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Toward an Understanding of the Mechanism of Nonphotochemical Quenching in Green Plants†

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ABSTRACT: Oxygenic photosynthesis in plants involves highly reactive intermediates and byproducts that can damage the photosynthetic apparatus and other chloroplast constituents. The potential for damage is exacerbated when the amount of absorbed light exceeds the capacity for light energy utilization in photosynthesis, a condition that can lead to decreases in photosynthetic efficiency. A feedback de-excitation mechanism (qE), measured as a component of nonphotochemical quenching of chlorophyll fluorescence, regulates photosynthetic light harvesting in excess light in response to a change in thylakoid lumen pH. qE involves de-excitation of the singlet excited state of chlorophyll in the light-harvesting antenna of photosystem II, thereby minimizing the deleterious effects of high light via thermal dissipation of excess excitation energy. While the physiological importance of qE has been recognized for many years, a description of its physical mechanism remains elusive. We summarize recent biochemical and spectroscopic results that have brought us closer to the goal of a mechanistic understanding of this fundamental photosynthetic regulatory process.

A green plant's ability to dissipate energy is, in many cases, as fundamentally important to its survival as its ability to harvest energy. Photoprotective energy quenching limits the production of harmful oxygen species that accumulate under conditions in which the input light intensity exceeds a plant's capacity for carbon fixation, a daily stress experienced by a variety of organisms (*1*). These species destroy vital proteins, such as the photosystem II (PSII)[1] D1 reaction center protein, as well as lipid bilayers, and pigments (*2*, *3*). The effects of such damage range from temporary decreases in photosynthetic efficiency to, in the worst case, death of the organism. Lessons learned from how plants carefully, and very successfully, balance photosynthesis and photoprotection may provide invaluable information about how to engineer and select plants that will be suited to specific environmental conditions.

The most common physical observable used to assess photosynthetic function and its subsequent downregulation in excess-light conditions is chlorophyll (Chl) fluorescence, because it is sensitive to a wide range of changes in the overall apparatus. Processes that decrease the overall Chl fluorescence quantum yield (Φ_{Chl}) are generally divided into two categories, photochemical quenching (qP), which is

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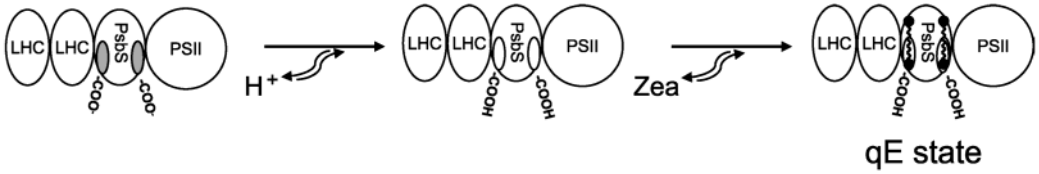

FIGURE 1: Schematic model for qE in PSII of plants. In excess light, a low thylakoid lumen pH leads to binding of H^+ to the carboxylates of two glutamate residues in PsbS, and Zea synthesis from Vio is induced. Binding of Zea to sites in PsbS results in the qE state in which de-excitation of ^{1}Chl* occurs. Modified from ref *29*.

exclusively associated with photochemical charge separation in the PSII reaction center (RC), and nonphotochemical quenching (NPQ). The latter process is broadly defined as all fluorescence quenching that is not directly related to charge separation. NPQ can be subdivided into three components, feedback de-excitation quenching or high-energy-state quenching (qE), photoinhibition (qI), and state transitions (qT), each of which has characteristic induction and relaxation kinetics (*4*). qE is the only NPQ component that is correlated with changes induced by rapid fluctuations, on the order of seconds to minutes, in input light intensity (*5*). The mechanism that gives rise to its Chl quenching kinetics is the focus of this review.

While qE is correlated with photoprotection (*6*, *7*), it is only the manifestation of the process by which plants and algae dissipate excess energy. When steady-state qE exists in an organism, under conditions of completely closed reaction centers (i.e., when the primary electron acceptor, Q_A, is fully reduced), the shortening of a specific Chl fluorescence lifetime component from ~2.0 to ~0.3 ns is the kinetic observable which characterizes qE (*8*). The decrease in the Chl fluorescence lifetime indicates that a nonradiative deactivation channel for singlet Chl molecules (^{1}Chl*) has been formed which can harmlessly emit the excess absorbed energy as heat. In the absence of this photoprotective quenching mechanism, there is a significant increase in the probability that the ^{1}Chl* molecules will form triplet Chl molecules (^{3}Chl*), a species that reacts with ground-state oxygen ($^3O_2$) to form the strongly photooxidative singlet oxygen ($^1O_2$*) in addition to other highly reactive oxygen species (*9*). In green plants and algae, qE can quench up to 80% of the ^{1}Chl* (*10*−*12*).

