Nonphotochemical Quenching by qE in Photosystem II

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Photosystem II (PSII) is a protein complex involved in the photosynthetic process, harvesting light and transforming excitation energy in chemical energy. Under high light conditions the system is able to dissipate the excess of energy as heat (NPQ), therefore avoiding major damage to the plant. One of these mechanisms is qE, the rapid component of NPQ. It involves de-excitation of the singlet excited state of chlorophyll in the light-harvesting antenna of PSII. The physiological importance of qE is known, but an unambiguous understanding of the physical mechanism remains elusive. Here a summation of the recent proposed mechanisms in qE has been made to get more perception in the mechanism of qE.

Plants need light to live by performing photosynthesis. In photosynthesis the energy received is used to convert CO_2 to organic compounds, especially sugars. This process starts in the photosystem II (PSII) (Fig.1), which provides the electrons for photosynthesis. The protons generated by the oxidation of water help to create a proton gradient, which is used by ATP synthase to generate ATP. But too much light can cause damage.

Light energy gets absorbed by chlorophylls (Chl), which are coordinated in the PSII by the antennae complexes (Fig. 1). The Chl is promoted to its first singlet excited state and transfers its energy by electron transport to the reaction centre, but with increasing light intensity this process in photosynthesis gets saturated. When this happens intersystem crossing (IC) can take place. This is the conversion, through spin inversion, of chlorophyll single excited state ($^1Chl^*$) into triplet excited state ($^3Chl^*$). $^3Chl^*$ can transfer energy to ground-state O_2 to generate singlet oxygen ($^1O_2{}^*$), an extremely damaging reactive oxygen species (ROS), which destroy vital proteins, such as the photosystem II (PSII) reaction centre proteins, as well as lipid bilayers, and pigments (Niyogi, K. K. 1999). When this damaging process keeps on going death of the organism will be the final consequence. So organisms which survive by photosynthesis have photoprotective mechanisms where the excess of energy gets dissipated with as little damage as possible.

There are three different processes known in plants with respect to photoprotection according to their relaxation kinetics in darkness (Horton, P. and Hague, A. 1988). The least defined and slowest (hours) is photoinhibitory fluorescence quenching (qI) where PSII gets inactivated. By state-transition quenching (qT) separates the LHCII complex from PSII, thereby reducing the amount of excitation energy in PSII. This process can takes place in minutes (Muller, P. et al. 2001). The major component, which takes place in seconds to minutes is energy-depended quenching also called feedback de-excitation (qE) and will be discussed here. This mechanism accounts for the quenching of more than 75% of $^1Chl^*$ (Bonente, G. et al. 2008a).

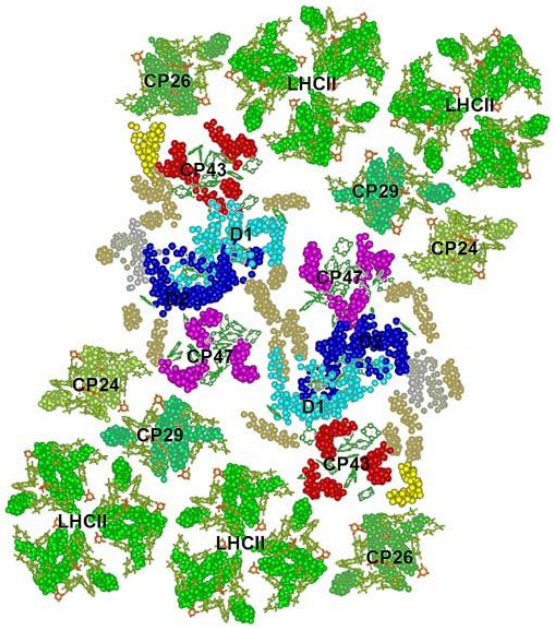

Figure 1. Simplification of the PSII. D1, D2, CP47 and CP43 form the reaction centre. LHCII is the major trimeric antenna complex and CP24, CP26 and CP29 are monomeric antennae (source: Bonente, G. et al. 2008a).

Some major players of the qE mechanism are known, because when a plant lacks violaxanthin de-epoxidase (VDE) (*npq1* mutant), the xanthophyll cycle is not functioning and no zeaxanthin is made and a decrease in quenching activity is observed (Niyogi, K. K. et al. 2001). In another mutant, *npq2*, zea accumulates. In this plant qE is equal the wild type, but the kinetics of the recovery are slower (Niyogi, K. K. et al. 1998). When the plant lacks lutein (lut2) also a decrease in qE amplitude and a slower induction are observed, but this effect is weaker then in the absence of zeaxanthin, (*npq1*). In the double mutant *npq1lut2* qE is absent and photo-oxidative stress appears under high light conditions (Niyogi, K. K. et al. 1998). Also a big decrease in qE is observed when the protein PsbS is lacking. It is known that all the processes are activated by a decrease of pH in the lumen.

So it is clear that for NPQ are needed a pH gradient, PsbS, xanthophyll cycle carotenoids, chlorophylls and antennae, but what the exact mechanism of qE at molecular level is, is not so obvious yet. Today there is research going on to get more insight in qE. There are several proposals and there seems to be some contradictions between them.

This essay will give an overview of the recent proposed mechanisms in qE to gain more understanding about this process.

