminoacridine fluorescence; Fig. 1a, left trace). ΔpH relaxes with similar kinetics in light chloroplasts, but here q_E is much slower to relax, taking 4–5 min (Fig. 1a, right trace). Addition of

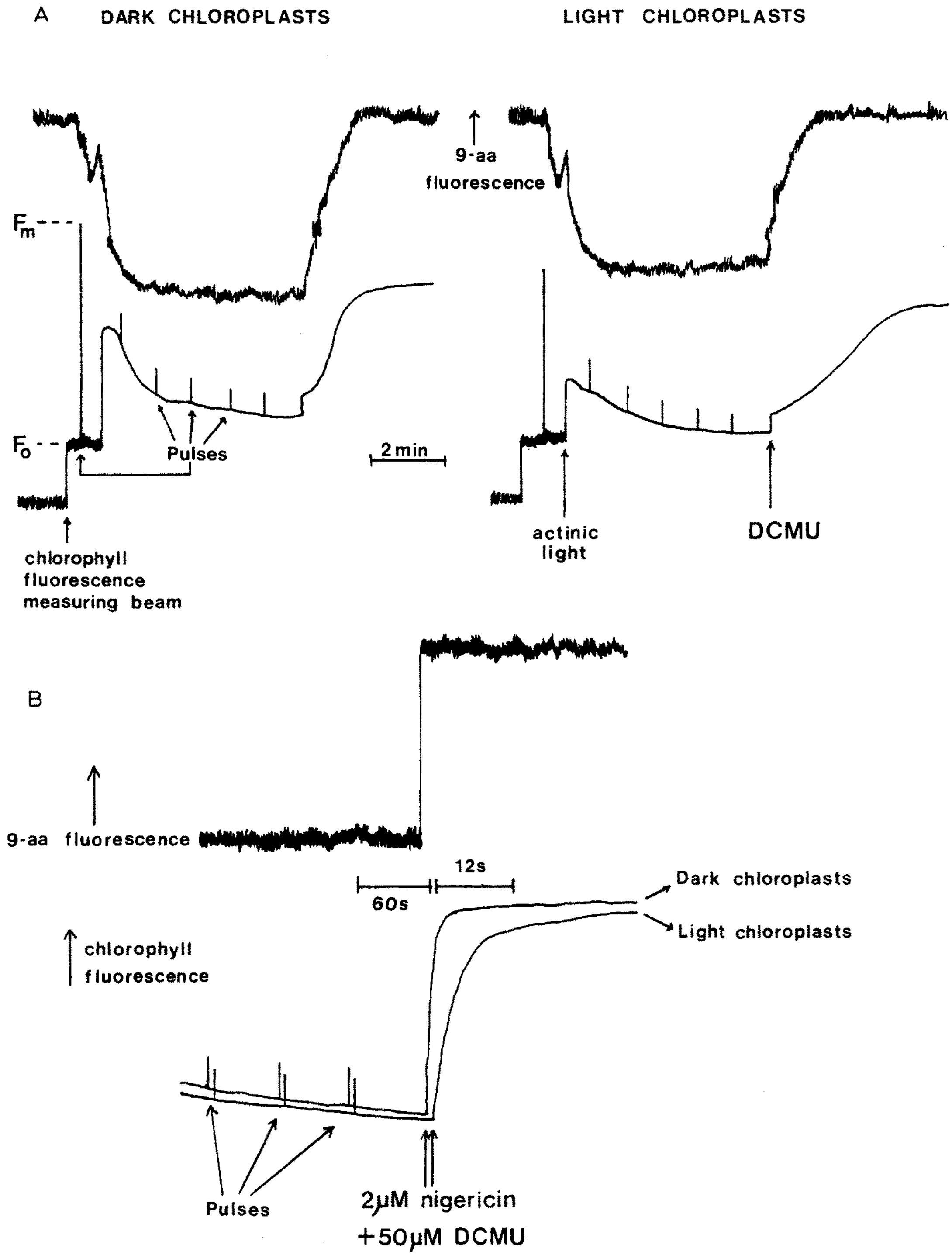

Fig. 1. Traces of chlorophyll and 9-aminoacridine fluorescence for chloroplasts isolated from darkened or preilluminated spinach leaves. Leaves were floated on water for 50 min in an atmosphere of 98% N_2, 2% O_2 while being darkened (dark chloroplasts) or preilluminated (light chloroplasts). Complete traces of chlorophyll and 9-aminoacridine fluorescence are shown in (a); actinic illumination was at a light intensity of 300 μmol m^{-2} s^{-1}. In this experiment, 50 μM DCMU was added after 5.5 min actinic illumination in order to check the rate of quenching relaxation. In chloroplasts from preilluminated leaves, zeaxanthin accounted for 39% of violaxanthin cycle pigments; no zeaxanthin was detected in chloroplasts from dark-adapted leaves. Part (b) shows that a difference in relaxation kinetics is still apparent when relaxation is followed upon rapid collapse of ΔpH with 2 μM nigericin. This concentration of nigericin caused apparently immediate collapse of ΔpH in both sets of chloroplasts and so only one 9-aa fluorescence trace is shown in (b). Note that chlorophyll fluorescence traces are staggered in the interests of diagrammatic clarity and that the time-scale changes after addition of nigericin and DCMU. In (b) light chloroplasts contained 35% of violaxanthin cycle pigments as zeaxanthin; dark chloroplasts contained no zeaxanthin.

