Direct Determination of the S_1 Excited-State Energies of Xanthophylls by Low-Temperature Fluorescence Spectroscopy†

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The xanthophylls, violaxanthin, lutein, and zeaxanthin, associated with the antenna protein assembly of Photosystem II (PS II) play roles as light-harvesting pigments and protective agents in the photosynthetic apparatus of higher plants. The dissipation of excitation energy exceeding that needed for photosynthesis is thought to be regulated by an enzymatic process known as the xanthophyll cycle where violaxanthin and zeaxanthin are reversibly interconverted, but the role of the cycle in controlling the process in vivo is not clear. The two hypotheses are (i) direct quenching of chlorophyll excited states by the xanthophylls and (ii) indirect quenching via carotenoid-mediated changes in the structure of the light-harvesting complexes. These mechanisms depend on the structures and/or energetics of the xanthophyll pigments, which have not yet been fully elucidated. In this work, fluorescence spectroscopy at 77 K has been used to determine the energies of the S_1 excited states of violaxanthin, zeaxanthin, and the major xanthophyll component of green plants, lutein. High performance liquid chromatography (HPLC) was carried out just prior to the spectroscopic experiments to obtain isomerically pure samples devoid of fluorescent contaminants. The experiments at cryogenic temperatures provide enhanced resolution compared to room-temperature studies, reveal clearly the vibronic features of the fluorescence line shapes, and allow precise, direct assignments of the spectral origins and electronic-state energies of the molecules. The results are important for broadening our understanding of the mechanisms of light-harvesting and nonphotochemical dissipation of excess energy in plants.

Introduction

When green plants are exposed to photon flux levels higher than required for photosynthesis, thermal dissipation of the excess energy occurs. This process, termed nonphotochemical quenching (NPQ), is an adaptation by photosynthetic organisms to environmental stress.[1−4] The efficient and safe removal of excess excited states of chlorophyll (Chl) is thought to be at least partially controlled by an enzymatic process known as the xanthophyll cycle.[1,2,5−9] In this cycle, violaxanthin and zeaxanthin molecules associated with the light-harvesting assembly of Photosystem II (PS II) are reversibly interconverted and correlate with different extents of Chl fluorescence quenching.[3,9−15] It is also known that pH plays a role in controlling the extent of quenching,[4,16] but the precise molecular mechanism by which xanthophylls participate in dissipating Chl excited states in vivo is still not clear. There are two main hypotheses: (i) direct carotenoid−Chl interactions involving either energy transfer or electron transfer that result in the quenching of Chl fluorescence and the dissipation of excitation energy[11,17−19] and (ii) indirect quenching involving carotenoid-mediated changes in the structure and/or organization of the light-harvesting complexes.[4,20] These mechanisms depend critically, but in different ways, on the structures and/or energetics of the xanthophyll pigments. Thus, it is important to understand the relationships between their structures, excited-state properties, and photochemical behavior.

† Abbreviations: Chl, chlorophyll; HPLC, High-pressure liquid chromatography; EPA, low-temperature glass made from ethanol, isopentane and diethyl ether.

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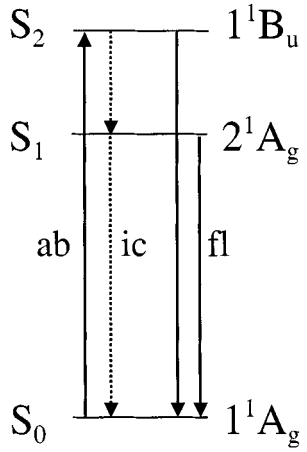

Figure 1. Energy level diagram denoting absorption (ab), internal conversion (ic), and fluorescence (fl) among the lowest lying singlet states of xanthophylls.

