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Dynamic behaviour of the light harvesting antenna of photosystem II

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Introduction

The light harvesting proteins of higher plants play a vital role in the regulation of photosynthesis. There are large differences in the size and composition of the light harvesting system of PSII (LHCII) depending on plant growth conditions (Bailey et al., 2001), and the individual complexes show striking structural and functional flexibility (Horton et al., 1996). This dynamic behaviour forms a part of key physiological functions of plants that provide adaptation to different environmental conditions (Horton et al., 2001). The purpose of this article is to describe the essential features of the dynamic properties of LHCII, concentrating on the development of the approaches taken in this laboratory over the last 15 years.

The need for control of the light reactions of photosynthesis

The recognition that the light and dark reactions of photosynthesis are tightly coupled (i.e. that there are interactions between light harvesting/electron transport and carbon fixation/metabolism) provided a breakthrough in understanding the effect of environmental factors on chloroplast function (Horton, 1985a). A challenge faced by plants during evolution was how to provide both ATP and NADPH at rates in keeping with the level of sunlight and the capacity for biomass accumulation. Building upon the insight of Osmond (1981), it became clear that whenever light input exceeds the dissipative capacity of electron transport and carbon assimilation, there is an excess of light which can destabilize (by over-reduction and over-energisation) and even damage (by generation of reactive oxygen species) the photosynthetic process. Conversely, it would be an inefficient use of resources to over-invest in photosynthetic capacity when light is limiting. In order to achieve balance and stability of the photosynthetic apparatus, and to enable the plant to capture energy and resources from the environment with maximum effectiveness, regulatory mechanisms are necessary. The plant cell has to assemble a chloroplast with the "correct" composition, and various parts of the photosynthetic process need to be able to adjust their activities in response to internal and external information. Thus the electron transport and carbon metabolism can be considered to be linked by a feed-back and feed-forward control network (Horton, 1985a). Always there is a compromise – between maximizing the collection and utilization of light, and the avoidance of instability when light is in excess. The most appropriate way of looking at the regulatory mechanisms is, given a fixed composition and a fluctuating environment, that they extend the range of conditions over which photosynthesis can remain in balance – they provide homeostasis of excitation energy level, redox state and ΔpH (Horton, 1985b).

Regulation of light harvesting – optimising ΔpH and redox potential

Regulation of light harvesting was first considered in terms of the phosphorylation of LHCII by the redox regulated protein kinase (Horton, 1983). Evidence was obtained that delivery of excitation energy to PSII was reduced by phosphorylation of LHCII. It was found that levels

of phosphorylation were lower at high light compared to low light (Fernyhough et al., 1984), consistent with the results of the deconvolution of the various nonphotochemical quenching (NPQ) processes in whole leaves (Walters and Horton, 1991) and protoplasts (Quick and Horton, 1984; Horton and Hague 1988). As a result of these observations we concluded that LHCII phosphorylation and the state transitions were mechanisms to optimise photosynthetic yield in limiting light, and this included the provision of optimal ΔpH and ATP/NADPH ratios by controlling the pathway of electron transfer (Horton, 1989).

It was apparent from this analysis of NPQ that the major form of quenching was rapidly reversible and sensitive to uncouplers, the so-called ΔpH dependent qE type of quenching. This work also established that over a wide range of conditions that the fluorescence yield was rather constant. It was concluded that all NPQ processes and the photochemical quenching resulting from photosynthetic electron transport caused the lowering of excitation energy levels in PSII, and this established the conceptual framework for regulation (Horton, 1987). When electron transport tends towards saturation and qP decreases, NPQ is induced, giving a homeostasis of the excitation energy density in the PSII antenna, and consequently in the electron transport system. Thus, NPQ processes are regulatory and photoprotective. The state transitions and qE should be regarded as operating together to provide coordinated ΔpHstats and redox potentiostats.