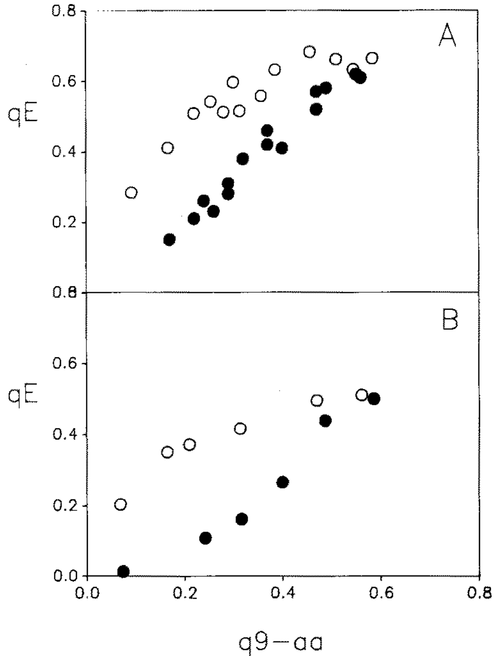

Fig. 2. Effect of preillumination of spinach leaves upon the relationship between q_E and ΔpH (q9-aa) in subsequently isolated chloroplasts. Chloroplasts were either broken immediately before assay by incubation in 30 mM $MgCl_2$/0.5 mM EDTA/10 mM Hepes (pH 7.6) for 30 s (A) or were assayed intact (B). Electron acceptor was methyl viologen in both cases. q_E and q9-aa were manipulated by changes in actinic light intensity. For chloroplasts from pre-darkened leaves, zeaxanthin was present at between 0 and 6% of violaxanthin cycle pigments (violaxanthin + antheraxanthin + zeaxanthin), while in chloroplasts from light-treated leaves zeaxanthin comprised 37–39% of these pigments.

saturating uncoupler simultaneously with DCMU causes rapid collapse of the pH gradient and accelerates q_E relaxation markedly in both sets of chloroplasts (Fig. 1b). Nevertheless, a difference in rate of relaxation between the chloroplasts is still evident (Fig. 1b): dark chloroplasts show fast relaxation of q_E (within 2-5s) while q_E in light chloroplasts takes 10–20 s for complete reversal. In the following experiments, q_E was determined by application of a saturating pulse given after 5 min dark subsequent to illumination, a protocol which allows measurement of F_0 immediately upon darkening (F_0'). The traces of Fig. 1a demonstrate the validity of this approach, since in both sets of chloroplasts reversible fluorescence quenching is fully relaxed within 5 min following DCMU addition. Values for q_E were similar whether calculated from data using DCMU or saturating pulse following dark recovery.

Fig. 2 shows titrations of q_E against ΔpH (q9-aa) for light and dark chloroplasts, where both parameters were manipulated by changes in intensity of actinic illumination. In (a) chloroplasts were assayed following osmotic shock in 30 mM $MgCl_2$ (final concentration during assay 15 mM), optimal concentration for q_E formation in these chloroplasts [33]. A clear difference in the ΔpH profile of q_E is evident, particularly at ΔpH values which are subsaturating for q_E formation. The shape of the curve changes from linear or slightly sigmoidal to hyperbolic following preillumination of leaves. It is noteworthy that the maximum extent of q_E was virtually unchanged by leaf preillumination. Fig. 2b shows a similar experiment, but here the titration was carried out with intact chloroplasts. The same phenomenon is observed; preillumination of leaves shifts the ΔpH profile of q_E in subsequently-isolated chloroplasts such that much higher values of q_E are formed at low ΔpH values. Control experiments in which saturating uncoupler was added to chloroplasts during actinic illumination (as in Fig. 1b) showed that no difference existed in the 9-aa fluorescence level prior to actinic illumination and that following addition of uncoupler. This demonstrates that no ΔpH existed in these chloroplasts preparations prior to actinic illumination. Violaxanthin cycle pigment content of these chloroplasts are given in the legend to Fig. 2. Zeaxanthin levels were significant only for the light chloroplasts. Zeaxanthin is expressed as % of violaxanthin cycle pigments, which together generally comprised 20–25% of total carotenoids. Values given for pigment contents are the mean of two measurements, one from a sample taken at the beginning of the experiment (within 30–60 min of chloroplast isolation) and one taken 3–4 h later, at the end of the experiment. These samples gave values in close agreement, showing that the pigment content of the chloroplasts did not change during the experiment. Violaxanthin and zeaxanthin were the only pigments which showed significant changes as a result of leaf pre-treatment. Antheraxanthin levels were generally the same in chloroplasts from darkened or preilluminated leaves (approx. 2–4% of total carotenoid; 8–15% of violaxanthin cycle pigments). Other carotenoids remained constant (neoxanthin at about 11–12%, lutein at 35–40%, and β-carotene at 28–30%, of total carotenoids).