All xanthophylls display very strong absorption in the visible spectrum where Chl *a* is not a very efficient absorber.[21] The energy is then transferred to Chl. Light absorption by the xanthophylls is associated with an electronic transition between the ground state, S_0, having A_g symmetry in the idealized C_{2h} point group, and an excited singlet state having B_u symmetry (Figure 1). The lowest excited singlet state, S_1, of these molecules also has A_g symmetry. Hence, electronic transitions between S_0 and S_1 are forbidden. Several technical problems have hampered assigning the energies of S_1 states of xanthophylls from fluorescence studies including difficulties in obtain-

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TABLE 1: Electronic Energies of the S_1 and S_2 States, Quantum Yields, ϕ_f, of $S_2 \rightarrow S_0$ and $S_1 \rightarrow S_0$ Emission, S_1 Lifetimes, τ_{S_1}, and Radiative Rate Constants, k_r, of the Carotenoids

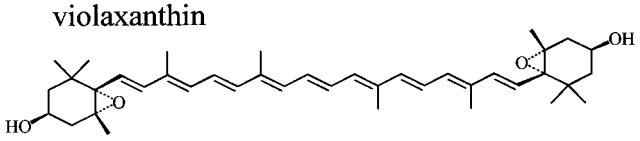
molecule	no. of conj −C=C− bonds	solvent	temp/K	spectral origin $S_1 \rightarrow S_0$ transition (cm^{-1})	spectral origin $S_0 \rightarrow S_2$ transition (cm^{-1})	$10^5\phi_f$ ($S_1 \rightarrow S_0$)	$10^5\phi_f$ ($S_2 \rightarrow S_0$)	τ_{S_1}/ps	$10^{-6}k_r$ ($S_1 \rightarrow S_0$)/s^{-1}	refs
violaxanthin	9	EPA	77	15 580 ± 60[a]	20 650 ± 80	2.3 ± 0.5	11 ± 1			
		EPA	293		21 310 ± 270	5.0 ± 3.0	14 ± 8		2.1	
		n-hexane	RT	14 880 ± 90[a]	21 230 ± 90			23.9		17, 18
		methanol	RT	14 470 ± 90[b]	21 420			24.6		32
lutein	10	EPA	77	14 570 ± 70[a]	20 360 ± 80	2.3 ± 0.5	8.5 ± 1			
		EPA	293		20 980 ± 110	2.0 ± 1.0	9.0 ± 0.6	14.6[d]	1.4	
		octanol	277	15 100 ± 300[c]						35
zeaxanthin	11	EPA	77	14 610 ± 40[a]	20 000 ± 80	1.5 ± 0.1	1.6 ± 0.5			
		EPA	293		20 490 ± 460	1.0 ± 0.7	4.0 ± 2.0		1.1	
		n-hexane	RT	14 550 ± 90[a]	21 010 ± 90			9.0		17, 18
		methanol	RT	14 030 ± 90[b]	21 010			8.6		32

[a] Fluorescence-detected. [b] Transient absorption-detected. [c] Two-photon excitation-detected. [d] In petroleum ether (ref 55).

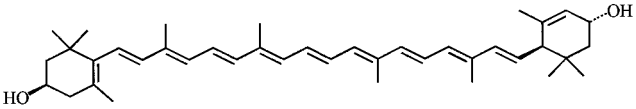
ing pure samples of the xanthophylls, low emission yields, and the lack of adequate detection sensitivity. S_1-state fluorescence yields of carotenoids are on the order of 10^{-4} or less and only a few reports of fluorescence from the S_1 states of carotenoids having more than nine conjugated carbon−carbon double bonds have appeared.[22−25] These technical problems have led to indirect experimental approaches of deducing the energies of the S_1 states of carotenoids and xanthophylls; e.g., by simply extrapolating the energies from short polyenes that exhibit fluorescence from their S_1 states,[26−28] or by measuring the dynamics of short carotenoids and then using the energy gap law for radiationless transitions to obtain the state energies.[29−31] Fluorescence spectroscopy clearly is a more direct manner in which to assign the energies of the S_1 states of carotenoids and xanthophylls. Recent work has solved many of the technical problems, and the S_1 energies of zeaxanthin and violaxanthin have been reported on the basis of room-temperature fluorescence spectroscopic experiments.[18]