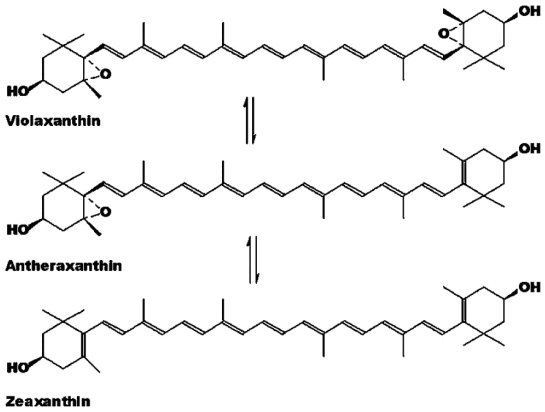

Figure 2. The violaxanthin cycle consists of the de-epoxidation of violaxanthin to zeaxanthin in high light via antheraxanthin, catalysed by VDE; ZE carries out the conversion in the reverse direction. (Source: Muller, P. et al. 2001)

Xanthophyll cycle

VDE catalysis the reaction of violaxanthin (vio) to antheraxanthin and finally zeaxanthin (zea) in plants (Fig. 2). VDE uses Vitamin C as a cosubstrate to reduce the epoxide groups of vio (Bratt et al., 1995). The enzyme is active at low pH (Eskling, M. et al. 1997), when its glutamic acids are protonated (Bugos, R. C. and Yamamoto, H. Y. 1996). So the trigger for this reaction is a decrease in pH in the lumen created by an excess of light. The reaction the opposite way is catalysed by an enzyme probably at the stromal side of the thylakoid membrane, because zeaxanthin epoxidase (ZE) has its optimum at pH 8. The balance between zea and vio is modified by the activity of VDE with respect to ZE, caused by the ΔpH (Bugos, R. C. et al. 1998).

Two roles for zeaxanthin

There are two conceptions for qE with respect to zea at the moment. One defines zea as a direct excitation quencher by accepting the excitation energy from PSII antenna Chl (Demmig-Adams, B. and Adams, W. W. 2002, Horton, P. and Ruban, A. 2005). The other classifies zea as an allosteric activator where vio acts as an inhibitor of structural change in the PSII light-harvesting antenna, activated by the xanthopyll cycle, stimulated by the trans-membrane pH gradient. It has been proposed that the latter process is associated with the trimeric LHCII antenna complex happening in the extern V1 binding site (Liu, Z. F. et al. 2004, Ruban, A. V. et al. 1999). Two other types of xanthophylls are coordinated to LHCII as well. One is lutein and binds at the internal L1 and L2 sites and a neoxanthin is placed in the N1 site (Fig. 3). Lutein is suggested to act as a direct quencher in LHCII (Liu, Z. F. et al. 2004).

CP29, CP26 and CP24 can all bind a xanthophyll cycle carotenoid. In all three monomers lutein is attached to the L1 site, furthermore CP29 and CP26 can bind a neoxanthin (Bassi, R. et al. 1993, Morosinotto, T. et al. 2002, Peter, G. F. and Thornber, J. P. 1991, Ruban, A. V. et al. 1994, Ruban, A. V. et al. 1999). It is thought by Ahn et al. and Avenson et al. that zea acts here as a direct quencher (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008).

Chlorophyll transition background (Blankenship, R. E 2002)

Chls have two major absorption bands, one in the vicinity of blue or near UV region and the other is in the red or near infra red. Because there is no absorption in the green region the Chls have a green color. The two absorption

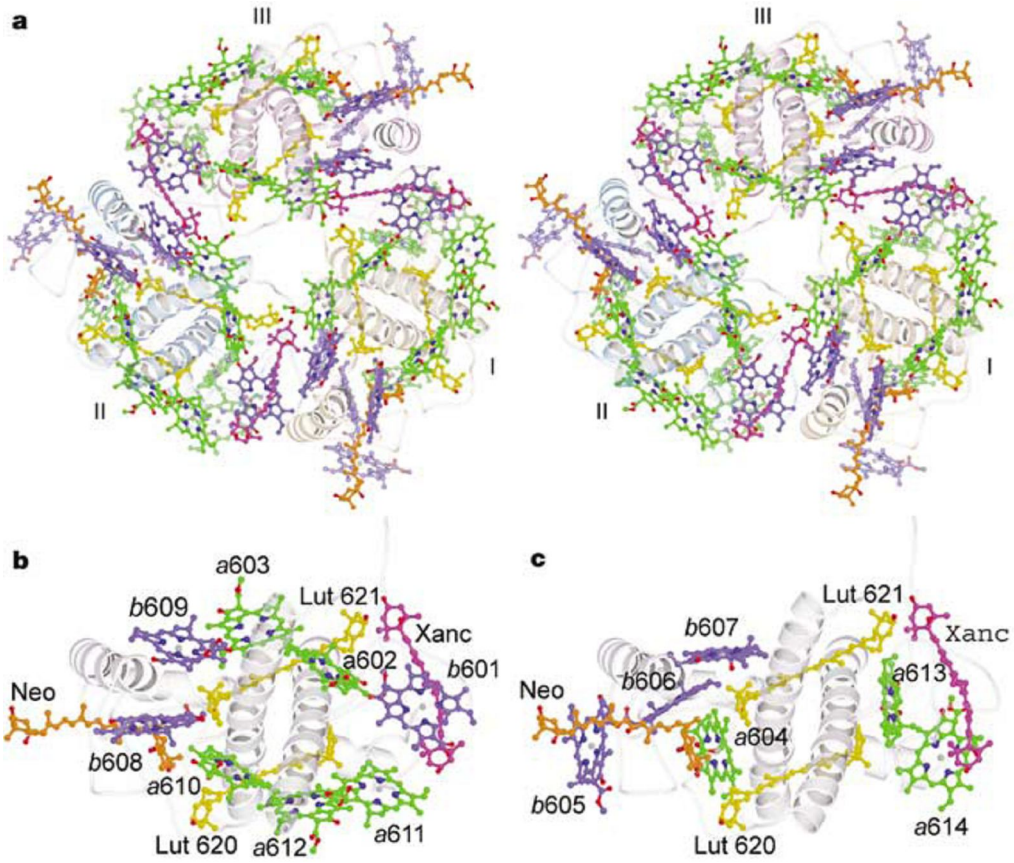

Figure 3. Pigments in the LHCII trimer and monomer. a, Stereo view showing the pigment arrangement pattern in the LHCII trimer, viewed along the membrane normal from the stromal side. Monomers are labelled I–III. For clarity, the Chl phytyl chains and lipids are omitted. Green, Chl*a*; blue, Chl*b*; yellow, lutein; orange, neoxanthin; magenta, xanthophyll-cycle carotenoids. b, c, Pigment pattern in a monomer at the stromal and lumenal sides, respectively. Colour designation the same as in a. (Source: Liu, Z. F. et al. 2004).