The link between zeaxanthin and the shift in ΔpH profile of q_E is strengthened by the data of Fig. 3. The open symbols again show the dependence of q_E upon q9-aa for light chloroplasts, in which over half of violaxanthin cycle pigments are present as zeaxanthin. The closed triangles also describe a curve for chloroplasts from leaves subjected to preillumination, but for these leaves light treatment was carried out following overnight infusion of DTT. The relationship between q_E and q9-aa is very similar to the relationship in chloroplasts prepared from darkened leaves. Zeaxanthin accounts for only 10% of violaxanthin cycle pigments in these chloroplasts, as expected from the known action of DTT as an inhibitor of violaxanthin de-epoxidation [22]. It is also noteworthy that, for chloroplasts from DTT-treated leaves, relaxation rates of q_E (Fig. 1) resemble dark chloroplasts more than light chloroplasts (not shown). Control experiments showed that the

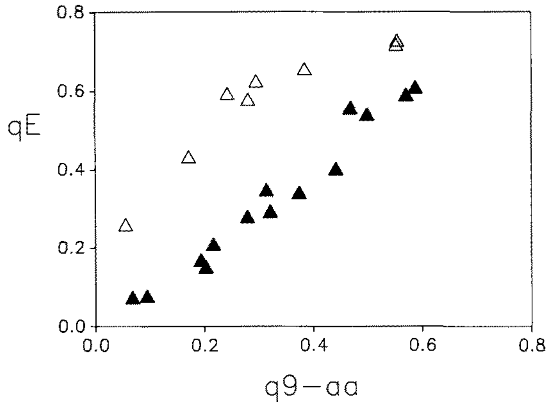

Fig. 3. Effect of dithiothreitol treatment upon the shift in ΔpH profile of q_E. Leaves were harvested and incubated overnight with petioles in either water (control chloroplasts, open triangles) or 1 mM dithiothreitol (chloroplasts from DTT-treated leaves, closed triangles). On the following morning leaves were illuminated as described for Fig. 1, prior to chloroplast isolation. Leaves which had been treated overnight with DTT were floated on 1 mM DTT solution during light treatment. Chloroplasts were osmotically shocked in 30 mM $MgCl_2$ prior to fluorescence measurements during electron transport to methyl viologen. The relationship between q_E and q9-aa was explored by changes in actinic light intensity. Control chloroplasts contained 51% of their violaxanthin cycle pigments as zeaxanthin; for chloroplasts from DTT-treated leaves this figure was 10%.

abolition of the shift in the ΔpH profile of q_E is not caused by a direct effect of DTT upon q_E formation in the isolated chloroplasts: light chloroplasts from leaves illuminated in the absence of DTT show the same hyperbolic relationship between q_E and ΔpH regardless of whether DTT is present or absent during the assay (results not shown).

Fig. 4 shows the results of an experiment designed to investigate whether the shift in ΔpH profile of q_E reverses on cessation of light treatment. Subsequent to illumination of leaves at 200 μmol m^{-2} s^{-1} in 2% O_2 for 50 min, the gas mix was changed to air and the light intensity reduced to 4–5 μmol m^{-2} s^{-1}. These latter conditions were found to favour the net conversion of zeaxanthin back to violaxanthin, and for the sake of simplicity will be referred to below as 'darkness'. For this experiment, three light intensities were chosen and assays were carried out at these light intensities for each chloroplast preparation. Relatively low light intensities were chosen to produce relatively low ΔpH values, where the observed stimulation of q_E by leaf preillumination is greatest. The change in relationship in chloroplasts isolated immediately after illumination of leaves (compare open squares, light chloroplasts, with closed squares, dark chloroplasts) was much less pronounced in chloroplasts isolated after darkening for a subsequent hour (being intermediate between dark and light chloroplasts; inverted triangles). Chloroplasts isolated after 3 h darkness following illumination (triangles) showed ΔpH profile for q_E indistinguishable from

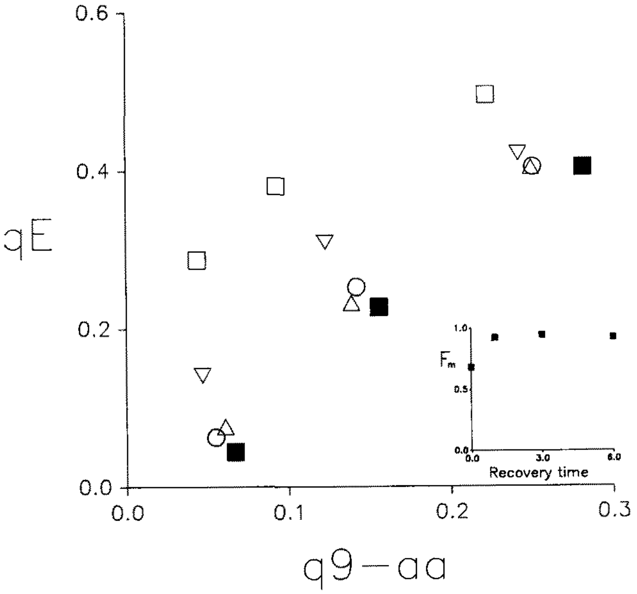

Fig. 4. Recovery of the shift in ΔpH profile of q_E. Closed squares show data obtained with chloroplasts from dark-adapted leaves. Other data were obtained for chloroplasts from leaves light-treated for 50 min at 200 μmol m^{-2} s^{-1} in an atmosphere of 98% N_2, 2% O_2 but given the following periods of time between the end of light-treatment and isolation of chloroplasts: open squares, 0 min; inverted triangles, 60 min; triangles, 180 min; circles, 360 min. Recovery was allowed to take place in air at low light (4–5 μmol m^{-2} s^{-1}). Table I shows violaxanthin cycle pigment content of these chloroplasts. The inset figure shows the recovery of maximum fluorescence yield (determined prior to actinic illumination) in the chloroplasts as a function of length of time of darkness of leaves (recovery time); each point is the mean of three measurements, normalised to yield in chloroplasts from dark-adapted leaves.

