Chapter 1

The Non-Photochemical Quenching of the Electronically Excited State of Chlorophyll a in Plants: Definitions, Timelines, Viewpoints, Open Questions

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Summary

Chlorophyll *a*-containing plants, algae and cyanobacteria absorb sunlight in order to perform oxygenic photosynthesis using two sequential photoreactions: Light Reaction II, which takes place in Photosystem II (PS II), oxidizes water to molecular oxygen (O_2) and reduces plastoquinone to plastoquinol; Light Reaction I, which takes place in Photosystem I (PS I),

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B. Demmig-Adams et al. (eds.), *Non-Photochemical Quenching and Energy Dissipation in Plants, Algae and Cyanobacteria*, Advances in Photosynthesis and Respiration 40,
DOI 10.1007/978-94-017-9032-1_1,

oxidizes plastoquinol to plastoquinone, via cytochrome b_6f complex, and reduces $NADP^+$ (nicotinamide adenine dinucleotide phosphate) to NADPH. In most cases, a large fraction of the electronic excitation acquired by absorbing sunlight is used for running the photoreactions of photosynthesis, a small fraction is emitted as chlorophyll fluorescence, and the remainder is degraded to heat and dissipated to the surroundings. These electronic excitation degradation processes encompass both spontaneous (i.e., "unprovoked") de-excitations (internal conversion) as well as de-excitations triggered and regulated by various physical and chemical signals. These signals involve photosynthetic electron transport (PSET) and are generated within and across the thylakoid membranes. Only the regulated dissipation of electronic excitation is assessed as *non-photochemical quenching* (NPQ) of chlorophyll fluorescence. Signals triggering NPQ include redox potential shifts of intramembranous electron transport intermediates, electrostatic potential shifts at membrane surfaces, and formation of trans-membrane ion concentration gradients, such as a proton concentration difference (ΔpH). Oxygenic photosynthetic organisms employ various processes to relieve the sensitive PS II from destructive effects of excess electronic excitation (excess excitation energy). The latter goal is achieved either by directly quenching excited states of pigments in the peripheral and the core antenna pigment-protein complexes of PS II, or by moving peripheral antenna complexes from the vicinity of PS II to PS I. In this chapter, we shall outline the remarkable and unprecedented

Abbreviations: A – antheraxanthin; ATM – atmosphere; ATP – adenosine triphosphate; CAC – Core antenna light harvesting Chl *a*-protein complexes; Chl – chlorophyll; CP22 – see PsbS below; CP24, CP26, CP29 – chlorophyll *a*-, *b*-, and xanthophyll-binding proteins of photosystem II with molecular mass of 24, 26 or 29 kDa, respectively; CP43, CP47 – Chl a binding protein of 43kDA, 47 kDa molecular mass; Cyt – cytochrome; D1, D2 – two major proteins of Photosystem II reaction center complex; DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Dd – diadinoxanthin; DDE – diadinoxanthin de-epoxidase; Dd-Dt – diadinoxanthin-diatoxanthin cycle; DGDG – digalactosyldiacylglycerol; Dt – diatoxanthin; ΔpH – trans-thylakoid proton concentration gradient; Fd – ferredoxin; FI – fluorescence induction; FNR – ferredoxin $NADP^+$ reductase; fs, ps, ns – femtosecond, picosecond, nanosecond; Ga – billions of years before present; hv – represents a photon of light (h = Planck's constant, and v is frequency of light); L – lutein; LHC – light harvesting chlorophyll protein complex; Lhcb – light harvesting protein complex of photosystem II; Lhcbm – m subunit of light harvesting protein complex of photosystem II; LhcbM5 – a specific subunit « M5 » of light harvesting protein complex of photosystem II; LHCSR – stress-related (SR) light-harvesting chlorophyll protein complex (LHC); Lhcx – a light harvesting protein complex found in diatoms equivalent to LHCSR of *Chlamydomonas*; Lhcx1-6 – different forms of Lhcx; LHCX6 – equivalent term for Lhcx6; LHCII – light harvesting complex of photosystem II; LHCIIb – specific light harvesting complex of photosystem II containing chlorophyll *b*; Lx – lutein epoxide; Lx-L – lutein epoxide-lutein cycle; MGDG – monogalactosyldiacylglycerol; $NADP^+$, NADPH – oxidized, reduced nicotinamide adenine dinucleotide phosphate; NPQ – non-photochemical quenching (lowering) of the singlet electronic excitation energy of Chl *a* in photosystem II; N – Neoxanthin; OEC – oxygen evolving complex; OCP, OCP^o, OCP^r – orange carotenoid protein, its orange (o) form, and its red (r) form; PAC – peripheral antenna light harvesting Chl *a*/*b*-protein complex(es); PAL – present atmospheric level; PBS – phycobilisome(s); PC – Plastocyanin; PGR5 – proton gradient regulator; Pheo – pheophytin; PMF – proton motive force; PQ, PQH_2 – plastoquinone, plastoquinol; PS I, PS II – Photosystem I, Photosystem II; PsbS or CP22 – A 22 kDa pigmentless protein of photosystem II, involved in NPQ; PSET – photosynthetic electron transport; Q_A, Q_B – first and second plastoquinone electron acceptors of Photosystem II, the former being a one-electron acceptor, and the latter being a two-electron acceptor; qE – NPQ dependent upon trans-thylakoid proton gradient (ΔpH); qT – related to state changes (see text); RC – reaction center; ROS – Reactive oxygen species; STT7 – LHCIIb specific kinase in *Arabidopsis* related to state changes; stt7 – LHCIIb specific kinase in *Chlamydomonas* related to state changes; V – violaxanthin; VAZ – violaxanthin-antheraxanthin-zeaxanthin cycle; VDE – violaxanthin de-epoxidase; WWC – Water-water cycle; Y_Z, Y_D – tyrosine-161 and tyrosine-160 on D1 and D2 proteins, respectively, Y_Z donates electrons to the oxidized reaction center $P680^+$ but Y_D is a very slow electron donor; Z – zeaxanthin; ZEP – zeaxanthin epoxidase

discoveries of the last 60 years that have led to the current understanding of processes leading to thermal dissipation of excess excitation energy by photosynthetic organisms.

I Introduction

With the rare exception of chemolithoautotrophs (organisms producing energy-rich molecules via oxidation of inorganic compounds; Pfannschmidt and Yang 2012), all life on Earth depends on *Photosynthesis*, a complex process by which plants, algae and cyanobacteria, as well as anoxygenic photosynthetic bacteria, convert the fleeting energy of sunlight into storable and transportable chemical energy on a massive scale. For the basics of photosynthesis and its potential for practical use, see Rabinowitch and Govindjee (1969) and Blankenship (2014). For an overview of the molecular mechanism of light harvesting, see Ruban (2013). Although the annually available energy from sunlight far exceeds the annual energy demand of our world, improvements in natural as well as in artificial photosynthesis must be vigorously pursued in order to meet the energy needs of the increasing human population (Blankenship et al. 2011; Najafpour et al. 2012).

The inner sanctum of plants, which oxidizes water to oxygen, and produces ATP and the reducing power for the reduction of CO_2 to sugars, is embedded in the thylakoid membranes of the chloroplasts (Fig. 1.1). Thylakoids are closed vesicles, which define an outer aqueous phase called the *stroma* and an inner aqueous phase called the *lumen*. The photosynthetic apparatus in the thylakoid membrane includes four protein supercomplexes: Photosystem II (PS II), Cytochrome (Cyt) b_6f, Photosystem I (PS I), and the ATP synthase. Both PS I and PS II collect sunlight and facilitate two energetically uphill steps at the reaction center chlorophylls; PS II decomposes H_2O photochemically to O_2 and protons (H^+s), and reduces plastoquinone; PS I oxidizes plastoquinol via Cyt b_6f, and produces the reductant NADPH that reduces CO_2 to carbohydrate. See Wydrzynski and Satoh (2005) for a discussion of PS II, and Golbeck (2006) for a discussion of PS I. Two facts are critical: (1) only the reaction-center chlorophylls a (Chls a) of PS II (P680) and PS I (P700) convert light energy into chemical energy, with all other reactions following from there; (2) during electron transfer a proton motive force is built up that leads to the synthesis of ATP (at the ATP synthase), which is essential for the conversion of CO_2 to carbohydrate. The latter occurs in the stroma using the enzymes of the Calvin-Benson cycle (details in the Fig. 1.1 legend; see Strand and Kramer, Chap. 18).

Each photosystem carries a reaction center complex (PS I_{RC}, PS II_{RC}) and ensembles of light-harvesting Chl-protein complexes, which are characterized as *Core Antenna*

Glossary of Chemical Terms: **Alkene** – hydrocarbon containing a single carbon-carbon double bond: >**C**=**C**<; **Allene** – hydrocarbon containing two adjacent carbon-carbon double bonds: >**C**=**C**=**C**< with planes of the double bonds normal to each other; **Conjugated double bonds** – two carbon-carbon double bonds separated by a carbon-carbon single bond: >**C**=**C**–**C**=**C**< with the bond-forming p electrons of the carbon atoms delocalized over the conjugate structure and often referred to as *π-electrons* or *pi-electrons*; the two double bonds are coplanar; **Alkyne** – hydrocarbon containing one carbon-carbon triple bond: –**C**≡**C**–; **Diol** – an organic compound containing two hydroxyl groups (a di-alcohol); **Epoxide** – a ring formed by two carbon atoms and an oxygen atom, the anhydritic product of a 1, 2 diol; Epoxides are strained structures; **Di-epoxide** – organic molecule with two epoxide groups; **Epoxidic** – property of an organic compound of being an epoxide; **Epoxidation** – addition of an oxygen atom to a double bond to form an epoxide; **De-epoxidation** – removal of an oxygen atom from an epoxide and reformation of the double bond; **Lipocalins** – family of multifunctional proteins that bind small lipophilic molecules

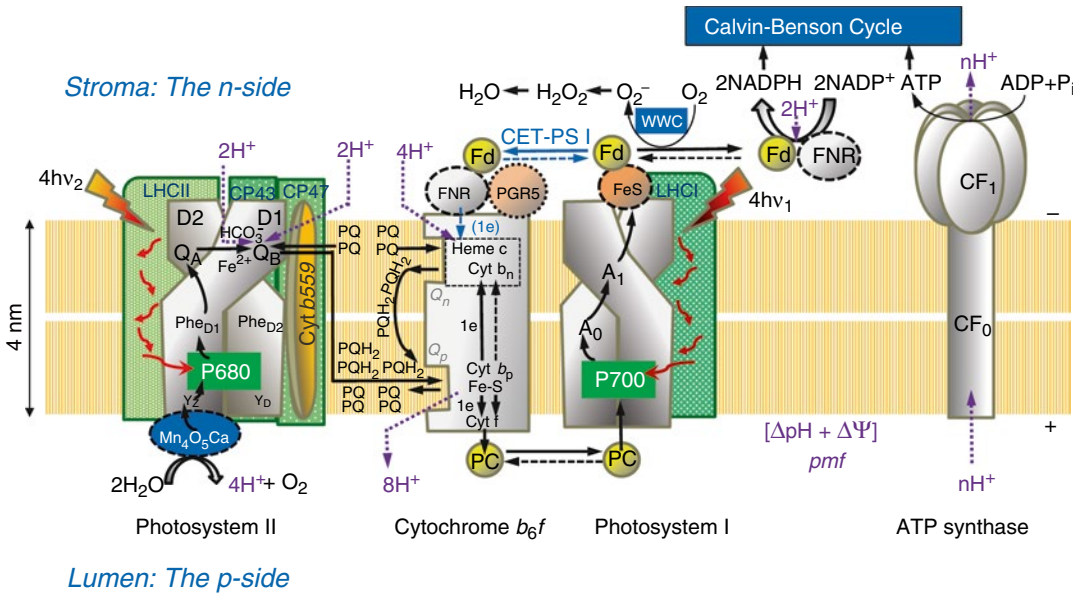

Fig. 1.1. A diagrammatic scheme of the thylakoid membrane of oxygenic photosynthetic organisms. Four major intramembranous protein complexes are shown: *From left to right*: Photosystem II (PS II; water-plastoquinone oxido-reductase); cytochrome (Cyt) b_6f (plastoquinol-plastocyanin-oxidoreductase); Photosystem I (PS I; plastocyanin-ferredoxin-oxido-reductase); and ATP synthase. Absorption of photons by each of the two photosystems by their respective light harvesting complexes (LHC) and other antenna subunits, and after excitation energy transfer, ultimately leads to charge separations within the PS I and the PS II reaction center complexes. hν stands for a photon of light (h = Planck's constant; and ν = frequency of light). **Photosystem II**: Primary radical pair in PS II is [$P680^+Phe_{D1}^-$], where P680 (represents the participation of a few Chl *a* molecules in the D1/D2 complex, not shown) and Phe_{D1} (pheophytin on the D1 protein) are the primary electron donor and acceptor of PS II, respectively. The electron is transferred from Phe_{D1}^- to Q_A (a one-electron accepting plastoquinone, tightly bound to a site on the D2 protein). The oxidized primary donor $P680^+$ receives an electron via Y_Z (tyrosine -161 on the D1 protein), which, in turn, receives an electron from the tetra-nuclear manganese–oxygen–calcium cluster (Mn_4O_5Ca) in the O_2-evolving complex (OEC). Further, the electron from Q_A^- is transferred to Q_B (a two-electron accepting plastoquinone, docking on the D1 protein); this plastoquinone is bound weakly to its protein site in its oxidized state, but tightly when it is reduced to Q_B^-. A bicarbonate anion (HCO_3^-) is bound to a non-heme iron (Fe^{2+}) that sits between Q_A and Q_B, which is suggested to participate in Q_B^{2-} protonation. The formed PQH_2 (plastoquinol) at the Q_B-site is released, and then replaced by a new PQ (plastoquinone) molecule from a mobile PQ-pool in the thylakoid membrane. **Cyt b_6f**: The intermediary electron transport, from PS II to PS I, takes place via the Cyt b_6f complex that contains the following intersystem components of the electron transport chain: an iron-sulfur (Fe–S) protein, known as the Rieske FeS protein, one cytochrome *f* (Cyt *f*), two cytochromes b_6 (i.e., Cyt b_p and Cyt b_n) and a heme c (the subscripts "p" and "n" refer to the electrically positive (*inner*) and negative (*outer*) sides of the thylakoid membrane). At the Cyt b_6f complex, PQH_2 is re-oxidized at the Q_p-site (toward the lumen, close to the electrically positive side of the membrane, the p-side), while PQ is reduced, during what has been termed the Q-cycle, at the Q_n-site (toward the stroma, close to the electrically negative n-side of the membrane). Associated with the Cyt b_6f complex there is also a ferredoxin $NADP^+$ reductase (FNR), as well as what has been termed the proton-gradient regulator (PGR5), which is involved in cyclic electron transport around PS I (CET-PS I) via ferredoxin (Fd). **Photosystem I**: The primary charge separation in PS I generates the primary radical pair [$P700^+A_0^-$], where P700 (a special Chl *a* pair) and A_0 (a special Chl *a* molecule) are the primary electron donor and acceptor of PS I, respectively. Plastocyanin (PC; there are more than one PC molecules per PS I), a mobile, water-soluble, copper protein situated on the lumen side of the membrane, transfers electrons from Cyt *f* to $P700^+$, while on the electron acceptor part of PS I the electron from A_0^- is transferred successively to: A_1 (vitamin K_1); three non-heme iron–sulfur centers (shown as *FeS*); and the mobile water-soluble non-heme iron protein ferredoxin (*Fd*), situated on the stroma (or the n) side of the membrane (there are more than one Fd molecules per PS I). The reduced Fd transfers electrons mainly to $NADP^+$ (nicotinamide–adenine dinucleotide phosphate), which is reduced to NADPH via FNR. However, the electrons on reduced ferredoxin may also go to Cyt b_6f leading to a cyclic electron transfer (*CET*). In addition, there is the possibility of other electron acceptors that may receive electrons from reduced Fd; one example is the water-water cycle (WWC), in which O_2 is reduced to

