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The xanthophyll cycle and carotenoid-mediated dissipation of excess excitation energy in photosynthesis

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Abstract: Light is a major stress factor in plants and algae, resulting in photoinhibition and photooxidation in photosynthetic tissues, with a concomitant loss in productivity. Protection from such light-mediated stress is therefore of key importance. Recent studies have determined a clear correlation between the dissipation of excess excitation energy and the formation of zeaxanthin from violaxanthin in the light-harvesting complexes of plants and some algae. Although the mechanism of carotenoid-mediated energy dissipation is still not fully understood, two main models have been proposed: the "Molecular Gear Shift Model" (singlet-singlet energy transfer from chlorophyll to carotenoid) and the "LHCII Model" (carotenoid-mediated changes to light-harvesting complex organisation leading to the formation of a 'quencher'). The main features of these two models are discussed.

INTRODUCTION

Carotenoids have a number of functions in photosynthetic systems: they act as (i) accessory light-harvesting pigments, (ii) quenchers of triplet state chlorophyll (Chl), and (iii) quenchers of singlet oxygen ($^1O_2$). These roles have been well documented (e.g. refs. 1-3). Studies on plastid biogenesis and *in vitro* reconstitution studies have also identified a key role for carotenoids in the structure/organisation of the photosynthetic apparatus (ref. 4). Some xanthophylls have further been implicated in the non-photochemical quenching of Chl fluorescence in plants and some algae, an important photoprotective process (ref. 5). The role of xanthophylls in this process, resulting in dissipation of excess excitation energy *via* quenching of Chl fluorescence, is a feature of the interconversion of carotenoids due to the xanthophyll cycle. This article will not cover other, more general, features of the xanthophyll cycle which are already well-documented (refs. 5-7) but will concentrate on the possible mechanism(s) of carotenoid-mediated quenching of Chl fluorescence.

THE XANTHOPHYLL CYCLE

The interconversion of violaxanthin ((3*S*, 5*R*, 6*S*, 3'*S*, 5'*R*, 6'*S*)-5,6;5',6'-diepoxy-5,6,5',6'-tetrahydro-ß,ß-carotene-3,3'-diol), antheraxanthin ((3*S*, 5*R*, 6*S*, 3'*R*)-5,6-epoxy-5,6-dihydro-ß,ß-carotene-3,3'-diol) and zeaxanthin ((3*R*, 3'*R*)-ß,ß-carotene-3,3'-diol) is commonly referred to as the 'violaxanthin cycle' or 'xanthophyll cycle' (Fig. 1). The violaxanthin content of leaves can be decreased by transfer into a high photon flux density (PFD) due to its de-epoxidation into zeaxanthin *via* antheraxanthin. This is reversed when the leaf is transferred back to a low PFD or to darkness. A different xanthophyll cycle, involving diadinoxanthin ((3*S*, 5*R*, 6*S*, 3'*R*)-5,6-epoxy-7',8'-didehydro-5,6-dihydro-ß,ß-carotene-3,3'-diol) and diatoxanthin ((3*R*, 3'*R*)-7,8-didehydro-ß,ß-carotene-3,3'-diol), is present in some groups of microalgae (Fig. 1). The one-step de-epoxidation of diadinoxanthin into diatoxanthin appears to serve the same function as the two-step de-epoxidation of violaxanthin into zeaxanthin does in higher plants (refs. 8-9).

A number of roles have been proposed for the xanthophyll cycle over the past 20-30 years (ref. 7), but it was not until 1987 that Demmig *et al.* (ref. 10) first proposed a now widely accepted role linking zeaxanthin formation with photoprotection. When photosynthetic tissues are exposed to light levels that are in excess of those capable of being utilised with maximum quantum yield, a process of non-photochemical thermal dissipation of the excess absorbed photons occurs. This permits short-term adaptation to changes in light intensity, allowing protection of the photosynthetic membrane against damage. This process is referred to as non-photochemical quenching of Chl fluorescence, qN (refs. 11-12).

