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# The lifetimes and energies of the first excited singlet states of diadinoxanthin and diatoxanthin: the role of these molecules in excess energy dissipation in algae

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**Abstract**

The lifetimes of the first excited singlet states ($2^1A_g$) of diadinoxanthin and diatoxanthin, carotenoids involved in the xanthophyll cycle in some genera of algae, have been measured by femtosecond time-resolved optical spectroscopy to be $22.8 \pm 0.1$ ps and $13.3 \pm 0.1$ ps, respectively. Using the energy gap law for radiationless transitions set forth by Englman and Jortner (Mol. Phys. 18 (1970) 145–164), these lifetimes correspond to $S_1$ excited state energies of 15210 $cm^{-1}$ for diadinoxanthin and 14620 $cm^{-1}$ for diatoxanthin. The lowest excited singlet state energy of Chl *a* has an energy of 14700 $cm^{-1}$. The fact that the $S_1$ state energy of diadinoxanthin lies above that of Chl *a*, whereas the $S_1$ state energy of diatoxanthin lies below that of Chl *a*, suggests that the xanthophyll cycle involving the enzymatic interconversion of diadinoxanthin and diatoxanthin may play a role in regulating energy flow between these molecules and Chl *a* in many species of algae, essentially fulfilling a role identical to that proposed for violaxanthin and zeaxanthin in higher plants and green algae (Frank et al. (1994) Photosyn. Res. 41, 389–395).

*Keywords:* Diadinoxanthin; Diatoxanthin; Energy dissipation; Excited state; Femtosecond; Optical spectroscopy; Xanthophyll cycle

## 1. Introduction

Energy transfer to and from carotenoids takes place in several different ways in the photosynthetic apparatus [1]. Light-harvesting by carotenoids in photosynthetic organisms involves the transfer of singlet excitation energy from carotenoids to chlorophylls in antenna pigment-protein complexes [2]. The quenching of excited triplet states of chlorophylls by carotenoid molecules is well documented in antenna complexes and in reaction centers [3]. Carotenoids also quench active oxygen species, most notably the $^1\Delta_g$ state of $O_2$ [4]. Carotenoids in higher plants and in some algal species may be involved in the dissipation of excess excitation energy from chlorophylls via singlet-singlet energy transfer to carotenoids, thereby preventing damage to the photosynthetic apparatus

Abbreviations: Chl *a*, chlorophyll *a*.

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that would otherwise occur. This last process involves carotenoids produced in the light-harvesting complexes in conjunction with the operation of the so-called 'xanthophyll cycle' [5].

In higher plants and in some species of algae, notably the Chlorophyta and members of the Phaeophyceae, the xanthophyll cycle consists of the enzymatic interconversion of violaxanthin into zeaxanthin via an intermediate, antheraxanthin. This process is now well-characterized, and the formation of zeaxanthin from violaxanthin has been clearly correlated with the non-photochemical quenching of chlorophyll (Chl) fluorescence (i.e., with the dissipation of Chl singlet energy) in a number of photosynthetic systems [6]. Although several plausible mechanisms have been suggested through which a preponderance of zeaxanthin may be associated with Chl fluorescence quenching [6,7], only recently have these mechanisms been investigated by spectroscopic means [8–12].

A different xanthophyll cycle involving the carotenoids, diadinoxanthin and diatoxanthin, is present in many species of microalgae, especially the Bacillariophyceae, Chyrsophyceae, Xanthophyceae and the Dinophyceae (see Fig. 1; [6,13,14]). Interestingly, within both the Xanthophyceae and the Chyrsophyceae, there are some reports of one or more species that possess the violaxanthin/zeaxanthin cycle [15,16]. In some species of algae (e.g., *Pleurochlorsis meiringenssi*-Xanthophyceae) diadinoxanthin may be the major xanthophyll, and, in this particular species, together with heteroxanthin, may effectively replace violaxanthin as a major light-harvesting pigment [17]. Diadinoxanthin may also be present in both the Photosystem I and Photosystem II light-harvesting complexes. Recent studies have suggested that the one-step de-epoxidation of diadinoxanthin into diatoxanthin may provide the same function of the dissipation of excess excitation energy in algae as the two-step de-epoxidation of violaxanthin into zeaxanthin does in higher plants [14]. This one step de-epoxidation process shares a number of characteristics with the equivalent higher plant/green algae xanthophyll cycle carotenoids. When an alga is exposed to high irradiances, diatoxanthin is formed at the expense of diadinoxanthin. This has been correlated with the quenching of Chl fluorescence [18–20]. On the return of the alga to lower irradiance (or complete darkness) diadinoxanthin is produced, albeit at a slower rate than the initial de-epoxidation. Similar to the xanthophyll cycle, the de-epoxidation of diadinoxanthin into diatoxanthin can be inhibited by incubation of the alga with dithiothreitol [19]. In dithiotreitol-treated algae, illumination with high photon flux densities will induce photodamage to the light-harvesting complexes as excess excitation energy can no longer be quenched by the presence of diatoxanthin. Another feature seen in the algal cycle is that, in common with violaxanthin, only a proportion (generally ~ 60%) of the chloroplast diadinoxanthin appears to be available for de-epoxidation [18,21]. Similarly, changes in the diadinoxanthin/diatoxanthin pool size has been correlated with irradiance levels during algal growth and in this shows similar behavior to that seen in sun/shade adaptation in higher plants [22].

