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Origin of Absorption Changes Associated with Photoprotective Energy Dissipation in the Absence of Zeaxanthin*

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To prevent photo-oxidative damage to the photosynthetic membrane in strong light, plants dissipate excess absorbed light energy as heat in a mechanism known as non-photochemical quenching (NPQ). NPQ is triggered by the transmembrane proton gradient (ΔpH), which causes the protonation of the photosystem II light-harvesting antenna (LHCII) and the PsbS protein, as well as the de-epoxidation of the xanthophyll violaxanthin to zeaxanthin. The combination of these factors brings about formation of dissipative pigment interactions that quench the excess energy. The formation of NPQ is associated with certain absorption changes that have been suggested to reflect a conformational change in LHCII brought about by its protonation. The light-minus-dark recovery absorption difference spectrum is characterized by a series of positive and negative bands, the best known of which is ∆A535. Light-minus-dark recovery resonance Raman difference spectra performed at the wavelength of the absorption change of interest allows identification of the pigment responsible from its unique vibrational signature. Using this technique, the origin of ∆A535 was previously shown to be a subpopulation of red-shifted zeaxanthin molecules. In the absence of zeaxanthin (and antheraxanthin), a proportion of NPQ remains, and the ∆A535 change is blue-shifted to 525 nm (∆A525). Using resonance Raman spectroscopy, it is shown that the ∆A525 absorption change in Arabidopsis leaves lacking zeaxanthin belongs to a red-shifted subpopulation of violaxanthin molecules formed during NPQ. The presence of the same ∆A535 and ∆A525 Raman signatures in vitro in aggregated LHCII, containing zeaxanthin and violaxanthin, respectively, leads to a new proposal for the origin of the xanthophyll red shifts associated with NPQ.

To ensure the efficiency of photosynthesis, even under low light conditions, the photochemically active Chl of the photosystem II (PSII) reaction center are served by additional antenna Chl bound to light-harvesting complexes (LHCII) (1). However, under certain environmental conditions, the amount of light absorbed by the LHCs is in excess of that which can be used in photochemistry. Left unchecked, the excess absorbed light energy can lead to the formation of Chl triplet states in the PSII reaction center that sensitize the production of singlet oxygen (2–4). Singlet oxygen damages the PSII reaction center and other components of the photosynthetic membrane, leading to a sustained decrease in photosynthetic efficiency that is known as photoinhibition (2). To mitigate this, plants have evolved a photoprotective mechanism, known as non-photochemical quenching (NPQ), whereby the excess excitation energy is safely dissipated as heat (4, 5). The major component of NPQ is controlled by the level of the trans-membrane proton gradient (ΔpH) formed as a result of photosynthetic electron transport and is known as qE (6). ΔpH formation triggers the protonation of LHCII (7) and of the PsbS protein (8), as well as activating the violaxanthin de-epoxidase enzyme, which converts the LHCII-bound xanthophyll violaxanthin into zeaxanthin (9). The interaction of these three factors brings about formation of dissipative pigment interactions within the PSII antenna, thus shortening the Chl excited-state lifetime (10, 11).

The precise nature of the quenching pigment interactions responsible for qE remain under debate (12–17). Despite this, many of the photophysical and photochemical features of qE are well characterized experimentally. It has been shown that qE is associated with a series of absorption changes in both the Soret and the Qy regions (18–24). These absorption changes have been suggested to reflect an altered environment of bound Chl and xanthophyll molecules, brought about by a protein conformational change within LHCII as a result of ΔpH formation (22). The light-minus-dark recovery absorption difference spectrum in the Soret region contains a series of three negative bands below 500 nm and a positive

