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Hypothesis

Control of the light harvesting function of chloroplast membranes: The LHCII-aggregation model for non-photochemical quenching

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Abstract Dissipation of excess excitation energy within the photosystem II light-harvesting antenna (LHCII) by non-photochemical quenching (NPQ) is an important photoprotective process in plants. An update to a hypothesis for the mechanism of NPQ [FEBS Letters 292, 1991] is presented. The impact of recent advances in understanding the structure, organisation and photophysics of LHCII is assessed. We show possible locations of the predicted regulatory and quenching pigment-binding sites in the structural model of the major LHCII. We suggest that NPQ is a highly regulated concerted response of the organised thylakoid macrostructure, which can include different mechanisms and sites at different times.
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Keywords: Non-photochemical quenching; Light harvesting complex; Thylakoid membrane; Chlorophyll fluorescence; Xanthophyll cycle; Photoprotection

1. Introduction

In higher plants and most algae, non-radiative dissipation of excess excitation energy protects the photosynthetic membranes by moderating deviations in the redox state of the electron carriers and reducing the rate of unwanted photooxidations by excited state chlorophylls [1,2]. The increase in non-radiative dissipation, detected as the non-photochemical quenching of chlorophyll fluorescence (NPQ), is a feedback regulatory mechanism induced upon exposure to a photon flux density in excess of that which can be used with maximum quantum yield by photosystem II (PSII). It consists of qE, which forms and relaxes (in the dark) rapidly, triggered by an increase in the ΔpH across the thylakoid membrane and qI, which has slower kinetics and is larger under higher light intensity. NPQ is correlated with the de-epoxidation of violaxanthin to zeaxanthin via the xanthophyll cycle [3]. In 1991, we proposed the first mechanistic model for NPQ [4]. The purpose of this paper is to re-visit this hypothesis, assessing the extent to which it has been affected by subsequent developments. We show that the recent elucidation of the high resolution structure of LHCII [5–7], together with a greater understanding of the macrostructure of PSII in the thylakoid membrane [8], provides strong support for this hypothesis. However, there are new challenges from data indicating an increased level of NPQ heterogeneity [9], from the genetic dissection of NPQ [2], and from spectroscopic approaches suggesting a possibility of direct involvement of zeaxanthin in the dissipation of chlorophyll excited states [10]. The hypothesis has nine tenets, outlined below.

2. The NPQ hypothesis

2.1. NPQ involves structural changes in the PSII light harvesting antenna

There is strong evidence that qE occurs in the light harvesting antenna of PSII [1] and the association between qE and "light scattering" and other changes in thylakoid ultrastructure implies that qE involves conformational change [4]. The hypothesis proposes that LHCII is the site of qE, the mechanism of quenching involving chl–chl and/or xanthophyll/chl interactions that occur only in a particular conformation. The model describes four different structural/functional states of LHCII (Fig. 1A) – unprotonated and protonated states binding either violaxanthin (vio) or zeaxanthin (zea). The protonation of the vio state and zea state both cause qE, but the pH dependency differs; hence de-epoxidation "activates" qE [11]. The zea-activated state is proposed to represent the qI component of NPQ. It was suggested that the antenna complexes behave like an allosterically regulated multi-subunit enzyme, with the extent of de-epoxidation controlling the affinity of the qE site for protons and the co-operativity of proton binding [12].

2.2. NPQ resembles in vitro quenching occurring upon LHCII aggregation

The fluorescence yield of detergent solubilised LHCII is high, but can be reduced several fold by dilution of the detergent concentration [1]. Quenching does not require oligomerisation of proteins, although quenching was always much larger when aggregates formed [13]. This type of quenching, found in LHCII, CP26 and CP29, was shown to resemble in vivo NPQ in several respects [1,14]. Most importantly, zea increased and vio decreased, the rate of formation of the quenched state and the amount of aggregation. Hence, the 4 states in Fig. 1A were originally proposed to represent different *aggregation* states of LHCII. Because of the complexity of the thylakoid membrane, neither the unquenched (solubilised) or fully