A number of additional characteristics of qE have been well documented. qE is regulated by the magnitude of the transmembrane pH gradient (ΔpH) across the thylakoid membrane, therefore making the quenching rapidly reversible (*5*, *13*). When the magnitude of ΔpH surpasses the value present when the amount of light does not exceed the photosynthetic capacity, the violaxanthin de-epoxidase enzyme is activated and triggers the interconversion of carotenoids (Cars), namely, violaxanthin (Vio) to zeaxanthin (Zea), via the xanthophyll (Xan) cycle (*14*). In addition, qE requires the presence of a functional version of the PsbS protein (*12*). Also, a light-induced absorbance change at 535 nm (ΔA_{535}) is always associated with qE (*15*).

While many of the physical observables of qE are well characterized and many of the requirements well-known, the most fundamental question, namely, the mechanism(s) of ^{1}Chl* deactivation (which will subsequently be termed the mechanism of qE), has eluded scientists. The identity of the quenching species, in terms of both pigment composition and location, and the means by which the quencher(s) dissipates the excess energy are unknown. To be able to utilize and manipulate this natural photoprotective process, a detailed, physical understanding of qE must be obtained. Accomplishing this goal has proven to be a significant challenge, because it concerns the response of an interconnected, complex system, the photosynthetic apparatus. In this review, we focus on recent progress in a variety of fields toward understanding the mechanism of qE.

PsbS and qE

PsbS is a PSII subunit with an apparent molecular mass of 22 kDa (*16*−*19*). The study of the subunit has been at the forefront of recent qE research since genetic screening work on *Arabidopsis thaliana* plants identified a mutant, *npq4-1*, which displayed normal photochemistry but lacked all pH-dependent quenching [characterized by both the absence of ΔA_{535} (*12*) and an ~0.3 ns Chl fluorescence lifetime during exposure to a high level of light (*7*)]. Analysis of the mutant showed that it was completely PsbS deficient, definitively establishing a photoprotective role for the protein, which is a member of the light-harvesting complex (LHC) superfamily (*18*, *19*). While these results demonstrate that PsbS is necessary for qE in plants, the exact role of the protein is unclear. The major mechanistic issue concerning PsbS is whether it binds the qE quenching pigment(s) and/or is an allosteric regulator of qE; i.e., its function is to interact with neighboring proteins leading to local or overall conformational changes in PSII which induce the production of quenchers.

Pigment Binding and PsbS

If PsbS is the site of nonradiative ^{1}Chl* quenching, it must bind the quenching pigment(s) under qE conditions *in vivo*. One clear distinction between PsbS and the Chl *a*/*b* binding antenna proteins (CABs), namely, Lhcb1−3 (LHCII), Lhcb4 (CP29), Lhcb5 (CP26), and Lhcb6 (CP24), is that PsbS is

[1] Abbreviations: ΔA_{535}, light-induced absorbance change at 535 nm; Anthera, antheraxanthin; CABs, Chl *a*/*b*-binding antenna proteins; Car, carotenoid; CD, circular dichroism; Chl, chlorophyll; ^{1}Chl*, singlet chlorophyll; ^{3}Chl*, triplet chlorophyll; CP29, CP26, and CP24, chlorophyll proteins of 29, 26, and 24 kDa, respectively; DCCD, dicyclohexylcarbodiimide; E, glutamate; IEF, isoelectric focusing; IP, ionization potential; LHCII, light-harvesting complex of photosystem II; L/P, lipid/protein; LHC, light-harvesting complex; Lut, lutein; Neo, neoxanthin; NPQ, nonphotochemical quenching; $^1O_2$*, singlet oxygen; Pc, phthalocyanine; ΔpH, transmembrane pH gradient; Φ_{Chl}, chlorophyll fluorescence quantum yield; Φ_{LHC}, chlorophyll fluorescence quantum yield of a light-harvesting complex; PSII, photosystem II; qE, feedback de-excitation quenching or high-energy-state quenching; qI, photoinhibition quenching; qP, photochemical quenching; qT, state transition quenching; RC, reaction center; S_0, ground electronic state; S_1, first singlet excited electronic state; TA, transient absorption spectroscopy; CT, charge transfer; Vio, violaxanthin; Xan, xanthophyll; Zea, zeaxanthin.

the only apoprotein that it is stable in the absence of Chl (*20*). Sequence analysis also raises concerns about the Chl binding ability of PsbS, because it shows that most of the amino acid residues, including all of the conserved histidine residues (*21*), that serve as Chl binding ligands in LHCII (*22*), CP26, and CP29 (*23*, *24*) are not present in PsbS.