Two extrapolations followed from this rationale. Firstly, the quenching associated with photoinhibition (qI) should be seen as itself providing another level of regulation, rather than being a coincidental result of damage to PSII (Horton, 1987). Indeed, subsequently it has been found that qI has all the features of a regulatory mechanism rather than a damage, with both qE and qI sharing many common features, and perhaps arising from similar molecular mechanisms. Secondly, the acclimation of photosynthesis to irradiance can be simply rationalized. The adjustment of the composition of the photosynthetic apparatus after growth in high light compared to low light strives toward reaching a state of maximum quantum efficiency – maintaining redox potential and ΔpH at the correct level. Thus, across a 10-fold range of growth irradiance we have found that qP measured at the growth irradiance is 0.9 or above, this being achieved by both decreases in light harvesting antenna size and an increase in the capacity for electron transport and carbon assimilation (S. Bailey, unpublished data). As growth irradiance further increases there are increases in the capacity for photoprotection and, eventually, decrease in the chlorophyll content of the leaf to lower light absorption. Even macroscopic events such chloroplast movements or changes in leaf orientation can be viewed in this way - the paraheliotrophic leaf movements of bean result in qP values of 0.9 being recorded at mid-day in full summer sunlight (Pastenes et al., 1998). Thus, plants do whatever they can, by a multitude of mechanisms, to limit the level of excitation energy in the PSII antenna, giving balance with the demands of photosynthesis, and optimising the redox state and ΔpH. At the thylakoid level, qE is the major one of these mechanisms.

Energy dependent NPQ (qE)

Initially we sought to obtain two kinds of information about qE. Firstly, where in PSII it occurred, and secondly the kinetic features of the process, as one might investigate the properties of an enzyme-catalyzed reaction. The fluorescence models developed by Warren Butler 10 years earlier (Butler and Kitajima 1975) were used to attempt to discover whether quenching was occurring in the reaction centre or in the antenna of PSII (Rees et al., 1990). Although qE did not exactly fit an idealized quencher of either type, its behaviour much more closely resembled antenna quenching. Most notably the quenching of the Fo level of fluorescence was symptomatic of an antenna process, and in fact qE was found to quantitatively resemble the quenching observed if an artificial dynamic antenna quencher was added to thylakoid membranes or algal cells. Refinements of the analysis suggested that qE is

best explained by the transition between two states of the PSII antenna, with different rate constants for energy dissipation (Walters and Horton, 1993). Subsequently, analysis of the chl fluorescence lifetimes by Gilmore *et al*. (1995) provided direct support for this suggestion.

We also sought to obtain spectroscopic data to identify the site of quenching. Using the approaches developed by Butler (Butler and Kitajima, 1975) and by Ruban (1991), we found that qE preferentially quenched excitation energy in the LHCII antenna of PSII (Ruban and Horton, 1994). Biochemical evidence was also obtained (see below) – striking dependency on [Mg^{2+}] was observed, inhibitors of qE were found to interact with LHCII proteins and the role of the xanthophyll cycle carotenoids, bound to LHCII, in qE was established. Although the possibility of some part of qE occurring in the RC can not be excluded, the sum of this evidence was that qE is principally occurring in the PSII antenna (Horton et al, 1996).

Simultaneous measurements of qE and the ΔpH in isolated chloroplast allowed titration curves to be constructed, and apparent pK values to be calculated (Noctor and Horton, 1990). Antimycin A was found to inhibit qE without affecting ΔpH (Oxborough and Horton, 1987), its effect later shown as resulting from a shift of the titration curve towards a higher pK (Ruban et al, 2001a). Data obtained from this type of experiment was later to play a major part in shaping our ideas about the mechanism of qE (see below).

The xanthophyll cycle

One of the major advances in understanding the regulation of light harvesting came from the connection made by Demmig-Adams and co-workers between NPQ and the xanthophyll cycle (Demmig-Adams, 1990). A vast amount of data, from a wide variety of plant species under many different environments showed correlations between the extent of NPQ and the de-epoxidation of the xanthophyll cycle pool. This applied both to qE and qI types of quenching. The implication that zeaxanthin was involved in qE has lead to a sustained and often controversial search to uncover the molecular mechanism underlying these correlations. The challenge was to distinguish between the proposal that zeaxanthin was directly involved in quenching Chl excited states by Chl/zea energy transfer (Owens et al., 1992), and the notion that zeaxanthin was working indirectly, modifying or inducing a quenching process intrinsic to the PSII antenna (Horton et al., 1991).