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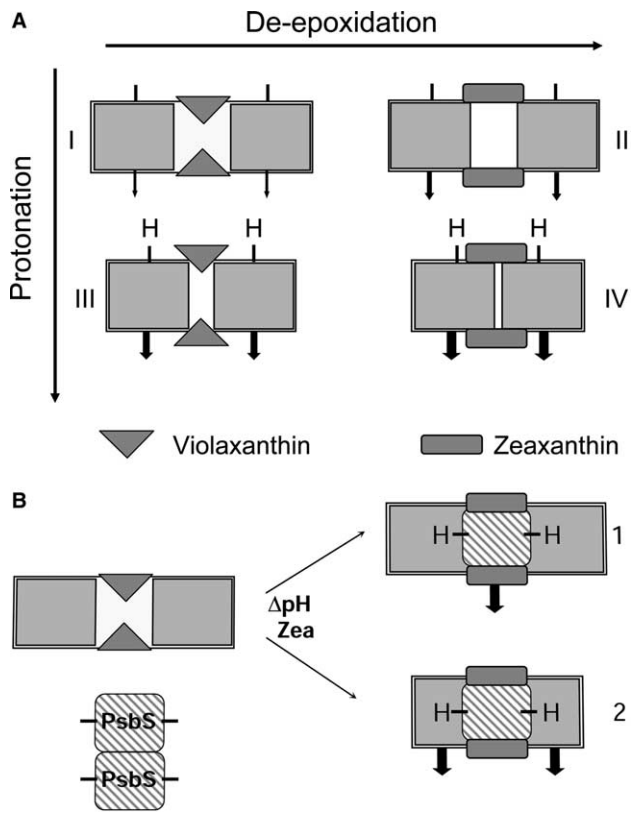

Fig. 1. (A) The LHCII conformation model for NPQ. The model depicts the way in which de-epoxidation and protonation control the conformation of the PSII antenna. The proximity between the inner rectangles represents the extent of conformational change (e.g., the changes in configuration of chl and xanthophyll in LHC that cause energy dissipation), which governs the efficiency of quenching (thickness of arrows). The outer rectangles may refer to a single PSII light harvesting antenna complex, a macrodomain of antenna complexes or a LHCII/PSII megacomplex. States I–IV refer to the difference quenching states – I is the dark-adapted unquenched state and IV is the predominant qE state reached after several minutes of exposure to excess light. State III is the qE state that may arise transiently immediately after illumination before de-epoxidation starts. State II is the "memory" state, found a few minutes after darkening a leaf previously exposed to excess light, and describes the remaining quenching frequently referred to as qI. (B) Possible mechanisms of action of PsbS. In both cases PsbS is considered to bind protons. In pathway 1, protonated PsbS binds zeaxanthin acting directly as a quencher of a chl in the LHCII antenna. In pathway 2, PsbS induces quenching in the antenna indirectly, catalysing the conformational changes between state I and III, and between II and IV as depicted in (A). To carry out this function it may also bind protons and zea, but this is not an obligatory requirement, and it may only have structural role in these transitions.

quenched (aggregated) states can exist in vivo, and NPQ modulates the fluorescence yield in a relatively narrow region between these two extreme states. In fact, high resolution EM [8] and CD spectroscopy [15] have shown a highly integrated in vivo supramolecular organisation of the PSII antenna, in which there are numerous interactions between proteins, giving the possibility that changes in these interactions could be the cause of NPQ. It has also been pointed out that the LHCII–LHCII contacts arising in vitro in aggregates are not possible in vivo [6]; instead, contact with another hydrophobic surface (e.g., lipids or the PsbS protein, see below) could induce the changes equivalent to in vitro "aggregation". Thus, the 4 states represent the different *conformational* states of individual the antenna complexes, caused by changes in their environment arising from protonation and de-epoxidation. Indeed, the recent observation that quenching is found in LHCII crystals with no close contact between pigments or protein shows that energy dissipation is an intrinsic property of individual complexes [7].

2.3. NPQ depends upon the macrostructure of the PSII antenna rather than a single light harvesting protein

It is proposed that qE depends upon the very precise macromolecular organisation of the PSII antenna found in the grana, because this provides the correct molecular environment to allow the necessary conformational changes [16]. Disruptions of this macrostructure, for example, as found in carotenoid biosynthetic mutants, cause reductions in qE [17]. Analysis of mutant plants has shown that no specific light harvesting protein is obligatory for qE [18,19], although conversely removal of particular proteins causes reductions in qE. In *Chlamydomonas* elimination of an LHCII protein was found to strongly inhibit qE [20]. In the absence of the main Lhcb1 and Lhcb2 proteins in *Arabidopsis* qE is formed, although at a reduced level. There is overexpression of the *Lhcb5* gene, partially replacing LHCII with CP26 [21] and the macrostructure of PSII is retained [21].