There are three important features of qN (refs. 11, 13): (i) the major part is induced as a result of the acidification of the thylakoid lumen associated with the formation of the proton motive force (qE); (ii) qE is a process by which energy is dissipated in the light-harvesting system of photosystem II; (iii) the formation of qN is correlated with the formation of zeaxanthin as a result of de-epoxidation of violaxanthin *via* the xanthophyll cycle. This correlation has been found to hold true for a wide range of environmental conditions (water stress, extremes of temperature etc.) over a wide range of PFDs (refs. 5-6). The role of zeaxanthin in qN has been further strengthened by the use of dithiothreitol (DTT) which inhibits violaxanthin de-epoxidation. In DTT-

treated leaves and in chloroplasts isolated from these leaves (in which zeaxanthin formation has been prevented) a large proportion of qN is inhibited.

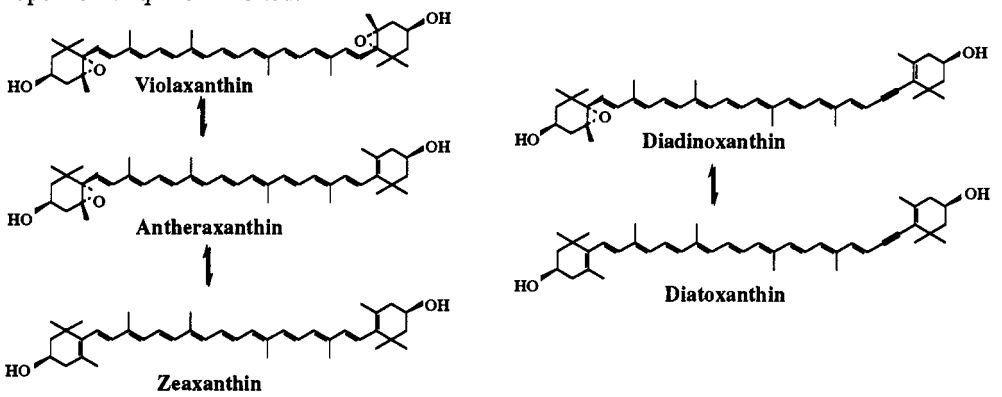

Fig. 1. Structures of the xanthophyll-cycle carotenoids.

In order to understand the role that the xanthophyll cycle carotenoids might play in photosynthesis it is important to establish their location and hence their possible site(s) of action. Unfortunately the electron crystallography study of higher plant LHCIIb has provided no information on the location of any xanthophylls other than lutein, but it does suggest that violaxanthin occupies a position on the periphery of the complex (ref. 14). High levels of de-epoxidation can be achieved when thylakoids are unstacked in the presence of Mg^{2+} (ref. 15), which supports the idea that violaxanthin availability may depend on the organisation of the complexes and suggests that it occupies a peripheral site in the LHC.

The bulk (>50%) of violaxanthin of PSII is associated with LHCIIb which binds 1.0 molecule of violaxanthin per trimer (refs. 16-17). Significant zeaxanthin formation has been observed for LHCIIb and this suggests that violaxanthin ↔ zeaxanthin interconversion in this complex regulates qE. In contrast, the minor LHCII complexes (a, c and d) are specifically enriched in violaxanthin (refs. 16-19). Each minor complex binds one violaxanthin per monomer (ref. 17) and recent evidence from DCCD-binding studies supports the view that these minor complexes are critical in the quenching process (ref. 20). The de-epoxidation states ([Z + 0.5A]/[Z +A + V]) of LHCIIb, c and d are similar to that reported for the thylakoid preparation from which they were isolated, but the de-epoxidation state of LHCIIa appears to be much lower by comparison (ref. 17). It is also crucial to consider the ratio of xanthophyll-cycle carotenoid : Chl for each complex. This applies whether quenching arises *via* a direct, singlet-singlet, interaction between carotenoid and Chl, or by an 'indirect' (amplification) action through carotenoid-mediated alterations to LHC organisation (see below). This ratio is consistently highest in the minor complexes and lowest in LHCIIb (refs. 16-18), providing further support for an important role for carotenoids in these minor complexes in controlling qE.