Two studies produced contrasting results with regard to the ability to isolate PsbS with bound pigments, either Chls or Xans. A study by Funk et al. (*20*) isolated PsbS from spinach with nonionic detergents and preparative isoelectric focusing (IEF), yielding a complex that was believed to bind 5 ± 1 Chls (which could be both Chl *a* and Chl *b*) and approximately one Car [which was most likely to be lutein (Lut)], but could also be neoxanthin (Neo), Vio, Zea, or antheraxanthin (Anthera). A more recent study by Dominici et al. (*24*), which used spinach and the same isolation procedure as Funk et al. (*20*), was unable to detect any pigment binding. The authors of this second study noted that the isolation procedure involving preparative IEF tends to isolate PsbS in a partially aggregated form, a possible indication of protein denaturation that may cause pigment loss. A second isolation procedure, ultracentrifugation in a detergent/lipid sucrose gradient, was employed that yielded PsbS in a nonaggregated form, but still lacked bound pigments. Dominici et al. also attempted to bind pigments to PsbS by overexpressing the *psbS* gene from *A. thaliana* in *Escherichia coli* and refolding the apoprotein in the presence of a mixture of Chl and Cars. The procedure also did not produce a PsbS subunit with bound pigments. The results imply that if PsbS is able to bind pigments, the method of pigment binding is substantially different from that of other CABs.

Recent work by Aspinall-O'Dea et al. (*25*) has shown that PsbS has the ability to bind a carotenoid that is significant for qE, Zea. The authors achieved this by means of reconstitution of the native, solubilized PsbS apoprotein, as opposed to the overexpressed apoprotein used by Dominici et al., in the presence of Zea. Approximately two Zea molecules were bound per monomeric PsbS subunit, identical to the value suggested by resonance Raman spectroscopy on leaves and thylakoid membranes (*26*), and the circular dichroism (CD) spectrum of the complex suggested that they were excitonically coupled. In agreement with the facts concerning qE, namely, that Zea and PsbS have a joint role in generating ΔA_{535}, no evidence for Vio binding was obtained.

Proton Binding to PsbS

The idea that protonation of PsbS is necessary for qE induction arose from two previous experiments with dicyclohexylcarbodiimide (DCCD), a chemical that covalently binds to luminal acidic amino acid residues located in hydrophobic environments. Ruban et al. found that DCCD inhibits qE (*27*), and Dominici et al. showed that PsbS binds DCCD (*24*). CP26 and CP29 bind DCCD, and a strong similarity has been shown between the two lumen-exposed loops in PsbS and the DCCD/Ca^{2+} binding domain of CP29 (*28*). However, PsbS is known to be significantly more hydrophobic than the other two proteins (*24*), implying that PsbS has the highest affinity for DCCD and is, therefore, the mostly likely protein of the three to bind the chemical *in vivo*. Recent work on wild-type *A. thaliana* thylakoids showed that at pH 5, DCCD binds to PsbS (*29*). Interestingly, the work by Aspinall-O'Dea et al. found that binding of Zea to PsbS *in vitro* was pH-independent (*25*). The results of Aspinall-O'Dea et al. and the DCCD work collectively suggest that *in vivo* Zea binding may be regulated by protonation of carboxyl amino acids during qE that induce conformational changes in PsbS.

Recent site-directed mutagenesis work attempted to identify the amino acid residues in PsbS that are necessary for the protein to function (*30*). The study identified two pairs of symmetric, conserved glutamate (E) residues on the luminal side of PsbS that were important for the protein's qE function, E131 and E235, and E122 and E226. Single mutations in the latter residue set resulted in the most dramatic decreases in qE (approximately 60−70%), and the double mutant, *npq4-E122Q/E226Q*, was virtually qE deficient (displaying a qE level of 0.05 ± 0.02 as compared to the value of 0.00 ± 0.09 obtained for the *npq4-1* mutant). The E122 and E226 residues are located in the central region of the two conserved loops in PsbS and were suggested to be potential proton binding sites. Further support for this hypothesis comes from DCCD binding studies on thylakoids from both the single mutants and the *npq4-E122Q/E226Q* double mutant. At pH values similar to those found in the lumen during qE, the plants with a single or double mutation bind ~50 or 100% less DCCD than the wild type, respectively (*29*).