Other "direct" spectroscopic methods have also been used to determine the S_1 energies of xanthophylls. Polívka et al.[32−34] used a femtosecond time-resolved laser pulse to excite the molecules from S_0 to S_2, which then relaxes to the S_1 state within approximately 200 fs. They then detected the excited-state absorption from the S_1 state to the S_2 state using a probe laser pulse in the infrared spectral region. Subtracting the energy of the spectral origin of the $S_1 \rightarrow S_2$ transition from the energy of the spectral origin of the strongly allowed $S_0 \rightarrow S_2$ absorption revealed the position of the S_1 level. This approach was applied to the xanthophyll cycle pigments, violaxanthin and zeaxanthin,[32] and also to spheroidene obtained from the photosynthetic bacterium, *Rhodobacter sphaeroides*.[33,34] Two-photon spectroscopy is another approach that has been used to obtain the S_1 energies of xanthophylls. Walla et al.[35] reported the two-photon excitation profile of Chl *a* fluorescence in the LHC II complex from *Chlamydomonas reinhardtii* and determined the S_1-state energy of the molecule (probably lutein) transferring energy to Chl *a* to be 15 100 ± 300 cm^{-1}. New work by this group has suggested that energy transfer to Chl may originate from a hot vibronic state of the carotenoid so that the actual S_1-state energy in the LHC II complex may be lower than this.[36] The significant aspects of the two-photon studies are that they allow direct experimental verification of energy transfer from the S_1 state of the carotenoid to chlorophyll and provide useful information on how the state energies of carotenoids may be affected by binding of the molecules to proteins. All of the values reported for the S_1 energies of zeaxanthin, lutein and violaxanthin are summarized in Table 1. There are substantial differences in the values reported by the different research groups. It is not clear whether these are due to systematic or random errors associated with the experimental conditions or the method of observation of the electronic origins of the transitions associated with the S_1 state.

violaxanthin



lutein



zeaxanthin

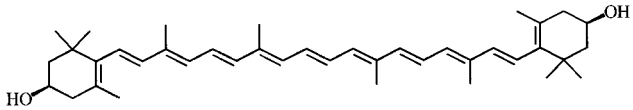


Figure 2. Molecular structures of the xanthophylls studied in this work.

In this paper we present an investigation using low-temperature fluorescence spectroscopy of the S_1-state energies of violaxanthin, lutein, and zeaxanthin (Figure 2). At room temperature, the $S_1 \rightarrow S_0$ and $S_2 \rightarrow S_0$ emission spectra are broad leading to considerable uncertainty in the assignment of the spectral origins (0−0 bands) for the optical transitions.[18] At low temperatures, the electronic spectra sharpen significantly and reveal substantial vibronic structure that greatly simplifies the interpretation of the spectra and the determination of the spectral origins. In this work the energies of the S_1 states energies have been determined at 77 K to be 15 580 ± 60 cm^{-1} for violaxanthin, 14 570 ± 70 cm^{-1} for lutein, and 14 610 ± 40 cm^{-1} for zeaxanthin. These data are consistent with trends in the results from previous studies on apo- and diapocarotenoids and other analogous compounds.[27,28,37] The results provide important information for considering the mechanisms of light-harvesting and nonphotochemical dissipation of excess energy (NPQ) in plants and further emphasize the importance of fluorescence spectroscopy in detecting and understanding the nature of the lowest energy S_1 states of xanthophyll molecules.