CAROTENOID STRUCTURE

The interconversion of violaxanthin ↔ zeaxanthin and diadinoxanthin ↔ diatoxanthin alters the extent of the conjugated double bond system as a result of the epoxidation and de-epoxidation reactions. The implications of this are: (i) the extent of the conjugated system in carotenoids affects both the energies and lifetimes of their excited states, from 9 to 11 C-C double bonds in violaxanthin and zeaxanthin, respectively (see below); (ii) for carotenoids in which the end-groups are in conjugation with the main polyene chain, a coplanar conformation is energetically favoured. In zeaxanthin, steric hindrance prevents it from being fully coplanar and a near-planar conformation is adopted. In contrast, in carotenoids such as violaxanthin, in which the conjugation is removed (by the presence of epoxide groups in the C5,6 position) the end-group occupies a perpendicular position relative to the main chain. Such conformational changes in the carotenoid molecule may, in turn, affect the organisation of the LHC (see below).

POSSIBLE MECHANISMS OF FLUORESCENCE QUENCHING

Although the operation of the xanthophyll cycle in plants and algae is now well documented (see above), the nature of the molecular mechanism whereby zeaxanthin mediates quenching of Chl fluorescence is still subject to considerable debate. Two main models have been suggested: (i) a direct carotenoid-Chl interaction involving singlet-singlet energy transfer resulting in quenching of Chl fluorescence and dissipation of excitation energy; (ii) an indirect quenching process involving carotenoid-mediated changes in the structure/organisation of the light-harvesting complexes.

(i) Singlet-singlet energy transfer

The photochemical and spectroscopic properties of carotenoids are derived from their low-lying energy states (see refs 21-22). The low-lying singlet states of carotenoids are denoted the 1^1B_u (S_2) and the 2^1A_g (S_1) states (Fig. 2). The energies and lifetimes of these singlet states are important in their roles in photosynthetic

systems. An electronic transition from the ground state (1^1A_g or S_0) to the S_2 state (which has B_u symmetry) gives rise to the familiar visible absorption spectra of carotenoids. The energy of the S_2 state can be readily determined from the carotenoid absorption spectrum and has been shown to be dependent upon the extent of π-electron conjugation of the carotenoid: an increase in conjugation results in a decrease in S_2 energy. The extremely fast rate of internal conversion between S_2 and S_1 has led researchers to focus on the role of the S_1 state in energy transfer. However, it has only recently become possible to determine these S_1 energies accurately. For a few carotenoids (with fewer than 9 conjugated double bonds) this has been done by measurement of their weak fluorescence spectra (due to the $S_1 \rightarrow S_0$ transition; refs. 23-25). Dual emission can be observed for compounds with eight and nine conjugated double bonds but, as the length of the conjugated system increases further, emission from the $S_2 \rightarrow S_0$ electronic transition dominates (ref. 23). The majority of carotenoids in photosynthetic systems have more than nine conjugated double bonds and, as a result, the role of this S_1 state in energy transfer to and/or from Chl has been difficult to establish. Locating the 2^1A_g states in long chromophore carotenoids such as ß-carotene has generally relied on the extrapolation of data obtained from either the $S_1 \rightarrow S_0$ fluorescence spectra of shorter polyenes (refs. 24-25), or the lifetimes of the S_1 states (refs. 21, 26-27) in conjunction with the energy gap law for radiationless transitions (ref. 28).