bands mentioned are the $\pi \rightarrow \pi^*$ transitions, where electrons are involved in the conjugated π system of the Chl. The spectra can theoretically be described by use of the "four orbital" model, where four π molecular orbitals are involved in the transition, which are the two HOMOs (Highest Occupied Molecular Orbitals) and the two LUMOs (Lowest Unoccupied Molecular Orbitals). The two lowest energy transitions are referred to as Q bands and the two highest are called Soret or B bandsThe diagram in figure 4 shows that in the electron transition only a jump of an electron from the HOMO to the LUMO takes place, but in fact this is an oversimplification of a complex relationship between electronic states and orbital energies (Hansen, L. K. 1991). Many electron configurations play part in the transition, so configuration interaction occurs.

The electronic transitions have transition dipole moments with differences in strength and orientation. The lowest energy transition is invariably polarised along the y molecular axis and for that reason it is called the Q_y transition (~680 nm) and a $^1Chl^*$ is formed. The other Q transition is called the Q_x and takes place at a shorter wavelength, but is above the scope of the essay.

The fluorescence of the Chl is coming from the Q_y transition, when the absorbed photon gets reemitted. The yield of this process is usually very low (below 1%), but it is very useful for scientists since it tells in any moment the level of excited states in the photosynthetic system (Bonente, G. et al. 2008a).

Energy transfer

A generally discussed and appealing molecular mechanism for the dissipation of the excess excitation energy in NPQ is the singlet excitation energy transfer from the excited Q_y state of the Chl to the S_1 state of zea (Bassi, R. and Caffarri, S. 2000, Frank, H. A. et al. 1994, Frank, H. A. et al. 2000, Gilmore, A. M. 1997).

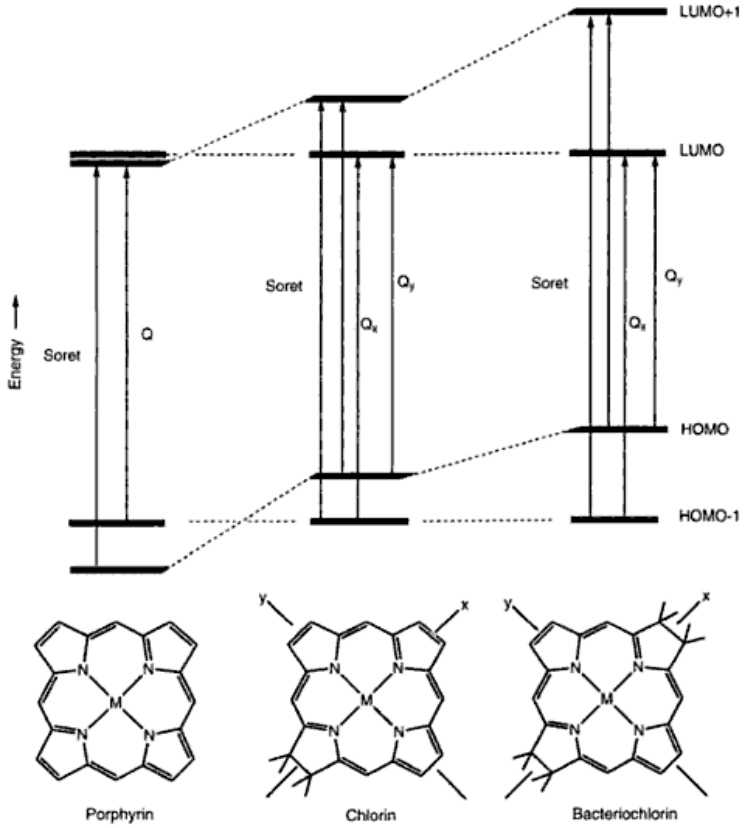

Figure 4. Molecular orbital energy level diagram of porphyrin, chlorin and bacteriochlorin. A very simplified representation of the different electronic transitions is indicated (source: Hansen, L. K. 1991).

It is known that the S_1 state of zea has a lifetime of 10 ps and decays non-radiative (Frank, H. A. et al. 1994). The S_1 state of vio has an higher energy than zea because zea can delocalise the accepted energy better than vio because of the difference in number of conjugated π-bonds, 11 and nine respectively. The fact that NPQ works with zea and not with vio is explained by the differences in S_1 energy of the xanthophyll cycle carotenoids. Because the Q_y state of Chl is supposed to be lower in energy than the S_1 state of vio and thought to be higher in energy than the S_1 state of zea. Experiments are suggesting that the S_1 state of zea is directly connected to NPQ (Ma, Y. Z. et al. 2003). However, the exact determination of S_1 energy of Cars is a night impossible task, because of two reasons. At first if energy transfer is happening from Chl Q_y to Car S_1, then the Car S_1 lifetime is significantly shorter than the energy transfer time from Chl to Car. This results in an almost undetectable Car S_1 population. Second, the Car S_1 state is one-photon-forbidden by absorption and emission from the ground and excited states respectively (Holt, N. E. et al. 2004).