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band at 535 nm (25). The formation and relaxation kinetics of the latter change known as $\Delta A_{535}$ are often monitored as a collective measure of these conformational changes. $\Delta A_{535}$ was originally believed to arise from selective light scattering brought about by a change in the membrane thickness linked to pH formation (26, 27). Later $\Delta A_{535}$ was shown to depend upon the presence of zeaxanthin and was closely correlated with the formation and relaxation of qE (18–20, 22). In the absence of zeaxanthin, qE is greatly reduced and the $\Delta A_{535}$ change has a much smaller amplitude and is blue-shifted to 525 nm ($\Delta A_{525}$) (28). The npq4 mutant of Arabidopsis that lacks the PsbS protein was found to lack rapidly reversible qE and also lacked any $\Delta A_{535}$ providing a further link between these two phenomena (29). Resonance Raman spectroscopy has proved of use in identifying the origin of absorption bands both in isolated light-harvesting complexes and in intact chloroplasts and even leaves (30). Resonance Raman spectroscopy confirmed that $\Delta A_{535}$ belonged to the 0-0 component of the S2 electronic transition of zeaxanthin (31). The size of the zeaxanthin resonance enhancement in the Raman quenched-minus-unquenched difference spectrum at 528 nm excitation was also found to depend upon PsbS (31). In addition, the negative absorption changes in the qE difference spectrum below 500 nm were associated with a loss of xanthophyll resonance confirming that they also arise from true electronic transitions rather than light scattering (31). Native isolated PsbS was found able to bind zeaxanthin, producing a red shift in its absorption spectrum mimicking $\Delta A_{535}$ (32). The putative binding simultaneously affected protein phenylalanine absorption and circular dichroism (CD), leading to the suggestion that $\Delta A_{535}$ may arise from zeaxanthin binding to this hydrophobic protein (32). Indeed, it was suggested that $\Delta A_{535}$ may represent the formation of a zeaxanthin-PsbS quenching complex responsible for qE (8). However, several observations have brought this proposal into question. Firstly, recombinant PsbS was consistently found unable to bind any xanthophylls (33, 34). Second, PsbS was still found to perform its function in the absence of zeaxanthin (35), and thirdly, the npq4 mutant lacking PsbS was found to possess $\Delta A_{535}$ and wild-type levels of NPQ, albeit both forming on a much longer timescale than the wild-type (36).

Recently, the origin of one of the main negative bands in the qE difference spectrum peaking at 495 nm ($\Delta A_{495}$) was shown to depend upon the presence of lutein (25). In Arabidopsis leaves lacking lutein, the band was shifted to 497 nm (in lut2−, lutein replaced by violaxanthin) and 501 nm (lut2npq2−, lutein replaced by zeaxanthin) (25). Very similar negative absorption changes to those observed in leaves below 500 nm have been observed upon aggregation of isolated LHCI and also in quenched but non-aggregated LHCI, suggesting that both in vivo and in vitro types of quenching may have a common origin (37, 38). However, the characteristic $\Delta A_{535}$ change was not observed upon LHCII aggregation, although this may be due to the absence of zeaxanthin in these samples (37). Thus, the question of the exact origin of all elements of the qE-related absorption changes remains. It is known that the energy of an electronic transition within a molecule is strongly dependent on the refractive index of the solvent environment (39, 40). However, such a strong red shift as seen during the $\Delta A_{535}$ change would require a very dramatic change in the solvent environment, possibly due to the arrival of a static dipole or a point charge in the vicinity of the zeaxanthin (41, 42). Alternatively, this shift in absorption may arise from excitonic interactions between pairs of zeaxanthin molecules (43). These zeaxanthin pairs would occur as a result of aggregation of LHCII trimers within the thylakoid membrane. Because zeaxanthin occupies a relatively peripheral position within the LHCII trimer (44), aggregation could result in close associations between xanthophylls belonging to neighboring trimers. Indeed, it has been shown that the formation of excitonically coupled J-aggregates of zeaxanthin (co-linear or “top-to-tail” chains of molecules) in water-ethanol mixtures gives rise to a red shift in the 0-0 transition to 535 nm (34, 45, 46), whereas lutein, antheraxanthin, and violaxanthin J-type aggregates possess red-shifted bands between 500 and 530 nm (45). In the following study, we specifically address the origin of $\Delta A_{525}$. Firstly, using resonance Raman experiments on intact chloroplasts lacking zeaxanthin, we identify the xanthophyll responsible for $\Delta A_{535}$ as violaxanthin. In addition, we show that the same Raman signature associated with $\Delta A_{535}$ and $\Delta A_{525}$ in vivo is observed upon aggregation of LHCII binding, respectively, zeaxanthin or violaxanthin in the peripheral V1 site.