Frank and colleagues (ref. 21) determined the S_1 energy levels of the xanthophyll-cycle carotenoids by measuring the dynamics of shorter chromophore carotenoids ($n \leq 9$) which fluoresce directly from their S_1 state and then using the dynamics of the longer chromophore carotenoids to deduce their S_1 energies by extrapolation. The S_1 lifetimes and energies for 3,4,7,8-tetrahydrospheroidene, 3,4,5,6-tetrahydrospheroidene and 3,4-dihydrospheroidene were used to apply the energy-gap law (ref. 28) to the xanthophyll cycle carotenoids. The lifetimes τ = 23.9 ps for violaxanthin, τ = 14.4 ps for antheraxanthin and τ = 9.0 ps for zeaxanthin were used to calculate energies for the S_1 levels (τ is the lifetime of the S_1 state). The precision in these values was later improved by explicitly considering the full functional dependence on ΔE of the energy gap law expression (ref. 26), including the recently determined S_1 energy of ß-carotene (14,200-14,500 cm^{-1}; ref. 29). The energies for the xanthophyll cycle carotenoids are therefore revised to 15,290 cm^{-1}, 14,720 cm^{-1} and 14,170 cm^{-1} for violaxanthin, antheraxanthin and zeaxanthin, respectively. In comparison, the energy of the lowest excited singlet state of Chl *a* (denoted S_1) in the LHC of PSII has been estimated to be ~14,700 cm^{-1} (based on a maximum fluorescence of 680 nm; ref. 30).

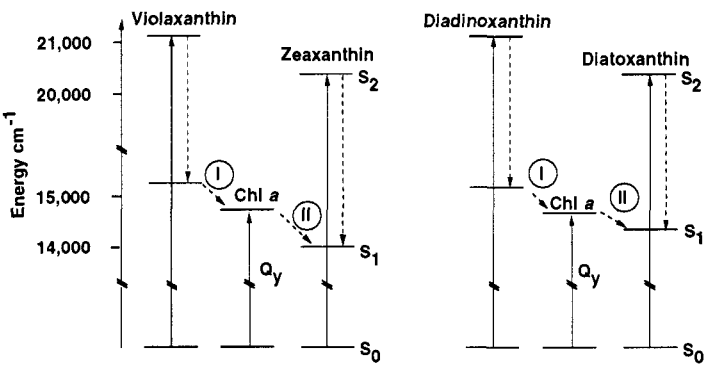

Fig. 2. The "Molecular Gear Shift" model showing the positions of the S_1 energies of the xanthophyll cycle carotenoids relative to Chl *a*: (A) violaxanthin and zeaxanthin (B) diadinoxanthin and diatoxanthin. I - light-harvesting, II - photoprotection. Antheraxanthin (not shown) is isoenergetic with Chl *a*; (see refs. 21, 31 for full details).

The significance of these values is clear from Fig. 2; the value of 14,700 cm^{-1} for Chl *a* is lower than that determined for the S_1 state of violaxanthin but higher than that of zeaxanthin. Thus, it is energetically possible for the S_1 state of zeaxanthin to quench Chl fluorescence *via* deactivation of the Chl excited singlet state. In contrast, the higher S_1 value obtained for violaxanthin would lead it to act preferentially as a light-harvesting pigment, transferring its excitation energy on to Chl *a*. It is possible that zeaxanthin may also function as a light-harvesting pigment using energy transfer from its S_2 state to Chl. The role of antheraxanthin is not clear as its S_1 energy is isoenergetic with that of Chl *a*, but it is possible that it may quench Chl fluorescence. The term 'Molecular Gear Shift' has been used to describe this process; at high PFDs when the dissipation of excess excitation energy is required, zeaxanthin is formed which serves to deactivate the excited singlet state of Chl *a* (resulting in a reduction in Chl fluorescence) and dissipate excitation energy harmlessly as heat (ref. 21). A similar relationship has been found for diatoxanthin and diadinoxanthin; their S_1 energies also occupy positions above and below that of the Q_y of Chl *a* , respectively (ref. 31). Thus it is energetically feasible for the S_1 state of diatoxanthin to quench the singlet excited state of Chl *a* but it is much less likely that diadinoxanthin would perform this same function (Fig. 2). These data support the notion that the enzymic de-epoxidation/epoxidation reactions of the xanthophyll cycle act as regulators of energy flow *in vivo*.