Some contrary results by determination of the S_1 state of Cars have been found. In 2004 Holt et al. tabled in an article the energy states of S_1 states of Cars found in published research articles. They stated that while precise knowledge of the energy levels of the relevant qE-related Cars and Chls cannot prove that the qE occurs by the direct quenching mechanism, it can assess if energy or electron transfer involving these molecules is feasible. Specifically, effective energy transfer from Chl(s) to Car(s) during qE requires that the S_1 state of Zea be lower in energy than the Q_y band of the energetically relevant Chl(s) (Holt, N. E. et al. 2004). All the calculations for the Chl-zea complex they found in literature have a lower energy than Q_y, but calculations made by Wormit in 2009 show that the transition state of zea lies just above Q_y and the one of lutein is just below (Wormit, M. et al. 2009). The method developed by Polivka et al. consistently found that the energies of both zea and vio are lower in energy than the Q_y state (Polivka, T. et al. 1999, Polivka, T. et al. 2002). This implies that if qE occurs by direct quenching, the differences in the distance

and/or orientations of Cars with respect to Chl must also be significant. It has to be noted that all these measurements are done *in vitro* and with respect to the LHCII complex, not with the minor antennae, which is where direct quenching with zea is thought to happen.
That the S_1 state of the Car for quenching has to be lower than the Q_y state of the Chl is not the only requirement for energy transfer, also the π-systems of both molecules have to overlap, so the orientation has to be right, otherwise quenching is in general not accessible (Dreuw, A. et al. 2003).

Does LHCII change conformation?
Almost all of the neoxanthin found in thylakoid membranes is bound to LHCII (Bassi, R. et al. 1993) and Ruban et al. used in 2007 this information to define in which configuration neoxanthin is in the LHCII, because when neoxanthin has its 9'-*cis* configuration (Fig. 5) it exhibits fingerprint Raman bands (Ruban, A. V. et al. 2000). They detected in the NPQ state *in vivo* in leaves and chloroplasts that when LHCII changes conformation this is manifested as a twist in the Raman configuration of the bound neoxanthin molecule. Their research showed that the Raman bands of the 9'-*cis* conformation visible at 953 cm^{-1} can be correlated with the magnitude of qE.
Since there were only small traces of vio and no zea present in the LHCII, those Cars are excluded to act as the quencher in this system *in vitro*. Lutein is likely to be the quencher, because it is located on a position near three Chl where the excitation has the highest possibility to be localised (Liu, Z. F. et al. 2004, van Grondelle, R. and Novoderezhkin, V. I. 2006). They supposed a Chl*a*-carotenoid energy transfer as quenching mechanism, where the energy is transferred from Chl*a* to a low-lying carotenoid excited state of lutein 1. This lutein is placed in the quenching position by the change in configuration of the LCHII (Ruban, A. V. et al. 2007).
Neoxanthin molecules are suggested to induce a conformational change triggered by the vio replacement of zea in LHCII. Two pigment domains within the complex appear to cooperatively interact in this conformational switch (Fig. 6). The neoxanthin domain contains Chls a_{604}, b_{606}, b_{607} and b_{608}, which show quenching-related changes in absorption and alterations in the CD signal structure. This domain has been previously highlighted *in vitro* and *in vivo* as an indicator of the conformational change (Horton, P. et al. 2005, Pascal, A. A. et al. 2005, Ruban, A. V. et al. 2007). The second domain is the suggested quenching site. It contains the terminal emitter group of pigments (Chls a_{610}, a_{611}, and a_{612}), which give rise to the red-shifted Chl*a* at 681 nm paralleled by changes in the Soret band around 438 nm. The absorption change peaking and CD transitions correspond to lutein 1, implying involvement of excitonic interactions between lutein and its neighbours (Ilioaia, C. et al. 2008). This confirmes the Chl*a*-carotenoid energy transfer suggested by Ruban et al. and the information obtained about the changes in pigment states in the quenching mode prove the involvement of neoxanthin and lutein 1 domains (Ilioaia, C. et al. 2008).

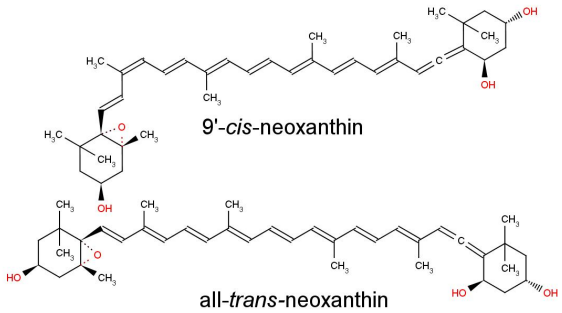

Figure 5. Molecule structures of two neoxanthin configurations. The 9'-*cis*-neoxanthin exhibits fingerprint Raman bands at 953 cm^{-1}.

Barros et al. assume that a conformational change in LHCII aggregates is not necessary to promote quenching. Barros hypothesises that qE is the result of a close interaction of LHCII with another pigment protein complex, most likely PsbS, that does not involve a conformational change within the antenna complex. They based this assumption on the finding that the change of vio into zea did not result in energy dissipation and they think PsbS is doing the quenching *in vivo*. They noticed that LHCII aggregates do quench and explained it by tight interaction of pigments exposed on the outer surface of the complex. This would create a large number of quenching centres that will effectively dissipate the excitation energy in aggregates (Fig. 7) (Barros, T. et al. 2009).
Ilioaia et al. have also done research at the LHCII, and found strong evidence against this hypothesis. The quenched complexes they used were in the trimeric state, not in the aggregated

state as in Barros' case. An energy transfer path to a low-lying excited state of lutein 1 was revealed in experiments on isolated quenched LHCII complexes (Ruban, A. V. et al. 2007).

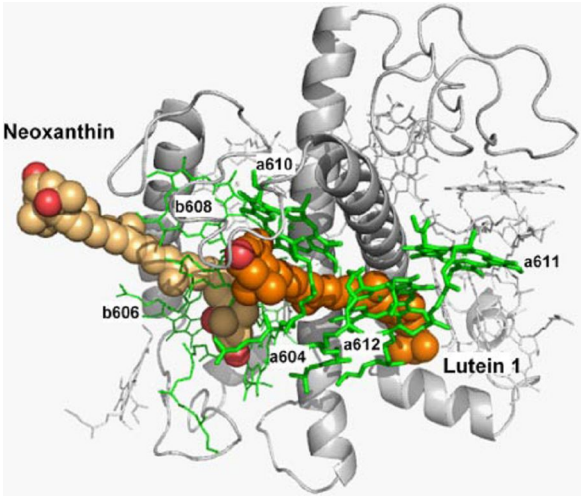

Figure 6. Neoxanthin and lutein 1 domains in the structure of LHCII. Neoxanthin is a Chl*b*-enriched domain interacting with the lutein 1 domain. The latter carries terminal emitter Chls (*a*610, *a*611, and *a*612) where the energy dissipation takes place (source: Ilioaia, C. et al. 2008).