Complexes (CAC) when they sit next to the reaction centers (PS I_{CAC}, PS II_{CAC}) and as Peripheral Antenna Complexes (PAC) when they sit at a distance from it (PS I_{PAC}, PS II_{PAC}). In the current literature, CACs are often called "inner antenna", and PACs "outer antenna". Electronic excitation transfer occurs from PACs to CACs in the two photosystems (Scheme 1; see, e.g., Ke 2001). We note that the terminology of PAC and CAC, used here, has not been in use in much of the current literature in photosynthesis, but we recommend that it be used because it clearly distinguishes between the three major components of the antenna system without having to constantly spell out their full forms.

$$\begin{gathered} \mathrm{PS\,I_{PAC}} \rightarrow \mathrm{PS\,I_{CAC}} \rightarrow \mathrm{PS\,I_{RC}} \\ \text{and} \\ \mathrm{PS\,II_{PAC}} \rightarrow \mathrm{PS\,II_{CAC}} \rightarrow \mathrm{PS\,II_{RC}} \end{gathered} \tag{1}$$

Energetically uphill (implying energy storage) photosynthetic electron transport (PSET) from H_2O to CO_2 occurs in a linear manner (Scheme 2), using the energy of photons absorbed by light-harvesting PACs and CACs. The end products are carbohydrates or sugars (from the reduction of CO_2) and O_2 (from the photochemical decomposition of H_2O). In addition to the uphill linear electron transport from H_2O to CO_2, using PS II and PS I, electrons on reduced ferredoxin and other post-PS I electron accepting intermediates move energetically downhill (implying energy release as heat) to plastoquinone or to Cyt b_6f. The redox energy made available in this way is used for the synthesis of energy-rich ATP in what is known as "cyclic electron flow". In fact, three such pathways exist (see, e.g., Bukhov and Carpentier 2004).

$$\begin{gathered} \mathrm{H_2O} \rightarrow \cdots \rightarrow \mathrm{PS\,II_{RC}} \rightarrow \left(\text{intersystem PSET}\right) \\ \rightarrow \mathrm{PS\,I_{RC}} \rightarrow \cdots \rightarrow \mathrm{CO_2} \end{gathered} \tag{2}$$

Ideally, in order to use its machinery safely and efficiently, a photosynthetic organism must absorb the exact amount of light needed to drive the PS II and PS I photoreactions (see, e.g., Barber and Andersson 1992; Ort 2001; Demmig-Adams et al. 2012). What is the right amount of light cannot be defined on an absolute energy scale, and instead depends on the organism, on its physiological state, as well as on environmental parameters (e.g., temperature). Furthermore, the photon flux falling on the system is subject to fast and slow, as well as

Fig. 1.1. (continued)

O_2^- by Fd^-; subsequently, the H_2O_2 formed can be converted to water. **ATP Synthase**: The enzyme ATP synthase, which is made up of intramembranous (but lumen accessible) CF_0 and stroma-exposed CF_1, lumen-exposed CF_1, synthesizes ATP from adenosine diphosphate (*ADP*) and inorganic phosphate (P_i) using the proton motive force (*pmf*) made up of the transmembrane electrical potential difference ($\Delta\Psi$) and the transmembrane proton concentration difference (ΔpH). ΔpH is built across the thylakoid membrane by protons originating from water "splitting" at the OEC of PS II, and by the translocation of protons from the stroma to the lumen during electron transfer from PS II to plastoquinone and from plastoquinol to Cyt b_6f. The number of protons transferred to the lumen (the *p*-side) from the stroma (the *n*-side) of the thylakoid membrane are represented in a stoichiometric relation with the number of electrons transferred after the absorption of 4 quanta of light by each photosystem (necessary to evolve one O_2 molecule and to reduce 2 $NADP^+$ molecules). We note that the above statement needs modification because the cyclic electron transferred envisaged in the scheme would increase the quantum requirement in PS I. Further, the scheme is not meant to show the details of the number of protons taken up from the "n" side, and released on the "p" side. However, the ATP and NADPH, produced during the process, are finally used, via the Calvin–Benson cycle, to fix CO_2 to produce sugars. The entry of protons into PS II may involve a role of bicarbonate (HCO_3^-; for a review see Shevela et al. 2012). (Source of the figure: Stirbet and Govindjee (2012), as modified by A. Stirbet and Govindjee (unpublished); it also includes information from Stirbet and Govindjee (2011), Cramer and Zhang (2006), Baniulis et al. (2008), and Hasan et al. (2013).

periodic and aperiodic, fluctuations. A photosynthetic organism must therefore be able to first assess the momentary excitation energy level generated by incoming radiation, and then to mobilize its machinery to deal with it.

In 1992, Barber and Andersson stated "Too much of a good thing: light can be bad for photosynthesis". Later, Ort (2001) discussed what plants do when there is too much light. We know that there can be either *too little* or *too much light* for a plant. At suboptimal illumination, a plant underperforms photosynthetically, while superoptimal light may trigger various photo-oxidative, and potentially damaging, processes. Particularly sensitive to inactivation is PS II because it generates a very strong oxidant and is relatively slower (compared to PS I) in using its electronic excitation. In contrast, PS I generates weak oxidant(s) and uses its excitation a bit faster. In other words, excited Chl *a* lives longer and is more prone to photo-oxidative inactivation in PS II than in PS I (see discussion in the context of photochemical damage in Renger 2008; for an interpretation of PS II inactivation as feedback downregulation in whole plants, see Adams and Demmig-Adams, Chap. 2; Adams et al. 2006, Chap. 23; Demmig-Adams et al., Chap. 24, who report an invariable association of photosynthetic inactivation in leaves with accumulation of sugar or starch produced in photosynthesis).

Experiments of Emerson and Arnold (1932a, b) on the green alga *Chlorella*, using repetitive brief and strong light flashes, interspersed by optimal dark periods, led to the conclusion that ~2400 chlorophylls co-operate in the evolution of one O_2 molecule. Soon thereafter, Gaffron and Wohl (1936) explained this result by suggesting that under these conditions, there is transfer of excitation energy, absorbed by many molecules, to a photoenzyme where oxygen evolution occurs. The concept of "antenna" and "reaction center" was born, without those particular terms being used then. We now know that, in addition to Chl *a* as present in all oxygenic photosynthetic organisms, excitation energy transfer (or migration) involves several other pigments, e.g., Chl *b* (in green algae and plants), Chl *c* (in diatoms), and Chl *d* (marine red algae), carotenoids (many organisms) and phycobilins (red algae and cyanobacteria); all these pigments serve as photon harvesters for antenna associated with PS II_{RC} and PS I_{RC} (see Ostroumouv et al., Chap. 4, and references therein; also see Govindjee 1999 for role of carotenoids).

Absorbed photons (in the form of excitation energy) that cannot be used for photochemistry represent excess excitation energy, which has the potential to cause damage to the system. Several mechanisms are known that deal with this excess excitation energy in PS II. In general terms, these are assessed and labeled as NPQ (Non-Photochemical Quenching) processes of the excited state of Chl *a*, the most prominent of which (under conditions conducive for cell division and growth of photosynthetic organisms) are: (a) qE quenching of the excess excitation energy in Chl *a*, and (b) the state 1 (high fluorescent) to state 2 (low fluorescent) transition ($qT_{1\rightarrow2}$). qE encompasses both non-enzymatic (i.e., physicochemical) and enzymatic processes that lead to the dissipation of excess excitation energy as heat; it is triggered by a high proton concentration in the lumen, which is a consequence of water splitting and proton transfer from the stromal space to the lumen space during electron transport (see Fig. 1.1); qE operates via enzymatic processes sensing the level of lumen acidity and ultimately leading to the dissipation of excess excitation energy as heat. Several xanthophyll molecules (see chemical structures in Fig. 1.2) have been shown to play a role in this process; there are several cycles involving different epoxidized and de-epoxidized forms of xanthophylls, each one characteristic of a particular class of photosynthetic organisms (see, e.g., Bungard et al. 1999; and Goss and Jakob 2010).

One of the most studied xanthophyll cycles is termed the VAZ cycle in this book, where V stands for violaxanthin, A for antheraxanthin, and Z for zeaxanthin (see chemical structures in Fig. 1.2). The VAZ cycle is typical of plants and green algae. Diatoms

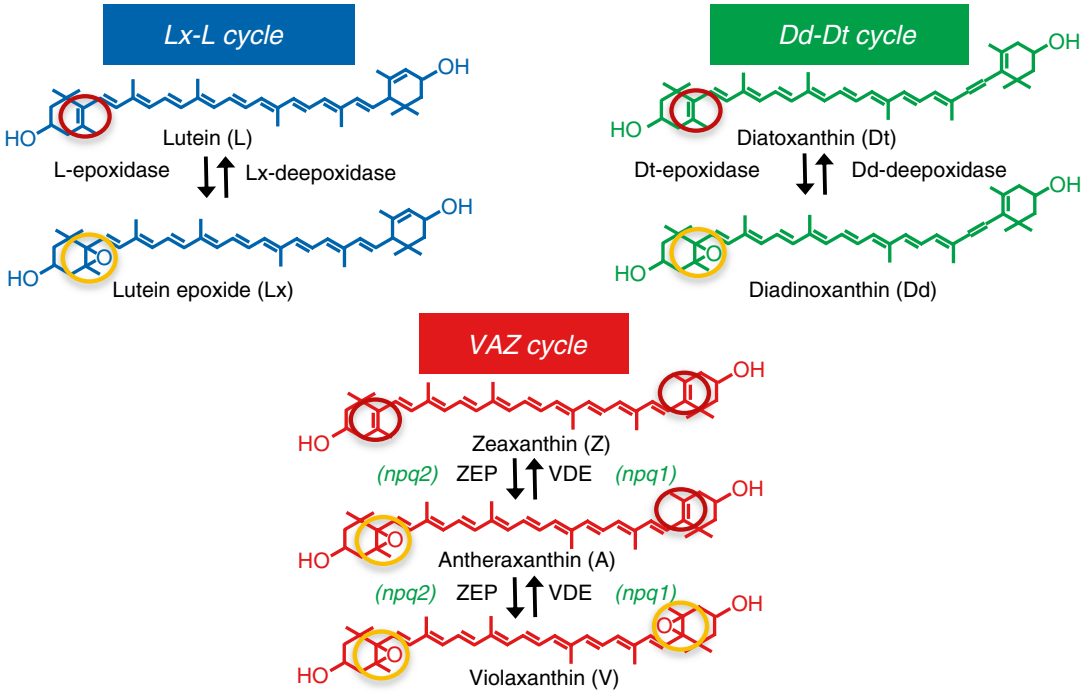

Fig. 1.2. The xanthophyll cycles, which serve to dissipate excess of electronic excitation, that momentarily resides on the Chls *a* of the peripheral antenna complexes of plants and algae, involve enzyme-catalyzed de-epoxidation of epoxidic xanthophylls in strong light and enzyme-catalyzed re-epoxidation in dim light or darkness. The figure illustrates the mechanism of the lutein epoxide-lutein (Lx-L) cycle of plants and green algae (*upper left*), the diadinoxanthin-diatoxanthin (Dd-Dt) cycle of Xanthophyceae, Euglenophyceae, Chrysophyceae, Bacillariophyceae, and diatoms (*upper right*) and the violaxanthin-antheraxanthin-zeaxanthin (*VAZ*) cycle of plants, green algae, brown algae and diatoms (*lower middle*).

possess the diadinoxanthin (Dd) – diatoxanthin (Dt) cycle (dinoxanthin in dinoflagellates) or, for short, Dd-Dt cycle. Finally, there is the lutein epoxide (Lx) - lutein (L) cycle, or Lx-L cycle. On the other hand, the state 1 to state 2 change (or state 1 to state 2 shift), $qT_{1\rightarrow 2}$, is a phenomenon where specific (mobile) subunits of the peripheral antenna complex of PS II (or PS II_{PAC}) migrate and attach to PS I. In state 1, there is more antenna in PS II, whereas in state 2, there is more antenna in PS I. These state changes are triggered by the redox state of intersystem intermediates (plastoquinones and Cyt b_6f) in the membrane phase (reviewed by Allen and Mullineaux 2004), and changes in NADPH/ATP ratio in the stroma space (reviewed by Cardol et al. 2011). In plants and green algae, the $qT_{1\rightarrow 2}$ process involves activation of kinases in the thylakoid membrane that phosphorylate specific light-harvesting complexes (LHCs) in the PS II-rich regions of the thylakoid membrane and enable their translocation, together with Cyt b_6f, to PS I-rich regions. In PS I, the extra excitation energy is used to generate ATP by driving cyclic electron transport around its reaction center ($PS\ II_{RC}$), or is dissipated non-photochemically as heat.

II The Reign of Photochemical Quenching

As late as 1960, it was firmly believed that Chl *a* in plants dissipates a constant fraction of its excitation energy as heat, and what remains is subject to a competition between photosynthetic utilization and Chl *a* fluorescence emission. The complementarity of

photosynthesis and Chl *a* fluorescence was in apparent agreement with Warburg's photolyte model (1Chl *a*: $1CO_2$: $1O_2$; Nickelsen 2012; Nickelsen and Govindjee 2012), and thus measuring fluorescence, by the then newly designed photoelectric methods, was a convenient way of measuring photosynthesis (Papageorgiou and Govindjee 2011). Experimental evidence for the photosynthesis – Chl *a* fluorescence complementarity model was obtained by McAlister and Myers (1940a,b). Characteristic of the prevalence and the longevity of this model are the following two statements. As late as 1971, the two of us wrote: ". . . *both Chl a fluorescence and photosynthesis draw on the excited Chl a population*, *and thus a change in the photosynthetic rate is reflected as a change in the yield of Chl a fluorescence* . . ." (Govindjee and Papageorgiou 1971). In the same spirit, a few years later, Myers (1974) asserted: ". . . *a Chl a molecule cannot use the same quantum of energy for both fluorescence and photochemistry* . . .".