2.4. The xanthophyll cycle carotenoids are allosteric effectors binding at peripheral allosteric sites on light harvesting complexes

Raman spectroscopy has proved that both vio and zea are bound to protein sites in the membrane, not free in the lipid phase [22]. The major part of the xanthophyll cycle pigments is rather weakly bound to trimeric LHCII [23]. The high resolution structural model of LHCII revealed that the binding site for vio was indeed on the periphery of the complex, the V1 site (Fig. 2, and [5]). Vio is more tightly bound to the monomeric complexes, including CP26 and CP29, and it has been suggested that here it binds at the lutein 2 (L2) site [24]. Alternatively, the de-epoxidisable fraction of vio may bind at the V1 site, whereas only that less efficiently epoxidised is at the L2 site [23]. The location of the zea site(s) is not yet proven. It has been suggested that when vio bound to LHCII is de-epoxidised, the zea replaces the xanthophyll bound to the L2 site in the minor complexes [24]. A simpler explanation is that zea is also bound to peripheral V1 sites, where its affinity is higher than for vio [23].

The configuration of the xanthophyll molecule, rather than the number of conjugated double bonds, is the key difference between them in terms of biological function [25,26], allowing only zea to be "activated" by interactions with itself or with neighbouring molecules. ΔA_{535} is the signature of this activation, a red shift in about 2 zea per PSII [27], possibly arising from head-to-tail dimerisation.

2.5. Quenching occurs by specific interactions between pigments in light harvesting complexes

It is proposed that NPQ arises from new pigment interactions intrinsic to the complex which occur upon a change in protein conformation. These interactions are similar to the phenomenon of "concentration quenching" that occurs whenever the concentration of chlorophyll is high enough that dimers or excited state dimers (excimers) are formed. Efficient

