Activation of Zeaxanthin Is an Obligatory Event in the Regulation of Photosynthetic Light Harvesting

Alexander V. Ruban, Andrew A. Pascal, Bruno Robert, and Peter Horton

When plants are exposed to high light intensities a sustained decline in the maximum photosynthetic quantum efficiency, called photoinhibition, frequently occurs. This inevitably leads to the drop in plant productivity, endangering the yield of agricultural crops (1). However, plants have acquired a process of non-radiative dissipation of the excess light energy. This process is controlled by features of the intact membrane, the transmembrane pH gradient, the organization of the photosystem II antenna proteins and the reversible binding of a specific carotenoid, zeaxanthin. Resonance Raman spectroscopy has been applied for the first time to wild type and mutant Arabidopsis leaves and to intact thylakoid membranes to investigate the nature of the absorption changes obligatorily associated with the energy dissipation process. The observed changes in the carotenoid Resonance Raman spectrum proved that zeaxanthin was involved and indicated a dramatic change in zeaxanthin environment that specifically alters the pigment configuration and red-shifts the absorption spectrum. This activation of zeaxanthin is a key event in the regulation of light harvesting.

By dynamic changes in protein structure and function, the photosynthetic membranes of plants are able to regulate the partitioning of absorbed light energy between utilization in photosynthesis and photoprotective non-radiative dissipation of the excess energy. This process is controlled by features of the intact membrane, the transmembrane pH gradient, the organization of the photosystem II antenna proteins and the reversible binding of a specific carotenoid, zeaxanthin.

Resonance Raman spectroscopy has been applied for the first time to wild type and mutant Arabidopsis leaves and to intact thylakoid membranes to investigate the nature of the absorption changes obligatorily associated with the energy dissipation process. The observed changes in the carotenoid Resonance Raman spectrum proved that zeaxanthin was involved and indicated a dramatic change in zeaxanthin environment that specifically alters the pigment configuration and red-shifts the absorption spectrum. This activation of zeaxanthin is a key event in the regulation of light harvesting.

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and (c) difference spectra between samples containing zeaxanthin and violaxanthin can be calculated using chlorophyll Raman bands.

This approach provides a new opportunity to investigate the origin of $\Delta A_{535}$. In this study we have been able to carry out RR spectroscopy on whole thylakoid membranes in the dark-adapted and dissipative states and most importantly to obtain data from leaves from wild type and the npq4 mutant of Arabidopsis.

MATERIALS AND METHODS

Pigment analysis and zeaxanthin purification was performed as described in Ref. 14. Reconstitution and purification of zeaxanthin in detergent/lipid micelles was carried out using a sucrose gradient method (15). Intact chloroplasts were obtained by the procedure described in Ref. 6. To induce maximum violaxanthin de-epoxidation, leaves were illuminated for 1 h, as described in Ref. 17.

Simultaneous measurements of chlorophyll fluorescence and absorption changes in leaves at 535 nm were performed using Walz fluorimeter combined with the SLM DW2000 spectrophotometer as described in Ref. 4. Chlorophyll fluorescence analysis of chloroplasts was carried out in a temperature-controlled cell with permanent stirring. Intact chloroplasts were osmotically shocked for 30 s. The reaction medium was with or without 5 mM Mg$^{2+}$ to obtain thylakoids either with or without grana stacks. Leaf and chloroplast samples were immediately frozen in liquid nitrogen after induction of NPQ. Analysis of standard thin sections of Arabidopsis leaf samples was performed using CM10 Phillips electron microscope. Low temperature resonance Raman spectra were measured using a helium flow cryostat (Air Liquide, France) and a Jobin-Yvon U1000 Raman spectrophotometer equipped with a liquid-nitrogen-cooled CCD detector (Spectrum One, Jobin-Yvon, France) as described in Ref. 18. Excitation was provided by Coherent Argon (Innova 190) and Krypton (Innova 90) lasers (457.9, 476.5, 496.5, 488.0, 501.7, 514.5 nm, and 528.7 and 413.1 nm, respectively) and a Liconix helium-cadmium laser (441.6 nm).