Aggregation State of PsbS

The knowledge that two amino acids, arginine and glutamate, known to be important for helix−helix interactions are conserved in PsbS led Jansson et al. (*31*) to predict that the protein may exist as a dimer in thylakoid membranes. Recent work by Bergantino et al. (*21*) has confirmed this hypothesis by producing an antibody that was selective to a region that was highly conserved among PsbS proteins, but clearly distinct from the other LHCs. Western blots of various membranes solubilized with digitonin in the presence of the antiserum revealed both 21 and 42 kDa bands, evidence which implies the presence of monomers and homodimers of PsbS, respectively. Additional support for the assignment of the 42 kDa band as a PsbS dimer came from experiments which showed that PsbS did not form heterodimers with other LHC proteins and from the observation that both Western blot bands could not be sequenced, a well-characterized property of PsbS but not of other LHCs.

Bergantino et al. also found evidence that the monomer-to-dimer ratio is light- and pH-dependent in experiments on intact chloroplasts and whole leaves. The ratio increased with increasing light intensity and was reversed when the pH was lowered. The ratio was zero at pH 4 (*21*), a value which would lead to dimer dissociation at luminal pH values of ~5.2−6.0 that are proposed to occur at high light levels (*30*).

Location of PsbS

A variety of results imply that PsbS is most likely associated with the additional LHCII pool that is not strongly attached to the PSII supercomplex, but is able to transfer energy to the PSII core (*20*, *32*). Recent immunoblotting work by Nield et al. (*33*) found that the PsbS protein was

present in PSII-enriched BBY membranes, but absent from the fraction of those membranes which contained the LHCII−PSII supercomplex structure. While it is possible that the PsbS protein is lost from the LHCII−PSII supercomplex during the isolation procedure (*34*), the immunoblotting results are further supported by analysis of the most recent, highest-resolution structure of the LHCII−PSII supercomplex which concludes that there is not enough space for the four-transmembrane helix PsbS protein. A study by Dominici et al. (*24*) on barley mutants with defects in PSII or LHC subunits also supports the idea that PsbS is a peripheral PSII protein. Since defects in LHC proteins do not affect the accumulation of other LHC proteins (*35*), but defects in PSII subunits affect the accumulation of the PSII core (*36*), the fact that the presence of PsbS was detected in PSII core mutants leads to the conclusion that PsbS is located on the periphery of PSII.

While it seems unlikely that PsbS is located in the LHCII−PSII supercomplex, there is mounting evidence for some kind of association between PsbS and the PSII core. Thidholm et al. showed association of PsbS with only the LHCII−PSII dimeric supercomplex and monomeric subcomplexes, but not with the additional LHCII fraction (*37*). Dominici et al. (*24*) found that in BBY particles the amount of PsbS depended more on the concentration of the inner-supercomplex proteins, namely, the core, CP26, and CP29, than on decreases in the Lhcb antenna proteins. Additional studies on G&Y preparations, which contain the core, CP26, and CP29, but little LHCII, support a similar conclusion.

The results on the location of PsbS seem to be potentially contradictory, since in some cases PsbS appears to be more closely associated with the additional LHC antenna and in other cases with the PSII core. The suggestion that PsbS links the core and the antenna can explain these observations (*32*). Recent results of Bergantino et al. (*21*) also provide an explanation for this apparent discrepancy by finding that the two different aggregation states of PsbS have different affinities for the core and the LHC antenna. At pH 4, the protein exists as a monomer and preferentially associates with the antenna LHCs. At pH 7, the favored PsbS aggregation state, a dimer, mainly comigrates with the PSII core. Coupling this information with the observation that dimers monomerize upon qE induction in intact systems because of acidification of the thylakoid lumen leads to the conclusion that monomers associated with the LHC antenna are responsible for *in vivo* energy dissipation.

Local Environment of Zea in PsbS

Ruban et al. showed that ΔA_{535} arises from a strong red shift (∼22 nm) of the Zea absorption band upon *in vivo* qE induction (*26*). A similar shift was observed upon binding of Zea to the native, isolated PsbS, suggesting that this event specifically accounts for ΔA_{535} during qE (*25*). The conclusion that an electronic absorption change is responsible for ΔA_{535} was further confirmed by resonance Raman spectroscopy which identified bands distinct for a red-shifted Zea for both the *in vitro* and *in vivo* samples. Furthermore, the ∼22 nm shift indicates that the bound Zea is situated in a highly polarizable medium with an index of refraction of ∼1.8 (*26*). Such a large red shift is not observed when Zea binds to the other Lhcb proteins. As a result, the fourth transmembrane helix of PsbS has been suggested to be the Zea binding site because it is unique to PsbS and contains a number of phenylalanine residues, which, as judged by changes in UV absorption, were influenced by Zea binding (*25*).