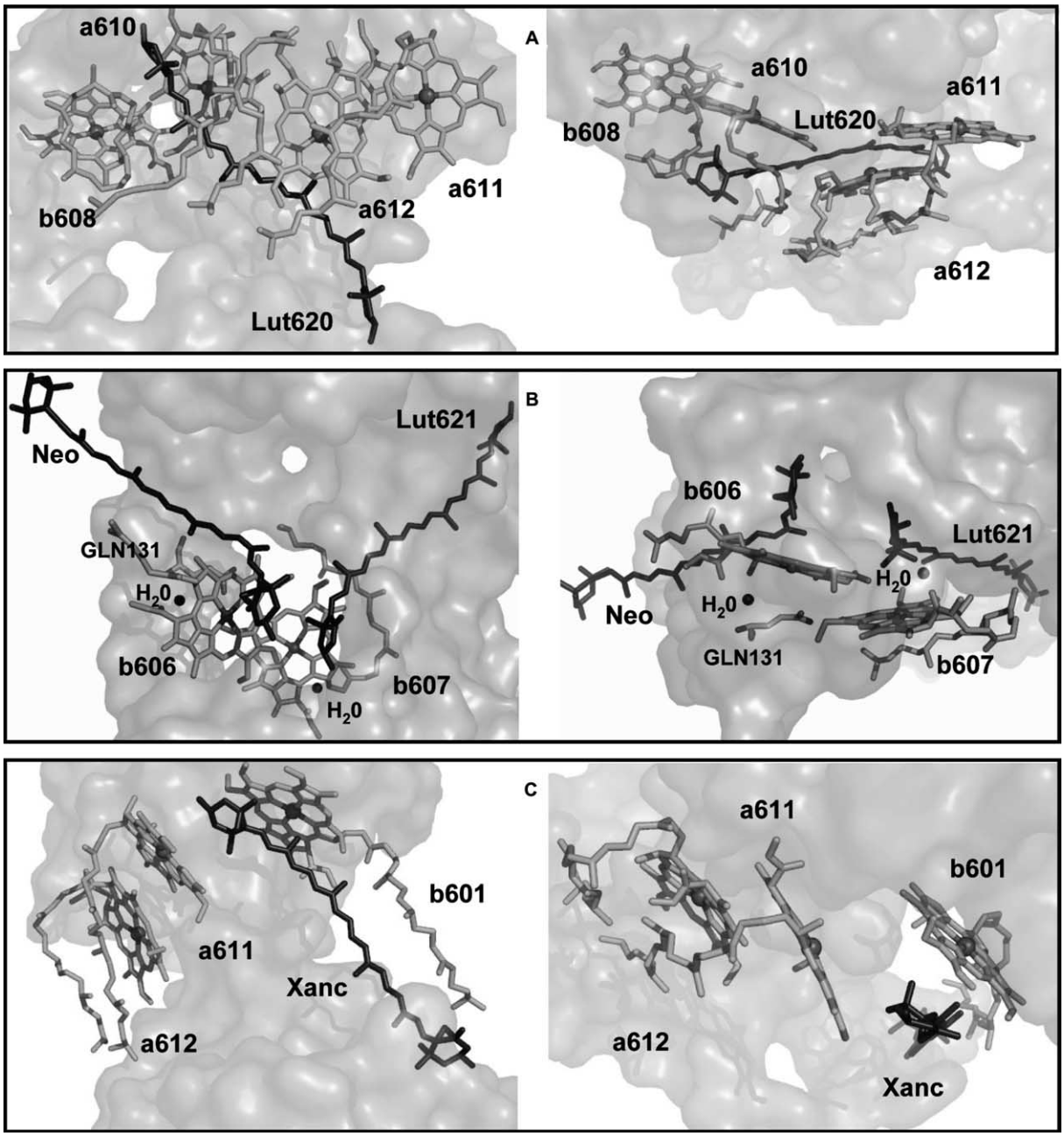

Fig. 2. The possible sites of quenching and allosteric regulation revealed in the structural model of LHCII. (A) The postulated quenching locus in the Lut620 domain for the side (exterior to trimer) and top (stromal) view. Pigment nomenclature is as described by Liu et al. [5], with a610, a611 a612, b608 and Lut620 being equivalent to a1, b2, a2, b1 and Lut1, respectively, in a previous model [26]. Closest pigment–pigment distances are 3.77 Å for a611–a612, 3.61 Å for a610–lut620 and 3.65 Å for a612–lut620. (B) An alternative quenching locus in the b606 and b607 domain, with the closest pigment–pigment distance of 3.5 Å. The ligand of the Mg of b607 is a water molecule, which H-bonds the formyl group of b606. The formyl group of b607 interacts with Gln131, a residue that also forms an H-bond to the coordinating water 310 of b606. Also shown is neoxanthin (Neo), the carotenoid whose configuration changes when LHCII adopts the dissipative state. (C) The postulated allosteric V1 binding site (Xanc), showing its proximity to the putative quenching locus shown in (A). b601, and a613 and a614 (not shown) may participate in direct quenching by zea. Shaded is the protein matrix of LHCII.

light harvesting requires that the configuration and separation of pigments be finely controlled by the protein structure, to prevent energy wastage by concentration quenching. Therefore, if the protein conformation is modified, such dissipative pigment interactions can occur. The second order kinetics of fluorescence decrease suggested that quenching was a bimolecular reaction between two fluorescing molecules or domains [12,14].

Specific changes in configuration of chlorophylls and carotenoids detected by absorption, fluorescence, CD and resonance Raman spectroscopies occur upon quenching (reviewed in [1]). New red-shifted fluorescence bands have been associated with quenching in vitro and in vivo [4,28]. An absorption change at 685–90 nm, possibly arising from the red-shifted terminal emitter chlorophylls of LHCII, correlates with quenching in vitro [29], is seen in plants, but is most pronounced in diatoms,

which have a several-fold higher NPQ capacity [30]. It is proposed that small changes in protein conformation, with low activation energy [31], cause pigments in specific domains to become reversibly configured in a way to favour specific quenching interactions between them – e.g., creation of a chl dimer/excimer or formation of chl–xanthophyll charge transfer states.

Recently, we have shown that the LHCII crystals used to provide the structural model of LHCII are quenched, similar to LHCII aggregates [7] and to 2D crystals [31]. Therefore, it is possible identify the potential site(s) of quenching within this structure. Analysis of the spectroscopic data had previously led to the suggestion that quenching may arise within the "terminal emitter domain" [31]. This domain is clearly seen in the structural model of LHCII, and comprises chls a610, a611 and a612 and lutein 620 (Fig. 2A). Interestingly, a612 is displaced slightly closer to a611 in the LHCII crystals analysed by Kühlbrandt and co-workers [6], which we predict are more highly quenched than those of Liu et al. [7]. Another domain involving chls b606 and b607 can also be highlighted in the structure (Fig. 2B). Raman spectroscopy showed changes in chl *b* interactions in the crystal compared to the trimer and it was suggested that these arise from the formation of the H-bond between water 308 and the formyl group of b606 [7]. This pair of chl molecules could be another site of excitation quenching in LHCII. Moreover, the changes in pigment configuration provide direct evidence for a conformational change in LHCII that is associated with the establishment the quenching state.