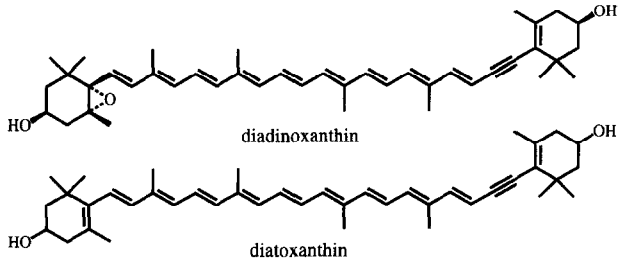



Fig. 1. Structures of diadinoxanthin and diatoxanthin.

The operation of the xanthophyll cycle and the subsequent dissipation of excess excitation energy is an important contribution to the protection of the photosynthetic apparatus from light-induced damage. This photoprotective mechanism is of particular relevance in microalgae which may during their lifetime experience large fluctuations in both the quantity and spectral quality of light in the water column. For the majority of planktonic microalgae their light-harvesting systems are tuned to obtain maximal efficiency from a relatively low level of light penetrating the water column. Thus, unless an effective method for the dissipation of excess energy is available to the alga, exposure to high irradiances may result in severe photodamage.

There have been two models proposed for the mechanism of carotenoid-, viz. zeaxanthin-mediated dissipation of excitation energy within the light-harvesting complex of Photosystem II of higher plants. The first model was originally suggested by

Demmig-Adams [6] in response to the findings of Snyder et al. [23] that the $2^1A_g$ states of carotenoids may be much lower than previously thought [24]. The details of the model were postulated by Owens et al. [7] who argued that the deactivation of excited Chl may occur by singlet-singlet energy transfer to the carotenoid molecule; i.e., effectively the reverse of energy transfer during light-harvesting. Frank et al. [9] investigated the energetic feasibility of this model for the xanthophyll cycle carotenoids and termed it the 'molecular gear-shift model'. In this model, the quenching of Chl excited states has its origin in the de-epoxidation of violaxanthin into zeaxanthin which results in an increase in the conjugated $\pi$-electron chain length from 9 conjugated double bonds to 11 conjugated double bonds. The length of the conjugated chain of a carotenoid in large part determines both the energy and lifetime of the low-lying excited state (the $S_1$ or $2^1A_g$ state). It has been suggested [9] that because of its longer conjugated chain length, the energy of the $S_1$ state of zeaxanthin is lower than that of violaxanthin and Chl *a*, whereas the energy of the $S_1$ state of violaxanthin is higher than that of Chl *a*. Therefore, zeaxanthin would be more efficient than violaxanthin at dissipating excess energy from Chl *a*. This mechanism would be strongly dependent upon the position of the $S_1$ energy level of the carotenoid relative to that of Chl *a*. For this reason, determining the $S_1$ energies of these carotenoids is important in testing this hypothetical mechanism. The energies of the $S_1$ states of the xanthophyll cycle carotenoids, zeaxanthin, antheraxanthin and violaxanthin, as well as numerous other carotenoids have been determined using either fluorescence or the dynamics of their $S_1$ states in conjunction with the energy gap law for radiationless transitions [25]. Also, the role of the $S_1$ energy levels of a series of carotenoid molecules has been studied in the isolated LHCIIb light-harvesting complex [26].

The second model, denoted the 'LHCII model', proposes that the formation of zeaxanthin from violaxanthin is indirectly responsible for energy dissipation by controlling the structure of the light-harvesting complexes of PSII [27]. In this model, zeaxanthin itself may not play a direct role in energy dissipation processes, but would promote the formation of quenchers (carotenoid, carotenoid-Chl or Chl-carotenoid-Chl associations).

A recent study has attempted to reconcile the differences between these two contrasting models by demonstrating that carotenoids may function in vitro as 'antiquenchers', inhibiting the formation of a quenched state of LHCII, possibly by forming strong associations between carotenoid and Chl, but additionally some carotenoids, with sufficiently low $S_1$ energies such as zeaxanthin, may be able to accept energy from Chl [26,28,29]. Whichever model is most appropriate to explain the zeaxanthin-associated energy dissipation, it is clear that the $S_1$ energy of the carotenoid is a key feature of this process in a protein environment [26]. Hence, any information regarding the energies and dynamics of the $S_1$ states of carotenoids will be useful in elucidating the precise details of the mechanism of the process of energy dissipation.