TABLE I

Rate of disappearance of zeaxanthin in leaves subsequent to light treatment

Spinach leaves were floated on water in the dark in an atmosphere of 98% N_2, 2% O_2. A sample of leaves was taken for chloroplast isolation (dark chloroplasts); the remaining leaves were illuminated at a light intensity of 200 μmol m^{-2} s^{-1}. After 50 min light, leaves were taken for chloroplast isolation (light chloroplasts); the gas blowing over the remaining leaves was changed to air and the light intensity was reduced to 4–5 μmol m^{-2} s^{-1}. Further samples were taken for preparation of chloroplasts after 1, 3 and 6 h. Chloroplasts were used to examine the relationship between q_E and q9-aa (Fig. 4) and samples were taken for pigment analysis by HPLC; these data are expressed as % of total carotenoids or as % of summed violaxanthin cycle pigments (violaxanthin (Viol) + antheraxanthin (Anth) + zeaxanthin (Zea)). All data are the mean of two measurements.

Treatment of leaves	Pigment content of chloroplasts (% of total carotenoids)				
	Viol	Anth	Zea	Zea : Viol	Zea/Zea + Anth + Viol
Dark chloroplasts	15.6	2.7	0	0	0
Light chloroplasts	6.8	2.4	13.5	1.99	0.59
Light + 1 h recovery	15.6	0	4.7	0.30	0.23
Light + 3 h recovery	16.9	2.8	4.9	0.39	0.20
Light + 6 h recovery	15.6	2.5	2.7	0.17	0.13

dark chloroplasts. Table I gives violaxanthin, antheraxanthin and zeaxanthin contents for these chloroplasts. No zeaxanthin was detected in dark chloroplasts, whereas it accounted for 13.5% of total carotenoids (59% of violaxanthin cycle pigments) in light chloroplasts, with violaxanthin showing a corresponding decrease in these chloroplasts. However, most of this zeaxanthin had disappeared within 1 h of darkness (Table I). Maximum fluorescence yield of the chloroplasts measured prior to actinic illumination (F_m; Fig.4 inset) showed complete recovery within 1 h of darkness. Leaves given 6 h darkness following illumination produced chloroplasts containing zeaxanthin at only 2.7% of total carotenoids and gave a q_E : q9-aa relationship essentially similar to that of dark chloroplasts.

Fig. 5 shows results of two experiments which investigated the rate of appearance of the shift in q_E profile upon illumination of leaves. Open and closed squares represent light (i.e., 50 min light) and dark chloroplasts respectively. Other symbols represent chloroplasts prepared from leaves illuminated for various intermediate periods. Levels of violaxanthin cycle pigments for all these chloroplast preparations are given in Table II. It is clear from Fig. 5 that 15–20 min illumination of leaves is sufficient to induce a maximal shift in ΔpH profile of q_E, and that 10 min light (bottom figure; circles) produces a profile for q_E closer to the profile of light chloroplasts than that of dark chloroplasts. Zeaxanthin synthesis seems to be complete within 20 min of illumination (Table II). After 15 min, at which point the shift in the profile of q_E has occurred, zeaxanthin accounted for 9.5% of total carotenoid. This figure rises to 13.3% after 20 min light. It seems there is increased accumulation of zeaxanthin past the point where the maximum effect of illumination upon q_E has occurred. The inset figures of Fig. 5 show the maximum fluorescence yield of the chloroplasts (F_m: determined prior to actinic illumination). Maximum decrease in F_m in the chloroplasts seems to take 15–20 min preillumination of leaves, similar to the increase in zeaxanthin content of the chloroplasts (cf: Fig. 5 insets and Table II). Taken together with the similarity in rate of recovery of F_m (Fig. 4, inset) and rate of disappearance of zeaxanthin in the dark (Table I), these data support the notion that zeaxanthin is involved in long-lived, slowly-reversing quenching [13,14].

The observation of continued zeaxanthin synthesis past the point where maximum shift in ΔpH profile of q_E has occurred (cf: Fig. 5 and Table II) suggests that the relationship between the two phenomena may not be a simple one. Other data further support this conclusion (not shown). Illumination of leaves in the presence of atmospheric oxygen levels does not lead to zeaxanthin synthesis as extensive as in leaves illuminated under low oxygen conditions (unless higher intensity of illumination is used [29]). High zeaxanthin synthesis in low oxygen may reflect a decreased rate of epoxidation, for which oxygen is a requirement [15]. Chloroplasts isolated from leaves immediately following illumination for 50 min at atmospheric levels of oxygen contained only 5.1% of carotenoids as zeaxanthin but nevertheless gave the same hyperbolic relationship between q_E and q9-aa as chloroplasts prepared from leaves illuminated under the same conditions at 2% O_2 (zeaxanthin 9.2% of total carotenoid).