The fact that qE had been observed in dark-adapted chloroplasts which would contain no zeaxanthin suggested that zeaxanthin was not required as an obligatory quencher of excitation energy. In order to try to explain the correlations observed in vivo we compared the qE/ΔpH titration curves for chloroplasts isolated from dark-adapted leaves and from leaves pre-treated to induce maximum conversion of violaxanthin to zeaxanthin. The result was striking - although the maximum qE was almost the same for the two chloroplast preparations, the pKa shifted from near 4.5 in the dark-adapted sample to well over 5.0 in the presence of zeaxanthin (Rees et al., 1989, Noctor et al., 1991). If we assume that the ΔpH in vivo does not fall below 5.5, then it was easy to see how qE and de-epoxidation state could be correlated. The light activation of qE by zeaxanthin also explained how the chloroplast could have a ΔpH high enough in limiting light to allow ATP synthesis without qE, yet in saturating light, how to have maximum electron transport rates and high qE simultaneously. Most significantly, however, these data prompted us to suggest that qE was an allosteric phenomenon - that a protein component of LHCII was under the control of the interacting effects of protonation and zeaxanthin binding, similar to a regulated enzyme (Horton et al., 1991). Later experiments have shown this conclusively, with qE exhibiting cooperativity with respect to H^+ binding, with zeaxanthin both increasing the apparent pK and reducing cooperativity (Ruban et al., 2001a).

The existence of protonation and zeaxanthin (which was known from previous work to be associated with antenna complexes) suggested the notion of multiple states of LHCII (Horton,

1989). Including the phosphorylated state, there could be 8 different states of LHCII. The question was posed whether all these states allowed (e.g. could the apparent complimentary behaviour between qE and qT arise at the level of LHCII). This question is rather complex however, since many different proteins comprise LHCII, not all can be phosphorylated, they bind differing amounts of de-epoxidizable xanthophyll cycle carotenoid, and not all have the DCCD-sensitive sites that may indicate the presence of active protonation residues.

The involvement of changes in conformation

The evidence of allostericity indicated that the key event in qE might be a change in conformation. Many years earlier is was found that the formation of the ΔpH leads to large changes in the structure of the thylakoid membrane, observed as the "light scattering change" around 535 nm (Krause, 1973). Examination of the ΔA_{535} indicated it was well correlated with qE both in leaves (Ruban et al., 1993a) and chloroplasts (Noctor et al., 1993). Interestingly, antimycin blocked ΔA_{535} formation, and the kinetics of formation of qE and ΔA_{535} were similar, and much slower than ΔpH. These data linked ΔA_{535} to qE rather than to the ΔpH directly. The origin of ΔA_{535} has not been elucidated, but we showed that the absorbance changes accompanying qE included changes in the chl and carotenoid absorption bands (Horton and Ruban, 1994). We have suggested that ΔA_{535} may not represent scattering from a macroscopic conformational change, but it may result from an electronic transition of LHCII-bound xanthophyll, possibly zeaxanthin (Ruban et al., 1993b, Noctor et al., 1993).

Quenching in isolated LHCII

Analysis of NPQ *in vivo* did not provide any indication of how the quenching process occurred. It was clear that the mechanism of quenching would only be uncovered after examination of isolated proteins. Our initial observations on the purification of LHCII "rediscovered" the observation of Arnzten and co-workers that this protein displays a remarkable ability to be quenched if the detergent solubilized protein is caused to aggregate (Mullet and Arntzen, 1980). A detailed quantitative analysis of this phenomenon indicated that this quenching was sufficient to explain NPQ *in vivo*, and the sensitivity of this *in vitro* quenching to antimycin suggested that the *in vivo* and *in vitro* quenching processes may be similar (Ruban and Horton, 1992). Strength was added to this assertion when long wavelength Chl forms were identified in both systems under quenched conditions (Ruban et al., 1992a).