The first evidence that PS II and PS I interact antagonistically was reported by Govindjee et al. (1960). These authors discovered that, when *Chlorella* cells are illuminated with Light 2 (light preferentially absorbed by PS II) and with Light 1 (light preferentially absorbed by PS I), the Chl *a* fluorescence emitted is lower than when they are illuminated with Light 2 alone, suggesting that Light 1 exerts a quenching effect on the Chl *a* fluorescence that Light 2 excites. Subsequently, Duysens et al. (1961) reported that illumination of algal cells in suspension with Light 2 causes photoreduction of an intersystem cytochrome (see Scheme 2), whereas illumination with Light 1 causes its photo-oxidation. Finally, Duysens and Sweers (1963) demonstrated that only the fluorescence emitted by Chls *a* of PS II upon Light 2 excitation competes with photochemical utilization of excitation energy at physiological temperatures, and confirmed that Light 1 causes fluorescence decline (or quenching). On the basis of these observations, Duysens and Sweers concluded that, when oxidized, PS II_{RC} (which they denoted as Q) quenches Chl *a* fluorescence of PS II, while, when reduced (denoted as QH), it does not (i.e., fluorescence is higher). In this newer concept, the complementarity between photosynthetic O_2 evolution and Chl *a* fluorescence of the McAlister and Myers (1940a, b) era was narrowed down to a complementarity between the rate of reduction of Q (now known as Q_A), i.e., photochemistry of PS II, and the yield of Chl *a* fluorescence of PS II.

III The Emergence of the Non-Photochemical Quenching (NPQ) Concept

Although the complementarity between photosynthesis and Chl *a* fluorescence was tenaciously upheld in the 1960s and beyond, its erosion had started much earlier. Already in the early 1950s, it became known that assimilation of CO_2 requires not only energy-rich reductant, reduced nicotinamide adenine dinucleotide phosphate NADPH, but also energy-rich adenosine triphosphate ATP that provides energy upon its hydrolysis. Frenkel (1954) demonstrated that chromatophores of photosynthetic bacteria produce ATP in the light, and Arnon et al. (1954) showed that chloroplasts of higher plants make ATP in the light, i.e., they photophosphorylate (also see Strehler 1953). How the light absorbed by photosynthetic cells is converted to the free energy stored in the ATP molecule was discovered by Jagendorf and his co-workers (Hind and Jagendorf 1963; Jagendorf and Uribe 1966; reviewed by Jagendorf 2002) and was explained on the basis of the chemiosmotic theory of Mitchell (1961) for mitochondria. According to Mitchell's theory, respiratory electron transport is coupled to the translocation of protons across the inner mitochondrial membrane. As hinted earlier, this creates a pH difference (ΔpH), whose free energy is subsequently conserved in the enzymatically synthesized ATP molecule as phosphate bond energy. Jagendorf and coworkers (Hind and Jagendorf 1963; Jagendorf and Uribe 1966; reviewed by Jagendorf 2002) succeeded in demonstrating

that an artificially created ΔpH across the thylakoid membrane is capable of driving ATP synthesis in the absence of light. Therefore, photosynthetic phosphorylation occurs in two sequential stages: **(i)** a light stage that produces ΔpH (acidic inside) via "splitting" of water and proton translocation-coupled PSET; and **(ii)** an enzymatic stage, during which relaxation of ΔpH is coupled to conversion of ADP to ATP. For a discussion of the involvement of the electrochemical potential gradient in ATP production, see Junge and Jackson (1982).

Since PSET is coupled to proton translocation across the thylakoid membrane, any interference with ΔpH would be expected to change the rate of PSET and, through it, to modify *photochemically* the yield of Chl *a* fluorescence. Accumulation of ΔpH (e.g., in the absence of ADP and/or of phosphate) will slow down PSET and lead to a rise of Chl *a* fluorescence from PS II. On the other hand, release of ΔpH by a protonophoric uncoupler will accelerate PSET and cause the Chl *a* fluorescence to drop. In addition to this photochemical quenching, PSET also affects the excited state of Chl *a* non-photochemically by two mechanisms: a faster one triggered by light-induced formation of trans-thylakoid ΔpH (known as "high energy" quenching, or X_E- or qE-quenching, see section "*High Energy Quenching of Chlorophyll a Excitation*") and a slower one triggered by the redox level of intersystem intermediates, e.g., plastoquinone (that leads to state 1 to state 2 transitions, section "*State Transitions*"; also see Krause and Jahns 2004).

In addition, a form of NPQ that is continuously maintained for days or weeks is seen in plants in response to stress (for recent reviews, see Adams et al. 2006, Chap. 23; Demmig-Adams et al. Chap. 24). Many plants experience days, weeks, or entire seasons, during which their green leaves or needles are exposed to intense sunlight while plant growth may be arrested and CO_2 fixation is either minimal or completely absent. Under such conditions, excitation energy is excessive and thermal dissipation of excess energy might be expected to remain high for as long as CO_2 fixation remains low or absent. Such a continuous maintenance of very high NPQ levels in whole leaves or plants is exactly what is observed under both natural and experimental conditions. Two forms of continuously high NPQ have been reported. One form involves continuous maintenance of trans-thylakoid ΔpH in low light and/or in darkness (over entire 24-h day/night periods in nature) and is observed, e.g., during intermittent freezing days in the fall. Using uncoupler treatments, this continuously maintained NPQ was demonstrated to be ΔpH-dependent under experimental (Gilmore and Björkman 1994, 1995) and natural (Verhoeven et al. 1998) conditions. A second form of continuously (24-h per day) maintained high levels of NPQ was reported, e.g., for leaves of the drought-tolerant shrub *Nerium oleander* under long-term drought stress (Demmig et al. 1988), for leaves of the highly salt-tolerant mangroves under a combination of high salinity and high light (Björkman et al. 1988), and for leaves or needles of evergreen shrubs and trees overwintering in areas where soil water remains frozen for months (see Adams and Demmig-Adams, Chap. 2). This continuously maintained strong NPQ in leaves or needles was shown (i) to be accounted for by increases in the rate constant of thermal energy dissipation (Björkman et al. 1988; Demmig et al. 1988), (ii) to correspond to a pronounced shortening of chlorophyll fluorescence lifetime (Gilmore et al. 1995; Gilmore and Ball 2000), and (iii) to be closely and positively correlated with long-term maintenance of high levels of zeaxanthin and arrest of the operation of the VAZ cycle (Adams and Demmig-Adams, Chap. 2; Demmig-Adams et al., Chap. 24). This strong continuously maintained NPQ in plants in nature is associated with strong continuously maintained decreases in F_v/F_m, and thus also corresponds to what is termed photoinhibition in whole plants.

Not all plants and/or natural conditions that inhibit plant growth induce strong continuously maintained NPQ; for a host of mechanisms that lower light interception by leaves, see Logan et al., Chap. 7; for

environmental conditions inducing a lowering of leaf chlorophyll content, see Morales et al., Chap. 27. Lastly, drought-deciduous and or winter-deciduous plant species simply drop their leaves altogether for entire seasons with severe drought or freezing, respectively.

A High Energy Quenching (qE) of Chlorophyll a Excitation

According to Krause and Jahns (2004), "high energy" quenching was recognized first by Papageorgiou and Govindjee (1968a, b) in studies with cyanobacteria and green algae, and by Murata and Sugahara (1969) and Wraight and Crofts (1970) with isolated thylakoids, via demonstratation that Chl *a* fluorescence can also be quenched non-photochemically, namely by the build-up of a "high-energy state" in thylakoids, consisting of a trans-thylakoid proton gradient and related structural alterations of the photosynthetic apparatus.

Demonstration of qE quenching would generally rely on evidence of a light-induced change of Chl *a* fluorescence level, while keeping the primary electron acceptor of PS $II_{RC,}$ Q_A, stably reduced by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). Papageorgiou and Govindjee (1968a,b) explained the light-induced changes of Chl *a* fluorescence in DCMU-treated green algae and cyanobacteria, and their reversal by protonophoric uncouplers, in terms of ΔpH-related changes in thylakoid membrane conformation (cf. Packer 1963; Packer et al. 1965). On the other hand, Murata and Sugahara (1969) ascribed the light-induced Chl *a* fluorescence decrease in DCMU-treated chloroplasts to photophosphorylation as a whole. Subsequent evidence, however, consolidated the concept that local changes in thylakoid membrane conformation mediate the effect of transmembrane ΔpH on Chl *a* fluorescence. This evidence includes the following: **(i)** results from the comparison by Krause (1973) of the kinetics of Chl a fluorescence and of the light-induced absorbance change at 535 nm (ΔA535; a measure of chloroplast shrinkage); **(ii)** the early interpretation by Crofts and Yerkes (1994) of the then existing experimental evidence in terms of a protonation of lumen-exposed glutamate residues of the minor Chl *a*/Chl *b* light harvesting complexes (minor LHCs) resulting in pigment dimers (for a more definitive experiment on the role of glutamate using specific mutants, see Li et al. 2004 and Niyogi and Jansson, Chap. 13); and **(iii)** the demonstration by Horton et al. (2000) that the double de-epoxidation of violaxanthin (V) to zeaxanthin (Z) can cause allosteric changes that convert peripheral PS II antenna (LHCII) from an emissive state to a quenched state.

B State Transitions

Oxygenic photosynthesis uses two pigment systems, PS II and PS I. The absorption spectrum of PS II does not overlap with the absorption spectrum of PS I, particularly at the longer wavelengths beyond, e.g., 690 nm. This is the cause of the "Red Drop" in the action spectrum of the quantum yield of photosynthesis, and the Emerson Enhancement Effect (Emerson and Lewis 1943; Emerson et al. 1957; see Govindjee and Björn 2012). Therefore, depending on the wavelength of the absorbed light, one photosystem may be overstimulated relative to the other. To rectify such an imbalance, oxygenic photosynthetic organisms evolved a light-adaptation mechanism, known as *state transitions*, with which they optimize photosynthesis by adjusting antenna sizes of PS II and PS I, and thereby the amount of excitation energy delivered to PS II_{RC} and PS I_{RC}. When a photosynthetic organism absorbs more PS II light (Light 2), it shifts to *state 2*; in this state, the excitation energy share of PS II decreases and that of the PS I rises. Conversely, when a plant absorbs more PS I light (Light 1) it shifts to *state 1*; in this state, the excitation energy share of PS I decreases and that of PS II rises.

Diagrammatically, these events can be represented as follows (Scheme 3):

$$
\begin{aligned}
&\textbf{\textit{State 1}} - \textit{to} - \textbf{\textit{State 2}}\ \textit{transition}\\
&\textbf{\textit{State 1}}\left[\textit{more}\ \text{excitation}\ \textit{to}\ \textbf{\textit{PS II}}, \textit{less}\ \textbf{to}\ \textbf{\textit{PS I}}\right] + \textbf{Light 2}\\
&\quad \rightarrow \textbf{\textit{State 2}}\left[\textit{more}\ \text{excitation}\ \textbf{to PS I, less to PS II}\right]\\
&\textbf{\textit{State 2}} - \textit{to} - \textbf{\textit{State 1}}\ \textit{transition}\\
&\textbf{\textit{State 2}}\left[\textit{more}\ \text{excitation}\ \textit{to}\ \textbf{\textit{PS I}}, \textit{less to}\ \textbf{\textit{PS II}}\right] + \textbf{Light 1}\\
&\quad \rightarrow \textbf{\textit{State 1}}\left(\textbf{more}\ \text{excitation}\ \textbf{to PS II, less to PS I}\right)
\end{aligned}
\tag{3}
$$

Light-adaptive redistribution of excitation energy between PS II and PS I in cyanobacteria and green algae was first reported in detail by the two of us (see Papageorgiou and Govindjee 1967, 1968a, b; reviewed by Govindjee and Papageorgiou 1971; Papageorgiou 1975). However, this redistribution was then attributed to light-induced conformational changes such as those that had been shown to occur in isolated chloroplasts (Packer 1963; Packer et al. 1965). The breakthrough interpretation of this phenomenon, in terms of the state transitions concept, and its terminology, is due to Murata (1969) and to Bonaventura (1969) and Bonaventura and Myers (1969). This interpretation was based on fluorescence spectra of the red alga *Porphyridium cruentum* in the first case (Murata), and on fluorescence spectra and oxygen evolution by the green alga *Chlorella pyrenoidosa* in the second (Bonaventura and Myers 1969).

In all oxygenic photosynthetic organisms, the chemical signal that triggers the state 1–to–2, and the state 2–to–1 transitions is the redox (oxido-reduction) state of a set of plastoquinone molecules involved in transporting electrons from PS II to PS I (the plastoquinone pool, or PQ pooi, see Fig. 1.1). We know that PS II (water-plastoquinone oxidoreductase) reduces the PQ pool, whereas PS I (plastocyanin-ferredoxin oxidoreductase) oxidizes the PQ pool by receiving electrons from it via the Cyt b_6f complex (Ke 2001; Wydrzynski and Satoh 2005; Golbeck 2006). Thus, there is an antagonistic regulation (feedback regulation, so-to-say) of the PQ pool by the photochemical activities of PS II and PS I. In addition to this regulation, transitions to state 1 or to state 2 can be realized in the absence of light by means of chemical manipulation of the oxido-reduction state of the PQ-pool (Vener et al. 1995).

Beyond these two first steps, however, the detailed molecular mechanism of the state transitions in green algae and plants, which contain only intrinsic Chl *a*/Chl *b*-binding antenna proteins (LHC proteins), is quite different from that in cyanobacteria and red algae that contain no intrinsic Chl *a*/Chl *b*-binding proteins. In plants and green algae, state transitions involve lateral displacement of the intramembranous specific mobile LHCII subunits and of the Cyt b_6f complex from PS II to PS I and back. In cyanobacteria, however, light harvesting is by phycobilisomes (PBS) that are extrinsic to the thylakoid membrane (i.e., they are extramembranous antenna) and can associate with PS II and with PS I. The PBS have three types of chromophores: phycoerythrins, phycocyanins and allophycocyanins. It has long been known that excitation energy is efficiently transferred from phycoerythrin to phycocyanin and from phycocyanin to allophycocyanin, and then from allophycocyanin to Chls *a* in PS II or PS I (see, e.g., Glazer 1989; Mimuro 2004; Adir 2005; and citations therein). Translational and/or rotational movements of the PBS brings them closer to one or the other photosystem and facilitate excitation energy transfer from the PBS to that respective photosystem. Since at room temperature, PS II emits more Chl *a* fluorescence than PS I, transition from *state 1* → *state 2* leads to a decrease in Chl *a* fluorescence intensity, and conversely, transition from *state 2* → *state 1* leads to an increase in fluorescence. At 77 K,

state 1 (also referred to as state I) and state 2 (also referred to as state II) can be recognized by the characteristic emission spectra: in state 1, one observes higher PS II (F686 and F696) emission bands, and a lower PS I (F730) emission band. For further background on these emission bands, see chapters in Govindjee et al. (1986) and Papageorgiou and Govindjee (2004); for LHCII subunits, see Kargul and Barber (2008), Iwai et al. (2008, 2010a, b), and Minagawa (2011); for the Cyt b_6f complex, see Cramer and Zhang (2006), Baniulis et al. (2008), Kallas (2012), and Hasan et al. (2013); for the PBS, see Allen and Mullineaux (2004).