The structural model also provides clues about the regulation of quenching. It was suggested that interaction with the second lutein domain provides modulation of quenching via changes in grosser features of LHCII organisation, including protein–protein interactions [32]. The structural model of LHCII reveals that the V1 site is also near to both the two potential quenching domains, giving the possibility of direct communication between these two sites (Fig. 2C). This supports the idea that V1 is the predicted zea allosteric site and that it controls the extent of NPQ by modulation of the conformation of the nearby quenching domain(s).

2.6. Xanthophylls may also participate directly in NPQ

qE can be observed in the absence of zea and therefore zea should be considered only as a modulator of qE. However, it cannot be excluded that it is also more directly involved in the quenching reaction [2,3,10,33]. It has been pointed out that the V1 site is close to a chl domain (a613, a614, b601) in LHCII (Fig. 2C) and this could be the site of quenching [5,6]. Evidence has been obtained for the formation of a chl–zea excited state dimer under qE conditions [33]. A carotenoid radical cation has also been detected, suggesting formation of a charge transfer complex in this putative chl–zea dimer, which could provide a route for excitation energy dissipation [10]. However, the meaning of the correlation between these phenomena and fluorescence quenching is still unclear, and they could merely suggest some alteration in the zea environment in the qE state (such as that responsible for ΔA_{535}). This may be a modification of its binding site or its transfer to a new binding site, resulting in the closer interaction with chl. Similarly, it has not yet been proven that the signals arise from zea rather than from another xanthophyll, such as the lut620 domain of LHCII. Another possibility is that zeaxanthin has a dual role – it may cause (weak) direct quenching at the V1 site (a qI type of quenching – see below) *and* allosteric control of the lut620 domain.

2.7. PsbS is the regulatory subunit of the qE "enzyme"

Mutation of the gene encoding the PsbS protein causes severe disruption of qE [34]. Two lumen-facing glutamic acid residues were identified, whose mutation caused a 50% decrease in NPQ and ΔA_{535} [35]. PsbS may be dimeric in the thylakoid membrane, and undergo a light-induced, ΔpH-dependent monomerisation that promotes its interaction with LHCII [36]. PsbS is an extremely hydrophobic protein, and this property may allow it to bind zeaxanthin, giving rise to the strong red-shift responsible for ΔA_{535} [37]. Indirect evidence that PsbS binds zea is that overexpression of PsbS relieves end-product inhibition of violaxanthin de-epoxidase by zea [38].

It has been proposed that PsbS is the site of quenching (Fig. 1B, pathway 1) – protonation of PsbS induces zea binding, and the PsbS/zea/H^+ complex then directly quenches a component at or near the PSII core antenna [33,35]. Alternatively, PsbS may be a regulatory subunit of the antenna (Fig. 1B, pathway 2) in effect, acting as a qE catalyst, lowering the activation energy for the transition to the quenched state [39]. It may do this by responding to ΔpH and violaxanthin de-epoxidation, sensing protons and acting as a vector concentrating zea at the qE site, or it may only have a structural role. In either case, the central idea is the same – the PsbS/H^+ complex interacts with an LHC protein, promoting the proposed conformational change that forms a quencher.

There are still significant gaps in our understanding of PsbS – principally what proteins it interacts with and where in the thylakoid membrane it is located. It does not form a part of the PSII supercomplex [40], but it may be localised in the peripheral domains, where CP24 and the external LHCII trimers are found. Its association with other proteins may only be transient, depending on the ΔpH for example [36]. Until such details are obtained it is impossible to begin to distinguish between the alternative models for its action.

