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A PHOTOPHYSICAL CONTROL MECHANISM FOR ZEAXANTHIN-ASSOCIATED RADIATIONLESS ENERGY DISSIPATION IN PHOTOSYNTHESIS

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Understanding the manner in which excess solar energy is dissipated by photosynthetic membranes under conditions of high light stress is a major problem facing researchers studying photosynthesis.¹ Light energy absorbed but not used to drive the photosynthetic light reactions must be deactivated by plants in a manner that does not lead to the photodestruction of the photosynthetic apparatus. The dissipation of this excess light energy can be measured in higher plants and algae by the extent to which chlorophyll fluorescence originating from the light-harvesting pigment-protein complexes is quenched under different chemical and physical conditions.¹ It has long been known that a specific group of carotenoids known as xanthophylls are associated with these light-harvesting complexes.² A correlation has been established between one such xanthophyll, zeaxanthin, and the magnitude of the fluorescence quenching.^{1,3,4} The nature of the specific molecular mechanism by which zeaxanthin is able to quench chlorophyll fluorescence and thereby dissipate excess absorbed excitation energy has eluded researchers for years.¹ Herein, we report femtosecond time-resolved, fast-transient optical spectroscopic analyses of three important xanthophylls. This work strongly supports the notion that the enzymatic reactions that interconvert these xanthophylls act as a kind of "molecular gear shift" controlling whether the molecules function as light-harvesting pigments performing forward energy transfer or as fluorescence quenchers performing reverse energy transfer.

Three specific xanthophylls, violaxanthin, antheraxanthin, and zeaxanthin, typically found in the light-harvesting apparatus of higher plants and algae, are interconverted into one another in the chloroplast membrane in a process referred to as the "xanthophyll cycle".⁵ In this cycle, exposure of the photosynthetic system to light levels exceeding those that can be used for photosynthesis induces the enzymatic de-epoxidation of violaxanthin to antheraxanthin and zeaxanthin. Limiting light levels or dark adaptation of the photosynthetic membranes reverses the process, reforming violaxanthin by epoxidation of zeaxanthin and antheraxanthin. See Fig. 1.

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A strong correlation between the zeaxanthin content of leaves or chloroplasts and energy-dependent quenching of chlorophyll fluorescence (qE) has recently been established.¹ In leaves, qE accounts for the majority of all non-photochemical quenching (qN) observed, with zeaxanthin accounting for 60-70% of this process. The remaining qE appears to be independent of zeaxanthin formation, although other xanthophylls may be involved. Some of the excess photon energy that is absorbed by chlorophyll, rather than being transferred to the reaction center, is dissipated by a form of radiationless energy transfer that involves zeaxanthin in the light-harvesting apparatus, but not the other xanthophylls. Interestingly, the effect is reversible. The fluorescence quenching typically relaxes within a few minutes upon returning the leaves to darkness. This finding is strongly suggestive of zeaxanthin playing a protective role of dissipating excess light energy which may otherwise lead to damage to the photosynthetic apparatus.

Previously, carotenoids (xanthophylls are one class of carotenoids possessing oxygen-containing functional groups) were thought to play only two roles in photosynthesis - that of light-harvesting pigment and triplet state quencher.⁶ As light-harvesting pigments, carotenoids supplement the light-capturing ability of chlorophyll by absorbing in regions of the visible spectrum where chlorophyll does not absorb. Carotenoids then transfer this energy to chlorophyll, which is capable of carrying out the primary photochemistry of photosynthesis. As triplet state quenchers, carotenoids are able to protect the photosynthetic apparatus by quenching highly reactive chlorophyll triplet states as soon as they are formed. Both of these processes, light-harvesting and triplet state quencher, require energy transfer. In the former process, carotenoids transfer singlet state energy to chlorophylls. In the latter process, carotenoids accept triplet state energy from chlorophylls. The two roles are thought to be made energetically possible by the fact that carotenoids, in general, have singlet states that are higher in energy and triplet states that are lower in energy than those of chlorophyll. Recent fluorescence studies on carotenoids have revealed, however, that their singlet states are not as high in energy as previously thought.^{7,8} Herein, it is demonstrated that a low-lying singlet state is responsible for the zeaxanthin-mediated fluorescence quenching of chlorophyll.