The present study presents the $S_1$ lifetimes and energies of the two algal carotenoids, diadinoxanthin and diatoxanthin, whose role in energy dissipation is currently undergoing scrutiny [18,19]. The significance of the $S_1$ energies in relation to the operation of the xanthophyll cycle will be discussed.

## 2. Materials and methods

Diatoxanthin and diadinoxanthin were extracted from a light-treated culture of *Phaeodactlyum tricornutum* using acetone. The carotenoids were purified from the total lipid extract of the alga using a combination of thin-layer chromatography (TLC), reversed-phase high-performance liquid chromatography (HPLC) and finally by passage through a small column of de-activated neutral alumina (Brockman Grade III). The elution of the pigments by silica TLC (0.25 cm thickness) or neutral alumina (Brockman Grade III) used redistilled diethyl ether (100%) and 5% (v/v) ethanol/diethylether. Reversed-phase HPLC was performed on a semi-preparative Spherisorb ODS2 column (25.0 cm × 10 mm, 10 $\mu$m particles). Pigments eluted on a gradient of 0–100% ethyl acetate in acetonitrile/water (9/1) at 1.5 ml/min over 25 min. Carotenoids were identified by their chromatographic behavior and by their absorption maxima compared to published values [30].

The apparatus used for the femtosecond transient absorption experiments has been previously described

[9]. The sample in petroleum ether solvent was contained in a 1-cm path length cuvette and was stirred. Changes in the transmission of the measuring probe light through the sample and changes in the reference probe beam were monitored by photodiodes, the output of which were integrated, digitized and recorded by a personal computer. Kinetic parameters were obtained by iterative reconvolution using the Levenberg-Marquardt algorithm. The instrument response time was 120 fs.

## 3. Results and discussion

The two low-lying excited states of carotenoids that are associated with most of their photophysical behavior are designated $2^1A_g$ and $1^1B_u$ according to their symmetry representations in the $C_{2h}$ point group. The ground state representation is $1^1A_g$. The strong visible absorption that is characteristic of all carotenoids is attributable to the $1^1A_g \rightarrow 1^1B_u$ (or $S_0 \rightarrow S_2$) transition. The $1^1A_g \rightarrow 2^1A_g$ (or $S_0 \rightarrow S_1$) transition is symmetry forbidden and therefore very weak. The low oscillator strength of the $1^1A_g \rightarrow 2^1A_g$ (or $S_0 \rightarrow S_1$) transition has made determinations of the energies of the $2^1A_g$ states of carotenoids extremely difficult. When carotenoids absorb light, rapid internal conversion within a few hundred femtoseconds occurs from the $1^1B_u$ to the $2^1A_g$ state [31]. The $2^1A_g$ state population then either decays to the ground state in a time on the order of 10 ps or is transferred to Chl. Because of their involvement in energy transfer to Chl, the energies of the $2^1A_g$ states of carotenoids are important in attempting to assess the energetic feasibility of the process. Fluorescence studies on a limited number of carotenoids have revealed their $2^1A_g$ state energies [2,32]. This has been accomplished because very weak fluorescence associated with the $2^1A_g \rightarrow 1^1A_g$ transition in these molecules can be observed from highly purified samples of some carotenoids. Carotenoids having conjugated chain lengths greater than nine carbon-carbon double bonds, with the exception of $\beta$-carotene, whose $S_1$ energy has recently been determined from time-resolved fluorescence spectroscopic techniques, have a tendency not to fluoresce from the $2^1A_g$ state, but rather exhibit fluorescence associated with the $1^1B_u \rightarrow 1^1A_g$ transition [2]. Thus, determining the $S_1$ energies of these longer carotenoids by fluorescence techniques is very difficult.

The $2^1A_g$ energies of many of the long chain carotenoids can be obtained by direct extrapolation from the shorter molecules whose $2^1A_g$ energies have been determined by fluorescence spectroscopy. This type of analysis has been carried out by Cosgrove et al. [33]. Another type of analysis uses a fit of the energy gap law for non-radiative transitions [25] to the $2^1A_g$ state dynamics of several short chain length carotenoids whose $2^1A_g$ energies are known from fluorescence studies. The energy gap law describes the dependence of the rate constant for internal conversion on the energy separation between the participating states. The appropriate expression is:

$$k_{ic} = \frac{C^2(2\pi)^{1/2}}{\hbar(\Delta E \cdot \hbar\omega_M)^{1/2}} \exp\left[-\frac{\gamma \Delta E}{\hbar\omega_M}\right] \quad (1)$$

where $k_{ic}$ is the internal conversion rate constant, $\Delta E$ is the $2^1A_g$–$1^1A_g$ energy gap, $C$ is a vibronic coupling matrix element, $\hbar\omega_M$ is the energy of the accepting vibrational mode, $\gamma = \ln(2\Delta E/d\Delta_M^2\hbar\omega_M) - 1$, where $\Delta_M$ is the relative displacement of the potential surfaces of the two electronic states, and $d$ is the number of degenerate modes. The parameters determined in the fit can be then used to deduce the $2^1A_g$ energies by extrapolation to longer carotenoids whose energies have not been determined from fluorescence studies. This analysis has been carried out by Chynwat and Frank [34] to deduce the energies of several carotenoids.