Quenching in Individual LHCs and the Connection to qE

The current lack of definitive evidence for Chl binding to PsbS casts some doubt on whether the protein is the quenching site for qE. A number of findings suggest that the binding of Zea to PsbS induces quenching in one or more of the LHCs. Studies of recombinant, monomeric LHC proteins provide evidence for intracomplex quenching and show that Vio and Zea have opposing effects on the fluorescence quantum yield of the complex (Φ_{LHC}). Binding Vio to the L1 and/or L2 sites increases the yield, whereas Zea binding decreases the fluorescence yield when compared with Lut binding (*38*). Additional studies have established that the L2 site is an allosteric regulator of Φ_{LHC} (*11*, *23*, *39*, *40*). Specifically, when the L1 and N1 sites of LHCII were saturated with Lut and Neo, respectively, binding of Zea to the L2 site decreased Φ_{LHC} by ∼44% with respect to samples which had either Vio or Lut in the L2 site, each of which had statistically identical values (*38*). More in-depth studies on isolated and recombinant minor LHCs showed similar decreases in Φ_{LHC} in addition to changes in the isoelectric point (R. Bassi, personal communication) and lifetime distribution (*41*) in the protein, leading the authors to conclude that these complexes assume L2 site Car-dependent quenched and unquenched states.

While the results show that the presence of Zea in an individual LHC can cause a decrease in Φ_{LHC} of ∼30−50%, these changes are too small to account for qE *in vivo* which can quench up to 80% of Φ_{Chl} (*10*−*12*). Aggregation-dependent fluorescence quenching in LHCII, which was induced by lowering the pH of the sample and was further enhanced by the presence of Zea in the protein with respect to Vio, led Horton et al. (*42*) to propose that interprotein interactions may account for the additional decrease in Φ_{Chl} observed during qE. These ideas were quantified by the dependence of the three Chl fluorescence lifetimes in LHCII on the protein concentration (*41*). With increases in the lipid/protein (L/P) ratio, the amplitude of the ∼0.2 ns component increased at the expense of the amplitude of the ∼2.3 ns component while the ∼1.0 ns component was unchanged. At specific L/P ratios, average fluorescence lifetimes were obtained that were similar to the values measured during qE *in vivo*. Furthermore, the transition from the detergent to lipid environment produced a decrease in the lifetime of each of the individual decay components, suggesting that membrane-dependent conformational changes may also play a role in qE.

Other observations, including the similarity of the loops between helices B and C of CP29 with the luminal loops of PsbS (*28*, *43*), the fact that CP26 and CP29 both bind the qE inhibitor DCCD (*44*), and the fact that CP26, CP24, and, to a lesser extent, CP29 and LHCII all bind Zea in a pH-dependent manner in the L2 site (*45*), point to these CAB proteins as potential quenching sites. The strong similarities between the kinetics observed during qE in intact chloroplasts and in the quenching in LHCII monomers and trimers, CP26,

and CP29 also favor this supposition (*46*, *47*). In addition, the *npq5* mutant of the green alga *Chlamydomonas* suggests that an LHCII subunit appears to be involved in qE (*48*). However, a significant number of findings appear to contradict this notion, including the well-characterized transcriptional repression of the LHCs under light stress (*49*, *50*) and Chl *b*-less *chlorina f2* barley (*51*−*54*) and *A. thaliana* (X.-P. Li and K. K. Niyogi, unpublished results) mutants which are highly deficient in all LHC antenna, but still display qE. The lack of DCCD binding in LHCII poses further questions about the role of this protein in qE. Furthermore, while aggregation induces quenching in these proteins *in vitro*, no study has ever observed such a phenomenon *in vivo* (*11*).

Role of Cars in the Mechanism of qE

While the results highlighted above represent significant advances in our phenomenological understanding of qE, unfortunately they do not provide definitive insight into its mechanism. Typically, the interconversion of Vio to Zea via the Xan cycle, known to be necessary for complete qE induction, is thought to be significant for two potential reasons. One hypothesis is that the first singlet excited electronic state (S_1) of Zea, and, to a lesser degree, Anthera and Lut (*55*), can nonradiatively deactivate ^{1}Chl* by means of either energy and/or electron transfer, while the S_1 state of Vio cannot (*6*, *56*, *57*). The second is that structural differences between the Cars allow for allosteric regulation of qE; i.e., Zea induces conformational changes in the pigment protein complexes that lead to the formation of either local or long-range excitonic Chl interactions that are responsible for the observed quenching and do not exist when Vio is present (*13*, *42*, *58*). The two ideas are commonly termed the direct and indirect quenching mechanisms, respectively, due to the nature of the role of the Car in each case. At present, no evidence unequivocally proves or excludes either mechanism, leaving the possibility that both the spectroscopic and structural properties of these Cars are significant for qE.