It has long been known that carotenoids quench Chl fluorescence: (i) ß-carotene in Chl *a*/benzene solution quenches Chl fluorescence (ref. 32); (ii) ß-carotene derivatives quench porphyrin fluorescence in covalently-linked synthetic carotenoporphyrins (ref. 33); (iii) quenching of Chl *a* fluorescence by ß-carotene in nematic

liquid crystals has been observed and results from the interactions between pigment molecules (ref. 34). A recent study suggests that two different types of quenching process may occur in Chl *a*/carotenoid/benzene solutions (ref. 35). The first occurs at low concentrations of the carotenoid and involves the formation of a complex or aggregate between the different pigment molecules; quenching was found to be dependent on the structure of the carotenoid. The second relates to the probability of carotenoid-to-Chl encounters at higher concentrations of carotenoid and gave rise to linear Stern-Volmer plots. The extent of this type of quenching may depend on the overlap between the Chl *a* fluorescence and the carotenoid absorption spectra. The data obtained showed zeaxanthin to be a slightly better quencher of Chl *a* fluorescence than violaxanthin. This may be attributed to the reduced spectral overlap between the Chl *a* fluorescence and the violaxanthin $S_0 \rightarrow S_1$ absorption compared to that for zeaxanthin. The spectral overlap of violaxanthin, however, is expected to be less owing to the fact that its S_1 state is thought to be higher in energy than that of zeaxanthin by ~1,200 cm^{-1}. The data did not show unambiguously that zeaxanthin was capable of preferentially quenching Chl *a* fluorescence *in vitro* and could not account for the correlation between zeaxanthin content and fluorescence quenching seen *in vivo*. Diadinoxanthin is 1.7 times more likely to transfer energy from its S_1 state to Chl *a* than is diatoxanthin. Conversely, it was estimated that Chl *a* would be 0.6 times less likely to transfer its energy uphill to diadinoxanthin than downhill to diatoxanthin (ref. 31). A similar trend would apply for violaxanthin and zeaxanthin.

It is important to emphasise that there is, as yet, no direct evidence to show that singlet-singlet energy transfer from Chl to carotenoid occurs *in vivo*. The data only suggest that such a direct quenching process may indeed be a possible route of deactivation. The interconversion of zeaxanthin ↔ violaxanthin would not act as an 'on/off' switch for light-harvesting/photoprotection *via* singlet energy transfer, but rather (due to changes in spectral overlap between carotenoid and Chl) one process would be preferred over the other.

(ii) Carotenoid-mediated alterations to LHC organisation

The LHCII Model

It has been suggested that zeaxanthin formation may serve simply to amplify fluorescence quenching in the LHC rather than acting as the sole driving force as suggested above (ref. 36). One of the main sources of experimental evidence to support this is that zeaxanthin is not required for qE *per se* (ref. 37). In fact, the zeaxanthin-associated quenching seen *in vivo* shares similar spectroscopic features with quenching brought about by LHCII aggregation. This suggests that qE occurs *via* a common mechanism. qE requires a ΔpH, and a number of studies (see ref. 13) have demonstrated 'light-activation' of such pH-dependent quenching so that quenching could be achieved at a lower ΔpH in thylakoids and chloroplasts in the presence of zeaxanthin than in its absence. Such 'light-activation' suggests an indirect role for the xanthophyll-cycle carotenoids in controlling qE. This has led to the development of an allosteric LHCII model for zeaxanthin-mediated regulation of qE (see Fig. 3). LHC aggregation has been shown to be associated with distinct changes in the properties of the bound Chl and carotenoid (ref. 38). The absorption changes associated with qE formation in isolated LHCII (in both the blue and red regions of the spectrum) are similar to the changes seen when both chlorophylls and xanthophylls are aggregated *in vitro* (ref. 39). This suggests that xanthophyll/Chl associations may be formed *in situ* within the LHC, giving rise to the quenched state of the complex. In this model, the carotenoids are proposed to control quenching by a mixture of quenching and anti-quenching effects (see below; ref. 40).