EXPERIMENTAL PROCEDURES

Fluorescence and Absorption Measurements—Arabidopsis thaliana cv Col0 (wild-type) and two mutants derived from it, npq1 (lacking zeaxanthin) and L17 (PsbS overexpressor) (47), were grown for 8–9 weeks in Sanyo plant growth rooms with an 8-h photoperiod at a light intensity of 100 μmol of photons m−2 s−1 and a day/night temperature of 22/18 °C. The composition of carotenoids was determined by HPLC for leaf disks rapidly frozen in liquid N2, as described previously (48). Leaves were vacuum-infiltrated with 20 mM HEPES buffer (pH 7.0) containing 5 mM dithiothreitol (DTT) to inhibit violaxanthin de-epoxidation, whereas control leaves were vacuum-infiltrated with buffer only. Chl fluorescence kinetic analyses of whole leaves was carried out using a Dual-PAM-100 fluorometer (Walz) using varying actinic intensity together with light saturation pulses (4000 μmol of photons m−2 s−1) as indicated in the figures. The maximum quantum yield of PSII ($F_{v}/F_{m}$) was defined as ($F_{m} - F_{o}$)/$F_{m}$, the yield of PSII (ΦPSII) as ($F_{m}' - F_{o}'$)/$F_{m}'$, qP as ($F_{m}' - F_{m}$)/($F_{m}' - F_{o}'$), and NPQ as ($F_{m}' - F_{m}$)/$F_{m}'$. qE-related absorption changes were recorded on whole leaves using an Aminco DW2000 spectrophotometer as described previously (25), and difference spectra were calculated by subtracting the “dark recovery” spectrum (5 min of preillumination at 1000 μmol of photons m−2 s−1 actinic light followed by 5 min of dark relaxation) from the “light” spectrum recorded in the presence of the actinic light following 5 min of preillumination.

LHCCI Isolation—Spinach trimeric LHCCI binding either violaxanthin or zeaxanthin was isolated from n-dodecyl β-o-maltoside solubilized spinach BBY membranes using sucrose gradient ultracentrifugation as described previously (49). Deperoxidation of violaxanthin to antheraxanthin and zeaxanthin
was achieved by incubating the thylakoids (from which BBYs were derived) for 30 min in 0.33 M sorbitol, 1 mM EDTA, 30 mM HEPES, 20 mM MES, 40 mM ascorbate at pH 5.5 at 20 °C. LHCII was desalted to remove sucrose in a PD10 desalting column (GE Healthcare) in a buffer containing 20 mM HEPES (pH 7.8) and 0.03% (w/v) n-dodecyl β-D-maltoside. Quenched LHCII was prepared by removal of detergent by SM-2 bioabsorbent beads (Bio-Rad) allowing for a 10× reduction in fluorescence yield as determined by a PAM-101 fluorometer (Heinz Walz). Chl concentration was determined according to the method of Porra et al. (50).

**RESULTS**

The well known light-minus-dark recovery absorption difference spectrum in Arabidopsis wild-type leaves is presented in Fig. 1, exhibiting the usual positive maximum at 535 nm consistent with the presence of zeaxanthin (Table 1). In the npq1 difference spectrum, the positions of the negative bands below 500 nm are virtually identical, whereas the 535 nm maximum is blue-shifted to 525 nm, consistent with the absence of zeaxanthin (and antheraxanthin) (Fig. 1, Table 1). The amplitude of all bands in the difference spectrum is also smaller in npq1 than in the wild type, consistent with the lower NPQ in the former sample (Fig. 1). Using resonance Raman difference spectroscopy, the origin of the 525 nm band in the npq1 absorption difference spectrum was probed. Two Raman spectra were recorded using 528.7 nm excitation, one for leaves frozen under illumination (5 min of preillumination at 1000 μmol of photons m⁻² s⁻¹) or alternatively preilluminated and then frozen following an additional period of 5 min of dark relaxation (dark recovery) and on isolated LHCII prepared in the trimeric and aggregated states as described above. Purified xanthophylls prepared as described previously (51, 52) were dissolved in pyridine for 77 K measurements. Samples were measured in a helium flow cryostat (Air Liquide, France) using a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon, France) as described (51). Excitation was provided by a Coherent Argon (Innova 100) laser (488.0 and 528.7 nm).