The study of Ilioaia provides direct evidence that the mechanism of the excitation quenching in LHCII is localised at the level of monomeric LHCII, involving a conformational change in the protein, and is associated with the specific alterations in configuration of pigments (Ahn, T. K. et al. 2008, Ilioaia, C. et al. 2008, Pascal, A. A. et al. 2005, Ruban, A. V. et al. 2007, Yan, H. C. et al. 2007).

The experiments were performed in presence and absence of a detergent. When the detergent was removed strong fluorescence quenching was the result, which was completely reversible (Fig. 8 black trace). Then the protein cross-linker glutaraldehyde was used to explore whether a conformational change was happening in LHCII. Non-quenched LHCII was incubated in a detergent-free medium after being cross-linked with glutaraldehyde. The quenching after 6 h of incubation had a k_d[1] of 1,6 (Fig. 8 red trace), compared with the control value of 10 (Fig. 8 green trace). When pre-quenched LHCII, cross-linked with

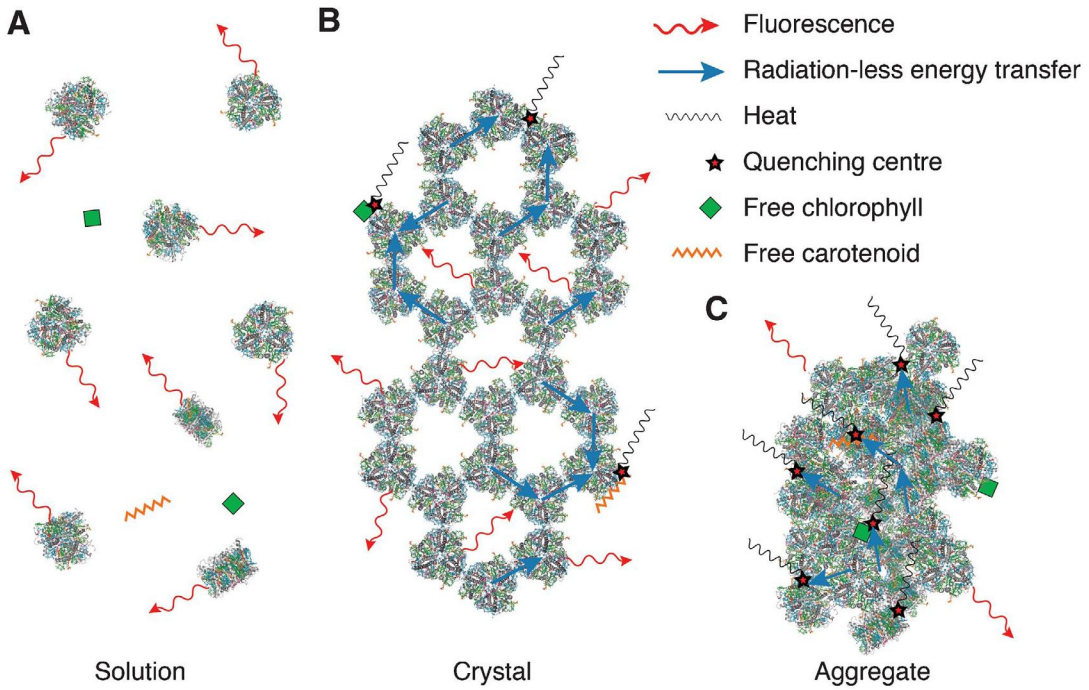

Figure 7. Energy transfer and dissipation by LHC-II *in vitro*. (A) Solubilized LHC-II trimers in detergent solution are functionally independent and re-emit absorbed light as fluorescence. (B) LHC-II crystals show similar fluorescence, although its intensity and spectral range are modified by re-absorption. Conceivably, occasional tight interaction of LHC-II trimers with one another, for example, at grain boundaries, or with contaminating free pigments can generate quenching centres, to which the energy flows through the excitonically coupled complexes. (C) Randomly compacted trimers in LHC-II aggregates generate a high density of quenching centres, resulting in reduced lifetimes and conversion of the absorbed energy into heat (source: Barros, T. et al. 2009).

[1] Fluorescence quenching strength or amplitude (k_d) was defined as $(F_u - F_q)/F_q$, where F_u and F_q are the fluorescence levels of unquenched and quenched samples, respectively

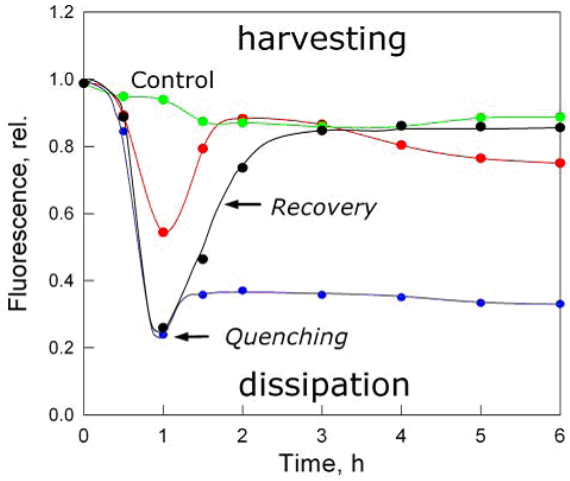

Figure 8. Time course of the Chl fluorescence relative yield of LHCII incorporated in polyacrylamide gel. Fluorescence was excited at 650 nm and measured in the 685–800 nm region. Without detergent no quenching of the gel containing LHCII takes place, and with detergent fluorescence quenching is observed (*black trace*). The *red trace* indicates incubation of the gel containing LHCII pretreated with glutaraldehyde for 5 min before setting the gel. After 60 min, recovery of the fluorescence of the samples was achieved by placing the gel fragment in medium containing 0,03% detergent; the control sample (*green trace*) was incubated in this buffer throughout the experiment. The *blue trace* indicate an LHCII gel placed in recovery buffer additionally containing glutaraldehyde (source: Ilioaia, C. et al. 2008).

glutaraldehyde was incubated in the detergent buffer, very little relaxation of quenching was observed compared with the control (Fig. 8 blue trace). Concerted both results draw the conclusion that the LHCII protein undergoes conformational changes during transition, both into and out of the dissipative state.