Here we apply the energy gap law approach to deduce the $2^1A_g$ energies of diadinoxanthin and diatoxanthin. First, a plot must be constructed which fits the energy gap law to a series of carotenoids for which both the $2^1A_g$ energies and lifetimes are simultaneously known [34]. The molecules used to generate this fit are $\beta$-carotene [32], fucoxanthin [35,36], 3,4,5,6-tetrahydrospheroidene [37], 3,4,7, 8-tetrahydrospheroidene [37], and two short $\beta$-carotene analogs with 7 (mini-7 carotene) and 5 (mini-5 carotene) carbon-carbon double bonds [38,39]. The fit of Eq. (1) to these molecules required the following parameters: $C = 2024\ cm^{-1}$, $\hbar\omega_M = 3250\ cm^{-1}$ and $\gamma = 2.0 \pm 0.2$. Values of $\gamma$ in this range are consistent with these carotenoid molecules being in the weak coupling limit for which the energy gap law is highly

appropriate to describe the singlet-to-ground state internal conversion [25,34].

Next, the dynamics of diadinoxanthin and diatoxanthin were measured using a 120 fs, 412 nm laser pulse to excite them 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. The decay of these absorption bands occurred with single-exponential kinetics corresponding to $S_1$ lifetimes, $\tau$, equal to $22.8 \pm 0.1$ ps for diadinoxanthin and $13.3 \pm 0.1$ ps for diatoxanthin (Fig. 2). Finally, these data were used in conjunction with the best fit to the energy-gap law expression to yield $2^1A_g$ state energies of 15210 $cm^{-1}$ for diadinoxanthin and 14620 $cm^{-1}$ for diatoxanthin (see Fig. 3).

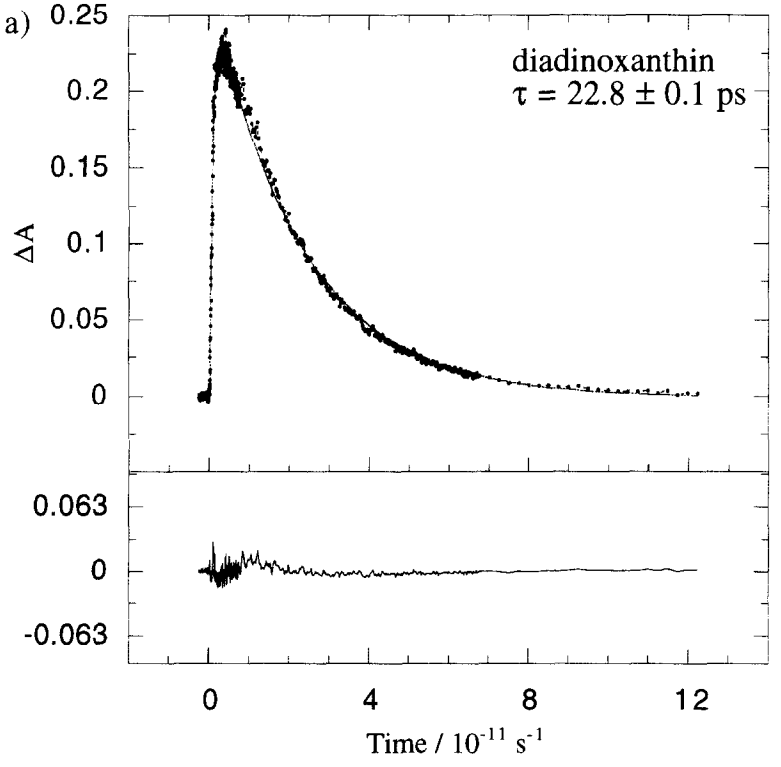
The most compelling feature of these data is revealed when one compares the energies of the $2^1A_g$ states of the xanthophylls to that of the lowest excited singlet state of Chl *a* (see Fig. 4). Chl *a* has an $S_1$ excited singlet state energy of $\sim 14700$ $cm^{-1}$ [40]. This lies below that for diadinoxanthin, but higher than the value for diatoxanthin. This energy ordering is similar to that seen from violaxanthin, antheraxanthin and zeaxanthin which are the carotenoids involved in the xanthophyll cycle in higher plants. The $S_1$ energies of violaxanthin, antheraxanthin and zeaxanthin were determined to be 15200 $cm^{-1}$, 14700 $cm^{-1}$ and 14200 $cm^{-1}$, respectively [9]. The energy of the $S_1$ state of Chl *a* was found to be isoenergetic with antheraxanthin, above that of zeaxanthin, and below that of violaxanthin. It is important to note, however, that the energy differences between the $S_1$ states of the carotenoids involved in either the xanthophyll cycle described previously [9], or in the diadinoxanthin cycle described here, are not large. Furthermore, these excited state energies should not be taken as limits determining whether forward or reverse energy transfer can occur to or from Chl *a* in an absolute sense. In assessing the extent to which diadinoxanthin or diatoxanthin may be more adept than the other at performing either light-harvesting (forward energy transfer) or quenching of Chl excited singlet states (reverse energy transfer), it is appropriate to examine the relative magnitudes of the spectral overlaps between the absorption and emission bands of the energy donors and acceptors. The transitions to and from the ground and $2^1A_g$ ($S_1$) states of diadinoxanthin and diatoxanthin involve transitions that are forbidden by symmetry. Hence, the appropriate spectral overlap integral derives from the Dexter formalism [41] where the rate constant for energy transfer is given by:

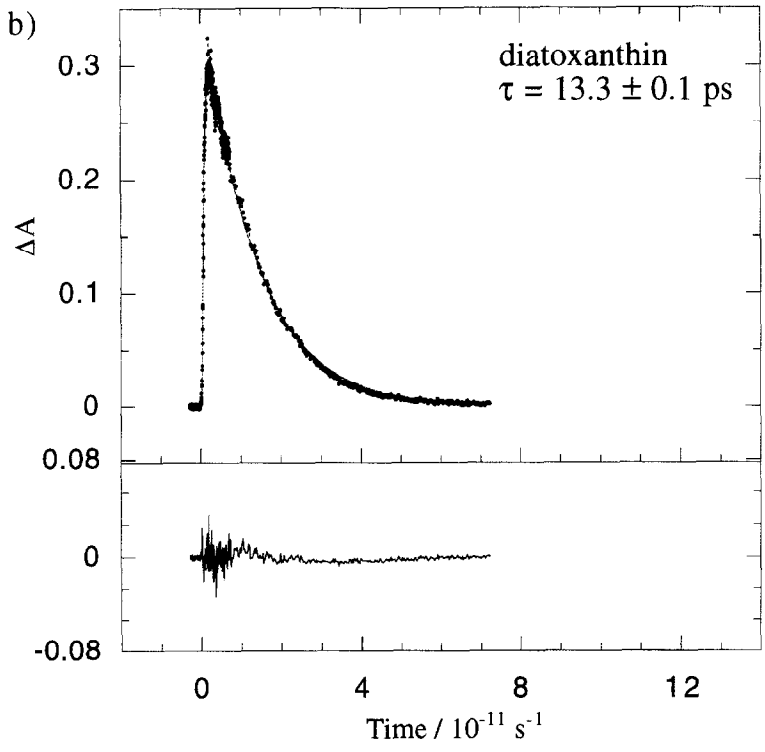
$$k_{ET} = K \exp\left(\frac{-2r}{L}\right) J_{exchange} \quad (2)$$

$K$ depends on the specific orbitals involved, $r$ describes the donor-acceptor distance relative to their Van der Waals radii, $L$, and $J_{exchange}$ is the overlap integral given by:

$$J_{exchange} = \frac{\int_{-\infty}^{\infty} F_d(\nu)\epsilon_a(\nu)\nu^{-4}\,d\nu}{\int_{-\infty}^{\infty} F_d(\nu)\,d\nu \int_{-\infty}^{\infty} \epsilon_a(\nu)\,d\nu} \quad (3)$$

$F_d(\nu)$ is the emission spectral lineshape function of the donor, $\epsilon_a(\nu)$ describes the absorption spectral lineshape for the acceptor and $\nu$ is the spectral frequency. The forbiddenness of the transitions has precluded direct observation of either the $1^1A_g \rightarrow 2^1A_g$ ($S_0 \rightarrow S_1$) absorption or the $2^1A_g \rightarrow 1^1A_g$ ($S_1 \rightarrow S_0$) emission from diadinoxanthin or diatoxanthin. However, a good approximation to the band shapes of the transitions can be obtained from the emission spectrum of 3,4,7,8-tetrahydrospheroidene previously reported [42]. This is because the profile of this spectrum is typical of all $S_1$ emission spectra from carotenoids and therefore represents a good generic emission profile for calculating spectral overlaps [32,43]. The magnitudes of the spectral overlap terms for the process of forward energy transfer from diadinoxanthin and diatoxanthin to Chl *a* were evaluated by first shifting the emission spectrum of 3,4,7,8-tetrahydrospheroidene to match the spectral origins determined for the $2^1A_g \rightarrow 1^1A_g$ ($S_1 \rightarrow S_0$) transitions of diatoxanthin and diadinoxanthin. Then the $J_{exchange}$ value was calculated according to Eq. (3) from the emission spectrum of the carotenoid and the absorption lineshape of Chl *a* (see Fig. 5a). For the process of reverse energy transfer, i.e., the quenching of Chl *a* excited singlet states by carotenoids, the shifted emission spectrum of 3,4,7,8-tetrahydrospheroidene was reflected about its spectral origin to give approximations to the $1^1A_g \rightarrow 2^1A_g$ ($S_0 \rightarrow S_1$) absorption profiles for diadinoxanthin and diatoxanthin. The $J_{exchange}$ value corresponding to the transfer of energy