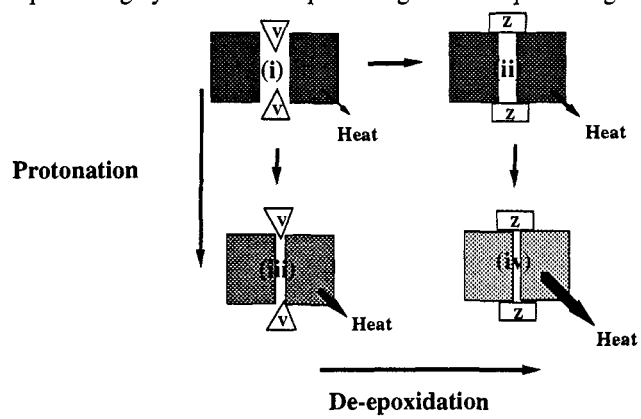

Fig. 3. LHCII model for carotenoid-mediated regulation of non-photochemical quenching (qE). In this model the rate of energy dissipation is controlled by structural changes to LHCII brought about allosterically by de-epoxidation of violaxanthin into zeaxanthin and by protonation. Four states for LHCII have been proposed; the equilibrium between these states depends on the ΔpH and zeaxanthin content: (i) unquenched, unprotonated, binds violaxanthin; (ii) slightly quenched (zeaxanthin/Chl), unprotonated, binds zeaxanthin; (iii) quenched (Chl/Chl), protonated, violaxanthin displaced from its binding site; (iv) highly quenched (zeaxanthin/Chl), protonated, binds zeaxanthin (see ref.13).

Carotenoids as anti-quenchers

The effect of exogenous pigments on quenching in the native bulk and minor LHC has been studied (refs. 41-43). These studies have demonstrated that the addition of violaxanthin and zeaxanthin affected both the aggregation state of the complex (as determined by sucrose-gradient centrifugation) and fluorescence quenching. The addition of violaxanthin inhibited both aggregation of LHC and quenching; this carotenoid could in fact be considered to be acting as an "anti-quencher". In contrast, zeaxanthin acted to stimulate both LHC aggregation and quenching. This effect was most pronounced in the minor complexes, again highlighting their possible key role in qE (ref. 43). These data are consistent with the LHCII model in which the roles of violaxanthin and zeaxanthin are to inhibit or stimulate aggregation of LHCII, respectively. A key observation was the pH-dependence of this effect *in vitro*, adding further support to the 'light-activation' (i.e. lowering of the pH requirement) of quenching by stimulating the formation of LHCII aggregates.

The effect of a range of carotenoids of different structures and S_1 energies on the extent of spontaneous quenching of Chl fluorescence in native LHCIIb has also been examined. From Fig. 4A. it is clear that only carotenoids with S_1 energies lower than that of Chl *a* (14,700 cm^{-1}) quench fluorescence, whilst carotenoids with higher S_1 energies have little, if any, effect. The transition from a 'quencher' to a 'non-quencher' is related to whether the carotenoid S_1 energy lies either below or above that of Chl *a*, respectively, and it is clear why violaxanthin and zeaxanthin play such an important role in photosynthetic systems. However, at a reduced pH (which itself induces strong quenching in LHCIIb; Fig. 4B), carotenoids with S_1 energies higher than the Q_y of Chl *a* (e.g. violaxanthin) cause a significant inhibition of quenching. At intermediate pHs quenching inhibition is less pronounced and a stimulatory effect of the lower-energy carotenoids more evident. This reveals a synergism between pH and carotenoid, similar to that observed *in vivo*, and it is difficult to explain inhibitory actions of carotenoids purely on the basis of their singlet-state energies.