Judging from the number of published research papers and reviews, we may say that the state transition concept has proven to be highly fertile in the area of photosynthesis research. Here we list only few selected post-2001 reviews: Allen and Forsberg (2001), Haldrup et al. (2001), Kruse (2001), Wollman (2001), Allen (2002), Zer and Ohad (2003), Allen and Mullineaux (2004), Bruce and Vasil'ev (2004), Finazzi and Forti (2004), Rochaix (2007), Murata (2009), Lemeille and Rochaix (2010), Minagawa (2011), Tikkanen et al. (2011), Papageorgiou and Govindjee (2011), Mohanty et al. (2012), Papageorgiou (2012), and Puthiyaveetil et al. (2012).

Quite often state transitions are listed as NPQ processes and are symbolized as qT, as we have often done ourselves. In our opinion, this designation, however, and its symbol are inaccurate and misleading. Here is the reason: State transitions are two processes, with different impact on the electronic excitation energy that momentarily resides on Chl *a* molecules of PS II. The state 2 →1 process provides excitation to PS II and therefore it cannot be a true qT or NPQ process. Since the state 1 → 2 transition depletes excitation energy from PS II and transfers it to PS I, it may be counted as an NPQ or $qT_{1\rightarrow2}$ process. However, the excitation energy that is transferred to PS I is conserved, for the most part, as ATP via the cyclic PSET pathway (see section on "Terminology and Semantics" for relevant citations).

Several reviews (see, e.g., Tikkanen and Aro 2012; Tikkanen et al. 2012) have discussed connections between (i) mechanisms serving to re-distribute excitation energy between PS II and PS I under limiting light and (ii) mechanisms serving in *net* energy dissipation under excess-light conditions and also involving interactions among PS II, LHCII, and PS I.

C Terminology and Semantics

As applied to photosynthesis, *non-photochemical quenching* (NPQ) is a scientific term whose semantics do not coincide sensu stricto with its literal content. Thus, *non-photochemical* excludes only the exciton trapping act (or the primary charge separation) at the PS II and PS I reaction centers but includes all other possible photochemical reactions. Secondly, the *quenching* part of the term does not necessarily pertain only to a stable population of Chl *a* molecules but may also involve population shifts, as for example in the state 1 to state 2 transition ($qT_{1\rightarrow2}$; see section on "State Transitions").

In addition, most groups researching NPQ processes focus on short-term physicochemical events (up to a few minutes; although see section "The Emergence of the Non-Photochemical Quenching (NPQ) Concept" on a form of NPQ continuously maintained for days, weeks, or months in certain plant species and certain growth-inhibiting environments; see also Adams and Demmig-Adams, Chap. 2 and Demmig-Adams et al., Chap. 24). The NPQ processes may **(a)** be triggered and regulated antagonistically by chemical or physical signals that PSET generates (e.g., low lumen pH, electrical polarization of the thylakoid membrane, oxidoreduction level of the intersystem intermediates), or **(b)** by PSET-independent signals, such as strong blue-green light as in the case of the PBS-containing cyanobacteria (reviewed by Kirilovsky and Kerfeld 2012; see also Kirilovsky et al., Chap. 22). In the latter case, blue-green light activates a stroma soluble carotenoid protein, known as the Orange Carotenoid Protein (OCP) that, after

light activation ($OCP^0 + h\nu \rightarrow OCP^r$, the superscripts "o" and "r" refer to orange and red forms) attaches to the terminal allophycocyanin emitter of the PBS and dissipates its (excess) excitation energy before it is transferred to the intra-membranous Chls *a* of the cyanobacterium. According to Gorbunov et al. (2011), the light activation leads first to an intermediate form, OCP^i, which in a subsequent dark step transforms to the active form OCP^r. The inactivation of OCP^r is effected by a stroma protein, known as the *fluorescence recovery protein*.

OCP quenching is not antagonistically regulated by PS II and PS I, but it is triggered by strong excitation energy and not necessarily by excess excitation energy. Characteristically, whereas in plants and algae, the excess excitation energy threshold depends on the physiological state of the cell, there is no physiologically set excitation energy threshold for OCP quenching. In fact, OCP-dependent quenching has been demonstrated to occur in a reconstituted system in vitro (Gwizdala et al. 2011) and in a S*ynechocystis* mutant that lacked both photosystems (Rakhimberdieva et al. 2011).

IV NPQ Mechanisms and Atmospheric Oxygen Content

The principal biological role of the qE mechanism (see section on "High Energy Quenching of Chlorophyll *a* Excitation") is protection from photo-oxidative damage. We first present a basic background on the energy levels of molecules: Molecules have different electronic excitonic energy levels: ground state, first singlet excited state, higher singlet-excited states, and corresponding triplet states. The spin multiplicity of the system equals $S = 2J + 1$, where J stands for the sum of electron spins in the molecule. The electron spin has a value either +1/2 or -1/2, and since all electrons in a molecule are paired, $J = 0$ and $S = 1$. This is the singlet state. However, if the spin of one electron is flipped by some means, then J becomes 1 and the multiplicity ($S = 2J + 1$) becomes 3. This is the triplet state (see Clayton 1970; Rabinowitch and Govindjee 1969). Excitation of ^{1}Chl *a* (in the ground state, the lowest singlet state) leads to excited Chl *a* singlet state (^{1}Chl *a**); and if by some means, the spin of one electron in an electron pair is flipped, it would give rise to longer-living excited triplet state (^{3}Chl *a**); the process that leads to the transition of excited singlet to a triplet state is called intersystem crossing. This triplet state can react with $^3O_2$ to produce excited O_2 singlets ($^1O_2$*) and ground-state reactive oxygen species (ROS), all potent oxidants. Triplet Chl *a* may also arise by charge recombination reactions, such as between a Chl *a* cation (Chl^+a) and a pheophytin anion ($Pheo^-a$) in the PS II reaction center (Pospisil 2012). We refer the reader to Fig. 1.1 and its detailed legend where the photosynthetic reactions are described; the primary photochemistry of PS II leads to the formation of Chl^+a and $Pheo^-a$, and it is recombination of these charges that is suggested to lead to the formation of ^{3}Chl *a** among other reactions.

Were such photo-oxidative reactions a problem for the earliest cyanobacteria on Earth? As conjectured from geochemical evidence, cyanobacteria must have appeared some time between 3.85 and 2.7 Ga (billions of years before the present time; Falkowski 2006; Buick 2008; Blankenship 2010; Hohmann-Marriott and Blankenship 2011). At that time, the Earth was warmer and covered by seawater, while its atmosphere was a mixture of methane, carbon dioxide, nitrogen and hydrogen. Free oxygen was essentially absent, well below 10^{-5} of its Present Atmospheric Level (PAL; see Holland 2006; Buick 2008, and citations therein), although it may have risen to $\sim 3 \times 10^{-4}$ PAL at ~3 Ga (Crowe et al. 2013). Nevertheless, the answer to the question posed above is probably yes. In view of the fact that there was always plenty of light and that cyanobacteria have survived, it seems logical that they must have evolved some sort of photoprotective mechanism (Blankenship 2010), essentially the Orange Carotenoid Protein (OCP) mechanism that dissipates excitation

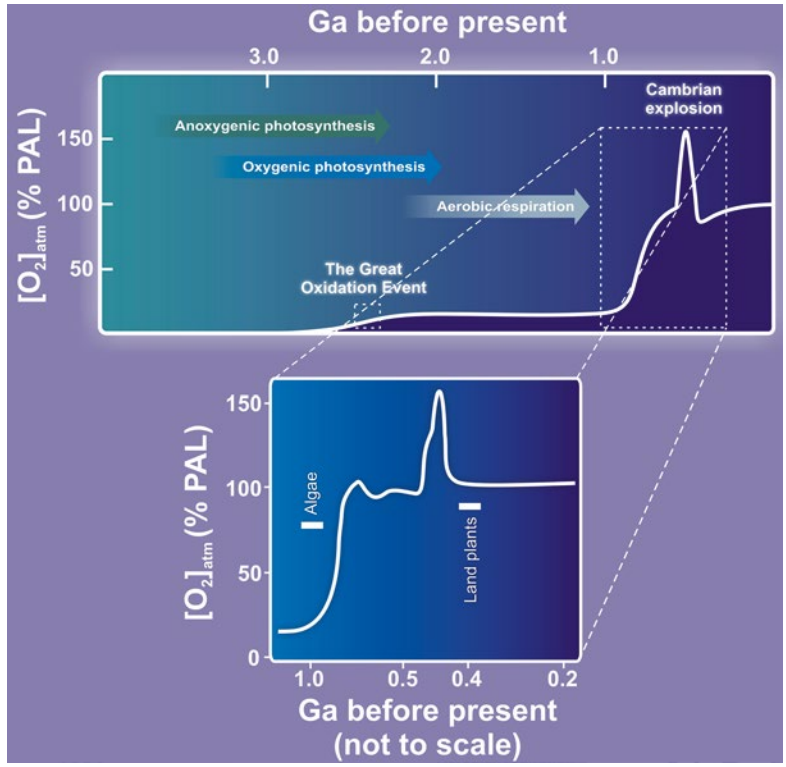

Fig. 1.3. A schematic view of the changing concentration of atmospheric O_2 as a function of geological time in billions of years (*Ga*) before the present time. Correlations between the estimated changes of O_2 concentration in the atmosphere (in % of present atmospheric level, PAL of O_2) and the evolution of metabolic pathways are based on numerous data (see, e.g., Falkowski 2006; Tomitani et al. 2006; Kump 2008; Blankenship 2002, 2010; Björn and Govindjee 2007, 2009; Hohmann-Marriott and Blankenship 2011 and references therein). The dates in the figure and in the zoomed insert are not to scale and are approximations. Uncertainties of the selected evolutionary events in the zoomed insert are depicted with bars. This figure was prepared by D. Shevela and Govindjee by modifying and adapting the figures published by Govindjee and Shevela (2011) and Shevela et al. (2013).

energy of the PBS before this energy is transferred to the core antenna complexes (Kirilovsky and Kerfeld 2012; Kirilovsky et al., Chap. 22).

As a result of oxygenic photosynthesis by cyanobacteria, the O_2 level in the atmosphere rose and by the time of the primary endosymbiosis event, estimated to have occurred at ~0.9 Ga (Shih and Matzke 2013), when a heterotrophic eukaryotic cell engulfed a cyanobacterium, the O_2 level rose to about 0.2 PAL (see Fig. 1.3). The engulfed cyanobacterium eventually became a chloroplast, having transferred most of its genome to the nucleus of the host, and after having replaced the extramembranous PBS and OCP complexes with intramembranous, Chl *a*/*b*/xanthophyll-binding complexes, the LHC proteins (see, e.g., Hohmann-Marriott and Blankenship 2011; Niyogi and Truong 2013).

Were the intra-membranous light-harvesting antenna of the oxygenic photosynthetic eukaryote an advantage, or a disadvantage, compared to the extra-membranous light harvesting antenna of the oxygenic prokaryote? First of all, internal antenna were structurally more stable than external PBS whose structural integrity is known to be temperature- and ionic strength-dependent (Gantt et al. 1979). Considering the structural integrity, therefore, the internalization of the

peripheral light harvesting antenna in the thylakoid membranes can definitely be viewed as an advantage.

On the other hand, since ROS primarily attack lipids, having ROS formed outside the thylakoid membrane by the excited PBS would be far less dangerous for the sensitive intra-membranous complexes. A simple mechanism, based on one and the same protein, the OCP, for sensing and dissipating the excitation energy of the PBS, would suffice for both early and present-day cyanobacteria. In contrast, in the case of the membrane-internal LHC proteins, the chance for photo-oxidative damage is more serious, taking into account the rise in atmospheric O_2 to 1 PAL (Fig. 1.3). We may speculate that, to meet the increased danger of photo-oxidative damage, LHC proteins evolved to serve not only for light-harvesting function, but also for sensing and dissipating excess excitation energy, e.g., the LHCSR (stress-related light-harvesting complex) proteins in green algae and the equivalent LHCX6 protein in diatoms; see section on "Xanthophyll-dependent "High Energy" Quenching of Chlorophyll *a* Excitation" for further information and citations.

Not much is known about the early evolution of NPQ, especially as oxygen concentration changed over time; in fact, lateral transfer of genes makes this exercise difficult. However, based on their studies with the moss *Phycomitrella patens*, Alboresi et al. (2010) have discussed the evolution of photoprotection mechanisms as land plants evolved. Gerotto et al. (2012) showed the co-existence of algal as well as higher-plant mechanisms of photoprotection in this moss. Since light-harvesting antennas are involved in photoprotection, their evolution is of interest to us (see Koziol et al. 2007). For a discussion of the evolution of photosynthesis, see, e.g., Blankenship (2002, 2010) and Björn and Govindjee (2007, 2009). Further research and insight is needed to deal with the important question about the evolution of NPQ. We must indeed wait.

Concerning the evolution of rapidly reversible NPQ versus continuously maintained NPQ, one may speculate that the ability to perform strong and continuous NPQ may have preceded the need to disengage NPQ as quickly as possible to facilitate rapid return to maximal photochemical efficiency whenever light was limiting photosynthesis of fast-growing land plants with very high maximal photosynthesis rates. The evolution of several xanthophyll cycles may, likewise, also have been driven by the need to quickly *remove* "dissipaters" rather than the need to *form* dissipaters (see also Adams and Demmig-Adams, Chap. 2).

V Timeline of Discoveries Relating to the Major NPQ Processes

There is a message in all timelines: they trace the evolution of ideas and are instructive in themselves. We can call this idea «*scaling from the past*». For us, these timelines are fascinating and useful in teaching as well as in research. We need to learn to weave in and weave out to grasp the insights of the process as we read through the timeline discoveries that follow. We recognize that a novice to the field may not always see connections upon first reading, but timelines will help highlight the evolution of ideas and thoughts over time. We refer the readers to the timeline of photosynthesis research by Govindjee and Krogmann (2004).

Going back to the topic of this chapter, the major NPQ process that dissipates most of the excess excitation energy of PS II as heat is triggered by physicochemical signals generated by intersystem PSET, and is subject to antagonistic regulation by PS II and PS I activities (feedback regulation). The "high-energy" state of the thylakoid membrane also encompasses an electrostatic potential difference ($\Delta\Psi$) across the membrane (lumen side positive), as well as concentration differences of ionic and non-ionic solutes ($\Delta\mu_i$). Two light-induced processes lead to accumulation of protons in the lumen: **(i)** decomposition of H_2O into electrons, O_2 and protons; and **(ii)** the translocation of stroma protons to the lumen by non-cyclic and cyclic PSET. The high-energy state of the

membrane is known to somehow quench singlet excited Chl *a* (^{1}Chl *a**). This excitation quenching process is usually described as "high-energy quenching" and is symbolized as qE (see section on "High Energy Quenching (qE) of Chlorophyll *a* Excitation").