The conclusion drawn by Ilioaia also explains the early reports on the lack of tight correlation between the level of fluorescence quenching and the extent of aggregation in isolated LHCII (Kirchhoff, H. et al. 2003). Although this model of LCHII can fully account for qE both qualitatively and quantitatively, qE may be a heterogeneous process in which quenching by lutein 1 in LHCII is only a part. The formation of a radical state of zea has been correlated with qE *in vivo*, suggesting that it is either the quencher or its formation is closely associated with the quenching process (Holt, N. E. et al. 2005, Ruban, A. V. et al. 2007, Yan, H. C. et al. 2007).

In recent studies it is shown that this radical can only be formed at the L2 site of the monomeric antennae, which assumes that only in the monomer antennae zea can act as a direct quencher by formation of a radical (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008).

Charge transfer

It is thought that zea can act as a direct excitation quencher of excited Chls by formation of a charge transfer state in the monomeric complexes. The proof of this theory is the formation of a zea radical cation absorbing around 1000 nm, which is correlated with the extent of qE (Holt, N. E. et al. 2005). This direct quenching through zea is happening via radical cation formation instead of energy transfer. This is possible when a Chl-Car dimer is formed. In this composition the molecules have a spatial overlap in the π-systems, and the process depends on the ionisation potential of the Car.

Calculations show that in the cases where electron transfer is possible, the CT state involves a transition from the HOMO of the Car to the antibonding LUMO of the Chl. Hence electron transfer results in a Car cation and Chl anion and is maximal when the geometry of the molecules have the π-systems of the Chl and Car parallel and the Chl is located above the centre of mass of the Car (Holt, N. E. et al. 2004). Holt et al. conclude by inspecting of the molecular structure of the Car that Chl-zea and Chl-anthera dimers could be formed, but that steric hindrance inhibits the formation of Chl-vio dimer. They only did not account the polar protein environments in their calculation and charge transfer is known to be extremely sensitive to solvent polarity (Holt, N. E. et al. 2004), because the polarity of the solvent can stabilise or destabilise the excited state, which results in moving to longer wavelengths as the solvent polarity increases (Anslyn, E. V. and Dougherty, D. A. 2006).

A difference between CT and energy transfer is that CT can be faster, because of the additional electrostatic attraction between the cation and the anion, the CT state can more rapidly dissipate energy than the S_1 state of a Car, and the CT state becomes the energetically lowest state. The CT decays nonradiatively into the ground state by thermal dissipation and back transfer of an electron (Dreuw, A. et al. 2003). The electron transfer of the acceptor back to the original donor and the emission of the exciplex formed will be at considerably longer wavelength, and thus at lower energy than the isolated excited molecule, which makes it a safe way for

dissipating energy (Anslyn, E. V. and Dougherty, D. A. 2006). Zea should have the lowest energy of the xanthophyll cycle molecules, because it has the most conjugated π-bonds, which results in more dislocation of the radical with a more stable state as effect and a lower energy than vio or antheraxanthin and a more stable state lower in energy.
Ahn et al. found evidence for charge-transfer in all three of the individual minor antenna complexes of PSII (CP29, CP26 and CP24), but their relative contributions to qE *in vivo* is not known. Ahn et al. excited the *in vitro* complexes at 650 nm and determined the absorption profile of the Car radicals, which was in agreement with transient zea radical cations. Zea binds specifically to the L2 domain, and they discovered that CT quenching in CP29 involves Chls A5 and B5, because no measurable zea formation signal was observed when these Chls were absent in the antenna. Chl A5 and B5 are reported to be strongly coupled (Cinque, G. et al. 2000, van Amerongen, H. and van Grondelle, R. 2001). The CP29 mutant lacking Chl A5, missed an additional Chl, and experimental data pointed out that this is B5 (Ahn, T. K. et al. 2008). Therefore it is thought that in CT quenching a coupled Chl dimer would be formed. This dimer will make the product formed in CT quenching more stable. And changing the distance between those two Chls might be the switch in CP29 to turn on and off the CT quenching, this can be achieved by PsbS interaction with CP29 (Teardo, E. et al. 2007).
In vitro is estimated that 0,5% of the minor complexes undergo charge transfer quenching, this would not be enough to count for the total qE *in vivo*, but the contribution for it *in vivo* still has to be determined (Avenson, T. J. et al. 2008).

Do zea-independent and zea-dependent mechanisms both contribution to qE?
The mechanisms standing out so far are not contradictory. They are triggered by a pH change and a PsbS-mediated conformation change is suggested in the zea radical formation in the minor antennae and quenching by lutein in the major (Ahn, T. K. et al. 2008, Ruban, A. V. et al. 2007). In fact it is possible that both mechanisms are involved with *in vivo* qE.
Johnson has investigated if both qE components are acting separate from each other or not. If so they hypothesised four consequences (Johnson, M. P. et al. 2009):

- The removal of certain Lhcb proteins by mutation would differentially affect the two components of qE.
- Both components would not be able to compensate for the loss of one another, hence they should each contribute a to discrete component of the kinetics for qE formation and relaxation (Niyogi, K. K. et al. 1998, Pogson, B. J. et al. 1998)
- When lutein is lacking, the zea-dependent component should stay unaffected.
- The two components are expected to be characterised by different absorption changes in the Soret region, which reflect changes in the absorption spectra of bound pigments brought about by conformational changes within the PSII antenna upon qE formation (Bilger, W. and Bjorkman, O. 1994).