2.8. Heterogeneity in NPQ arises from a common mechanism at multiple sites

NPQ is heterogeneous, but the delineation of qE and qI is probably an oversimplification. Since qE shows different features under different conditions, or in different organisms, the distinction between qE and qI can be blurred. Modifications to the antenna and reaction centre may be involved in both qE and qI, which have also both been linked to zea formation and protonation. Although previous work provided no evidence that qE occurred by more than one mechanism [1], it has been found that reversible inactivation of PSII charge separation occurred during the transient ΔpH burst that follows the onset of illumination, correlating with qE formation, and suggesting some quenching in the reaction centre [9]. This qE was absent in *npq4* – either quenching was only co-incidentally correlated to PSII inactivation, or the PsbS protein has a role in mediating the low pH inactivation of PSII, i.e., PSII inactivation also results from changes in conformation of the PSII macrostructure. Therefore, perhaps there are multiple protonation and zea binding sites in PSII [1], and/or multiple poten-

tial quenching sites distributed throughout the photosystem. These sites would principally involve pigment binding sites, in which quenching may arise by a mixture of chl–chl and chl–car processes arising from concerted changes in protein conformation. Which are the dominant quenching sites will depend upon the extent of overexcitation, the duration of the period of overexcitation, the previous history of the organism, and its genetic make-up. Critical are the numerous factors (ΔpH, de-epoxidation state, PsbS concentration, light intensity) which catalyse the change from the unquenched to the quenched conformation.

Upon the onset of illumination, the ΔpH immediately senses the balance between irradiance and metabolic capacity. Only low levels of de-epoxidation are present, but the ΔpH may still be sufficient for PsbS-dependent activation of qE. This could be augmented by quenching in the reaction centre [9] through low pH effects on the donor side [41], together with alterations in PSII electron transfer pathway [42]. Here, the state III in Fig. 1, qE may be focussed around the PSII supercomplex. As the xanthophyll cycle becomes fully activated, sensing the longer-term trend in excitation level, a redistribution of quenching sites towards the peripheral LHCII antenna may occur (state IV). As overexcitation persists, or at even higher levels of excess light or low temperature, more sustained responses come into play giving rise to more "qI", depicted collectively by state II, and consisting of a gradient of responses: stable conformational change in antenna proteins [1]; direct quenching by zea bound to the V1 site [5,6]; occupation of internal xanthophyll binding sites in light harvesting complexes by zea [24], and finally, direct effects of light on LHCII promoting the conformational transitions to quenched states [43]. Further redistribution of NPQ sites will result from acclimation of thylakoid composition [44]: an increase in xanthophyll pool size, degradation of LHCII and an increase in the number of PSII centres. In more extreme circumstances, such as in overwintering evergreen plants, very stable, deeply quenched states of the antenna are formed [28], with fluorescence properties very similar to aggregates of LHCII.

2.9. The proposed mechanism of NPQ allows metabolic regulation and ecological adaptation

NPQ is integrated into the physiology and metabolism of plants. The hypothesis predicts that only small changes in ΔpH would be needed to induce qE, allowing optimisation of electron transport and ATP synthesis, and metabolic control of light harvesting [1,4]. Control over ΔpH will be critical [45]: not only does it respond to the demand of carbon assimilation, but it will be modulated by the extent of cyclic electron transfer around PSI, the activation state of the ATP synthase and the partitioning of the protonmotive force between ΔpH and the membrane electrical potential. The hypothesis also explains how NPQ is adapted to the variety of changes in light environment occurring in nature [46]. The ΔpH-dependency of qE allows tracking of short-term changes in irradiance occurring in sunflecks, whilst the de-epoxidation state acts as a molecular memory of the medium-term trends in light conditions. Dynamic acclimation to the long-term trends allow its extent and stability to adjust to whether there is a need for both energy dissipation and photosynthesis (as in saturating light) or only dissipation (as in saturating light in the presence of another stress such as very low temperature). Finally, by genetic variation in the amount and type of PSII antenna proteins, of xanthophylls and of proteins such as PsbS, this simple process of NPQ can be adapted to particular ecological niches – its capacity can be large or small and its kinetics slow or fast.

3. Concluding remarks

The key predictions of the LHCII-aggregation hypothesis for NPQ have been confirmed by much subsequent multidisciplinary experimentation. Although several important issues remain unresolved, the model in Fig. 1 is still a valid description of NPQ and its central theme is upheld – changes in pigment interactions intrinsic to light harvesting complexes, caused by conformational change. NPQ should be considered a fluid and dynamic process that is both dependent upon, and occurs throughout, the LHCII–PSII macrostructure.

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