a)
diadinoxanthin
τ = 22.8 ± 0.1 ps
ΔA
0.25
0.2
0.15
0.1
0.05
0
0.063
0
-0.063
0
4
8
12
Time / 10^-11 s^-1


b)
diatoxanthin
τ = 13.3 ± 0.1 ps
ΔA
0.3
0.2
0.1
0
0.08
0
-0.08
0
4
8
12
Time / 10^-11 s^-1

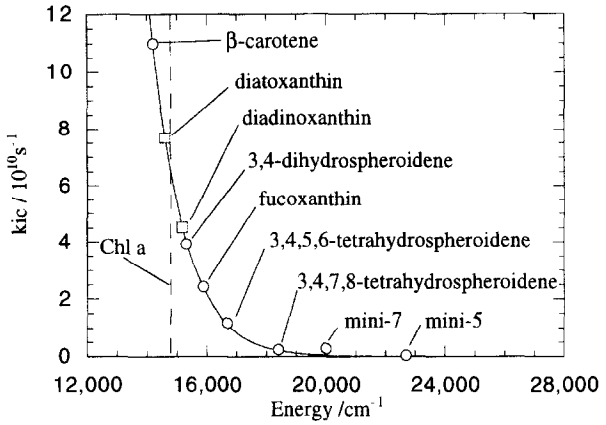



Fig. 3. The best fit (solid line) of the energy gap law expression given by Eq. (1) to the dynamics and energetics data (circles) from β-carotene [32], fucoxanthin-[35,36], 3,4,5,6-tetrahydrospheroidene [37], 3,4,7,8-tetrahydrospheroidene, and two short β-carotene analogs with 7 (mini-7 carotene) and 5 (mini-5 carotene) carbon-carbon double bonds [38,39]. The fitting parameters are described in the text. The squares represent the energies of diadinoxanthin and diatoxanthin extrapolated from the energy gap law and corresponding to their $S_1$ decay dynamics measured in this work. The vertical dashed line represents the $S_1$ energy of Chl *a*. The uncertainties in the values of the rate constants are smaller than the sizes of the symbols used in the plot.

from Chl *a* to the carotenoid was then determined using Eq. (3) and the fluorescence spectrum of Chl *a* (see Fig. 5b). The ratio of the overlap integrals for the two carotenoids carrying out forward energy transfer reveals that diadinoxanthin is 1.7 times more likely to transfer energy from its $2^1A_g$ ($S_1$) state to Chl *a* than diatoxanthin. For the reverse process of singlet state quenching by the carotenoid, Chl *a* was 0.6 times less likely to transfer the energy uphill to diadinoxanthin than downhill to diatoxanthin. Although the present data suggest that the $2^1A_g$ state of diatoxanthin is preferentially able to quench the excited singlet state of Chl *a*, the data do not support the idea that forward or reverse energy transfer is impossible in either case. That is, although the spectral origin of Chl *a* lies between the spectral origins of diadinoxanthin and diatoxanthin, both forward (light-harvesting) and reverse (quenching) energy transfer processes between either carotenoid and Chl *a* are still achievable for both diadinoxanthin and diatoxanthin. Also, despite diadinoxanthin being energetically preferred over diatoxanthin in the role of light-harvesting via its $2^1A_g$ state, either carotenoid may supplement its light-harvesting ability by transferring energy from its $1^1B_u$ state to Chl *a*. This mechanism of light harvesting has been gaining support from recent experiments [31].

Although the energy difference between the $2^1A_g$ ($S_1$) states of diadinoxanthin and diatoxanthin are not large, the data do support the notion that the enzymatic de-epoxidation/epoxidation reactions of the diatoxanthin/diadinoxanthin and zeaxanthin/violaxanthin xanthophyll cycles may be acting regulators of light flow in the pigment protein complexes. Under conditions of low light, the carotenoid could act preferentially as a light-harvesting pigment, transferring energy from its $2^1A_g$ state to the $S_1$ state of Chl. Under excess light conditions, de-epoxidation of the carotenoid occurs, and the system could then preferentially transfer the $S_1$ energy of Chl *a* back to the now low-lying $2^1A_g$ state of the carotenoid.