On the other hand, during state 1-to-state 2 transition, Lhcb subunits of peripheral PS II antenna shift (move) to the vicinity of PS I; this happens when intersystem PSET intermediates (e.g., plastoquinones) are reduced when PS II activity outpaces PS I. The Lhcb subunit is shifted (moved) back to the PS II vicinity from PS I during the state 2 to state 1 transition, which occurs when PSET intermediates become oxidized as a result of PS II being outpaced by PS I. The important process for relieving PS II from excess excitation energy is the state 1-to-state 2 transition.

If we define NPQ to be a process that facilitates dissipation of excess excitation energy, state transitions can hardly be termed as such, as mentioned earlier in this chapter. The extra excitation energy that PS I receives upon going from state 1 to state 2 is used primarily to make ATP via cyclic electron transport around PS I. On the other hand, the reverse process, that of state 2-to-state 1 transition, supplies additional excitation energy to PS II.

A High-Energy Quenching of Chlorophyll a Excitation

1 Xanthophyll Cycles: Biochemistry and Occurrence

1957

Discovery that light lowers, and that darkness restores, the violaxanthin content of plant leaves

Sapozhnikov et al. (1957) reported that, during a period of high light illumination, the violaxanthin (V) content of leaves decreased and that of lutein (L) increased. A subsequent dark period reversed these changes, i.e., L content was decreased and V content increased. These reciprocal changes in the oxidation state of plant xanthophylls were interpreted as indicating a light-induced, single step, de-epoxidation of di-epoxide V to the 1, 2-diol L. See reviews by Sapozhnikov (1973), Pfündel and Bilger (1994), and Yamamoto (2006b).

1959

Light-induced de-epoxidation of violaxanthin in green algae is quantitatively reversed in darkness

Working with the green algae *Chlorella pyrenoidosa* and *Scenedesmus obliquus*, and using ^{14}C-labeling for quantitatively tracking xanthophyll changes, Blass et al. (1959) determined that light-induced de-epoxidation of V and its dark-induced re-epoxidation are fully reversible. Further, they postulated an enzymatic dark resynthesis of V from Z via A.

1962

Discovery of the xanthophyll cycle

Yamamoto et al. (1962) had well-founded doubts about the light-induced conversion of V to L proposed by Sapozhnikov et al. (1957): first because of the implied double de-epoxidation in a single-step, and second, because of the implied isomerization of a *β*-carotene-type xanthophyll (V) to the *α*-carotene-type xanthophyll (L). Experimenting with spinach and bean leaves and using two-stage column chromatography, Yamamoto et al. (1962) succeeded in isolating the mono-epoxide antheraxanthin (A) and diepoxide zeaxanthin (Z), both *β*-carotene-type xanthophylls, as the products of light-induced de-epoxidation of V, while L remained unchanged; to interpret these light-induced/dark-reversed changes, they proposed the following mechanism:

$$\text{Light} \Rightarrow \quad \text{V} \Leftrightarrow \text{A} \Leftrightarrow \text{Z} \quad \Leftarrow \text{Dark}$$

Since additional light-dark induced de-epoxidation/epoxidation cycles, involving other xanthophylls (e.g., diadinoxanthin and lutein epoxide; see below Fig. 1.2) were subsequently discovered, the first xanthophyll cycle of Yamamoto et al. (1962) is now widely designated as the *violaxanthin cycle* (VAZ cycle, where V stands for violaxanthin, A for antheraxanthin and Z for zeaxanthin, as noted above). For a review, see Yamamoto (2006b).

1967

The VAZ cycle is associated with light-induced acidification of the thylakoid lumen

Hager (1967a, 1969) demonstrated that the VAZ cycle occurs in plant leaves (e.g., spinach) as well as green algal cells (e.g., *Chlorella pyrenoidosa*) and showed that V de-epoxidation required an acidic lumen and the presence of ascorbate.

In spinach chloroplasts, de-epoxidation of V can be triggered (a) in the light, by acidification of the lumen via H^+-translocating PSET (both non-cyclic and cyclic; see Fig. 1.1), (b) in the dark, by hydrolysis of exogenous ATP, and (c) in the dark and in the absence of ATP, by lowering pH of the suspension to ~5.0 (after the experiment of Hind and Jagendorf 1963). In *Chlorella* cells, V-to-Z conversion occurs upon lowering suspension pH to below 7.0. Protonophoric uncouplers, eliminating trans-membrane ΔpH, abolish both photophosphorylation and light-induced Z formation. Sonicated chloroplasts that cannot maintain a trans-membrane ΔpH are incapable of photophosphorylation as well as V-to-Z de-epoxidation. This suggests that a closed thylakoid membrane, capable of maintaining a proton concentration difference between the external and the internal aqueous phases, is a necessary condition for light-induced V de-epoxidation.

Both the forward (V → A → Z) and the backward (V ← A ← Z) reactions are enzymatic

Heating *Chlorella* cells for a short time stops the de-epoxidation reaction (V → A → Z), implicating the involvement of an enzyme (Hager 1967a); a Cu-containing enzyme was invoked to catalyze the epoxidation reaction (V ← A ← Z; Hager 1967b).

1970

Isolation of the enzyme violaxanthin de-epoxidase (VDE)

Hager and Perz (1970) isolated a VDE complex from spinach chloroplasts that, in the presence of ascorbate, de-epoxidizes V to Z. Using lettuce chloroplasts, Siefermann and Yamamoto (1974) established that de-epoxidation of V is a first order reaction, suggesting diffusion control for the process. Observing that VDE is isolated together with one monogalactosyl diacylglycerol (MGDG) molecule, Yamamoto and Higashi (1978) proposed that MGDG molecules in the vicinity of VDE are necessary for solubilizing the substrates (carotenoids being water-insoluble) and that the MGDG molecules act as functional components at the enzyme's active center. Since VDE de-epoxidizes only straight-chain all-*trans* carotenoids, such as V and A, but not bent-chain carotenoids, such as the 9-*cis* neoxanthin (N), Yamamoto and Higashi (1978) visualized the active center of VDE to be situated within a hollow well-like structure into which only straight chain carotenoids can fit. Lastly, Hager and Holocher (1994) found that VDE moves freely in the lumen at near-neutral pH and binds maximally to the lumen-exposed side of the membrane at pH 5.0. Thus, the two enzymes of the VAZ cycle are located on the opposite sides of the membrane, VDE in the lumen-exposed side and ZEP (zeaxanthin epoxidase) in the stroma-exposed side. See a review by Hager (1980).

Discovery and occurrence of the diadinoxanthin-diatoxanthin (Dd-Dt) cycle

The alkyne (one triple bond) mono-epoxidic carotenoid diadinoxanthin (Dd) is dominant in

yellow-green algae, the *Xanthophyceae*. Stransky and Hager (1970) discovered that, during illumination, the epoxy oxygen of Dd is cleaved in a single step and a –C = C– double bond is formed in its place, yielding diatoxanthin (Dt) (also see Fig. 1.2). The de-epoxidation reaction is reversed in the dark. The Dd-Dt cycle was also identified in *Euglenophyceae*, *Chrysophyceae* and *Bacillariophyceae* (Stransky and Hager 1970; Demers et al. 1991), and in diatoms (Arsalane et al. 1994). Later, however, Lohr and Wilhelm (1999, 2001) discovered that both the VAZ cycle and the Dd-Dt cycle operate in diatoms. In fact, it appears that V (double bond at carbons 7′,8′) is the precursor of both Dd (triple bond at carbons 7′,8′) and fucoxanthin (allenic double bonds at carbons 6′,7′ and 7′, 8′). For further information, see Goss and Jakob (2010); and Büchel, Chap. 11 and Lavaud and Goss, Chap. 20.

1974

Enzyme kinetics of V de-epoxidation by VDE

Siefermann and Yamamoto (1974) established that light-induced de-epoxidation of V in lettuce chloroplasts, in the presence of ascorbate, obeys 1st-order kinetics. The extent of de-epoxidation depends on availability of the substrate, which was also shown to be light-dependent, and, indirectly, on PSET (Siefermann and Yamamoto 1975a; Bilger and Björkman 1990; Demmig-Adams and Adams 1990).

1975

Characterization of zeaxanthin epoxidase (ZEP)

ZEP, the enzyme that epoxidizes Z to A and A to V, was characterized by Hager (1975) and Siefermann and Yamamoto (1975b, c) in chloroplasts. ZEP is located on the stroma-facing side of the thylakoid membrane, requires O_2 and NADPH (and flavin-adenine dinucleotide (FAD) as shown later by Büch et al. 1995) as co-substrates, and exhibits optimal activity at pH ~7.3; it has no activity below pH 5.5. Further, ZEP was characterized as a mono-oxygenase because it donates one O-atom of the O_2 molecule to substrates (Z and A) and forms H_2O with the other. The ZEP-catalyzed epoxidation was found to be much slower than VDE-catalyzed de-epoxidation; further, it did not require any light if supplied with the required co-substrates. However, of the two consecutive back reactions, Z → A was shown to be faster than the A → V reaction (Goss et al. 2006).

1978

The two consecutive de-epoxidations V → A and A → Z occur at different rates

Both de-epoxidations are catalyzed by VDE that, however, has a higher affinity for A than for V (Yamamoto and Higashi 1978; Grotz et al. 1999; Goss 2003).

1994

At neutral pH VDE is freely mobile in the lumen; it binds to the thylakoid membrane at ~pH 5

Hager and Holocher (1994) detected VDE in the supernatant of pelleted chloroplasts isolated from dark-pretreated spinach leaves. VDE, that must have been released from the pellet to go into the supernatant, decreased in quantity at pH < 6.5 and was absent at pH ~5 (near the optimum of enzyme activity). These findings support the view that the two enzymes of the VAZ cycle are located on the opposite sides of the thylakoid membrane, i.e., the ascorbate-dependent VDE on the lumen side and the NADPH-dependent ZEP on the stroma side, while V is located within the lipid phase of the membrane. See also Bratt et al. (1995) for supporting evidence.

VDE activity is controlled by the pH in sequestered membrane domains

Using high- and low-salt suspensions of broken pea chloroplasts, and conditions with and without ATP synthesis, Pfündel et al.

(1994) succeeded in differentiating between lumen pH and the pH in sequestered membrane domains. It is the latter pH that determines the activity of VDE that can be active even when bulk lumen pH is above 6.0.

1996

Purification and characterization of VDE

Rockholm and Yamamoto (1996) purified and characterized VDE from lettuce. Its apparent molecular mass was determined to be 43 kD, its optimal pH at ~5.2 and its isoelectric point at 5.4. Purified VDE could be precipitated by centrifugation only after addition of MGDG which is a Hex-II lipid containing mostly unsaturated fatty acids, and therefore forms tubular structures, not bilayers, with the hydrophobic fatty acid tails pointing outwards. The affinity of VDE to MGDG is specific; other thylakoid membrane lipids do not precipitate VDE. The molecular mass of VDE purified from spinach was determined to be 41 kD (Kuwabara et al. 1999).

1998

The VAZ cycle enzymes are members of the lipocalin family of proteins

Amino-acid-sequence analyses of VDE and ZEP, using cDNA libraries from several plants, enabled Bugos et al. (1998; see the review by Hieber et al. 2000) to classify these two VAZ cycle enzymes as lipocalins. Lipocalins are multifunctional proteins capable of carrying small lipophilic substrates (see, e.g., Boca et al. 2013). In the case of the VAZ cycle enzymes, the lipophilic substrates V and Z enter into hydrophobic, barrel-like cavities of the enzymes VDE and ZEP where they are processed by the active centers. Only straight chain (*all-trans*) carotenoids can fit into these cavities. A, the intermediate in the reversible V → Z transformation, fits into the active-center cavities of both VDE and ZEP. A hollow, well-like structure, such as the active center of VDE, was anticipated earlier by Yamamoto and Higashi (1978; see above) in view of VDE's inability to de-epoxidize the 9-cis carotenoid neoxanthin.

Interestingly, isolated LHCII, the major light-harvesting complex of PS II, also epoxidizes Z to V (Gruczecki and Krupa 1993a, b); this observation needs further confirmation.

1999

The majority of the violaxanthin available for de-epoxidation binds to LHCII

Isolated trimeric LHCII complexes from the annual plant spinach contain the majority of the VAZ cycle carotenoids (estimated at 15–19 molecules per reaction center; see Ruban et al. 1999 for detailed experimental conditions). With a mild detergent extraction, it was further established that V binds more loosely than the other xanthophylls (2 L and 1 neoxanthin, N) of the LHCII monomer. The latter result anticipated the subsequent elucidation of the crystal structure of LHCII monomers (Liu et al. 2004) according to which the VAZ cycle carotenoids attach to the periphery of the complex, making nearly no contact with the protein backbone, in contrast to the two L, which do; N is an intermediate between the two. Also see Fig. 1.2.

2000

Monogalactosyldiacylglycerol (MGDG) domains of the thylakoid membrane are the likely sites for VDE– and DDE–catalyzed de-epoxidation

To be de-epoxidized, all-*trans* V must fit into the barrel-like cavity, where the active center of the VDE is located (Bugos et al. 1998; also see Bugos and Yamamoto 1996). This requires V to dissociate from its site on an Lhcb antenna protein and to move to the surrounding lipid phase for an encounter with a VDE molecule. According to this picture, the fluidity of the lipid phase and the

solubility of the V in it are expected to be important determinants for the rate and extent of VDE-catalyzed de-epoxidation. A number of important in vitro studies with V-containing liposomes of known lipid composition substantiate the following expectation: An increase in MGDG content will increase both fluidity of the liposome lipid bilayer (Latowski et al. 2000, 2002) and solubility of V and of Dd in it (Goss et al. 2005; reviewed by Goss and Jakob 2010).

The phase structure of membrane lipids determines the de-epoxidizing activity of VDE

In vitro studies with liposomes and micelles made from thylakoid membrane lipids showed higher V solubility and VDE activity in micelle-forming lipids like MGDG than in bilayer-forming lipids like digalactosyldiacylglycerol (DGDG; Latowski et al. 2004; Goss et al. 2005; Yamamoto 2006a; Vieler et al. 2008). MGDG and DGDG are the major lipids of thylakoid membranes.

2001

VDE activity in primitive green algae (Prasinophyceae)

Frommolt et al. (2001) discovered that the prasinophycean alga *Mantoniella squamata* accumulates A upon illumination instead of Z that is accumulated by green algae and plants. The reason is that in this alga, the reaction V → A runs much faster, in either way, compared to the rate of Z formation in Z-accumulating plants and algae. Compared to spinach, the A → Z rate in *Mantoniella* is 20-times slower. In *Mantoniella* VDE has a reduced affinity for A, as well as for other mono-epoxides (e.g., Dd, Lx, and N) compared to plants (Goss 2003).