The singlet state energy diagram of carotenoids consists of at least two excited states that account for many of their photochemical properties.⁶ These are denoted 1^1B_u and 2^1A_g according to the idealized group theoretical (C_{2h} point group) designations. The ground state designation is 1^1A_g . The easily observed, prominent, visible, absorption spectrum that characterizes all carotenoids, is attributable to a strongly allowed transition from the ground 1^1A_g state to the 1^1B_u state. Lying below the 1^1B_u state is the 2^1A_g state into which absorption from the ground state is symmetry forbidden. The low oscillator strength of this $1^1A_g \rightarrow 2^1A_g$ transition has made the determinations of the precise energies of the 2^1A_g

states of carotenoids somewhat difficult. Upon absorption of light, rapid internal conversion (on the order of a few hundred femtoseconds) occurs from the 1^1B_u to the 2^1A_g state.⁹ This process represents a primary route of energy dissipation of carotenoids and of energy transfer to chlorophylls.⁶ Thus, the energies of the 2^1A_g states are extremely important in assessing the energetic feasibility of energy transfer from carotenoids to chlorophyll. Fluorescence studies have revealed the 2^1A_g state energies of some shorter carotenoids. However, the longer chain carotenoids (> 9 carbon-carbon double bonds) tend not to fluoresce from the 2^1A_g state, but rather exhibit fluorescence originating from the 1^1B_u level.⁸

One method of obtaining the 2^1A_g energies of the long chain carotenoids is by extrapolation from the shorter molecules whose 2^1A_g energies have been determined by fluorescence spectroscopy.⁸ This type of analysis is somewhat uncertain since only very few short chain length carotenoids have been characterized, and variations in their structures, primarily due to differences in their attached functional groups, make correlations between long and short carotenoids difficult. Recently, we have used picosecond time-resolved transient absorption spectroscopy to measure the 2^1A_g state energy of the carotenoid, spheroidene.¹⁰ In these experiments, the 2^1A_g state lifetimes of spheroidene and three *all-trans* analogs of spheroidene, 3,4,7,8-tetrahydrospheroidene, 3,4,5,6-tetrahydrospheroidene, and 3,4-dihydrospheroidene were measured. These molecules are structurally identical except for having π -electron conjugations that systematically increase from 7 to 10 carbon-carbon double bonds. Because the three spheroidene analogs exhibit fluorescence from their 2^1A_g states,⁸ and therefore, determine the energies of those states, a measurement of the 2^1A_g state lifetimes of these molecules "calibrates" the effect different 2^1A_g state energies of carotenoids have on their decay dynamics. Furthermore, the application of the "energy gap law" for nonradiative transitions,¹¹ which describes the magnitude of change in internal conversion rate constant with changes in state energy, makes it possible to determine the energies of the 2^1A_g states of molecules such as spheroidene, which do not exhibit fluorescence from their 2^1A_g levels.¹⁰ The use of the energy gap law for carotenoids is valid because the quantum yields of carotenoid fluorescence are typically very low, leading to a dominance of the nonradiative pathway of singlet state decay from their 2^1A_g states. The 2^1A_g state energies of carotenoids can be determined by fitting the generalized energy gap law equation

$$k_{ic} = c \cdot \exp(-\gamma \Delta E / \hbar \omega_M)$$

to the dynamics data.¹⁰ In this equation k_{ic} is the internal conversion rate constant, for carotenoids, very well approximated by $1/\tau$ because k_{ic} for polyenes is much larger than the radiative rate constant, $k_f \sim 10^9 \text{ s}^{-1}$. ΔE is the $2^1A_g - 1^1A_g$ energy difference, c is a

constant, $\hbar\omega_M$ is the energy of the high frequency "acceptor" modes, and γ can be related to the relative displacement of the potential surfaces in the two electronic states. The best fit to the data on the series of spheroidene molecules yielded a value of 8.98×10^{-4} cm for $\gamma / \hbar\omega_M$ and 3.71×10^{16} for c . The values of these parameters are applicable to all carotenoids because, in general, C-C stretching vibrations, $\hbar\omega_M \sim 1300\text{-}1600$ cm^{-1} , have been shown to govern internal conversion from 2^1A_g .¹² Also, it is likely that all 2^1A_g states of carotenoids are characterized by a reasonably narrow range of γ values.¹¹

In the present work, the energies of the 2^1A_g excited states of violaxanthin, antheraxanthin and zeaxanthin were determined. Similar to the experiments on the spheroidene analogs, a 300 fs, 420 nm laser pulse was used to excite the xanthophylls into their 1^1B_u states. After subsequent relaxation to the 2^1A_g state, the $2^1A_g \rightarrow S_n$ electronic absorption bands in the region 510-540 nm were probed and observed to decay with single exponential kinetics: $\tau = 23.9$ ps for violaxanthin, $\tau = 14.4$ ps for antheraxanthin and $\tau = 9.0$ ps for zeaxanthin. See Fig. 2. These data used in conjunction with the energy-gap law expression given above yields 2^1A_g state energies of $15,200$ cm^{-1} , $14,700$ cm^{-1} and $14,200$ cm^{-1} for violaxanthin, antheraxanthin and zeaxanthin, respectively.