RESULTS AND DISCUSSION

Fig. 1B shows the spectrum of the absorption changes associated with qE (open circles). To overcome possible problems from light-induced absorption artifacts in intact systems such as leaves and thylakoids, this spectrum is the calculated difference spectrum between illuminated wild type and npq4 mutant samples (see inset). In addition to the maximum at 538 nm there are minima at 501, 468, and 438 nm. To initially test the hypothesis that all or part of this change arises from zeaxanthin, we calculated the difference spectrum that would arise from a strong red shift in its absorption spectrum. Firstly, this calculation used the recently estimated $\alpha_{10}$ energy of zeaxanthin and violaxanthin can be calculated using chlorophyll Raman bands.

If $\Delta A_{535}$ originated from an electronic absorption change and belongs to a carotenoid, then it is predicted that there will be a qE-related change in the RR spectra of carotenoids. Indeed, the red-shifted zeaxanthin should resonate with the excitation lines 514.5 and, in particular, 528.7 nm of the argon laser. For such an experiment it was necessary to apply this technique to intact systems, either chloroplasts and/or leaves. The RR spectrum of carotenoids shows a number of characteristic features, an important one being the $\nu_1$ region ($\sim 1510–1530$ cm$^{-1}$) assigned to the C=C stretching vibrations (19, 20). The $\nu_1$ energy was found to be different for each of the chloroplast xanthophylls (13). This is partially due to a variation in the number of conjugated double bonds (CDB) (21) and the 9-cis conformation of neoxanthin, which makes the corresponding $\nu_1$ energy distinctly higher than that of the all-trans violaxanthin, possessing the same number of CDB. Fig. 2 (A and B) shows the $\nu_1$ region of the RR spectrum of Arabidopsis leaves using 528.7 nm resonance frequency. Leaves were illuminated for 15 min to induce zeaxanthin synthesis and qE formation, and then allowed to relax in darkness for 10 min, to remove qE but with insufficient time to bring about any zeaxanthin de-epoxidation. The spectra recorded for illuminated (L) leaves and those subjected to subsequent dark relaxation (R) were different; there was a significant shift in the peak position from 1526 to 1523 cm$^{-1}$, and the appearance of a shoulder around 1520 cm$^{-1}$ (arrow). The difference spectrum, L-R, has a peak at around 1520 cm$^{-1}$, the peak position of zeaxanthin When the complete RR spectrum was recorded, remarkable similarity was observed between the qE-related spectrum and that of zeaxanthin, either dissolved in pyridine or in detergent micelles. The both types of spectra had a $\nu_1$ maximum at 1520 cm$^{-1}$ and also very close $\nu_2$ energy (Fig. 3). Zeaxanthin dispersed in the detergent micelles appears to have an even more similar RR spectrum to that of the qE-associated change, particularly in the 1160–1280 cm$^{-1}$ region, normally assigned to the C-C stretching modes, variably coupled to the xanthophyll electronic excited state.
It is important to point out that the increase in RR intensity at 1520 cm$^{-1}$ is not due to the synthesis of zeaxanthin. Because an excitation wavelength of 528.7 nm is being used, a shift to longer wavelength by zeaxanthin would give rise to the increased RR intensity. Indeed if a shorter excitation wavelength was used, no enhancement, or even a decrease in intensity was observed (see below).

Four important control experiments were carried out to relate this RR spectral change to zeaxanthin and qE. Firstly, the experiment was repeated with the npq4 mutant of Arabidopsis. Secondly, the experiment was repeated using isolated chloroplasts, prepared in such a way that they contained no zeaxanthin or were enriched in zeaxanthin (58% de-epoxidation state). For the
chloroplasts containing zeaxanthin, a qE-related RR spectral shift was observed as for leaves (Fig. 2C); in contrast, for those containing no zeaxanthin, and very little qE, there was no change in the RR spectrum (Fig. 2D, top traces). Thirdly, it is possible to inhibit qE and $\Delta A_{335}$ in chloroplasts by depletion of the bound Mg$^{2+}$ ions. Again, in the depleted chloroplasts light-dependent changes in the RR spectrum were almost absent (Fig. 2D, two lower traces and Table I). Finally, the RR spectra were recorded for leaves of Impatiens glandulifera, a plant, which had been found to exhibit stronger qE formation than Arabidopsis; in these leaves the amplitude of the 1520 cm$^{-1}$ change was enhanced (Table I).