As argued above, if both mechanisms contribute to qE, there would be discrete kinetic components from each mechanism. If zea acts as a quencher its level would not affect the relaxation kinetics, but when it has an allosteric purpose a change in relaxation kinetics would be observed. They found one rate of relaxation depending on the level of zea. The effect of zea in slowing qE relaxation has been reported previously (Noctor, G. et al. 1991). Here, it was found that the relaxation of qE in *npq1* (no VDE) was faster than in the wild type, whereas in *npq2* (accumulation of zea) and *lut2npq2* (without lutein), qE relaxation was significantly retarded.
Measurement of fluorescence lifetimes confirmed the suggestion that the extra zea in *npq2* and *lut2npq2* caused a prequenching of fluorescence in the dark-adapted state (Dall'Osto, L. et al. 2005). In these mutants, all of the vio, including that bound to the L2 site in the minor antenna complexes, is replaced by zea.
Both zea-dependent and zea-independent qE were affected, the latter being almost completely eliminated. There was difference in the kinetics between the mutants and the wild type and still zea-dependent qE took place in the absence of lutein, indicating that lutein is not an obligatory Car for qE. It is clear that when either zea or vio replaces lutein in the internal binding sites, quenching still occurs,

but both the dynamics and amplitude of qE are affected. So lutein is important for fully functional qE.
The evidence that both the zea-independent and zea-dependent components of qE arise from the same mechanism within the PSII antenna has been strengthened. Both components of qE are reduced in amplitude in the absence of lutein (Niyogi, K. K. et al. 2001, Pogson, B. J. et al. 1998). Johnson et al. demonstrated that both were affected by changes in the minor monomeric and trimeric LHCII protein composition and both were accompanied by conformational changes leading to very similar absorption changes in the Soret region. Feedback de-excitation was found to consistently relax as a single component, even though the half-times differed more than 15-fold when the xanthophyll and/or the Lhcb protein composition was changed. All of these findings are consistent with zea as allosteric regulator involving the xanthophyll at the L1 binding site, which is activated by the LHCII conformational change that leads to neoxanthin distortion (Ruban, A. V. et al. 2007) and is regulated by the deepoxidation state at the V1 site (Horton, P. and Ruban, A. 2005).
Johnson et al. also put forward an alternative possibility. That zea can act as a direct quencher in both trimeric LHCII and the minor monomeric antenna, and when no zea is present a lutein cation can take its place as a quencher. However, independent of which mechanism is involved, it is clear that the natural xanthophyll composition and Lhcb protein content within the PSII antenna are necessary for a fully functional NPQ, in which the conformational dynamics are tuned to create maximum flexibility between efficient light harvesting in low light and rapid formation and relaxation of photoprotection in fluctuating light. (Johnson, M. P. et al. 2009)

Importance of PsbS

In higher plants, subunit S (PsbS) of PSII plays an essential role in qE. When it is eliminated by mutation this part of NPQ gets strongly reduced. Jansson at al. predicted in 1999 that PsbS may exist as a dimer in thylakoid membranes because of the conserved amino acids, arginine and glutamate, which are known to be important for helix-helix interactions (Jansson, S. 1999). Berganino et al. confirmed this hypothesis in 2003 and found evidence that the monomer-to-dimer ratio is pH dependent (Bergantino, E. et al. 2003). It is proposed that two glutamates E122 and E226 can get protonated at low pH which accounts for the monomerisation of the PsbS dimers (Li, X. P. et al. 2004). Single mutations in the mentioned residues set result for a dramatic decrease in qE (~60-70%) and double mutations resulted in qE deficient plants (Li, X. P. et al. 2002). The mechanism of how PsbS causes this strong regulatory effect on NPQ is not known, but some suggestions have been made. It is has been proposed that it provides the site of direct quenching, because two zea can bind to the protein *in vitro*. This binding of zea results in a change in the absorption spectrum of zea that reconstituted the ΔA_{535}, which is associated with qE (Aspinall-O'Dea, M. et al. 2002). If PsbS is the site of direct NPQ, it must be able to bind pigment(s) *in vivo* or to have them nearby. It is proposed by Barros et al. that the dimer-to-monomer transition uncovers the zea binding site on the hydrophobic surface of PsbS and proposed that zea is bound in a position where it is brought in contact with a Chl in LHCII or a minor antenna by Van der Waals interaction to perform quenching. Interestingly, the work by Aspinall-O'Dea et al. found that binding of Zea to PsbS *in vitro* was pH-independent (Aspinall-O'Dea, M. et al. 2002).
Sequence analysis showed that most of the amino acid residues that serve as Chl binding ligands in LHCII (Remelli, R. et al. 1999), CP26 and CP29 (Bassi, R. et al. 1999, Dominici, P. et al. 2002) are not present in PsbS and it has recently been shown not to bind pigments (Bonente, G. et al. 2008b), which brings us to the other thought where PsbS and zea act indirectly as allosteric regulators (Horton, P. et al. 2005). PsbS is shown to associate either with an outer antenna (CP29) (Teardo, E. et al. 2007) or with the PSII core as a monomer and dimer respectively (Bergantino, E. et al. 2003).
LHCII can act independently from PsbS as quencher *in vitro*, but *in vivo* quenching can not take place without PsbS. Recent research is pointing in the direction where PsbS is having a very important role in the organisation of the PSII antenna. Bode et al. showed that the PsbS lacking mutant *npq4* is the only one that almost completely lacks Car S_1-Chl coupling and concluded that PsbS is essential for the formation of these interactions *in planta*