**TABLE 1**

| Plant        | Neo | Lut | Vio | Ant | Zea | DEPs | Chl a/b % | Neoxanthin | Lutein | Violaxanthin | Antheraxanthin | Zeaxanthin | DEPs |
|--------------|-----|-----|-----|-----|-----|------|----------|------------|---------|-------------|---------------|------------|------|
| WT dark      | 5.2 ± 0.5 | 17 ± 1 | 4.4 ± 0.2 | 0.2 ± 0.1 | 0.9 ± 0.9 | 3.1 |
| WT light     | 5.1 ± 0.9 | 16 ± 1 | 4.0 ± 0.4 | 0.8 ± 0.3 | 4.6 ± 1.2 | 3.1 |
| npq1         | 5.3 ± 0.9 | 19 ± 2 | 4.4 ± 0.8 | ND | ND | 3.1 |
| LHCII + DTT dark | 5.2 ± 0.6 | 16 ± 1 | 4.4 ± 0.2 | ND | ND | 3.1 |
| LHCII + DTT light | 5.2 ± 0.5 | 17 ± 2 | 4.6 ± 0.4 | ND | ND | 3.1 |

Consistent with this enhanced ΔA_{525}, the light-minus-dark recovery resonance Raman difference spectrum for L17 leaves treated with DTT had a significantly improved signal-to-noise ratio.
ratio. Thus, a meaningful comparison with individual pigment spectra was possible, allowing us to attribute the band(s) (see below).

Fig. 2 presents the total light-minus-dark recovery resonance Raman difference spectrum using 528.7 nm excitation of L17 leaves treated with DTT. The four characteristic frequency regions of carotenoids have been assigned as follows: $v_1$, C=C stretching mode; $v_2$, C–C stretches coupled with C–H in-plane bending or C–CH$_3$ stretching; $v_3$, CH$_3$ in-plane rocking vibrations; $v_4$, C–H out-of-plane bending modes (30). The light-minus-dark recovery resonance Raman difference spectrum at 528.7 nm excitation of wild-type leaves containing zeaxanthin had a $v_1$ position at 1522 cm$^{-1}$ (Fig. 2), consistent with the position of isolated zeaxanthin in pyridine as observed previously (31). However, the $v_1$ position in spectra of L17 + DTT leaves was significantly different, peaking at 1528 cm$^{-1}$. Because the $v_1$ position is highly dependent upon the conjugation length of the C=C chain (conjugated double bonds) of a carotenoid, lutein, violaxanthin, and neoxanthin have respectively higher $v_1$ positions (51). In the case of neoxanthin, the presence of a 9-cis configuration results in a further up-shift in $v_1$ when compared with violaxanthin, despite them both possessing nine conjugated double bonds. The $v_1$ position is thus a unique “molecular fingerprint” of the xanthophyll species involved in a particular resonance enhancement in the Raman difference spectrum resulting from an absorption change (30). Therefore, the up-shifted $v_1$ position in L17 + DTT leaves when compared with the wild-type confirms that a xanthophyll other than zeaxanthin is involved in the $\Delta A_{525}$ change (Fig. 2).