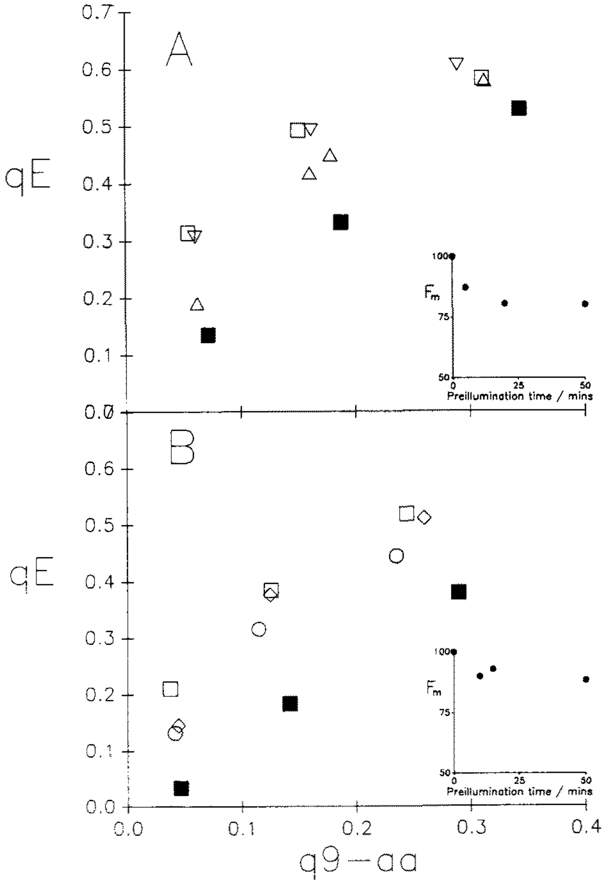

Fig. 5. Time-course for manifestation of the shift in ΔpH profile for q_E. Data in top and bottom figures are from two separate experiments. Chloroplasts were isolated from dark-adapted leaves (closed squares) or from leaves preilluminated for 5 min (triangles), 10 min (circles), 15 min (diamonds), 20 min (inverted triangles), or 50 min (open squares) prior to chloroplast isolation. Preillumination was at 200 μmol m^{-2} s^{-1} in an atmosphere of 98% N_2, 2% O_2. Corresponding pigment data is given in Table II. Inset figures show maximum fluorescence yield, determined prior to actinic illumination, for each chloroplast preparation. In these figures, each point is the mean of three data, except for chloroplasts from leaves given 5 min light which is the mean of four. F_m is normalised to the yield obtained in chloroplasts from dark-adapted leaves.

The extent of q_E-associated quenching of the F_0 level of fluorescence (q_0) is dependent upon the site of action of q_E F_0 can be quenched considerably by high q_E if q_E occurs primarily in light-harvesting and antenna pigments of PS II, while equivalent quenching at the PS II

TABLE II

Time-course for changes in violaxanthin cycle pigments upon illumination

Spinach leaves were floated on water in an atmosphere of 98% N_2, 2% O_2 while being illuminated at a light intensity of 200 μmol m^{-2} s^{-1} for the indicated periods of time immediately prior to chloroplast isolation. Chloroplasts were either used to obtain the fluorescence quenching data shown in Fig. 5 or were used for analysis of carotenoids. Pigment contents are expressed as % of total carotenoids or as % of summed violaxanthin cycle pigments (violaxanthin (Viol) + antheraxanthin (Anth) + zeaxanthin (Zea)). Data are the mean of two measurements.

Time of preillumination of leaves (min)	Pigment content of chloroplasts (% of total carotenoids)				
	Viol	Anth	Zea	Zea : Viol	Zea/Zea + Anth + Viol
	(% of total carotenoids)				
0	15.5	2.7	0	0	0
5	12.6	2.9	3.5	0.28	0.18
10	10.6	4.3	8.7	0.82	0.37
15	11.8	4.9	9.5	0.81	0.36
20	8.5	2.8	13.3	1.56	0.54
50	6.6	3.4	13.3	2.02	0.57

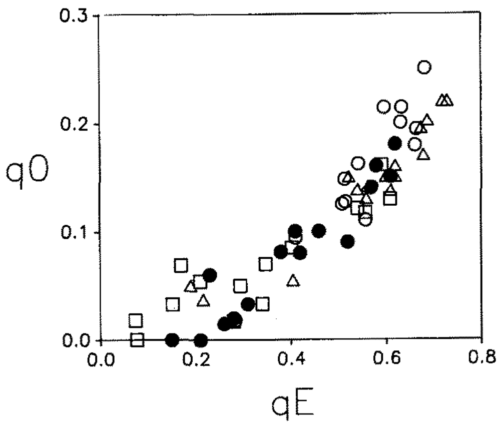

Fig. 6. Relationship between q_E and quenching of F_0 fluorescence for chloroplasts containing different amounts of zeaxanthin. All data for chloroplasts broken in 30 mM $MgCl_2$, isolated intact from leaves subjected to the following treatments: preilluminated for 50 min in 98% N_2, 2% O_2 (open circles); preilluminated 50 min in 80% N_2, 20% O_2 (triangles); preilluminated 50 min in 98% N_2, 2% O_2 after overnight infusion of DTT (squares); darkened 50 min in 98% N_2, 2% O_2 (closed circles). The percentages of violaxanthin cycle pigments present as zeaxanthin in these chloroplasts were 37–39%, 22–25%, 10%, and 0–6% respectively.

reaction centre is expected to exert a smaller effect upon the F_0 level [35,36]. Failure to observe F_0 quenching associated with q_E in the absence of zeaxanthin has prompted proposals that under these conditions quenching proceeds at the reaction centre [23,25]. Fig. 6 shows the relationship between q_E and q_0 for four sets of chloroplasts, isolated from leaves treated as described in the legend. These treatments produced chloroplasts showing the relationship between q_E and q9-aa previously described (i.e., light treatment at low or high O_2 concentration gave a hyperbolic relationship; darkening or light treatment in the presence of DTT gave linear relationships (Fig. 2a and 3)). Despite the variation in zeaxanthin content in these chloroplasts from 0% to 39% of violaxanthin cycle pigments, an identical relationship between q_E and q_0 is obtained (Fig. 6). Some similarity of mechanism for q_E in light and dark chloroplasts, containing disparate amounts of zeaxanthin, is also supported by the susceptibility of the quenching in both types of chloroplast to antimycin A (Table III), a known inhibitor of q_E in chloroplasts [37].