Materials and Methods

Sample Preparation. Violaxanthin was extracted from spinach as follows: Approximately 250 g of fresh spinach leaves were chopped to small pieces and mixed with ~500 mL of acetone. The mixture was then homogenized using a blender and filtered using cheesecloth. An equal volume of diethyl ether was added to the acetone solution, and extraction was carried out three times. The diethyl ether layer was then washed with distilled water four times. Subsequently, 25 mL of 2% NaCl solution was added to the mixture with gentle swirling to remove emulsions formed during the extractions.

The diethyl ether solution was then evaporated to dryness on a rotary evaporator. Ten mL of acetone were added and the sample saponified by adding an equal volume of 6% KOH in ethanol and incubating for 15 min. This process cleaves any ester linkages to the xanthophylls and also destroys the chlorophylls. Violaxanthin was then separated from the other pigments using an alumina column and *n*-hexane containing increasing percentages of acetone from 0.5 to 50% as an eluant. The absorption spectra of the fractions were obtained using a Cary 50 UV/vis spectrometer. The fraction containing violaxanthin had a λ_{max} of 440 nm and was obtained from the alumina column after elution with ~30% acetone in *n*-hexane. The violaxanthin solution was dried completely with a gentle stream of nitrogen prior to further purification using high performance liquid chromatography (HPLC). (See below.)

Synthetic (3*R*,3′*R*)-zeaxanthin was a gift from Roche Vitamins Ltd. and was further purified using HPLC as described below. Lutein was purchased as a dietary supplement from Douglas Laboratories, Inc. Two 6 mg capsules were cut open and the contents dissolved in ~5.0 mL of diethyl ether. The mixture was then saponified using the procedure for lutein developed by Khachik et al.[38] in which 10 mL of 30% methanolic KOH was added to the sample. The solution was then stirred for 3 h under nitrogen. Five milliliters of a saturated solution of sodium chloride and 20 mL of petroleum ether were then added to the solution, and the pet. ether layer was separated. The aqueous layer was partitioned several times with pet. ether to recover additional material from the organic layer left after the previous extractions. The combined ether layer was then washed three times with water, dried over magnesium sulfate, filtered using Whatman No. 1 filter paper, and evaporated to dryness using nitrogen. One milliliter of acetone was added to all of the xanthophylls prior to HPLC.

HPLC separations were carried out using a 250 × 4.6 mm YMC carotenoid column (5 mm) on a Millipore Waters 600E HPLC equipped with a photodiode array detector. The HPLC instrument was operated using Waters Millenium software version 3.20. The xanthophylls were separated using an isocratic mixture of 85:15 v/v acetone/borate buffer (1 mM, pH 8.2). Flow rates were 0.6 mL/min for violaxanthin and 1.0 mL/min for lutein and zeaxanthin. The chromatographic separation was monitored at 440 nm for violaxanthin and 450 nm for lutein and zeaxanthin. The all-trans-isomers, identified by their very low-intensity cis bands at ~340 nm, were collected and dried using a gentle stream of nitrogen. The purified xanthophylls were then dissolved in EPA (ether/isopentane/ethanol, 5/5/2, v/v/v). Absorption and fluorescence spectra were taken immediately thereafter at room temperature and 77 K.