2 Xanthophyll-Dependent "High-Energy" Quenching of Chlorophyll a Excitation

1987–1989

Zeaxanthin is correlated with qE in plant leaves

Demmig-Adams et al. (1989) demonstrated that the extent of qE (at the time quantified as the rate constant for thermal energy dissipation, k_D), upon illuminating plant leaves with excess actinic light, is linearly related to the Z content of leaves. On this basis, they proposed that Z, derived from V in the VAZ cycle, is a link between the "high-energy" state of thylakoids and the dissipation of excess excitation energy of Chl *a* as heat. This demonstration of a correlation between Z and qE followed the initial establishment of a correlation between Z and a dark-sustained form of NPQ (Demmig et al. 1987).

1991

qE has two distinct kinetic components in isolated chloroplasts

Using isolated chloroplasts, Gilmore and Yamamoto (1991) obtained evidence for two kinetically distinct components of ΔpH-dependent quenching of Chl *a* fluorescence: a faster (~1 min) Z-independent, and a slower (~10 min) Z-dependent quenching component. The faster quenching component was suggested to reflect quenching by the mono-epoxidic antheraxanthin (Gilmore and Yamamoto 1993) and by non-epoxidic xanthophylls (Gilmore et al. 1994). See also Niyogi et al. (1997) for evidence supporting dependence of the rapidly reversible ΔpH-dependent quenching on lutein of the LHC proteins.

qE quenches singlet excited Chl a in the LHCII antenna of PS II

Quenching of singlet-excited Chl *a* can be monitored by the effect of qE on Chl *a* fluorescence in isolated LHCII complexes as reduction in its fluorescence intensity. In fact, one can look at the entire emission spectrum either at room temperature or after cooling to 77 K; further, since qE appears in the light, evidence for this quenching can be obtained by comparing 77 K fluorescence emission spectra of a dark treated (minus qE) and a light-treated leaf (plus qE; Ruban et al. 1991; Ruban and Horton 1994). Results obtained by these authors support this expectation. Another technique that directly monitors

the loss of energy as heat is photoacoustic spectroscopy (see its use, e.g., by Carpentier et al. 1985). Mullineaux et al. (1994), using laser light–induced photoacoustic spectroscopy, showed that qE dissipates Chl *a* excitation within 1.4 μs, much more slowly than expected for excess excitation energy dissipation by reaction center complexes. Thus, the qE effect was interpreted to occur in the antenna complexes of PS II.

1992

Zeaxanthin and lumen acidity are sufficient for quenching Chl a fluorescence in darkness

Schreiber and Avron (1979) had already shown that hydrolysis of exogenous ATP in isolated chloroplasts induces a back-flow of electrons that reduces the primary quinone acceptor (Q_A) of the PS II_{RC}, and thus causes an increase in Chl *a* fluorescence within ~1 min. However, it was Gilmore and Yamamoto (1992) who demonstrated, in isolated chloroplasts, that hydrolysis of exogenous ATP leads to a slower (~10 min) ΔpH-dependent NPQ of Chl *a* fluorescence that includes both Z-dependent and Z-independent components. Further, the light-independent Z-quenching lasted for longer periods (under conditions of little or no reverse electron flow). This shows that actinic light is only indirectly involved in qE (i.e., by driving acidification of the lumen).

1993

qE can reflect aggregation of isolated LHCII proteins due to trans-thylakoid membrane ΔpH

By comparing 77 K fluorescence emission spectra of dark-treated (−qE) and light-treated (+qE) leaves, Ruban et al. (1993) established that the difference emission spectrum had a maximum at 700 nm, suggesting that it may originate from an aggregated form of LHCII complex (see chapters in Govindjee et al. 1986 for location of emission peaks in vivo). Furthermore, it was determined that the extent of qE, upon illumination of dark-pretreated leaves, correlates linearly with selective light scattering at 535 nm (ΔA535), which was shown earlier to reflect LHCII aggregation (Ruban et al. 1992). These results are consistent with the hypothesis formulated by Horton et al. (1991), according to which aggregation of isolated LHCII complexes in PS II can enable dissipation of excess excitation energy by qE.

qE in the monomeric peripheral minor antenna complexes CP29 and CP26

On the other hand, Bassi et al. (1993) determined that 80 % of V in dark-adapted maize leaves is contained in the minor monomeric LHC proteins CP24, CP26, and CP29. On the basis of this result, they proposed that these proteins are the sites of qE. Gilmore et al. (1996a) showed that both intensity and lifetime of Chl *a* fluorescence of PS II (the fluorescence lifetime is a direct measure of the quantum yield of fluorescence) were independent of antenna size differences between wild type barley and the chlorina mutant that lacked LHCIIb. [Note that change in fluorescence intensity can simply be due to change in the concentration of Chl; thus, measurement of lifetime of fluorescence is crucial in reaching firm conclusions; see, e.g., Holub et al. 2000, 2007]. This suggested involvement of the innermost peripheral antenna complexes (i.e., CP24, CP26, and CP29) in qE. This proposal was further supported by the lowering of Chl *a* fluorescence lifetimes upon binding of Z to recombinant CP26 (~10 % quenching; Frank et al. 2001) and CP29 (~30 % quenching; Crimi et al. 2001) proteins.

1995

qE lowers both intensity and lifetime of Chl a fluorescence

Gilmore et al. (1995) established that the extent of qE quenching and of the lifetime of Chl *a* fluorescence change in parallel, thus demonstrating that qE quenching is a true quenching in the physico-chemical sense. In the unquenched state, lifetime values centered

at ~2 ns, but in the quenched state lifetime dropped to ~0.4 ns (~80 % quenching). In the authors' own words, "the fluorescence quenching [was] interpreted as the combined effect of the pH gradient and xanthophyll concentration, resulting in the formation of a quenching complex with a short (approximate to 0.4 ns) fluorescence lifetime." Upon lowering chloroplast Z level with dithiothreitol, the Chl *a* fluorescence fraction with lifetime ~0.5 ns decreased and that with lifetime ~1.6–1.8 ns increased. It was concluded that binding of Z to LHC proteins (most likely to CP26) acts like a "*dimmer switch*" for excitation energy on Chl *a* (Gilmore et al. 1996a, b, 1998, 2000; Holub et al. 2000, 2007; for reviews, see Gilmore 1997; Gilmore and Govindjee 1999; and Govindjee and Seufferheld 2002).

PsbS (*or PS II-S*), *an LHC-like protein of PS II*, *does not bind chlorophylls and xanthophylls in vivo*, *and does not harvest light*

PsbS (or PS II-S) is a 22 kDa nucleus-encoded protein of PS II that is homologous to the nucleus-encoded Lhcb1-6 peripheral antenna complexes, although it has four α-helices instead of three (see citations in Funk et al. 1994, 1995). The PsbS protein, unlike the Lhcb proteins, is present in etiolated spinach leaves and it does not require any pigments in order to be a stable molecule (see, e.g., Dominici et al. 2002). However, it was shown that, in vitro, PsbS binds Z (Aspinall-O'Dea et al. 2002). Funk et al. (1994) showed that PsbS binds Chl *a* and Chl *b*, although not stably; however, this concept was not supported by recombinant protein experiments (Dominici et al. 2002). Thus, although PsbS had been named CP22 (Funk et al., 1994), in analogy to the minor antenna Chl-protein complexes CP29, CP26 and CP24, it seems unlikely that PsbS has any light-harvesting role. It was only later that Li et al. (2000, 2002, 2004) demonstrated that PsbS is a key participant in qE, the ΔpH-dependent component of NPQ. For reviews, see Niyogi (1999), Govindjee (2002), Golan et al. (2004), Bonente et al. (2008a, b), Kiss et al. (2008), and Kereïche et al. (2010).

1997

Mutants come into the picture for unraveling qE: *for violaxanthin and zeaxanthin*

Niyogi et al. (1997, 1998), using video imaging of Chl *a* fluorescence in *Chlamydomonas reinhardtii* cells and *Arabidopsis thaliana* plants exposed to excess light, succeeded in isolating and characterizing VDE-deficient (*npq1*) and ZEP-deficient (*npq2*) mutants, which proved to be useful in analyzing the biochemical pathway of qE. The mutant *npq1* lacks Z, as it is unable to de-epoxidize V, while the mutant *npq2* accumulates Z as it is unable to epoxidize it to A and V. See reviews by Baroli and Niyogi (2000) and Müller et al. (2001).

Significantly, Pogson et al. (1998) established, in the *Arabidopsis npq1* mutant, that the absence of Z does not fully suppress the fast-relaxing qE, indicating the presence of a Z-independent component in this mutant. Pogson and Rissler (2000) and Niyogi et al. (2001) reported that, in L-deficient *C. reinhardtii* and *A. thaliana* mutants, both extent and induction rate of qE were depressed. A full suppression of qE was achieved in a double mutant *npq1 lut2* that lacks both Z and lutein, implicating, thereby, a possible involvement of lutein in qE. Li et al. (2009) concluded that, in a Z-deficient mutant, lutein can substitute for the role Z plays in the wildtype.

Further, Matsubara et al. (2011), using fluorescence lifetimes for estimating qE, established that in leaves of the tropical plant avocado (*Persea americana*), both A and photoconverted L, formed from lutein epoxide, enhance the qE associated with the 0.5-ns lifetime component of Chl *a* fluorescence. Unlike Z, however, retention of A and photoconverted L were not involved in continuously maintained NPQ in avocado leaves. See reviews, e.g., by Niyogi (1999, 2000); Müller et al. (2010); and Jahns and Holzwarth (2012); Esteban and García-Plazaola, Chap. 12.

1999

Discovery of a lutein epoxide-lutein (Lx-L) cycle that runs in parallel with the VAZ cycle

An Lx-L cycle that operates in parallel with the VAZ cycle was identified by Bungard et al. (1999) and Matsubara et al. (2011) in two parasitic plants. Lx is a mono-epoxide of the α-branch (L 5,6-epoxide), while V is a di-epoxide of the β-branch. Lx is de-epoxidized under strong irradiance to L, and L is epoxidized to Lx under weak irradiance or in darkness. This epoxidation is much slower compared to the epoxidation of Z to V. While direct evidence for an involvement of the Lx-L cycle in qE is not available to date, Esteban and Garcia-Plazaola (Chap. 12) postulate such a function.

2000

PsbS, a PS II protein, is a crucial component of qE in plants

PsbS, a 22 kDa membrane protein of PS II (CP22; four trans-membrane helices; pigment free in vivo) was described earlier (see above) by Funk et al. (1994, 1995) but its role in photosynthesis had remained unknown. Analysis of PsbS-minus (Li et al. 2000, 2002) and PsbS-defective (Peterson and Havir 2001) *Arabidopsis* mutants led to the recognition that PsbS participates quantitatively in qE, and that availability of PsbS determines the extent of qE. In view of the fact that in vitro PsbS is capable of binding Chls and xanthophylls, it was proposed that it is a potential site for the dissipation of the excess excitation energy of Chl *a* by qE; see Brooks et al., Chap. 13.

Protonation of VDE at acidic pH enables its attachment to the thylakoid membrane

Using anion-exchange chromatography, Kawano and Kuwabara (2000) obtained evidence that protonation of VDE at acidic pH changes not only its surface charge but also its conformation.

2001

Peripheral antenna complexes of PS II are the sites of qE

Andersson et al. (2001) showed that antisense *Arabidopsis* plants lacking CP26 or CP29 have normal qE, suggesting that these complexes are unnecessary for photoprotection. By modifying the Chl *b* and LHCIIb content of *Phaseolus vulgaris* plants by subjecting etiolated seedlings to a regime of intermittent and continuous illumination, Chow et al. (2000) established a positive correlation between increasing Chl *b* and LHCII content and qE levels, suggesting a role for LHCII in the thermal dissipation of excitation energy from ^{1}Chl *a**.

In contrast, Moya et al. (2001) reported that Chl *a* fluorescence is quenched more in purified monomeric CP29, CP26 and CP24 antenna complexes than in purified LHCII, and Morosinotto et al. (2002) reported that newly synthesized Z appears primarily in CP24 and CP26.

2002

Mutants come into the picture for unraveling qE: for Lhcb in Chlamydomonas

Elrad et al. (2002) identified and characterized a *Chlamydomonas reinhardtii* mutant (*npq5*) lacking Lhcbm1, a light-harvesting polypeptide present in the LHCII trimers. The *npq5* mutant showed less than one-third reversible qE compared to the wild-type cells, and most of qE was not reversed by the addition of nigericin (a protonophore that dissipates trans-membrane ΔpH), suggesting that a defect in thermal dissipation is the cause of diminished qE. This mutant of *Chlamydomonas* was normal for the phenomenon of state transitions (see, e.g., Papageorgiou and Govindjee 2011), for high light–induced de-epoxidation of V, and for growth in low light, but was more prone to photoinhibition than wild-type *Chlamydomonas*. The PS II antenna in the *npq5* mutant has one-third fewer LHCII trimers

compared to the wild-type cells. Elrad et al. (2002) interpreted these results to suggest that a large part of thermal dissipation within PS II antenna of *Chlamydomonas* occurs in LHCII. For reviews, see Govindjee (2002) and Golan et al. (2004).

Proton efflux rate through the ATP synthase of the thylakoid membrane conductivity regulates qE

Protons are transferred from the stroma to the lumen by non-cyclic electron transport through PS II and PS I and by cyclic electron transport through PS I (see Ke 2001) and are exported from the lumen to the stroma through the ATP synthase of the thylakoid membrane during ATP synthesis. Kramer and co-workers (Kanazawa and Kramer 2002; Takizawa et al. 2008) analyzed the observation that lumen acidification is enhanced when the level of inorganic phosphate in the stroma is lowered, and established that this is caused by a decrease in proton conductivity through the ATP synthase. Govindjee and Spilotro (2002) showed that an *Arabidopsis thaliana* mutant, altered in the γ subunit of the ATP synthase, had a different pattern of intensity dependent changes in NPQ, confirming the relation of the functioning of ATP synthase with NPQ (see also Strand and Kramer, Chap. 18).

2004

Plants use PsbS, a pigment-free LHC protein of PS II, to sense lumen pH and initiate the qE process

A double *Arabidopsis* mutant (*npq4*), lacking two lumen-exposed glutamates (E122, E226) of the PsbS protein, was found by Li et al. (2004) to be defective in qE quenching and in the absorption change at 535 nm (ΔA535), which monitors a conformational change within PS II, while it resembled the qE–defective mutant *npq1* with respect to Chl *a* fluorescence lifetimes. On the basis of this result, Li et al. (2004) proposed PsbS to be the sensor that perceives lumen acidity level (and thereby the level of excess Chl *a* excitation energy) and initiates membrane protein conformation changes, which, together with Z, enable dissipation of the excess excitation as heat. For further information, see Brooks et al., Chap. 13.