These results suggest that the capability of carotenoids to regulate the energy flow among chlorophylls may be a general feature of all xanthophyll cycle carotenoids regardless of their species of origin. It is important to stress, however, that the present data only demonstrate the energetic feasibility of energy flow regulation. The data do not imply that other factors such as a trans-thylakoid pH gradient and/or aggregation state of the light-harvesting complex are not important in controlling this action [44,45]. The interconversion of violaxanthin-to-zeaxanthin or diadinoxanthin-to-diatoxanthin results in changes to both the physico-chemical and photophysical properties of these molecules, and it is perhaps an oversimplification to consider only one of these changes in isolation. The de-epoxidation of violaxanthin to zeaxanthin and the resulting increase

Fig. 2. The decay of the transient absorbance excited at 420 nm and probed at 550 nm and 532 nm for (a) diadinoxanthin and (b) diatoxanthin, respectively, in petroleum ether. These wavelengths correspond to the maxima of the $S_1$ to $S_n$ absorption of each xanthophyll. $\tau$ is the $S_1$ lifetime obtained by fitting the decay curves to single exponential rate expressions. The points are the experimental data, and the solid lines represent the fitted curves. The traces at the bottom of each plot are the residuals for the fitting of the theoretical curves to the experimental traces.

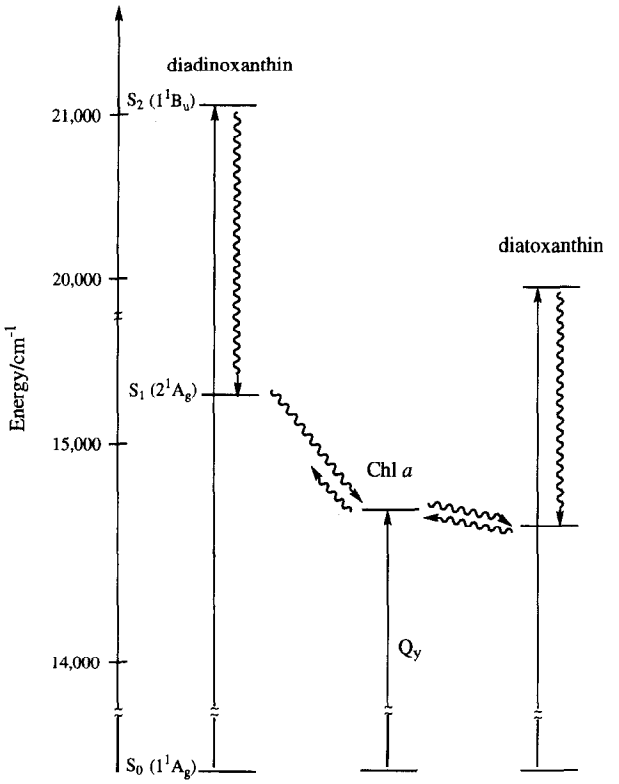



Fig. 4. Energies of the $S_1$ and $S_2$ states of diadinoxanthin and diatoxanthin relative to the excited state energy of Chl *a*. The vertical lines represent the allowed absorption transitions for the molecules and the wavy lines represent paths of internal conversion or energy transfer between the pigments.

in conjugation from 9 to 11 carbon-carbon double bonds directly affects the energies and dynamics of the singlet excited states. Importantly, it also affects directly the ring-to-chain conformation of the carotenoid molecule at carbon positions, C-6,7; from a perpendicular arrangement in violaxanthin to the near-planar conformation adopted by zeaxanthin. Similar changes in conformation would also be seen for diatoxanthin and diadinoxanthin. The consequences of this are not yet fully understood, but it has been observed that such conformational changes control the ability of different carotenoids to form aggregates from their monomeric state; e.g., zeaxanthin forms aggregates in vitro much more readily than violaxanthin [46]. Differences in carotenoid conformation may be related to the control of LHCII macro-organization, i.e., aggregation aid hence fluorescence quenching (see Ref. [47] for more details). It has also been suggested that changes in xanthophyll composition may affect thylakoid membrane fluidity [48,49].

It is difficult to reconcile the current, apparently conflicting, theories on xanthophyll-mediated quenching of Chl fluorescence. Nevertheless, the data from the present study clearly demonstrate that diatoxanthin-to-diadinoxanthin interconversion is a process which alters the properties of the $2^1A_g$ ($S_1$) state of the carotenoid so that energy transfer from Chl *a* to