Spectroscopic Methods. *Steady-State Absorption and Fluorescence.* Absorption spectra were recorded on a Cary 50 UV/vis spectrometer using a 4 mm path length Suprasil square quartz cuvette (Starna Cells 29F-Q-10) suspended in a cylindrical custom-made liquid nitrogen cryostat. A gentle stream of He gas was bubbled near the cuvette during the experiment to minimize agitation of the cryogenic liquid due to bubbling. The fluorescence experiments used the same cuvette and cryostat and were carried out using an SLM Instruments, Inc. Model 8000C spectrofluorometer equipped with a Hamamatsu R928 photomultiplier (PMT) tube as the detector. An SLM Instrument Model WCTS-1 thermostatically cooled housing was used to minimize the PMT dark current. A Spectra-Physics argon ion laser Model 164 operating at a power of ~60 mW for violaxanthin and lutein, and ~100 mW for zeaxanthin was used to excite the molecules. A 488 nm interference filter was placed between the laser and the sample to eliminate the emissive glow from the plasma tube. The band-pass setting for the emission monochromator was 16 nm. The SLM Aminco 8100 version 4.0 software automatically selects either signal averaging or integration, whichever gives a better signal-to-noise ratio over the scanned wavelength range. The emission spectrum was then scanned at a relatively low amplifier gain and PMT voltage to obtain the maximum signal intensity that would occur in that spectral range. The wavelength at which the maximum signal was observed was used by the software to set the PMT voltage and amplifier gain to achieve a signal that registered 80% of the saturation maximum of the PMT. The spectrum was then scanned using these instrumental parameters. A spectral scan of the solvent blank was taken under identical conditions and subtracted from the spectral trace of the sample to remove contributions from fluorescence and Raman scattering by the solvent. A correction curve generated by a spectral Irradiance 45W quartz−halogen tungsten coiled filament lamp standard was used to correct the fluorescence spectra.

For the violaxanthin and lutein fluorescence experiments, a 470 nm high-pass, cutoff filter was placed between the interference filter and the sample and a 495 nm high-pass, cutoff filter was placed between the sample and the emission monochromator. Zeaxanthin was excited at 497 nm using a 495 nm high-pass, cutoff filter placed between the excitation light source and the sample and a 515 nm high-pass, cutoff filter between the sample and the emission monochromator. To maximize the signal-to-noise ratio of the very weak S_1 emissions of the three xanthophylls, the fluorescence spectrometer acquisition mode was set to photon counting when scanning above 600 nm in the region corresponding to the $S_1 \rightarrow S_0$ emission.

Fluorescence Excitation Spectroscopy. Fluorescence excitation spectroscopy was done by scanning the excitation monochromator from 300 to 500 nm while monitoring the fluorescence emission at any one of a number of different emission wavelengths. The excitation spectra were corrected for variations in the excitation profile by dividing the spectra of the xanthophylls by a spectrum from a Rhodamine 610 standard.

Quantum Yield Measurements. The fluorescence quantum yields of the xanthophylls were measured relative to Rhodamine 590 in methanol ($\phi_F = 0.95$). The quantum yields were calculated according to the equation

$$\phi_c = \phi_r \left(\frac{1 - 10^{-A_{r\lambda}}}{1 - 10^{-A_{c\lambda}}}\right)\left(\frac{I_{r\lambda}}{I_{c\lambda}}\right)\left(\frac{n_c^2}{n_r^2}\right)\left(\frac{D_c}{D_r}\right) \quad (1)$$

where ϕ_c and ϕ_r are the quantum yields of the xanthophyll and reference solutions, respectively. $A_{c\lambda}$ and $A_{r\lambda}$ are the optical densities of the xanthophyll and reference solutions at the wavelength, λ. $I_{c\lambda}$ and $I_{r\lambda}$ are the relative intensities of the excitation light at the wavelength, λ, for the xanthophyll and reference solutions. n_c and n_r are the refractive indexes of the solvent for the xanthophyll and the reference, Rhodamine 590.

TABLE 2: Fitting Parameters for the Gaussian Deconvolutions of the $S_1 \rightarrow S_0$ Emission Spectra of Violaxanthin, Lutein and Zeaxanthin in EPA at 77 K[a]

		$S_1 \rightarrow S_0$			$S_0 \rightarrow S_2$
	vibronic band	$0 \rightarrow 0$	$0 \rightarrow 1$	$0 \rightarrow 2$	$0 \rightarrow 0$
violaxanthin	energy	15 580 ± 60	14 390 ± 40	12 890 ± 120	20 650 ± 85
	λ	642	695	776	484
	δ	1100 ± 490	1340 ± 430	1260 ± 210	455 ± 20
lutein	energy	14 570 ± 70	13 700 ± 250	12 910 ± 40	20 360 ± 85
	λ	686	729	775	491
	δ	760 ± 300	1000 ± 250	817 ± 230	490 ± 25
zeaxanthin	energy	14 610 ± 40	13 770 ± 30	12 470 ± 150	20 000 ± 80
	λ	684	726	802	500
	Δ	415 ± 160	760 ± 260		610 ± 30