Energy Level of the Car S_1 State

While precise knowledge of the energy levels of the relevant qE-related Cars and Chls cannot prove that qE occurs by the direct quenching mechanism, it can assess if energy or electron transfer involving these molecules is feasible. Specifically, effective energy transfer from Chl(s) to Car(s) during qE requires that the S_1 state of Zea be lower in energy than the Q_y band of the energetically relevant Chl(s). Although the transition energy for the specific Chl(s) involved in qE is another unknown parameter, a reasonable estimate of this value was obtained from low-temperature fluorescence spectroscopy on LHCII at 4 K and equals $\sim$14700 cm^{-1} (*59*). However, determination of the S_1 energy of Cars, in both solution and the protein environment, has turned out to be a rather formidable task due to the fact that the state is, for all intents and purposes, one-photon-forbidden by absorption and emission from the ground and excited states, respectively.

A variety of traditional and novel spectroscopic techniques have been used in attempts to determine the spectral origin of the $S_1 \rightarrow S_0$ transition. The results of such measurements for Cars in solution and in LHCII are summarized in Table 1. While the measurements of the $S_1 \rightarrow S_0$ transition energy show some agreement, there are also some noticeable technique- and sample-dependent differences. In four of the five measurements which were performed jointly on Zea and Vio, the S_1 energy of Vio was greater than the S_1 energy of Zea, and in three of those four cases, the S_1 energy of Zea and that of Vio were lower and higher than the approximate value for the Chl Q_y band, respectively. The unique method developed by Polívka et al. consistently found that the energies of both Zea and Vio were lower than that of the Chl Q_y band (*60*, *61*). If the measurements of Polívka et al. accurately represent the Car S_1 energy, they imply that if qE occurs by the direct quenching mechanism, differences in the distance and/or orientation of the these Cars with respect to the relevant Chl(s) must also be significant.

Table 1: Values Obtained for the S_1 Energy of Chl *a* and the "qE-Relevant" Cars[a]

molecule	conjugation length	sample conditions [solvent or protein *T* (K)]	ref	transition energy (cm^{-1})
Chl *a*	−	LHCII/4	*59*	14700
Zea	11	*n*-hexane/RT	*75*	14550 ± 90
		n-hexane/RT	*57*	14200
		methanol/RT	*61*	14030 ± 90
		L1 site of LHCII/RT	*60*	13850 ± 200
		EPA/77	*76*	14610 ± 40
Anthera	10	*n*-hexane/RT	*57*	14700
Lut	10	*n*-octanol/RT	*62*	14300 ± 300
		L1 and L2 sites of LHCII/RT	*60*	14050 ± 300
		EPA/77	*76*	14570 ± 70
Vio	9	*n*-hexane/RT	*75*	14880 ± 90
		n-hexane/RT	*57*	15200
		methanol/RT	*61*	14470 ± 90
		L1 site of LHCII/RT	*60*	13700 ± 300
		EPA/77	*76*	15580 ± 60
mixture of Lut, Neo, and Vio	−	WT LHCII from *Chlamydomonas reinhardtii*/RT	*62*	15100 ± 300

[a] Conjugation length is the number of conjugated carbon double bonds in the molecule. RT is room temperature (298 K). EPA is a low-temperature glass composed of ethanol, isopentane, and diethyl ether.

Energy Transfer and the Car S_1 State

There is at present no indisputable evidence for dissipative Chl $Q_y \rightarrow$ Car S_1 energy transfer in any pigment protein complex which suggests either that energy transfer occurs but the Car S_1 lifetime is significantly shorter than the energy transfer time from Chl to Car, resulting in an undetectable Car S_1 population, or that no energy transfer takes place. The former case poses questions since there is a significant amount of experimental evidence of efficient Car $S_1 \rightarrow$ Chl Q_y light-harvesting energy transfer, on the order of $\sim$250 fs (assigned to transfer from vibrationally "hot" S_1 states) (*62*) to a few picoseconds, in a variety of pigment−protein complexes (*63*−*65*). Since dissipative and light-harvesting energy transfer must be governed by the same fundamental laws, with the efficiency of both processes being explicitly dependent on the relative energy difference between the states, they can, in principle, lead to the same rates. Consequently, if such fast rates exist for Chl $Q_y \rightarrow$ Car S_1 transfer, they should produce an observable amount of Car S_1 population in transient absorption (TA) measurements.