Several features of the Raman difference spectrum using 528.7 nm excitation in L17 + DTT leaves give clues as to the identity of the xanthophyll species responsible for the $\Delta A_{525}$ change. Firstly, the $v_1$ position at 1528 cm$^{-1}$ is identical to that of isolated violaxanthin dissolved in pyridine (Fig. 3), whereas $v_1$ for isolated lutein is at 1524 cm$^{-1}$ and $v_1$ for neoxanthin is at 1533 cm$^{-1}$ (Fig. 3). Examination of the $v_3$ region of the spectrum provides further evidence of the involvement of violaxanthin in the $\Delta A_{525}$ change (Fig. 4). The $v_3$ peak position is at 1006 cm$^{-1}$ in the L17 + DTT Raman difference spectrum (Fig. 4), again consistent with the position of isolated violaxanthin (Fig. 4, trace 2), whereas for both isolated lutein and isolated zeaxanthin, $v_3$ is at 1003 cm$^{-1}$ (Fig. 4). It should be noted, however, that the satellite of the main $v_3$ band in the L17 + DTT difference spectrum is at $\sim$1022 cm$^{-1}$, deviating slightly from the corresponding band for isolated violaxanthin.
(1030 cm⁻¹), whereas it is still higher than that of isolated lutein at 1020 cm⁻¹ (Fig. 4). To account for this deviation, the L17 + DTT spectrum was compared with the previously reported in vivo violaxanthin Raman spectrum (dark adapted−minus-dark recovery spectrum of wild-type leaves, where a preillumination period has caused violaxanthin de-epoxidation). The resulting Raman difference spectrum at 488.0 nm excitation reveals a selective loss of resonance from violaxanthin, thus allowing the in vivo features of this pigment to be observed. Comparison of the in vivo violaxanthin spectrum with that of the L17 + DTT leaves presented here reveals that all features of the ν₄ region are identical, including the position of this satellite band (Fig. 4).

The ν₄ region of the Raman spectrum has been shown to provide information on the molecular distortion of xanthophylls upon binding to proteins because the C−H wagging modes are formally resonance-forbidden in planar carotenoids (30). In L17 + DTT leaves, the ν₄ region of the spectrum shows the two sharp modes at 949 and 962 cm⁻¹, characteristic for the in vivo violaxanthin Raman spectrum previously reported (51) and in contrast to the structureless ν₄ region of violaxanthin dissolved in lipid micelles (Fig. 5). In addition, the ν₄ region in L17 + DTT leaves is slightly broadened with additional modes appearing (Fig. 5, arrows). The data therefore indicate that the red-shifted violaxanthin responsible for ΔA₅₂₅ is selectively distorted through protein binding.

FIGURE 5. ν₄ region of the qE-associated Raman spectrum (difference spectrum of light−treated−minus−dark recovery) induced by 528.7 nm excitation in Arabidopsis leaves from L17 plants. The ν₄ region of the Raman spectra induced by 528.7 nm excitation (difference spectrum of wild-type dark adapted−minus−dark recovered leaves) (trace 1) is presented in comparison with the spectrum of isolated violaxanthin dissolved in lipid micelles (trace 2), rel., relative.

TABLE 2

Pigment composition of isolated LHCII antenna proteins

LHCII trimers enriched in either violaxanthin or zeaxanthin were obtained from sucrose gradients following solubilization of BBY membranes in n-dodecyl β-D-maltoside (see "Experimental Procedures"). De-epoxidation of violaxanthin to antheraxanthin and zeaxanthin was achieved by incubating the thylakoids (from which BBYs were derived) for 30 minutes in 0.33M sorbitol, 1 mM EDTA, 30 mM HEPES, 20 mM MES, 40 mM ascorbate at pH 5.5 at 20 °C (de-epoxidation state of thylakoids following this procedure was ~80%). Neo, V, Ant, Lut, Zea, DEPs, and Chl a/b ratio. Carotenoid data are presented normalized to 14 molecules of Chl a + b ± S.E. from four replicates, DEPs (Zea + 0.5Ant)/total Vio + Zea + Ant)% and Chl a/b presented as molar ratio; ND, none detected.