The LHCII model for qE

The location of qE in LHCII, the allosteric features of qE kinetics, and the behaviour of isolated LHCII gave rise to a new model for qE (Horton et al., 1991). It proposed that:

1. energy dissipation occurred in one or more of the proteins that constituted the light harvesting system of photosystem II (LHCII);
2. it was induced by a conformational change in one or more of these proteins, possibly involving also interactions between subunits of the LHCII;
3. it was controlled by the synergistic effects of protonation of amino acid residues on these proteins and de-epoxidation of the carotenoid violaxanthin *via* the xanthophyll cycle;
4. energy was dissipated because excitation energy absorbed by LHCII was converted into heat as a result of an altered Chl-Chl interaction in the system, which is known from model systems to strongly decrease the chlorophyll *a* excited state lifetime.

Formally this was model was presented as 4 states of LHCII in which the pKa of the violaxanthin binding form was lower that with bound zeaxanthin (Horton et al., 1991).

Alternatively the model can be represented as just two different states (unquenched and quenched), the latter binding protons and zeaxanthin (Horton et al., 1999). Cooperativity can be introduced into these models by invoking either multiple H^+ binding sites, or interactions between LHCII subunits (Horton et al., 2000). For the latter, the model then becomes recognizable as the classical allosteric representation of Wyman, Changeux and Monod.

Approaches to the experimental investigation of NPQ

A model is useful if it promotes thought, criticism and most importantly new experimentation; the LHCII model for qE has had a major impact on research in this area. We have sought to test the model using several very different approaches.

Development of an in vitro system to study quenching. We have extended the initial work on LHCII aggregation to develop a more refined and stable *in vitro* model in which we demonstrated the effect of zea as a quenching stimulator and viol as a quenching inhibitor (Ruban et al., 1994a; 1996; Philip et al., 1996) and explained this in terms of carotenoid structure (Ruban et al., 1998a). The effects of antimycin A, dibucaine, DCCD and pH were similarly comparably to their effects on *in vivo* qE. Shifts in pK resulting from altered de-epoxidation state were particular impressive demonstrations of the similarity between the *in vitro* and *in vivo* quenching (Ruban and Horton, 1999; Wentworth et al., 2001). The effects of carotenoid were observed whether added exogenously or endogenously by pretreatment of the source leaf material. It was shown that whilst quenching could be induced without causing protein aggregation, conditions which promoted protein oligomerization always brought about an enhancement of quenching (Wentworth et al., 2000). Direct evidence of the ability of zea and viol to control LHCII oligomerization was obtained (Ruban et al., 1997a). Similar behaviour was observed for each of the complexes tested, although CP29 and CP26 showed more exaggerated quenching behaviour than LHCIIb.

Spectroscopic investigation of the quenched state of LHCII.

We have sought to identify the changes in pigment interactions that are the basis of the quenching mechanism by spectroscopic analysis of the quenched and unquenched state of LHCII. Absorption spectra have shown that changes in chl a, chl b and xanthophylls occur upon quenching (Ruban et al., 1996). Resonance Raman spectroscopy (Ruban et al., 1995a) and linear dichroism (Ruban et al, 1997b) have shown changes in pigment configuration, resulting from new interactions, and orientation. Fluorescence spectroscopy at 4K have shown the existence of multiple red shifted spectral forms of Chl in the quenched state (Ruban et al., 1995b). Clearly the changes in pigment properties are significant, and could provide the explanation for quenching, but it has not been possible so far to definitively identify the quenching mechanism.