The most compelling feature of these data is revealed when one compares the energies of the 2^1A_g states of the xanthophylls with that of the lowest excited singlet state of chlorophyll a in the light-harvesting complexes of Photosystem II (LHCII). See Fig. 3. In this complex, chlorophyll a exhibits its red-most maximum absorption (Q_y transition) at ~ 680 nm. This translates into a value of $14,700$ cm^{-1} for the S_1 excited singlet state energy of chlorophyll which lies below that for violaxanthin but higher than the value for zeaxanthin. These data demonstrate that it is energetically possible for the 2^1A_g state of zeaxanthin to quench the excited state singlet of chlorophyll a *in vivo*. It is not energetically possible for violaxanthin to perform this same function. Conversely, it is energetically possible for violaxanthin to act as a light-harvesting pigment, absorbing light energy and transferring it from its 2^1A_g state to chlorophyll a. It is not energetically possible for zeaxanthin to transfer energy via its 2^1A_g state to chlorophyll a. Zeaxanthin may still act as a light-harvesting pigment, however, by transferring energy from its 1^1B_u state to chlorophyll. Thus, the enzymatic de-epoxidation/epoxidation reactions of the xanthophyll cycle appear to be acting as a sort of "molecular gear shift". Under conditions of low light, with violaxanthin as the primary xanthophyll present, the carotenoid acts as a light-harvesting pigment transferring energy forward from its 2^1A_g state to the S_1 state of chlorophyll. Under high light conditions, de-epoxidation of violaxanthin to zeaxanthin occurs, and the system shifts into reverse, transferring the S_1 energy of chlorophyll back to the 2^1A_g state of zeaxanthin. The possibility of reverse energy transfer from chlorophyll to the 2^1A_g state of carotenoids was

suggested by Demmig-Adams¹ in response to the findings of Snyder *et al.*⁷ that the 2^1A_g states of carotenoids may be much lower than previously thought.¹³ The low-lying nature of the 2^1A_g states of carotenoids was confirmed by the work of DeCoster *et al.*⁸ These ideas have also been elaborated on recently by Owens *et al.*¹⁴ and are supported by two-photon spectroscopic experiments from this group. The data presented here demonstrate conclusively the energetic feasibility of this "molecular gear shift" model and provide a reasonable explanation for the correlation of the presence of zeaxanthin with the occurrence of non-photochemical quenching of chlorophyll fluorescence. It is important to note that these data do not exclude other controlling factors such as aggregation being important as well.¹⁵ For energy transfer to occur into and out of the carotenoid 2^1A_g state, which has a low oscillator strength for a transition from the ground state, the exchange mechanism is likely to be operative.¹⁶ This mechanism requires close proximity (essentially van der Waals contact) between donor and acceptor molecules for efficient energy transfer. This may explain why the simple presence of zeaxanthin in the membrane does not necessarily result in radiationless energy dissipation.¹ Although our data demonstrate conclusively the energetic feasibility of zeaxanthin quenching chlorophyll a fluorescence, structural factors may also place constraints on determining in which systems zeaxanthin will be an effective quencher of the absorbed photon energy not used for photosynthesis.

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Figure legends

Fig. 1. Schematic representation of the xanthophyll cycle showing the de-epoxidation of violaxanthin to zeaxanthin and the epoxidation of zeaxanthin to violaxanthin. Both of these reactions occur by way of antheraxanthin as an intermediate.

Fig. 2. The decay of the transient absorbance excited at 420 nm and probed at 540 nm, 529 nm, and 512 nm for violaxanthin, antheraxanthin and zeaxanthin, respectively. These wavelengths correspond to the maxima of the $2^1A_g \rightarrow S_n$ absorption of each xanthophyll. τ is the 2^1A_g state lifetime obtained by fitting the decay curves to single exponential rate expressions.

Fig. 3. Schematic representation of the "molecular gear shift" mechanism of violaxanthin-to-chlorophyll a *forward* energy transfer (light-harvesting function) and chlorophyll a-to-zeaxanthin *reverse* energy transfer (non-photochemical fluorescence quenching function) induced by the enzymatic xanthophyll cycle.

Time/ps

"Molecular Gear Shift" Mechanism *Forward Energy Transfer*

Reverse Energy Transfer

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