| Complex  | Neo ± 0.1 | Vio ± 0.1 | Ant ± 0.04 | Lut ± 0.1 | Zea ± 0.1 | DEPs ± 0.1 | Chl a/b ± 0.1 |
|----------|-----------|-----------|------------|-----------|-----------|------------|---------------|
| LHCII V  | 1.04 ± 0.1| 0.69 ± 0.1| ND         | 2.1 ± 0.1 | ND        | 0          | 1.33 ± 0.1    |
| LHCII Z  | 1.05 ± 0.1| 0.08 ± 0.03| 0.08 ± 0.04| 2.1 ± 0.1 | 0.58 ± 0.1| 84%        | 1.33 ± 0.1    |
be required, such as the appearance of a static dipole or point charge near the pigment responsible (41, 42).

An alternative explanation for a large red shift would be an excitonic splitting of the $S_2$ excited state of the xanthophyll (46). This so-called Davydov splitting is a result of strong Coulombic coupling between the excited states of closely associated molecules. Such Coulombic coupling causes a mixing of the excited states of the individual molecules to form new excited states (excitonic states) that are delocalized across the whole molecular ensemble (53). The result of this quantum mechanical mixing is that the original single-molecule excited states are split symmetrically about the original $S_2$ state, and the energy ordering depends entirely on the relative geometry of the two molecules. An H-type (parallel or “card-packed”) geometry yields a dark state lower in energy than the bright state, whereas a J-type (collinear or top-to-tail) geometry results in the bright state being the higher in energy. It is clear that the formation of an H-aggregate is associated with an overall blue shift of the $S_2$ absorption peak because the bright state lies above the original $S_2$ state. By the same logic, the formation of a J-aggregate is associated with a corresponding red shift. We therefore propose that both $\Delta A_{535}$ and $\Delta A_{525}$ are the result of the formation of xanthophyll J-aggregates (zeaxanthin in the case of $\Delta A_{535}$ and violaxanthin for $\Delta A_{525}$) within the membrane.

Indeed, it has previously been shown that isolated zeaxanthin can mimic $\Delta A_{535}$ upon formation of J-type aggregates in vitro (45). A casual formation of J-type aggregates without protein interaction can be excluded for both $\Delta A_{525}$ and $\Delta A_{535}$ in vivo because the structure of the $v_4$ region of the Raman difference spectrum indicates that the pigments remain bound to protein. The distinct lack of structure of the $v_4$ region in J-type aggregates formed between zeaxanthin molecules isolated in vitro when compared with in vivo supports this view (32, 34). The data are rather more consistent with the hypothesis made recently, that upon LHClI aggregation, pairs of either zeaxanthin or violaxanthin, depending upon the de-epoxidation state, may come into contact in such a way that a J-type aggregate is formed at the trimer-trimer interface (43).

In vitro, zeaxanthin enhances LHClI aggregation and violaxanthin restricts it, consistent with an enhancement of the qE-related structural changes by the former (22, 54). A notable feature regarding both $\Delta A_{525}$ and $\Delta A_{535}$ is that neither depends upon the presence of a specific LHClI, minor antenna complex, or PsbS; rather they are features of the ensemble of LHCl(-like) proteins in the membrane, just like NPQ (25, 36). This would be anticipated given the high level of homology between LHClI and the minor antenna proteins (55). Indeed, CP26 and possibly CP29 are predicted to have V1 sites occupied by xanthophyll cycle carotenoids, according to biochemical studies (49). Thus, involvement of the V1 site in both $\Delta A_{525}$ and $\Delta A_{535}$ formation could explain why a signal is observed at $\Delta A_{530}$ in leaves of the Arabidopsis szl1 mutant, which lacks zeaxanthin but compensates by greatly increasing levels of lutein at the expense of violaxanthin (56). In this mutant, we predict that the extra lutein would replace violaxanthin in the V1 site, providing an explanation of the slight red shift of $\Delta A_{525}$ to $\Delta A_{530}$ in these plants (56).

The correlation between $\Delta A_{525}/\Delta A_{535}$ and qE has led to the suggestion that this change may monitor directly the forma-
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