Electron Transfer and the Car S_1 State

Direct quenching by Cars during qE may occur by electron transfer. Unlike energy transfer, electron transfer requires the formation of a Chl−Car dimer, a species defined by spatial overlap of the π-systems of the Car and the Chl, and depends on the ionization potential (IP) of the Car. Recent work on the *Rhodobacter sphaeroides* LH2 complex revealed ultrafast (∼200 fs) formation of a Car radical cation with a lifetime of 8 ps after excitation of the Car S_2 state (*66*). Furthermore, ultrafast TA measurements by Kodis et al. (*67*) on a covalently linked Car−phthalocyanine (Pc) triad, in which two Cars with either nine or 10 conjugated double bonds were oriented nearly perpendicular to the tetrapyrrole plane of a Pc derivative, detected a $Car^{\bullet+}-Pc^{\bullet-}$ charge-separated state after selective excitation of the lowest singlet excited state of the Pc. The time constant of charge separation was 2.5 and 2.2 ps for complexes in which the linked Car contained 9 and 10 conjugated double bonds, respectively. The ∼200 fs time scale for charge separation observed in LH2 as compared with the results for linked Car−Pc complexes in solution suggests that fine-tuning of the local environment, orientation, and distance by the protein may make electron transfer effective for energy dissipation.

Recent calculations by Dreuw et al. (*68*) using a hybrid theoretical approach involving time-dependent density functional theory and configuration interaction singles, which was able to capture the salient features of charge transfer (CT) states, investigated whether electron transfer could occur between a model Chl *a* chromophore, which contained the chlorin ring but lacked the side chain, and the three Xan cycle carotenoids. The calculations show that in all cases where electron transfer is possible, the CT state involves a pure highest occupied molecular orbital to lowest unoccupied molecular orbital transition, strictly confined to the Car and Chl, respectively. Therefore, electron transfer results in Car cation and Chl anion radicals. In good agreement with the known requirement of π-system overlap between the Car and the Chl, the calculations show that the electron transfer strongly depends on the molecular geometry and is maximal when the π-system of the Chl and the Car are parallel and the Chl is located above the Car's center of mass. In this geometry, the distance at which the CT state becomes the lowest-energy excited state of the complex is 5.5, 5.1, and 4.8 Å for dimers containing Zea, Anthera, and Vio, respectively. The differences in distance occur because a separate calculation on the individual Cars shows that the IPs vary in the following order: Vio > Anthera > Zea. By inspecting the molecular structure, the authors conclude that Chl−Zea and Chl−Anthera dimers could form *in vivo* and create a CT state that is responsible for qE quenching; however, steric hindrance may inhibit the formation of a Chl−Vio dimer. It should be noted that these calculations do not include the polar protein environments, which should stabilize the CT states nearly uniformly because of their large static dipole moments and relax the calculated distance constraints.

S and Its Potential Role in qE*

The LH2 *Rb. sphaeroides* complex (*69*) and the covalently linked Car−Pc triad (*67*) showed ∼80% Car energy transfer efficiency from a state that until recently was considered to be exclusively S_1. In-depth spectral analysis of the TA results in both complexes suggests a revision of the traditional idea that S_1 is the only excited state below S_2 in Cars. The studies find that the observed high efficiency involves a second Car donor state, termed S*, which accounts for 10−15% of the energy transfer in LH2 (*69*) and ∼20% in the triad (*67*). In addition, the appearance of the Car radical in LH2 from *Rb. sphaeroides* was accompanied by the formation of a singlet excited state in the visible that was not S_1 and is believed to be S* (*66*). The involvement of the S* state in energy transfer and possibly electron transfer suggests that its energy is comparable to that of the Chl Q_y band and, therefore, should be considered as a possible acceptor in the direct quenching mechanism.

Indirect Quenching

The observation that Chl aggregation, either at high concentrations or in polar solvents, leads to a decrease in the Chl fluorescence lifetime (*70*) is the source for the proposal of exclusively Chl-based quenching during qE (*13*, *42*, *58*). While the exact molecular level description for this finding is poorly understood, it probably involves one or more low-lying CT states (*71*) that are formed when the two Chls interact at an intermolecular separation distance of less than ∼12 Å (*70*).

Unlike in the direct quenching mechanism, where the spectroscopic properties of Zea are significant for qE, indirect quenching depends on the structural and chemical differences between the Cars which allow only Zea, and, to a lesser extent, Anthera and Lut, to induce the formation of Chl quenchers. Inspection of the Cars indicates that the presence or absence of conjugated double bonds in the β-ring affects the planarity of the molecule. Accordingly, Zea is the most planar of the three, Anthera less planar than Zea, and Vio the least planar. The ϵ-ring of Lut causes approximately the same deviation from planarity as the epoxy ring(s) in Vio and Anthera, making its overall three-dimensional structure analogous to that of Anthera, a finding which may explain the similarity between their observed functional roles in qE (*55*). An alternate explanation is that the polarity of the Car allosterically regulates qE through the removal or addition of epoxy group(s). Correspondingly, Zea and Vio are more and less hydrophobic, respectively, than Anthera, suggesting that differences in amphipathic properties may control the formation of Chl quenchers (*13*).