For earlier evidence for the involvement of lumen-exposed carboxylates in acidic pH-induced quenching of Chl a fluorescence, see Walters et al. (1996).

Excitation-energy transfer from bulk Chls a to a Chl a-zeaxanthin heterodimer followed by charge transfer within the latter

The excited heterodimer (Chl *a*-Z)* may also relax to Chl *a*-Z by way of reversible charge transfer (Holt et al. 2004). Supporting evidence has been obtained from observations of qE in LHCII crystals (Pascal et al. 2005) and from LHCII complexes embedded in solid gel (Ilioaia et al. 2008). Both systems preclude displacement and the aggregation of LHCII trimers. For further discussion of photo-physical mechanisms of thermal dissipation involving energy transfer, charge transfer states, and/or excitonic coupling, see Ostroumov et al. (Chap. 4), Polivka and Frank (Chap. 8) and Walla et al. (Chap. 9).

2005

In which antenna complex and how may Z quench singlet excited Chl a (^{1}Chl a)?*

The quenching of the excited ^{1}*Chl a** (the superscript "1" on the left of "Chl" means that we are talking about a singlet state, and the "*" means that it is in an excited state) by Z may not only occur by different mechanisms, but it may also involve different antenna complexes. Holt et al. (2005) showed that, in monomeric LHC proteins of PS II, quenching of Chl fluorescence can occur by means of excitation-energy transfer from excited Chl *a* dimer of the reaction center to the radical cation of Z (Z^+). The latter was suggested to act as a direct quencher of singlet Chl *a* excitation (see Ahn et al. 2008).

Another suggested mechanism involves conformational changes in the PS II antenna that cause de-excitation of ^{1}Chl *a**, and this is suggested to be triggered by the protonation of the PsbS protein (see Ahn et al. 2008; Horton et al. 2008; Avenson et al. 2009). Still another proposed mechanism involves Z acting as an allosteric regulator, with Z altering the conformation of the LHC protein, enabling excitation energy transfer from ^{1}Chl* to L and leading to a loss of energy from ^{1}L* as heat (Pascal et al. 2005). For further discussion, see, e.g., Walla et al., Chap. 9, Pascal et al., Chap. 10, and Morosinotto and Bassi, Chap. 14.

The excitation-dissipative state of isolated LHCIIb crystals involves changes in the orientation of specific pigment molecules

Pascal et al. (2005) presented spectroscopic data on isolated LHCIIb crystals, and concluded that specific changes occur in the configuration of its pigment population that allow LHCII to regulate the flow of excitation energy in the system. For example, one of the possible quenching sites may be a pair of specific Chl *a* molecules (Chl *a* 611 and Chl *a* 612) along with lutein 620 (where the numbers indicate their positions in the crystal structure of LHCIIb; see also Pascal et al., Chap. 10).

Excitation energy dissipation by qE can occur via the transient formation of a $Chl^{-\cdot}Z^{+}$ biradical

Detecting ultrafast absorption changes, generated upon selective excitation at 664 nm of light-adapted (+qE) and of dark adapted (−qE) spinach thylakoids, Holt et al. (2005) established that the + qE state is associated with the transient appearance of a Z cation radical ($Z^{+\cdot}$) whose absorbance at 1000 nm ($\Delta A_{\sim 1000\ nm}$) rises within ~10 ps (picoseconds, 10^{-12} s) and decays in ~150 ps. [This means that $Z^{+\cdot}$ is formed within 10 ps and then disappears since it must transfer its "+" charge to another intermediate within 150 ps.] A correlation of qE with formation of $Z^{+\cdot}$ was supported by the presence of ΔA at ~1000 nm in thylakoid preparations from wild-type *A. thaliana* and in qE-capable and Z-containing mutants (*npq2; npq2-Lutein2*) thereof; further, this interpretation is consistent with the absence of the $Z^{+\cdot}$ absorption difference signal in Z–minus (*npq1*) and PsbS–minus (*npq4-1*) *A. thaliana* mutants. In addition, charge transfer from Chl a^- to Z^+ (and/or L^+) has been shown to occur in minor antenna complexes (Lhcb4-6), but not in the major LHCII antenna complexes; see also Polivka and Frank, Chap. 8 and Walla et al., Chap. 9.

2006

qE is more pronounced in plants than in green algae

Studies on a green alga *Chlamydomonas reinhardtii* (Finazzi et al. 2006) revealed that its ability to generate NPQ is less pronounced than that of higher plants, and that $qT_{1\rightarrow 2}$, but not qE, contributes predominantly to the quenching in the alga. The diminished role of qE is due both to an intrinsically smaller ΔpH and to a limited capacity to translate it into a quenching response in this green alga. However, when photosynthesis is impaired, e.g., at low temperature (0 °C) or in mutants lacking Rubisco activity, the qE contribution increases, but its nature is different from that observed in higher plants, being characterized by a long-lived reaction center-based quenching that is inefficiently converted into an antenna-based quenching.

2007

Lutein 620 (Lut 1) may be the site of qE in isolated Lhcb antenna complexes

On the basis of the 2.72 Å resolution structure of the LHCII monomer (Liu et al. 2004), and of 77 K fluorescence emission spectra of LHCII in crystal and in solution, Yan et al. (2007) identified L620 (L at position 620 in the structure) as the likely site of

qE. This conclusion assumes an intra-subunit conformational change in the LHCII monomer that leads to the formation of a heterotrimer consisting of Chl *a* 612, Chl *a* 611 (specific chlorophyll molecules as numbered in the atomic resolution structure) and L620 (also see Pascal et al. 2005). The close proximity of the π-electron clouds in the trimer enables transfer of excitation energy from Chls *a* to L where it is downgraded to heat. [We note that π (pi) electrons are those that are involved in π (pi) bonds; these are double bonds; see the *glossary*.

That intra-Lhcb conformational changes accompany the establishment of qE was also concluded by Ruban et al. (2007) from the application of optical spectroscopic methods. Resonance Raman spectra of light-treated (+qE) and dark-treated (−qE) LHCII preparations isolated from spinach enabled detection of light-induced conformational changes in the vicinity of the lutein binding site of LHCII monomers. By measuring femtosecond (fs, 10^{-15} s) transient absorption changes at selected wavelengths, it was established that Chl *a* transfers singlet excitation energy to lutein in isolated LHCII. These results led Ruban et al. (2007) to propose that conformational changes occurring under qE conditions bring Chl *a* 610, Chl *a* 611, and Chl *a* 612 closer to lutein and facilitate dissipation of excitation energy as heat.

Furthermore, using absorption, fluorescence excitation and fluorescence emission spectra, Johnson and Ruban (2009) observed red shifts in the spectral bands of Chl *a* and a corresponding shift in the L band in the excitation spectrum when LHCII complexes (both isolated and in-situ) were placed in the +qE state. These spectral shifts were interpreted to indicate a closer approach of the trimeric cluster pigments that enables the emitting Chl *a* to transfer excitation energy to *Lut 1* where it is downgraded to heat.

Using absorption and circular dichroism spectroscopy (differential absorption by left and right circularly polarized light, a measure of the structure of proteins) to examine isolated wild-type and mutagenized Lhcb complexes, Mozzo et al. (2008) established that identical interactions occur in the Chl *a* 611/Chl *a* 612/L620 pigment cluster of the LHCII, CP29 and CP26 antenna proteins of PS II (but not in CP24), indicating that qE may occur indiscriminately in nearly all the light-harvesting subunits.

qE in isolated LHCII cannot be accounted for solely by replacement of violaxanthin by zeaxanthin

Amarie et al. (2007) observed that replacing V by Z in isolated LHCII samples has no effect on the lifetime of excited Chl *a*, although both V^{+} and the Z^{+} carotenoid cation radicals are detectable by means of resonance two-color, two-photon spectroscopy.

Zeaxanthin may have other functions besides that in qE: *it is also an antioxidant*

Havaux et al. (2007) examined the antioxidative activity of Z and other xanthophylls with a Chl *b*-less and LHCII-deficient *Arabidopsis* mutant (*ch1*) that has a very low qE capacity. Double mutants with different xanthophyll compositions were obtained by crossing *ch1* with xanthophyll mutants. This study showed that, under high light, suppression of Z (in the *ch1 npq1* double mutant) increased photo-oxidative stress relative to that in the mutant *ch1*, while constitutive accumulation of Z (in the *ch1 npq2* mutant) increased photo-tolerance. Further, the protection of polyunsaturated lipids by Z was enhanced when L was present. Suppression of other xanthophylls, or of PsbS-dependent qE, did not increase photo-oxidative stress. Havaux et al. (2007) concluded that the antioxidant activity of Z (distinct from its function in qE) is higher than that of all other xanthophylls in *Arabidopsis*, and that it occurs even in the absence of LHCII. For further

information, see Havaux and García-Plazaola, Chap. 26.

The qE mechanism in brown algae differs from that of green algae and plants

Comparing the brown alga *Macrocystis pyrifera* with the plant *Ficus* sp., García-Mendoza and co-workers (see, e.g., García-Mendoza et al. 2011; Ocampo-Alvarez et al. 2013) concluded that this brown alga displays only a slower (presumably Z-dependent) component of qE and lacks a faster ΔpH-dependent (and presumably Z independent) component. In this alga, the strong light-induced ΔpH serves only to activate VDE and to convert V to Z and has no other effect on the excitation energy of Chl *a*. Several other brown algae are known to behave in a similar manner (Rodrigues et al. 2002; Fernandez-Marin et al. 2011). A comparison of these results with those of green algae and plants suggest that the VAZ cycle may have preceded the evolutionary appearance of brown algae. On the other hand, Chl *c*-containing diatoms display both xanthophyll-independent and xanthophyll-dependent components of qE (Goss and Jakob 2010). Further experiments and analysis is needed before any picture of evolution of qE mechanism will be available.

2009

Lutein can replace zeaxanthin as the quencher of singlet excited Chl a (^{1}Chl a) in qE of a Z-deficient Arabidopsis mutant*

Using a Chl *a* fluorescence video imaging system, Li et al. (2009) isolated suppressors of the *npq1* mutant of *Arabidopsis* (lacking Z) that shows a much higher qE capacity compared to the *npq1* parent. One of these suppressors (named suppressor of Z–less1, *szl1*) had a very small xanthophyll cycle pool size, and instead accumulated twice as much L. Wild-type, *npq1*, *szl1*, and *szl1npq1* plants showed that L, in higher amount, was able to substitute for Z in qE in this Z-deficient mutant. Li et al. (2009) interpreted these results by suggesting that L cation radicals also play a direct role in the dissipation of Chl *a* excitation by qE in Z-deficient mutants.

A common mechanism may underly zeaxanthin-dependent and zeaxanthin-independent components of qE in various mutants

To address the question whether both Z and L quench singlet excited Chl *a* independently, operating at separate sites, or whether L is the quencher and Z only an allosteric regulator that modifies only the conformation of the of Lhcb protein, Johnson et al. (2009) compared qE-related conformational changes (ΔA535) in antisense *Arabidopsis* plants with altered contents of the minor and the major antenna complexes. Data on conformational changes within an Lhcb protein, on Lhcb-Lhcb interactions, and on the effects of externally and internally bound xanthophylls suggested that both Z-dependent and the Z-independent components of qE may arise from a common quenching mechanism based on intra-Lhcb conformational changes, inter Lhcb-Lhcb interactions, and on internally-bound xanthophylls.

Algae employ different LHC proteins as NPQ effectors than plants

Peers et al. (2009) constructed the qE-deficient mutant *npq4* of *Chlamydomonas reinhardtii* that could grow normally under weak light, but was inhibited under strong light. The *npq4* mutant lacked a specific *Light-Harvesting Complex Stress-Related* (LHCSR) protein, known also as the LI818 protein. According to Bonente et al. (2010), LHCSR protein serves both as lumen-acidity sensor and as Chl *a* excitation energy dissipator in *Chlamydomonas*. Unlike the PsbS protein, the pigment-free lumen acidity sensor of plants (Li et al. 2004), the LHCSR protein binds Chl *a*/Chl *b* and xanthophylls. The very

short Chl *a* fluorescence lifetime (t < 100 ps) of LHCSR strongly implies that it may function as an excitation-energy quenching center. Quenching was proposed to proceed via transient formation of an L radical cation, instead of the Z radical cation formed in plants, suggesting that L, not Z, is important for qE in this green alga. In addition, Niyogi and Truong (2013) have shown that, as a result of phosphorylation by the STT7 kinase, LHCSR moves from PS II-rich regions to PS I-rich regions of the thylakoid membrane, leading to state 1-to-state 2 transition.

In the marine diatom *Thalassiosira pseudonana*, two light stress-related proteins have been described, Lhcx1 and Lhcx6, both closely related to the LHCSR protein of *Chlamydomonas* (Zhu and Green 2010). The Lhcx1 protein accumulates in both light-stressed and unstressed cells, and its amount is unrelated to the Dd content of the cell. The Lhcx1 protein is presumed to have a role in assembly and stability of thylakoid membranes. The Lhcx6 protein accumulates only in strong light, and in parallel to a rise in the Dd content, and is thus suggested to have a role in the Dd-Dt cycle of diatoms. An Lhcx1 protein, with the same properties, was described in the diatom *Pheodactylum tricornutum* by Bailleul et al. (2010).

2010

An alternative suggestion is that qE occurs via Chl: *Chl interactions and does not involve xanthophylls*

Using an extremely fast (femtosecond) absorption spectrophotometer, Müller et al. (2010) studied ultrafast dynamics in isolated LHCII oligomers obtained from the *Arabidopsis thaliana* mutants *npq1* and *npq2*. The data were described kinetically by a two-state compartment model assuming that only Chl-excited states (^{1}Chl a^*) are involved in the quenching process. These results challenge earlier proposals, in which excitation-energy dissipation in Chl *a* in LHCII was assumed to involve direct energy transfer to carotenoids (e.g., ^{1}Chl $a^* \rightarrow$ Z, Frank et al. 2001; or ^{1}Chl $a^* \rightarrow$ L, Ruban et al. 2007). A Chl – Chl charge-transfer state is suggested in this paper to play an important role in the quenching process. For further information, see Holzwarth and Jahns, Chap. 5. However, further research is needed to reach a firm conclusion, especially to find if different mechanisms may operate in different systems, such as mutants with different pigment composition versus wildtype, isolated complexes versus intact leaves, different species with different NPQ features, and plants grown in the absence or presence of regular exposure to clearly excessive light.

May lutein epoxide (*Lx*) *be involved in qE*?

Leaves of the tropical plant species avocado (*Persea americana*) contain high concentrations of Lx in addition to V, A, and Z. Like V, Lx is also de-epoxidized in the light. In contrast, however, to Z and A that are rapidly epoxidized in darkness, L is epoxidized only slowly, such that illumination of avocado leaves gives rise to a persistent pool of photoconverted L (ΔL). Matsubara et al. (2011) showed in avocado leaves that qE is correlated with the sum of L, Z and A, while Förster et al. (2011) analyzed the qE development in avocado in three phases: **(i)** before Lx de-epoxidation, **(ii)** after Lx de-epoxidation, and **(iii)** after A and Z epoxidation but in the presence of the persistent ΔL pool. For further details, see the reviews by Jahns and Holzwarth (2012), Holzwarth and Jahns, Chap. 5, and Esteban and García-Plazaola, Chap. 12.