Investigation of the xanthophyll binding to LHCII. In order to understand the role of the xanthophyll cycle in NPQ it was necessary to determine where viol and zea are located. Using the widely tested methods of preparing chlorophyll protein complexes it was found that LHCIIb bound sub-stoichiometric amounts of these xanthophylls (approx. 0.3 or less mol per monomer) whereas CP29 and CP26 bound approx. 1 mol/monomer (Ruban et al., 1994b). An audit of the xanthophyll composition of PSII based on these binding ratios however indicated much less than that obtained from the composition of PSII membranes. Using very gentle detergent solubilization it was possible to recover all of the xanthophyll cycle carotenoid bound to LHCII (Ruban et al., 1999). It was found that each LHCIIb trimer could bind 3 mols, and that CP29 and CP26 could bind over 1. Our conclusion was that all LHCII components contain a peripheral low affinity binding site for viol, and it is this pigment that is available for de-epoxidation. These sites may not always be occupied depending upon the xanthophyll cycle pool size. On the other hand the tightly bound violaxanthin in CP29 and CP26 appeared

not to be active in the xanthophyll cycle – this carotenoid was assumed to occupy one of the two internal lut sites. There was no evidence of any selection between the LHCII components for de-epoxidation at these peripheral sites, suggesting that the regulatory role of the xanthophyll cycle in NPQ is dispersed throughout the PSII light harvesting system.

Putative proton binding sites on LHCII. The carboxyl modifying reagent DCCD has been widely used to identify functional proton binding amino acids in membrane proteins. Following the report that DCCD binds to an Lhc protein (Jahns and Junge, 1990), we showed that this reagent inhibits qE when incubated with thylakoids (Ruban et al., 1992b). It was subsequently proven that DCCD binds to the LHCII components CP29 and CP26 (Walters et al., 1994). For CP29 there appeared to be two DCCD binding sites, one of which could be removed by mutagenesis of E166, buried in the hydrophobic transmembrane domain (Ruban et al., 1998b). For CP26, DCCD binding occurred at two residues close to the lumen surface (Walters et al., 1996). These sites might be where protons are bound as a first step in the induction of qE, although the data may be explained in other ways – DCCD binding may block the structural changes in the proteins required for quenching.

Kinetic analyses of quenching in leaves, chloroplasts and proteins. The kinetics of a reaction can give clues about the mechanism. We have examined the kinetics of formation of quenching *in vitro* and *in vivo*. Whether qE is measured in leaves, or thylakoid, or whether quenching is measured in isolated LHCII, the decay of fluorescence with time obeyed second order kinetics (Ruban and Horton, 1999). This is a very robust observation – for qE at different irradiances, in the presence of inhibitors and enhancers and at different de-epoxidation states, the kinetics were always second order, only the rate constant and the amplitude varying (Ruban et al., 2001a). *In vitro*, quenching was second order for all complexes tested under all conditions of induction (Wentworth et al., 2001). The linearity of the reciprocal of fluorescence against time plots suggested a common mechanism for quenching of the reaction type $A + A \rightarrow 2A$. Mostly simply we suggested this could be explained by the interaction between two chl molecules forming a dimer or excimer. The effect of zea in increasing the rate of qE formation raises the question as to whether *in vivo* levels of this carotenoid are saturating. Using *Arabidopsis* plants in which the xanthophyll cycle pool size has been increased by over expression of β-carotene hydroxylase we have found that the rate of quenching is indeed not saturated for zea (Davison, Hunter and Horton, unpublished data). The rate of viol de-epoxidation is also faster. These data suggest that the xanthophyll composition of the thylakoid may not be optimised for rapid NPQ formation.

Genetic manipulation of light harvesting. A powerful way forward to determine which proteins are involved in NPQ is to investigate plants deficient in components of the PSII antenna. In our hands, the Chl b less mutant gave unequivical results – qE was reduced but still present. We have analysed plants in which CP29 and CP26 have been reduced to less than 5% protein by antisense technology (Andersson et al., 2001). These proteins had been suggested to be primary qE sites, but in each case NPQ is either unchanged (antiCP26) or reduced by only 30% (antiCP29). Particularly powerful have been the *npq* mutants of *Arabidopsis thaliana* isolated Niyogi *et al*. (1998). A major landmark in NPQ research was the characterisation of the *npq4* mutant, a mutant lacking <u>all</u> the rapidly forming qE and which is deficient in the Lhc-related protein PsbS (Li et al., 2000).