The addition of carotenoid to LHC *in vitro* may disrupt the existing Chl-Chl interactions seen at low pH and which are responsible for quenching. This would result in a rise in Chl fluorescence, i.e. anti-quenching. For carotenoids of sufficiently low S_1 energies, this effect would compete with their ability to quench Chl fluorescence by singlet-singlet de-excitation. It has been suggested that carotenoids may actually act as "anti-quenchers" by preventing Chl-Chl interactions (ref. 44). Such behaviour has also recently been demonstrated *in vitro* in organic solvents for ß-carotene-mediated quenching of Chl fluorescence when the addition of carotenoid initially de-aggregates Chl, resulting in a rise in fluorescence (ref. 35).

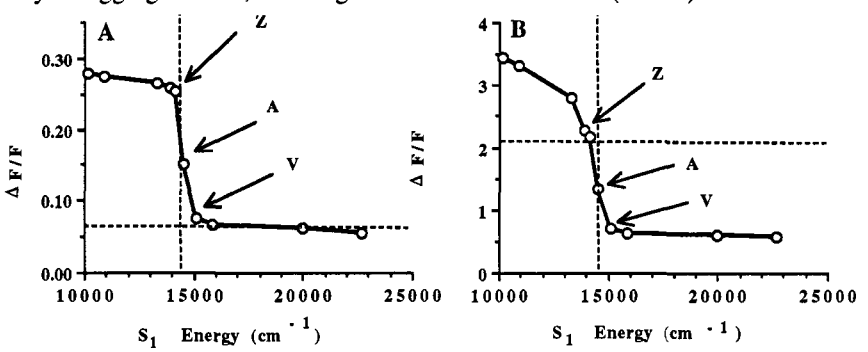

Fig. 4. Quenching of chlorophyll fluorescence (ΔF/F) in isolated spinach LHCIIb at (A) pH 7.8 and (B) pH 5.5 by exogenous carotenoids. Each point is a separate carotenoid of known S_1 energy. The vertical line shows the position of the Qy band of Chl *a* and the horizontal line the level of quenching in the control (solvent only). Values lying above this line indicate stimulation of quenching and those below an inhibition of quenching. The data are the means of 3-5 replicates (ref. 42).

The inhibitory effect of violaxanthin on quenching leads to the conclusion that physico-chemical factors other than S_1 energy (e.g. ring-to-chain conformation) must be involved in controlling qE. In addition, violaxanthin was the only carotenoid (of 19 tested) that was able to reverse dibucaine-induced quenching in isolated LHCIIb, suggesting a specific role for this carotenoid in binding to LHCIIb. Although the mechanism whereby dibucaine mediates such quenching is still unclear, both low pH and dibucaine have been shown to be linked to aggregation of the complex (ref. 41). This behaviour of violaxanthin is clearly unrelated to its S_1 energy and it has been suggested that this has a significant role in LHCIIb organisation and hence the control of quenching of Chl fluorescence *in vivo*; i.e. it is the removal of violaxanthin (as a result of de-epoxidation) and not zeaxanthin formation *per se* that may be the critical determining factor in controlling quenching, possibly by promoting organisational changes to the LHC.

The process of zeaxanthin-mediated dissipation of excess excitation energy is a key process in the photosynthetic systems of higher plants. The interaction between carotenoid and Chl is undoubtedly complex but it is clear that factors other than direct de-excitation by carotenoids are important in the control of quenching of Chl fluorescence *in vivo*. The data presented here are consistent with the LHCII model (see above): (i) a ΔpH is necessary to bring pigments into close proximity; (ii) violaxanthin acts as an 'anti-quencher', preventing the complex from forming a highly quenched state; (iii) such 'anti-quenching' may be overcome at a high

ΔpH; (iv) when zeaxanthin is present, quenching may be amplified by direct de-excitation.

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