B State Transitions

1967

Pre-illumination of algae and cyanobacteria changes the spectrum and yield of Chl a fluorescence

Papageorgiou and Govindjee (1967, 1968a, b) reported that pre-illumination of a cyanobacterium, *Anacystis nidulans*, and of a green alga, *Chlorella pyrenoidosa*, with light mainly absorbed by PS II or by PS I causes characteristic changes in the yield and spectrum of Chl *a* fluorescence of these organisms (also see Mohanty et al. 1970). These changes were attributed to conformational modifications of the pigment-protein complexes in the thylakoid membrane.

1968

Algae adapt to the color of ambient light by shifting excitation energy to go to the less favored photosystem

As discussed earlier, the regulatory adaptation of photosynthetic organisms to the quality of ambient light was independently discovered by Murata (1969, 1970) and by Bonaventura and Myers (1969; also see Bonaventura 1969) who named this phenomenon *light state transition* or, simply, *state transition*. At 77 K, photosynthetic samples give rise to three major fluorescence emission bands: at 685 nm (F685, which originates from CP43 of PS II), at 696 nm (F696, which originates from CP47 of PS II) and at 720–740 nm (F720-740, which originate mostly in PS I) (see Fig. 1.1 and chapters in Govindjee et al. 1986, Papageorgiou and Govindjee 2004 and the review by Papageorgiou 2012, and citations therein). When a photosynthetic sample is excited with light absorbed in PS I, one observes a high ratio of F720-740/F685-F696; this ratio is lower when one excites with PS II light. From these ratios, we can thus estimate the fraction of excitation energy going to PS II or PS I. Murata (1969) observed that pre-illumination of the red alga *Porphyridium cruentum* with Light 2 shifts excitation energy from going to PS II to PS I (labeled as state 1 to state 2 transition). Conversely, upon pre-illumination with Light 1, *P. cruentum* shifts excitation energy from going to PS I to PS II (state 2 to state 1 transition). Measuring photosynthetic O_2 evolution and Chl *a* fluorescence in the green alga *Chlorella pyrenoidosa*, Bonaventura and Myers (1969) estimated that after illumination with Light 2 less excitation energy is delivered to PS II (state 2) than after illumination with Light 1 (state 1). Both research groups suggested that these light quality adaptations improve the efficiency of photosynthesis in weak light.

1977

In plants, state transitions relate to reversible light-induced phosphorylation of LHCII

Bennett (1977) was the first to report light-dependent and dark-reversed phosphorylation of thylakoid membrane proteins. When isolated pea chloroplasts are illuminated, several thylakoid membrane proteins become phosphorylated, with the most conspicuous being the Chl*a*/Chl*b*-binding LHCII complex. Light-induced phosphorylation of LHCII was subsequently found by Bennett et al. (1980) to decrease the amount of excitation energy that goes to PS II and to increase that going to PS I (as inferred from fluorescence spectra at 77 K), to be inhibited by DCMU and to be insensitive to protonophoric uncouplers. Bennett et al. (1980) concluded that phosphorylated LHCII alters the properties and the interactions of PS II and PS I, such as to redistribute excitation energy in favor of PS I.

1981

The redox state of the PQ-pool regulates the light-induced phosphorylation of LHCII

The reduction of the PQ pool by PS II activates a membrane-bound kinase that phosphorylates LHCII and, thereby, causes a redistribution of excitation energy in favor of PS I (state 2). Oxidation of PQH_2 to PQ by PS I inactivates the kinase, phosphorylated-LHCII is de-phosphorylated by a phosphatase, and excitation energy is redistributed in

favor of PS II (state 1; Allen and Bennett 1981; Allen et al. 1981). The critical role of the PQ-pool in state transitions was also inferred from the fact that electron donors to PS I do not reverse LHCII phosporylation-dependent quenching of chloroplast fluorescence (Horton and Black 1981). Similar to plants, light-induced phosphorylation of LHCII was shown to accompany the transition from state 1 to state 2 in the green alga *Chlorella vulgaris* (Saito et al. 1983).

1983

Lateral mobility of phosphorylated LHCII complexes

Using freeze-fracture electron microscopy, Kyle et al. (1983) detected that the light-induced phosphorylation of LHCII causes a partial de-stacking of the grana accompanied by a redistribution of LHCII particles from the PS II-rich grana to the PS I-rich stroma lamellae.

1984

Is protein photophosphorylation required for state 1-to-2 transition of red algae?

According to Biggins et al. (1984), the light-induced protein phosphorylation patterns of thylakoid membrane proteins in state 1 and state 2 *Porphyridium cruentum* were the same. Thus, in this alga, protein phosphorylation was not implicated in state 1-to-state 2 transition. Only energy *spillover* seems to regulate the redistribution of excitation energy between PS II and PS I.

1988

Cyt b_6f is indispensible for state 1 to state 2 transition in Chlamydomonas reinhardtii

Wollman and Lemaire (1988) obtained evidence that, whereas a *Chlamydomonas reinhardtii* mutant lacking the proteins of the core complex of PS II was fully capable of state 2 to state 1 transition, a mutant lacking the Cyt b_6f complex was locked in state 1.

1991

The state 1-to-state 2 transition shifts not only LHCII complexes but also the Cyt b_6f complex from grana thylakoids to stroma thylakoids

Experimenting with maize chloroplasts and *Chlamydomonas* cells, Vallon et al. (1991) found that a transition to state 2 shifts not only LHCII complexes but also the Cyt b_6f complex from PS II-rich grana thylakoids to PS I-rich stroma thylakoids. Thus, state 1-to-state 2 transition should not be viewed simply as a light-adaptation mechanism for optimizing non-cyclic PSET; it should also be regarded as a re-routing of PSET from the non-cyclic to the cyclic path to enable cells to meet changes in ATP demand. Importantly, the state 1-to-2 transition in *Chlamydomonas* also involves a shift of the pH-sensing and excitation-energy dissipating LHCSR protein to PS I, suggesting the likelihood of qE operating in PS I-associated LHC antenna (Niyogi and Truong 2013).

Compelling evidence that state 1-to-state 2 transition involves a shift from non-cyclic PSET to cyclic PSET was obtained by Finazzi et al. (1999, 2002), who showed that DCMU inhibits the turnover of Cyt b_6f in state-1 *Chlamydomonas* cells, but not in state-2 cells. Further, the shift of LHCII from PS II to PS I is indeed linked to a shift from non-cyclic PSET to cyclic PSET since an LHCII-minus mutant of *Chlamydomonas* was unable to perform it (see reviews by Finazzi and Forti 2004 and Minagawa 2011).

1997

A reduced PQ-pool is not sufficient to activate the LHCII kinase; *it must be sensed and transduced by Cyt b_6f*: *Studies with Chlamydomonas reinhardtii*

According to experiments with *C. reinhardtii* by Vener et al. (1997), the kinase that phosphorylates LHCII is activated by the binding of one plastoquinol (PQH_2) molecule to the quinol-binding site of Cyt b_6f (i.e., Q_0). [We remind the reader that PS II reduces PQ to PQH_2 that is oxidized back to PQ by Cyt b_6f (see Fig. 1.1).] When PQH_2 is replaced with 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone, a reduced PQ-pool fails to activate the kinase. The kinase is also inactive after oxidation of bound PQH_2 by a single, light flash-induced turnover of PS I. It was further established that binding of PQH_2 at the Q_0 site of Cyt b_6f causes a structural change in the Rieske Fe-S protein of the complex (Carrell et al. 1997; Zhang et al. 1998; Breyton 2000; reviewed by Vener et al. 1998; Wollman 2001; Finazzi and Forti 2004).

What forces are behind the diffusion of LHCII from PS II to PS I

Allen and Nilsson (1997) used a synthetic polypeptide with identical sequence to the N-terminus of LHCIIb (where threonine at position 5 is phosphorylated) and found (by circular dichroism spectroscopy) that phosphorylation entails a structural change in the synthetic peptide. From this result, Allen and Nilsson (1997) proposed that a photophosphorylation induced structural change reduces the affinity of phosphorylated-LHCII for PS II, causing it to diffuse away toward PS I.

2000

In the state 1-to-state 2 transition, phosphorylated LHCII subunit docks directly on the H subunit of the core PS I complex

Using transgenic *Arabidopsis* mutants lacking specific subunits of PS I, Lunde et al. (2000) established that, when subunit PS I-H and, to a lesser extent, subunit PS I-L were missing, state 1 to state 2 transition was impaired. Further, Lunde et al. (2000) proposed that migrating phospho-LHCII complex docks on the PS I-H on the side of the PS I core complex, opposite to the side where the LHCI proteins are attached. For a review, see Haldrup et al. (2001).

2002

Identification of LHCII-specific kinases in Chlamydomonas and Arabidopsis

LHCII-specific kinases have been identified in the green alga *Chlamydomonas* (stt7; Depège et al. 2002) as well as in *Arabidopsis* (STN7; Bellafiore et al. 2005). Mutants deficient in these kinases were locked in state 1, unable to phosphorylate the LHCII proteins, and unable to drive their translocation or the translocation of the Cyt b_6f complex from the PS II-rich region of the thylakoid membrane to the PS I-rich region.

2006

Isolation and characterization of state 1- and state 2-specific PS II and PS I supercomplexes from Chlamydomonas

Employing centrifugal and chromatographic methods for protein isolation, immunoblotting for composition analyses, and spectroscopy for activity assays, Jun Minagawa and co-workers (see below) demonstrated that the molecular mass and the composition of PS II and PS I supercomplexes, isolated from *Chlamydomonas*, depend on whether they had been derived from state 1-adapted or state 2-adapted cells. These differences relate to migration of light-harvesting proteins CP29, CP26, LhcbM5 as well as of trimeric LHCIIs from PS II-rich appressed lamellae to PS I-rich unappressed lamellae as a result of a state 1 to-state 2 transition. State 2 cells thus have a smaller PS II super-complex than state 1 cells, whereas the same cells have a larger PS I super-complex than state-1 cells (Takahashi et al. 2006; Iwai et al. 2008). Further, using fluorescence lifetime microscopy to identify PS II-bound

LHCII from its characteristic lifetime of Chl *a* fluorescence (170 ps) and free phospho-LHCII from its characteristic fluorescence lifetime (270 ps), Iwai et al. (2010a) succeeded in visualizing dissociation of phosphorylated-LHCII from PS II in live *Chlamydomonas* cells. Further, Iwai at al. (2010b) isolated a 1.4–1.6 MDa PS I supercomplex from state-2 *Chlamydomonas*, which consisted of PS I, LHCI, LHCII, Cyt b_6f, Ferredoxin NADP$^+$ Reductase (FNR) and the integral membrane protein PGRL1. This super-complex was shown to perform cyclic PSET upon illumination; electrons on the electron-acceptor end of PS I were transferred to Cyt b_6f, and from Cyt b_6f back to PS I. For recent reviews, see Minagawa et al. (2011), Papageorgiou and Govindjee (2011), and Mohanty et al. (2012); also, Finazzi and Minagawa, Chap. 21.

VI Concluding Remarks

Until about the middle of the twentieth century, scientific research had focused on how plants optimize the use of absorbed light for photosynthesis. Starting in the 1960s, it became evident that how plants dispose of the excess of absorbed light was also an intellectually challenging and practically relevant pursuit. In the course of the following 50 years, intensive research led to formulation and refinement of new ideas and concepts on how oxygenic photosynthetic organisms perceive and assess any absorbed light in excess of their needs, and on how they remove it non-photochemically to avoid any photo-oxidative damage. In fact, the existence of multiple regulatory mechanisms, at the individual cell level as well as at the level of the whole plant (see, e.g., Logan et al., Chap. 7), that control the allocation of absorbed light to photosynthesis versus non-photochemical removal, is one of the key targets of photosynthesis research today.

The most widely applied technique that allows quantitative estimation of the non-photochemically disposed fraction of absorbed light has been the *pulse amplitude modulation* method (PAM) of Ulrich Schreiber and co-workers (see Schreiber 2004; Logan et al., Chap. 7, and citations therein). Another widely applied method to detect non-photochemical dissipation of the excitation energy of Chl *a* is fluorescence induction (FI), namely the kinetic trace of the Chl *a* fluorescence of a dark-adapted sample after transfer to continuous exciting illumination. The FI trace responds to both the qE and qT variants of NPQ and, historically, has been the method that led to the discovery of the regulated non-photochemical quenching of Chl *a* fluorescence (Papageorgiou et al. 2007; Papageorgiou and Govindjee 2011). For a relationship of slow fluorescence changes (termed P-to-S decay) with ΔpH, which is related to NPQ, see Briantais et al. (1979). For recent applications and theoretical treatments of this method, see Kaňa et al. (2012), and Stirbet and Govindjee (2011, 2012).

Of the many revelations from NPQ research (and which are detailed in this volume), perhaps the most central is the discovery of the multifunctionality of the LHC proteins and of the xanthophylls they bind. While formerly, LHC proteins were assigned only photon harvesting and excitation-energy supplying roles, it has now become apparent that they also sense, assess and degrade excess excitation energy to heat that is dissipated to the surroundings before it can be transferred to core complexes and reaction centers. Likewise, while formerly LHC xanthophylls were assigned only anti-oxidant and structural roles, and with no particular distinction for the epoxidic xanthophylls, it is now apparent that the latter are involved in enzyme-catalyzed, light-induced and dark-reversed transformations that convert an LHC protein from an excitation-energy supplying conformation to an excitation energy-dissipating conformation (see, e.g., Ruban and Mullineaux, Chap. 17).

We also note also that a qE-dependent, but xanthophyll-independent, dissipation of the excitation energy of Chl *a* has been suggested to occur in the core PS II complexes of plants (Finazzi et al. 2004, 2006; Ivanov et al. 2007). In contrast to the qE effects in peripheral LHC antenna, which materialize only under strong light, the pure qE effects in the PS II core complexes occur in weak light. Recently, qE of Chl a fluorescence has been shown to occur in the PS II core complexes of cyanobateria (Stamatakis and Papagerogiou 2014). The difficulty in detecting qE in cyanobacteria arises from the state 2-to-state 1 transition (and the resulting fluorescence rise), which masks qE effects (Papageorgiou et al. 2007).

Acknowledgments

We thank Sandra Stirbet for providing Figs. 1.1 and 1.2 and their legends, and Dima Shevela for providing Fig. 1.3 and its legend. In addition, Sandra Stirbet has generously checked our references with reference list and vice versa. We thank Barry Logan for helping us improve our chapter, particularly by suggesting addition of explanations of technical terms, and Beverly Green for providing evolutionary insights for the green, red and brown algae. We are thankful to William W. Adams III for his help during proof-reading of this chapter.

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