[a] The energies and widths (Δ) are in cm^{-1} and the wavelengths (λ) are in nm. The uncertainties represent standard deviations derived from multiple fits to the experimental spectra. The spectral origins of the $S_0 \rightarrow S_2$ transitions and the line widths (cm^{-1}) based on the absorption spectra are also given.

In this study, EPA was used as solvent for both the xanthophyll and reference solutions, so the ratio of the indexes of refraction was unity. D_c and D_r are the integrated areas of the corrected emission spectra of the xanthophyll and reference solutions obtained under identical conditions of slit width and gain, and were derived from Gaussian fits to the emission line shapes. To obtain comparable intensities of fluorescence from the weakly fluorescent xanthophylls and the strongly fluorescent reference under these experimental conditions, the optical density of the Rhodamine 590 reference had to be diluted approximately 4 orders of magnitude more than the xanthophyll sample. This precluded a direct measurement of the optical density of the reference. Thus, a linear calibration plot of the integrated area of Rhodamine fluorescence versus optical density was made in the region from 1.00 to 0.001 absorbance where the optical density could be measured directly by absorption spectroscopy. The value of the Rhodamine optical density used in eq 1 was derived from a linear extrapolation of the calibration plot to the point corresponding to the measured integrated fluorescence area of the very dilute Rhodamine 590 solution.

Gaussian Deconvolution of Spectra. Gaussian deconvolutions of the fluorescence spectral line shapes were performed using Origin version 6.1 software. The fluorescence spectra were converted from a fixed band-pass wavelength scale to a fixed band-pass wavenumber scale by multiplying the intensity by the square of the detection wavelength at each point. Starting values of the vibronic bands in wavenumbers were taken from estimates of the peaks and shoulders evident in the experimental spectra. The position, area, width, spacing and baseline of all the Gaussian components were allowed to vary using the Levenberg−Marquardt algorithm with a maximum of 50 iterations. Several Gaussian functions were used to fit the line shapes and the mean and standard deviation of each of the Gaussian components was determined. The final values of the calculated parameters for the fits are given in Table 2.

Results and Discussion

HPLC Analysis and Purification of the Xanthophylls. HPLC provides the means of purifying the xanthophylls and analyzing the integrity of the samples before and after the spectroscopic experiments. Although the samples were reasonably pure prior to HPLC, the protocol described above removed any remaining trace amounts of oxidative and thermal breakdown products of the xanthophylls that can be highly fluorescent and lead to unfavorable effects on the fluorescence and the fluorescence excitation spectra. The protocol also separates the all-trans isomers of the molecules from cis-geometric configurations that have rotations about carbon−carbon double bonds in the conjugated π-electron chain. The features of the $S_0 \rightarrow S_2$ absorption and $S_2 \rightarrow S_0$ fluorescence spectra of the xanthophylls may shift substantially upon trans-to-cis isomerization. A distribution of configurational isomers in the samples would result in broad spectral line shapes and difficulty in assigning the vibronic features and spectral origins. For these reasons, HPLC was carried just moments prior to the spectroscopic experiments. Also, freezing the purified samples immediately after HPLC inhibits geometric isomerization and thermal degradation.