Discussion

The extent of light-dependent quenching of 9-aminoacridine fluorescence is assumed to reflect the extent of the drop in intrathylakoid 'bulk-phase' pH on *illumination* [28]. Briantais et al. [6] obtained a linear relationship between q_E and q9-aa in pea chloroplasts, but more recently there have been several reports of flexible relationships between the two parameters [10,37–40]. These effects have been observed as a result of changes in conditions in isolated chloroplasts, and have been attributed to influence on q_E formation by the redox state of the electron transport chain [37] or

TABLE III

Antimycin a inhibits ΔpH-dependent quenching in chloroplasts from both dark-adapted leaves and preilluminated leaves

Spinach leaves were dark-adapted for 24 h before being either light-treated or darkened for 50 min as described in the legend to Fig. 1. Intact chloroplasts were isolated and samples were taken for analysis of carotenoids. 9-Aminoacridine fluorescence and chlorophyll fluorescence were measured in freshly shocked chloroplasts with methyl viologen as electron acceptor at a light intensity of 300 μmol m^{-2} s^{-1}. Illumination was for 5.5 min; a subsequent dark period of 5 min allowed relaxation of ΔpH and associated quenching of chlorophyll fluorescence and so enabled estimation of q_E.

Treatment of leaves	Zeaxanthin (% total carotenoid)	Zea/ Zea + Anth + Viol	Control		+1 μM antimycin A	
			q9-aa	q_E	q9-aa	q_E
Dark	4.6	0.14	0.56	0.65	0.53	0.06
Light	12.0	0.51	0.40	0.70	0.42	0.11

dependence of q_E on localised proton domains not in equilibrium with the measured bulk-phase pH [39,40]. Data presented here show that the relationship can be changed in chloroplasts by pre-treatment of the parent tissue. Pre-illumination of leaves allows q_E formation at significantly lower ΔpH in isolated chloroplasts assayed under identical conditions and so induces an 'amplification' of q_E. This is true whether the relationship is examined by means of titration against uncoupler [29] or by changes in light intensity (results shown here). Here, high zeaxanthin levels were induced by preillumination of leaves at comparatively low light intensities in low O_2 and in the absence of CO_2. Equivalent levels of zeaxanthin can be induced by preillumination of leaves at high light intensity in air [29]; such treatment of leaves produces a similar effect on the ΔpH profile of q_E in subsequently-isolated chloroplasts to that shown here [29]. Under conditions used here, this effect is complete within 15–20 min illumination of leaves (Fig. 5) and reverses in the dark within 1–3 h (Fig. 4), suggesting that it could represent physiological regulation of energy dissipation.

The similarity of the kinetics of induction and reversal of this effect (Figs. 4 and 5) to those of zeaxanthin synthesis and degradation (Tables I and II), together with the prevention of the effect by DTT (Fig. 3), provide evidence that zeaxanthin is involved in some way in q_E formation [19–21,23–25]. It is also worth noting that zeaxanthin levels were stable in isolated chloroplasts for a period of at least 3–4 h, as was the shift in the ΔpH profile of q_E. Our data, however, differ from those of Demmig-Adams et al. [19–21] in that we do not find a simple quantitative correlation between zeaxanthin content and total dissipation. At high ΔpH, q_E reaches the same values in chloroplasts containing or lacking zeaxanthin. Calculation of the parameter k_D (a rate constant for energy dissipation in the PS II pigment bed) from our chlorophyll fluorescence data as in Refs. 19–21 also shows that chloroplasts lacking zeaxanthin can dissipate energy to the same extent as chloroplasts with high zeaxanthin levels, if ΔpH is high enough (not shown). The equivalent effects of q_E in both sets of chloroplasts upon the F_0 level of fluorescence (Fig. 6) and their common susceptibility to inhibition by antimycin A (Table III) suggest some mechanistic features common to q_E formation in chloroplasts containing and lacking zeaxanthin. On the other hand, the different rates of relaxation of q_E, even upon immediate collapse of ΔpH with uncoupler (Fig. 1b), is suggestive of some difference in q_E mechanism between these chloroplasts. The relationship derived between q_E and q_0 is most compatible with energy dissipation occurring among the antenna and light-harvesting chlorophylls associated with PS II [35], irrespective of zeaxanthin content of the chloroplasts (Fig. 6). Such a site would be expected if zeaxanthin were involved in q_E and is in agreement with our previous study of the relationship between q_E and q_0 [36] and with other recent data [11]. Gilmore and Yamamoto [41] have suggested that in the absence of zeaxanthin lutein, an isomer of zeaxanthin, which is the main carotenoid found in the PS II pigment bed, may be the quencher responsible for q_E. If q_E occurs principally in the pigment bed, one may view its requirement for low intra-thylakoid pH as a requirement for binding of protons by pigment-protein complexes exposed to the intra-thylakoid space [6,38]. It is then possible to construct hypotheses attempting to explain the zeaxanthin-associated change in relationship between q_E and internal pH in terms of conformational changes which alter the affinity of these complexes for protons. Such hypotheses, however, are at present necessarily vague and would involve speculation to a degree unjustifiable by the present data. Whether the $q_E : q$9-aa relationship in the presence of zeaxanthin describes a true hyperbola or is rather the result of several quenching processes with differing dependence upon ΔpH is not unequivocally discernible from these data. Nevertheless, an apparently sigmoidal relationship between q_E and internal pH is consistent with the occurrence of co-operative conformational alterations associated with proton binding; a hyperbolic relationship suggests that this requirement for cooperative binding of protons disappears in the presence of zeaxanthin.