Using RR spectroscopy it is possible to determine the wavelength dependence for the resonance effect by recording spectra induced by various resonance lines (argon laser lines: 457.9, 476.5, 488.0, 496.5, 501.7, and 514.5 nm). Such excitation spectra are determined by the absorption spectrum of the pigment(s), giving rise to the Raman effect. Therefore, the qE-related change in intensity of the $v_1$ region was measured (Fig. 4). For both chloroplasts and leaves, the excitation spectrum showed features consistent with the qE-associated absorption change (Fig. 1): along with the positive, 535-nm maximum, a negative region below 505 nm was clearly detectable. These data established beyond doubt that this change was due to electronic transitions rather than light scattering. The $v_1$ energy for each excitation wavelength in the qE-associated RR spectrum matched that of isolated zeaxanthin, clearly proving involvement of this pigment in the $\Delta A_{335}$.

It was possible to estimate the number of zeaxanthin molecules that are responsible for the alteration in the RR spectrum. First we compared resonance Raman spectra obtained with 528.7 nm excitation for chloroplast samples containing only violaxanthin (Fig. 2D, top black trace) to those treated to induce de-epoxidation (Fig. 2C, black line). A maximum increase of 30% in the 1520 cm$^{-1}$-amplitude took place upon formation of the estimated 15 molecules of zeaxanthin per both photosystems I and II. Although the maximum of absorption of zeaxanthin in vitro is located at 503 nm (13), the pigment is still capable of absorbing light at 528.7 nm. Calculations show that the corresponding optical density at 528 nm is ~25% of the maximum at 503 nm. If all zeaxanthin would undergo the red shift to 525 nm after induction of qE, we should have observed at least a 16-% rise in the RR signal, taking into account that the Raman intensity is proportional to the square of the oscillator strength. However, instead only a 1.7-% rise was observed.

This corresponds to (1.7/16)×15 = 1.6 molecules of the red-shifted zeaxanthin, contributing to the RR increase upon formation of qE. Thus only ~11% of the zeaxanthin pool could be sufficient to account for the change in the RR spectrum associated with qE. How many molecules of zeaxanthin account for the size of the $\Delta A_{335}$ associated with qE under these conditions? With an $\Lambda_{335}$ of 1.5, an $\Delta A_{335}$ of ~0.01 OD units was observed at a de-epoxidation state of 0.58 (15 molecules of zeaxanthin). If 360 chlorophylls give an OD of 1.5, 0.01 OD could arise from 2.1 zeaxanthin molecules, given the extinction coefficient ratio of chlorophyll/zeaxanthin = 1.25/1.44. Thus, the estimations made here show that just about two red-shifted molecules of zeaxanthin could explain both the RR spectrum and absorption spectrum associated with qE, thus proving that the absorption change is of an electronic nature.

To shift the 0–0 absorption transition from around 503 to 525 nm would involve a large change in the local environment. For zeaxanthin dissolved in various organic solvents the energy of this transition is clearly dependent upon the refractive index of the solvent (22–24). In ethanol the maximum is at 482 nm shifting to 511 nm in carbon disulfide. A shift to from 503 nm to 525 nm would require a refractive index of about 1.8. Such a highly polarizing environment could also arise from a strong electric field of a dipole or charge in the zeaxanthin vicinity or binding site (25, 26). The signature of the specific binding of this red-shifted zeaxanthin can clearly be seen as a pronounced and characteristic enhancement of the $v_1$ region of qE-associated RR spectrum (Fig. 3, inset). Six new transitions at 942, 949, 954, 959, 964, and 969 cm$^{-1}$ appear here, originating from wagging vibrations of the various C–H groups (27, 28), which became allowed due to a strong and apparently specific distortion of zeaxanthin molecule in the binding locus. Thus, the region contains a “fingerprint” of the binding site of the red-shifted zeaxanthin. We suggest that the highly hydrophobic photosystem II antenna complexes such as CP24, 26, 29 which are enriched in zeaxanthin (29, 30) and particularly the PsbS protein (CP22), known to be required for qE and $\Delta A_{335}$ (7) may provide such a specific protein environment. Thus the possible participation of zeaxanthin in the qE-associated conformational changes of the photosystem II antenna could be an inclusion of it into the quenching locus involving the PsbS protein. This activation red-shifts the $S_0$ excited state of a subpopulation of zeaxanthin molecules by at least 22 nm. This