(Bode, S. et al. 2009) and Kiss et al. gathered data which is suggesting that PsbS controls the association between LHCII and the PSII core (Kiss, A. Z. et al. 2008). They made observations on restacking of unstacked grana membranes. They observed that Mg^{2+} on isolated thylakoid membranes exhibited a high PSII fluorescence yield and the concentration of PsbS showed a dramatic effect on the Mg^{2+}-dependence for the increase in fluorescence as well as the shape of the Mg^{2+}-titration curve. In the unstacked state LHCII is dissociated from PSII (Arntzen, C. J. and Ditto, C. L. 1976, Barber, J. 1982) and an increase of energy transfer from PSII to PSI is observed (Butler, W. L. and Kitajima, M. 1975, Murata, N. 1969). They observed a significant increase in fluorescence without restacking, which indicates that the association of LHCII with PSII is a major factor determining the PSII fluorescence yield. The association of those two complexes is depending on Mg^{2+} and mostly spontaneous. This process is also observed in the *npq4* mutant and thus not depending on PsbS, but it has a kind of molecular chaperone function. The results Kiss obtained were suggesting that PsbS provides a template for the assembly process, where groups of LHCII components are organised by PsbS allowing concerted assembly into the macrostructure (Fig. 9). This organising function of PsbS does not depend on qE, because mutations in PsbS, resulting in decrease of qE have no effect on the assembly process.

They concluded that pH-dependent action of PsbS triggers a change in LHCII interactions (Fig. 9). This confirms the hypothesis that qE is formed after a conformational change in the subunits of the PSII antenna (Pascal, A. A. et al. 2005, Ruban, A. V. et al. 2007) and that interactions between these subunits are needed for energy dissipation (Horton, P. et al. 2005, Horton, P. and Ruban, A. 2005).

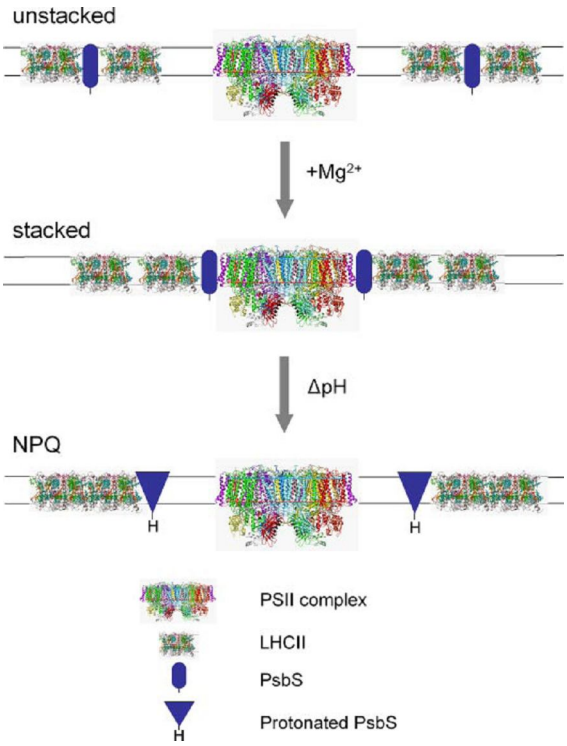

Figure 9. PsbS modulation of the structure and function of the PSII antenna. In *unstacked* membranes LHCII and PsbS are dissociated from the PSII complex. Mg^{2+} causes PsbS-facilitated lateral rearrangement of LHCII around PSII (*stacked*). Protonation of PsbS resulting from the ΔpH induces conformational changes in PsbS leading to its detachment from PSII along with aggregating LHCII (NPQ) (source: Kiss, A. Z. et al. 2008).

Conclusion

All the present suggestions on how feedback de-excitation can occur with respect to the molecular level have been discussed. In this process zea is forming a cation in the monomeric complex and lutein is acting as the direct quencher in the LHCII and it is discussed whether LHCII has to undergo a configuration change to be able to quench *in vivo* and the importance of PsbS in the whole quenching process has been put forward.

When the ΔpH triggers VDE to change vio into zea and protonation of the PsbS dimer, which causes it to form monomers are the first two important processes to start quenching (Bergantino, E. et al. 2003). When zea replaces vio in the monomeric complex it can act there as a direct quencher via cation formation (Ahn, T. K. et al. 2008, Avenson, T. J. et al. 2008) and PsbS can alter the coupling strength between two Chls in CP29 which causes qE in this monomeric antenna.

It might be that the zea cation formation in the monomeric complex is not part of the major quenching, but only to prevent the minor monomeric complexes from photodamage, which would explain why there is only a low quenching yield observed *in vitro*. It would also explain why Johnson et al. found only one kinetic contribution in qE (Johnson, M. P. et al. 2009).

The aggregate formation of the LHCII is enough to cause quenching *in vitro* (Barros, T. et al. 2009), but this configuration might not be possible *in vivo*. So the quenching in the latter process can only take place when the PsbS monomer coordinates the PSII in the right arrangement in the grana membranes, as shown in Fig. 10 (Kiss, A. Z. et al. 2008). It has been proved that neoxanthin and lutein are involved in the quenching mode of LHCII as well. When LHCII changes to its quenched conformation lutein is probably coordinated so that it is able to quench by accepting energy from a Chl*a* (Ruban, A. V. et al. 2007, Ilioaia, C. et al. 2008).

But because quenching and correlated Car S_1-Chl interactions are not entirely vanishing in any mutant that is deficient in a certain Car. It can be concluded that replacement of the missing pigment by other Cars can, at least partially, compensate for the formation of quenching excitonic Car S_1-Chl states (Bode, S. et al. 2009).

The account of the different mechanisms in qE *in vivo* is not clear yet, and still a lot of discussion is going on in this area. But it is quite clear how important every compound is in non-photochemical quenching and that it is an ingenious system with many backup systems, which makes it unambiguous and a challenge to discover how the whole process works exactly.

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