Some variability was observed in pigment content of leaves. Light chloroplasts used in the experiment of Fig. 2 contained zeaxanthin at 8–10% of total carotenoid whereas in the data of Tables I and II, zeaxanthin accounted for 13–14% of total carotenoid in light chloroplasts. Such variation in the zeaxanthin content of control chloroplasts means that conclusions regarding any quantitative relationships between zeaxanthin and the extent of amplification of q_E must be drawn with caution. Nevertheless, comparison of the rate of onset of amplification with that of zeaxanthin synthesis showed that maximal amplification was manifest before zeaxanthin content reached its maximum (cf: Fig. 5 and Table II). Maximal amplification was also observed for chloroplasts from leaves preilluminated in 2% or 20% O_2, treatments which produced different amounts of zeaxanthin (results not shown). These data suggest that the relationship between zeaxanthin content and the extent of shift in ΔpH profile of q_E is not a simple one, and perhaps allows three interpretations. If the effect can be related to an action of zeaxanthin as a quencher of excited chlorophyll, it may indicate that amplification of q_E displays saturation kinetics with regard to zeaxanthin, such that the effect is maximal at less than maximal zeaxanthin content. Secondly, it could indicate that the link between increased zeaxanthin levels and the extent of quenching amplification is an indirect one. Both may be qualitatively coincident phenomena arising, perhaps, from conformational changes in the antenna and light-harvesting chlorophylls. Lastly, it might reflect the fact that only a fraction of synthesized zeaxanthin is formed within PS II. On electrophoretic analysis of extracted native pigment-protein complexes, about half of complex-associated zeaxanthin is found within PS I complexes (Ref. 42; authors' unpublished results). A significant fraction of zeaxanthin may also be formed in the chloroplast envelope [43]. Clearly, zeaxanthin formed within PS I and the envelope is not expected to contribute to energy dissipation at PS II.

Some speculation concerning the physiological significance of the changed dependence of q_E upon ΔpH is warranted. Such light-induced amplification of q_E means that large q_E values are possible in the absence of a large pH gradient across the membrane. This is important since there is a potential conflict of interests in photosynthesis with regard to the magnitude of the light-induced ΔpH [44]. Electron transport rates to exogenous acceptors in isolated chloroplasts only approach rates of O_2 evolution commonly observed in vivo if uncoupler is included to lower ΔpH and the accompanying restriction over electron flow [30,33,44]. On the other hand, high q_E can only be observed in chloroplasts in the absence of uncoupler. Maximal rates of ATP synthesis might also be expected to require relatively high ΔpH. Such potentially paradoxical requirements can be reconciled as far as ATP synthesis is concerned by recourse to the fact that reduction of CF_1 allows this enzyme to remain active and able to catalyse high rates of ATP synthesis at lower ΔpH than that required by the oxidised enzyme [45,46]. Such reduction occurs upon illumination of leaves [47] or intact algae [48] and is thought to be due to the in vivo reductant thioredoxin. We propose that illumination of leaves allows a physiologically analogous mechanism to operate for energy dissipation, such that high q_E can be formed in the steady state without the need for ΔpH of a magnitude prohibitive to the operation of high rates of electron transport. Recent data in leaves is consistent with the saturation of steady-state ΔpH at low light intensities [49], whereas the steady-state level of rapidly-relaxing fluorescence quenching (q_E) only becomes saturated in leaves at higher light intensities [1,2]. If this is so, the extent of q_E must be controlled by a factor or factors additional to the magnitude of ΔpH. If, as suggested here, the primary physiological function of zeaxanthin is to act as a 'quenching amplifier', such additional control would be exerted by the zeaxanthin content of the leaf PS II pigment bed. Also, for chloroplasts containing a constant amount of zeaxanthin in the steady-state, the steeper relationship between q_E and ΔpH observed in light chloroplasts containing zeaxanthin will allow finer control over q_E by ΔpH. In the presence of zeaxanthin, large changes in q_E can be produced by smaller changes in ΔpH than in the absence of zeaxanthin.

Acknowledgements

We thank Dr. George Britton for helpful discussion and for the use of HPLC facilities for pigment analysis. G.N. and D.R. were supported by SERC and AFRC grants to P.H.