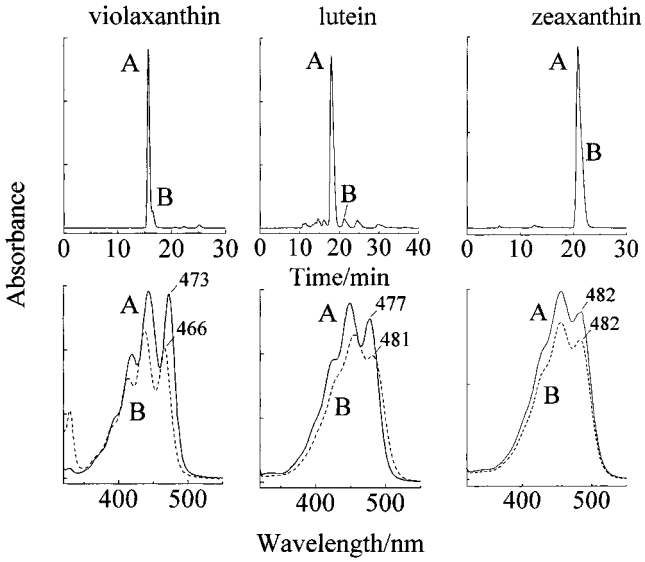


Figure 3. HPLC chromatograms of the xanthophylls taken just prior to the spectroscopic experiments. The chromatographic protocol is described in detail in the text. The peaks labeled A correspond to the all-trans-isomers of the molecules.

Typical HPLC traces are shown in Figure 3. The absorption spectra corresponding to the major peaks in all the chromatograms display strongly allowed ($S_0 \rightarrow S_2$) transitions in the 350−500 nm region. The retention times of the all-trans isomers of the different xanthophylls were very similar and eluted at ~18 min. The absorption spectra associated with some of the minor chromatographic peaks show varying intensity of a band found ~140 nm to shorter wavelength of the lowest energy spectral feature; e.g., see the spectrum of peak B in the chromatogram of violaxanthin (Figure 3). This so-called "cis peak" at approximately 320 nm indicates a cis-geometric isomers of the molecule.[39,40] Note that the spectral origin of the cis-isomer of violaxanthin is shifted by 7 nm compared to the all-trans isomer (peak A), which is distinguished by its extremely small cis-peak. For all of the xanthophylls, the all-trans-configurations

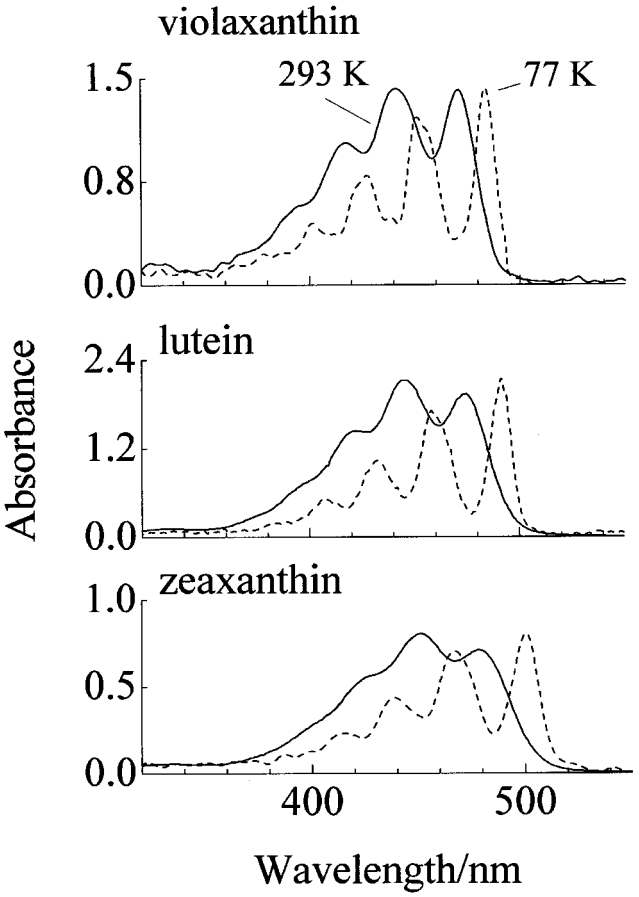


Figure 4. Absorption spectra of violaxanthin, lutein, and zeaxanthin in EPA at 293 (solid line) and 77 K (dashed line). The instrumental conditions are described in detail in the text.