Car−Chl Interactions

While the indirect and direct quenching hypotheses assume that the quenchers are exclusively Chl(s) and Car(s), respectively, an additional mechanism may exist in which Car−Chl excitonic interactions are responsible for qE. Evidence that such interactions exist in a pigment−protein complex comes from TA work by Gradinaru et al. (*72*) on LHCII which showed that upon excitation of Chl *a* instantaneous bleaches, within the 100−200 fs time resolution, on the order of 1−2% were observed for the two spectrally distinct Luts. A simple yet insightful calculation was carried out by van Amerongen et al. (*73*) to determine the effect of coupling between the Chl *a* Q_y and Car S_1 states on the observed Chl excited-state lifetime. The lowest excited state of this complex has a composition of 2% Car S_1 and 98% Chl *a* Q_y. The lifetime of the complex is ∼650 ps, a value

which is dramatically shorter than the Chl lifetime of ~5 ns in the absence of such interactions. While these finding are only rough approximations, it should be noted that in this quenching scenario only the relative energy difference between the Car S_1 state and the Chl Q_y band is significant and not, as in the case of energy transfer, the absolute energies of the states.

Investigation of the Mechanism of qE by TA Spectroscopy

The qE quenching species must have a lifetime that is shorter than the nanosecond Chl fluorescence lifetime, making it potentially detectable by femtosecond TA spectroscopy. One study to date has attempted to utilize this technique on intact thylakoid membranes that are capable of exhibiting qE (*74*). Upon selective excitation of the Chl Q_y band, qE-dependent changes were observed that could be attributed to Car excitation. Interestingly, the Car excitation was instantaneous (with respect to an instrument response of ~120 fs); however, a variety of additional experiments gave no indication that direct one-photon Car excitation was possible under the experimental conditions. These experiments provide the first and most convincing evidence to date that the excited states of Zea, either S_1 or S*, have some direct involvement in qE. The instantaneous rise of the Zea signal suggests that if the species observed in the work is the qE quencher, it may be a product of a Zea−Chl excitonic interaction.

Concluding Comments

While a significant number of advances have deepened our understanding of qE, the results also present clear challenges for future work in this field. Specifically, with respect to PsbS, clarification of its pigment binding properties is needed, including experiments to test if the native, isolated PsbS protein which was shown to bind Cars can also bind Chls. In addition, conclusive pigment binding results *in vivo* are vital for identifying the protein complex which binds the quenchers. Furthermore, the connection between Zea binding and the aggregation or protonation state of the protein must be elucidated. Finally, a consensus about the location of PsbS monomers and dimers and their functional role in qE is necessary.

Recent experimental and theoretical studies on the multiple types of quenching that can occur between photosynthetic pigments suggest new directions for the study of the mechanism of qE. Since the interactions in the LHCs may represent the actual qE quenching pigments or, at least, good models for them, further studies are needed to definitively establish the mechanism responsible for their fluorescence quenching. In addition, while measurements of the energy of the Car S_1 state in solution and in isolated pigment−protein complexes have inherent limitations, namely, that they may not account for the local environment experienced by the quenching pigments and specific interactions that occur in the membrane during qE, it is clear that general agreement on these values would provide a much better assessment of the feasibility of the direct quenching mechanism. Such knowledge may be able to determine if a Car−Chl quenching pair could be correctly characterized by weak coupling. Covalently linked Car and "Chl-like" systems also offer a number of novel opportunities to study the involvement of Cars in quenching since the Pc lifetime of ~1.4 ns measured in the triad containing Cars with 10 conjugated double bonds, which showed no energy transfer from the Car S_1 state, was clearly shortened with respect to the Pc in solution and the Pc in the triad containing Cars with nine conjugated double bonds, which both have a lifetime of ~5.3 ns (*67*). The system is of particular interest since the sample concentrations strongly disfavor any exclusively Pc-based quenching. Finally, extensive utilization of ultrafast TA spectroscopy on intact systems capable of qE could be very effective in the study of the mechanism of quenching and/or for elucidating the specific kinetics of the quencher(s).

Self-regulation is perhaps the most definable feature that separates a living system from the sum of its parts. As a result, the goal of this work, not simply the elucidation of the mechanism of qE but also "natural" and artificial manipulation of it, ultimately rests on both an understanding of quenching *in vitro* and *in vivo* and the connection between them.

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