References

1 Quick, W.P. and Stitt, M. (1989) Biochim. Biophys Acta 977, 287–296

2 Walters, R.G. and Horton, P. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 1, pp. 631–634, Kluwer, Dordrecht.

3 Horton, P. and Hague, A. (1988) Biochim. Biophys. Acta 932, 107–115.

4 Quick, W.P. and Horton, P. (1984) Proc. R. Soc. Lond. B. 220, 371–382.

5 Krause, G.H., Vernotte, C. and Briantais, J.-M. (1982) Biochim. Biophys. Acta 679, 116–124.

6 Briantais, J.-M., Vernotte, C., Picaud, M. and Krause, G.H. (1979) Biochim. Biophys. Acta 548, 128–138.

7 Krause, G.H. and Behrend, U. (1986) FEBS Lett. 200, 298–302.

8 Weis, E. and Berry, J.A. (1987) Biochim. Biophys. Acta 894, 198–208.

9 Krause, G.H. and Laasch, H. (1987) Z. Naturforsch. 42, 581–584.

10 Krause, G.H., Laasch, H. and Weis, E. (1988) Plant Physiol. Biochem. 26, 445–452.

11 Genty, B., Briantais, J.-M. and Baker, N.R. (1989) Biochim. Biophys. Acta 990, 87–92.

12 Horton, P., Noctor, G. and Rees, D. (1990) in Perspectives in Biochemical and Genetic Regulation of Photosynthesis (Zelitch, I., ed.), pp. 145–158, Alan R. Liss, New York.

13 Demmig, B., Winter, K., Kruger, A. and Czygan, F.-C. (1987) Plant Physiol. 84, 218–224.

14 Demmig, B., Winter, K., Kruger, A. and Czygan, F.-C. (1988) Plant Physiol. 87, 17–24.

15 Siefermann, D. and Yamamoto, H.Y. (1975) Arch. Biochem. Biophys. 171, 70–77.

16 Yamamoto, H.Y. (1979) Pure Appl. Chem. 51, 639–648.

17 Siefermann, D. and Yamamoto, H.Y. (1975) Biochem. Biophys. Res. Commun. 62, 456–461.

18 Hager, A. (1967) Planta 89, 224–243.

19 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 887–893.

20 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 894–898.

21 Demmig-Adams, B., Winter, K., Kruger, A. and Czygan, F.-C. (1989) Plant Physiol. 90, 881–886.

22 Yamamoto, H.Y. and Kamite, L. (1972) Biochim. Biophys. Acta 267, 538–543.

23 Demmig-Adams, B., Adams, W.W., Heber, U., Neimanis, S., Winter, K., Kruger, A., Czygan, F.-C., Bilger, W. and Bjorkman, O. (1990) Plant Physiol. 92, 293–301.

24 Bilger, W., Bjorkman, O. and Thayer, S.S. (1989) Plant Physiol. 91, 542–551.

25 Adams, W.W., Demmig-Adams, B. and Winter, K. (1990) Plant Physiol. 92, 302–309.

26 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 634–70.

27 Kraayenhof, R. and Arents, J. (1977) in Electrical Phenomena at the Biological Membrane Level (Roux, E., ed.), pp. 493–505, Elsevier, Amsterdam.

28 Pick, U. and McCarty, R.E. (1980) Methods Enzymol. 69, 538–546.

29 Rees, D., Young, A., Noctor, G., Britton, G. and Horton, P. (1989) FEBS Lett. 256, 85–90.
30 Noctor, G. and Horton, P. (1990) Biochim. Biophys. Acta 1016, 228–234.
31 Walker, D.A. (1980) Methods Enzymol. 69, 94–104.
32 Lilley, R.M^{c}C., Fitzgerald, M.P., Rienits, K.G. and Walker, D.A. (1975) New Phytol. 75, 1–10.
33 Rees, D. and Horton, P. (1990) Biochim. Biophys. Acta 1016, 219–227.
34 Barry, P., Young, A.J., and Britton, G. (1990) J. Exp. Bot. 41, 123–129.
35 Butler, W.L. and Kitajima, M. (1975) Biochim. Biophys. Acta 376, 116–125.
36 Rees, D., Noctor, G. and Horton, P. (1990) Photosynth. Res. 25, 199–211.
37 Oxborough, K. and Horton, P. (1988) Biochim. Biophys. Acta 934, 135–143.
38 Noctor, G., Rees, D. and Horton, P. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 1, pp. 627–630, Kluwer, Dordrecht.
39 Laasch, H. and Weis, E. (1988) Biochim. Biophys. Acta 936, 99–107.
40 Laasch, H. and Weis, E. (1989) Photosynth. Res. 22, 137–146.
41 Gilmore, A.M. and Yamamoto, H.Y. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 2, pp. 495–498, Kluwer, Dordrecht.
42 Siefermann-Harms, D. (1984) Photochem. Photobiol. 40, 507–512.
43 Siefermann-Harms, D., Joyard, J. and Douce, R. (1978) Plant Physiol. 61, 530–533.
44 Horton, P. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. 4, pp. 111–118, Kluwer, Dordrecht.
45 Mills, J.D. and Mitchell, P. (1984) Biochim. Biophys. Acta 764, 93–104.
46 Junesch, U. and Graber, P. (1985) Biochim. Biophys. Acta 809, 429–434.
47 Vallejos, R.H., Arana, J.L. and Ravizzini, R.A. (1983) J. Biol. Chem. 258, 7317–7321.
48 Noctor, G. and Mills, J.D. (1988) Biochim. Biophys. Acta 935, 53–60.
49 Harbinson, J. and Hedley, C.L. (1989) Plant Cell Environ. 12, 357–369.