### Table I

| Sample          | qE Amplitude | $\Delta A_{335}$ |
|-----------------|--------------|-----------------|
| A. thaliana, wt | 20.9 ± 1.21  | 12.1 ± 1.0      |
| A. thaliana, npq4| 21.3 ± 1.35  | 20.0 ± 0.94     |
| Chloroplasts + Mg$^2+$ + Zea| 57.8 ± 2.82  | 47.8 ± 1.51     |
| Chloroplasts + Mg$^2+$ + Zea| 57.8 ± 2.82  | 47.8 ± 1.51     |
| I. glandulifera, leaves| 48.0 ± 1.52  | 23.0 ± 2.22     |

$\Delta$ is the desopxidation index defined as $(Z + 0.5 A)/(Z + V + A)$, where $Z$, $A$, and $V$ are the relative concentrations of zeaxanthin, violaxanthin, and antheraxanthin, respectively. $\Delta A_{335}$ is the reversible component of the photoprotected nonphotochemical chlorophyll fluorescence quenching, calculated as $(Fm/Fm' - Fm'/Fm')$, where $Fm$, $Fm'$, and $Fm''$ are the levels of chlorophyll fluorescence before illumination, after illumination, and after subsequent dark recovery, respectively.

* DES is the desopxidation index defined as $(Z + 0.5 A)/(Z + V + A)$, where $Z$, $A$, and $V$ are the relative concentrations of zeaxanthin, violaxanthin, and antheraxanthin, respectively. $\Delta A_{335}$ is the reversible component of the photoprotected nonphotochemical chlorophyll fluorescence quenching, calculated as $(Fm/Fm' - Fm'/Fm')$, where $Fm$, $Fm'$, and $Fm''$ are the levels of chlorophyll fluorescence before illumination, after illumination, and after subsequent dark recovery, respectively.

a $\Delta A_{335}$ is the relative RR amplitude increase at 1520 cm$^{-1}$ obtained by dividing the difference spectrum (treatment-control) by the spectrum of control.
suggests the possibility of a similar, albeit smaller shift (31, 32) of the forbidden S₁ level, which was first implicated as having a central role in qE (33) and recently declined, because it was found to have very similar energy to that of violaxanthin (34, 16). However, the S₁ energy of the qE-associated red-shifted zeaxanthin may well be significantly lower than that of violaxanthin and therefore the activated zeaxanthin could indeed serve as a quencher. It should be noted, that the experiment with magnesium showed, that along with ΔpH, zeaxanthin and PsbS, an appropriate conformational state of the thylakoid membrane is required for qE. This implies a requirement for a specific arrangement of the quenching locus within the PSII antenna, which leads to the formation of an efficient dissipative state.

In conclusion, we have shown that resonance Raman spectroscopy can be used to probe molecular events involved in complex regulatory phenomena in intact biological samples such as a plant leaf. It has particular potential when employed in the differential mode, comparing wild type with mutants and transgenic plants, and therefore will become an important postgenomic tool. Using this approach we have been able to explain for the first time the origin of spectral changes accompanying the photoprotective dissipation of excitation energy in plants. We have obtained direct evidence that zeaxanthin plays a specific role in qE, that only 1–2 molecules are needed and, most importantly, that qE requires activation of zeaxanthin by a dramatic change in its environment. This latter discovery provides a new primary focus for future research into the control of light harvesting in plants and will direct the search to determine the physical mechanism involved including the role of the forbidden excited singlet state and the ability of the red-shifted zeaxanthin to promote conformational changes within antenna complexes.

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