Current research objectives

The nature of xanthophyll binding sites in vivo. Using resonance Raman spectroscopy and ultra low temperature absorption of thylakoid membranes it has been possible to probe the state of zea and viol *in vivo* (Ruban et al., 2001b). Both carotenoids are in well coordinated environments and not free to move. Zea appeared to be more distorted than viol and probably in a tighter association with protein than viol. Some heterogeneity in the xanthophyll cycle

pool was noted, and this may arise from different types of binding site. We have recently been able to apply this approach even to whole leaves, and this promises to expose exactly how zeaxanthin behaves when NPQ is induced (A.V. Ruban., unpublished data).

The role of PsbS A major goal of current research is to understand more about PsbS and how its removal can inhibit qE. We have sought to determine whether it binds pigment as suggested by previous data – our conclusion following purification by IEF is that it may tightly bind a small amount of Chl a, but not carotenoid. However, whether it has weak "peripheral" xanthophyll binding sites can not be determined by this method. The *npq4* mutant has also been exploited to attempt to determine by EM where PsbS is located in the thylakoid membrane (Yakushevska et al., 2001). We are also seeking to explore the role of PsbS by examining its effects on the *in vitro* quenching behaviour of LHCII – such experiments will give direct evidence of the modulator role of this protein.

The origin of 535 nm change.

It can be argued that if the origin of ΔA_{535} can be discovered, the mechanism of qE will be understood. We have recently sought to use resonance Raman spectroscopy to explore the nature of this change. Comparing wt and npq4 leaves it should be possible to identify qE-related changes – to test our hypothesis that at least a part of ΔA_{535} arises from a very strongly red-shifted population of zea.

The architecture of LHCII.

As noted above, the monomeric CP29 and CP26 complexes more readily quench than the trimeric LHCIIb. In fact LHCIIb monomers prepared after phospholipase treatment behave identically to CP26. Trimerization results in a stabilization of LHCII in the light harvesting unquenched state. It seems that the intrinsic quenching ability of LHCII subunits can be constrained (or enhanced?) by the protein-protein interactions. EM studies of thylakoid membranes are now revealing the molecular architecture of PSII (Boekema et al., 2000), showing the protein the many possibilities for protein-protein interaction, which may be not only two dimensional but three dimensional across the appressed membranes of the granum. Although quenching may reside within a protein subunit, modulation of these interactions may provide control over NPQ (Horton, 2000). The capacity of qE may be determined by the macroscopic organization of the system, and different types of NPQ may result from different types of organization change. For example, the sustained type of qI quenching may result from direct effects of light on LHCII structure as observed by Garab and co-workers (Barzda et al., 1996). Undoubtedly the phosphorylation of LHCII contributes to this dynamic behaviour of the thylakoid membrane. Structural analysis of PSII in vivo, in different physiological states and in plants genetically manipulated to contain different LHCII composition promises to deliver new insights into these processes. Such information will compliment more refined approaches and systems for investigating protein-protein interaction in vitro (e.g. Ruban et al., 1999).

The bigger picture

Whilst it is vital to discover the details of the molecular events underlying NPQ it should not be forgotten that it is just one of many physiological regulatory mechanisms that are involved in maximizing photosynthesis under different conditions. NPQ reflects the state of the light harvesting as it responds and adjusts to these conditions. But what of the state of the electron transport system, of the ΔpH and indeed of the metabolic processes of the stroma and cytoplasm? How do these change during leaf development, how are they affected by the switch from vegetative to reproductive growth? There are surprising and major gaps in our knowledge. It is crucial now that we understand how all of these regulatory mechanisms are

integrated (Horton 2000) – this is necessary if the efficiency of radiation conversion of the world's major crops is to be increased to a level sufficient to offset the food shortages predicted for later in this century.

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