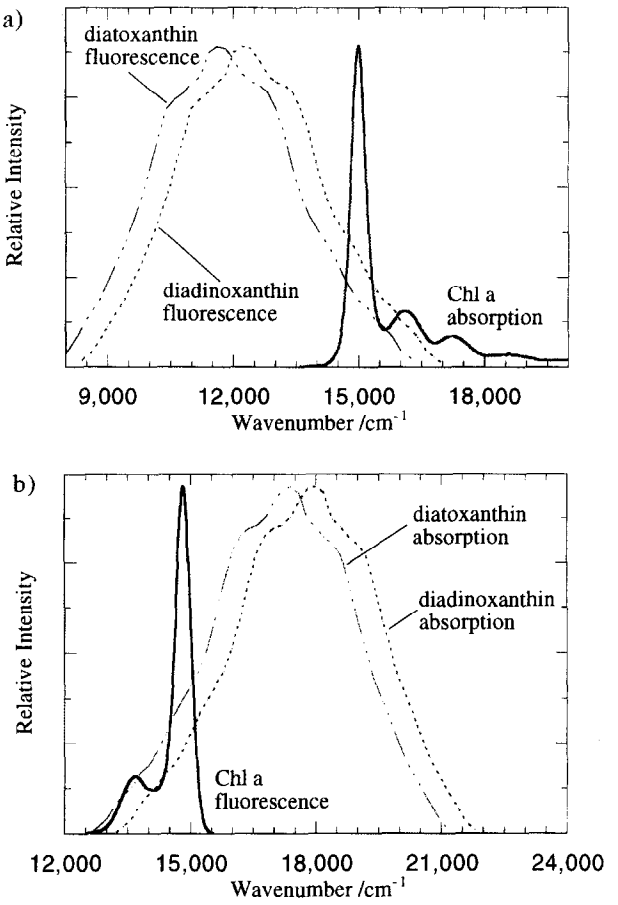



Fig. 5. (a) The hypothetical diadinoxanthin and diatoxanthin $S_1$ emission profiles generated by shifting the generic $2^1A_g \rightarrow 1^1A_g$ emission trace from 3,4,7,8-tetrahydrospheroidene to correspond to the spectral origins of diadinoxanthin and diatoxanthin. The hypothetical emission traces are overlaid with the absorption spectrum of Chl *a* for the purpose of calculating spectral overlaps according to Eq. (3) given in the text. (b) The emission spectrum of Chl *a* overlaid with the hypothetical $1^1A_g \rightarrow 2^1A_g$ absorption profiles of diadinoxanthin and diatoxanthin. The hypothetical absorption spectra of the carotenoids were generated by reflecting the emission traces of diadinoxanthin and diatoxanthin given in (a) about their spectral origins. The spectral curves are plotted on an energy (cm$^{-1}$) scale.

the carotenoid may become an enhanced route of de-excitation. A recent study [26] showed that the energies of the $S_1$ states of carotenoids may play a role in accepting energy from Chl *a* in isolated LHCIIb complexes, but it was also clear that the inhibition of fluorescence quenching seen at pH 5.5 by some carotenoids could not be accounted for purely in terms of energy transfer between carotenoid and Chl. In that study it was shown that a range of exogenous hydrocarbons and xanthophyll carotenoids have the ability to quench Chl fluorescence in the isolated complex provided their $S_1$ energies are lower than that of Chl *a*. At pH 7.8 the distinction between a quencher and a non-quencher was purely related to whether the $S_1$ energy of the carotenoid molecule lay either below or above the $Q_y$ of Chl *a*. Although diatoxanthin was not specifically tested in that study, the data presented here support the view that this carotenoid will indeed act as a quencher of Chl *a* fluorescence in many algal species.

The synergistic relationship between ΔpH and de-epoxidation of carotenoids is apparent from a number of studies (see Ref. [47] for a review). The data are consistent with an amplification role for zeaxanthin formation with the ΔpH initially being necessary to bring the pigments into close proximity. In that case, the presence of the epoxidized violaxanthin or diatoxanthin may act as an 'anti-quencher', preventing the complex from achieving a quenched state. In the presence of a ΔpH, the antiquenching may be overcome and the quenching would result from Chl-Chl interactions. When the de-epoxidized carotenoids are present, even at a moderate ΔpH, quenching may result from direct energy transfer from Chl *a* to the carotenoid via their $S_1$ states. A study by Holzwarth and colleagues [50] on fluorescence decay kinetics in isolated thylakoids has demonstrated that ΔpH and zeaxanthin are involved in different mechanisms contributing to excess energy dissipation. Zeaxanthin-associated quenching was related to increased thermal dissipation in the antenna.

The epoxidation/de-epoxidation of these xanthophyll cycle carotenoids appears also to influence their binding to the light-harvesting complex [26]. Increasing attention is focusing on the role of violaxanthin, and, by inference, diadinoxanthin as anti-quenchers; i.e., it is the *removal* of violaxanthin from its binding site(s) in the light-harvesting complex, and not the formation of antheraxanthin or zeaxanthin per se which may be the key process in excess energy dissipation. These carotenoids may simply serve to amplify quenching in vivo. Clearly, further studies will be required to determine the precise mechanism by which de-epoxidized carotenoids participate in the quenching of Chl *a* excited states.

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