dominated the isomeric mixture. Cis-isomers that could potentially broaden the vibronic features in the fluorescence spectra were removed. The HPLC trace associated with the purification of lutein shows the presence of a small amount of zeaxanthin (peak B in Figure 3) that was present in the original source. The zeaxanthin was easily removed from the lutein sample due to the fact that baseline separation was achieved between the peaks in the chromatogram. The HPLC chromatogram of synthetic zeaxanthin shows two features labeled A and B in Figure 3, which eluted extremely close to one another. The absorption spectra of these two components were indistinguishable, suggesting that they correspond to zeaxanthin molecules having different geometries of the hydroxyl groups on the isoprenoid rings. The synthetic protocol for this molecule yielded the (3*R*,3′*R*) stereoisomer as the dominant component (peak A) (A. Giger, private communication). Peak B may be the (3*R*,3′*S*) stereoisomer, resolved by the HPLC protocol, but spectrally identical to the other form. Stereoisomers of this type have no deleterious effect on the resolution of the vibronic components of the optical spectra, and therefore, need not be removed from the mixture.

Absorption Spectra. The absorption spectra of the xanthophylls in EPA at 77 and 293 K are shown in Figure 4. The positions of the spectra, relative intensities of the vibronic components, and line widths of the vibronic bands of the molecules change dramatically with the extent of π-electron conjugation and with temperature. The absorption spectrum of zeaxanthin is red-shifted compared to lutein, and the absorption spectrum of lutein is red-shifted compared to violaxanthin. This trend is due to the increasing number of conjugated carbon−carbon double bonds, which is nine for violaxanthin, ten for lutein, and eleven for zeaxanthin (Figure 2). At 293 K, the first and second vibronic features in the spectrum of violaxanthin have roughly equal intensities, whereas for lutein and zeaxanthin the most intense band in the absorption spectra corresponds to the second vibronic feature. Also, at 293 K the absorption spectrum of violaxanthin shows a higher degree of resolution in its vibronic bands than lutein or zeaxanthin; i.e., the troughs between the peaks in the violaxanthin spectrum are deeper than for lutein and zeaxanthin, and lutein is better resolved than zeaxanthin. This may be explained by the fact that the epoxide functional groups in violaxanthin uncouple the relatively bulky isoprenoid rings from the π-electron conjugation, thereby reducing conformational disorder in the polyene chain.[41] Because lutein has only one ring directly associated with the π-electron conjugation (Figure 2), it is expected to have a better resolved spectrum than zeaxanthin, which has two rings in the conjugated chain. This is observed in Figure 4.

Lowering the temperature to 77 K results in a pronounced (>10 nm) red shift of the spectra due to an increase in the index of refraction, and hence the polarizability, of the solvent in going from the liquid to the solid state. (See also Table 1.) Lowering the temperature also results in considerable narrowing of the vibronic features. The effect of solvent polarizability and temperature on the absorption spectra of carotenoids have been well documented.[42−49] The redistribution of the absorption spectral intensity to the lower energy vibronic features and the spectral narrowing resulting from lowering the temperature may be attributed to a reduction in the thermal population of higher energy vibronic states.[50,51]

Fluorescence Spectra. The fluorescence spectra of the xanthophylls are shown in Figure 5 and can be thought of as being comprised of two components. The first component exhibits a small Stokes shift between the origins of emission and absorption, indicating that it corresponds to the $S_2 \rightarrow S_0$ ($1^1B_u \rightarrow 1^1A_g$) transition. It is also largely a mirror image of the $S_0 \rightarrow S_2$ absorption suggesting a small horizontal displacement of the potential energy surfaces associated with S_0 and S_2. The second component of the emission is significantly red-shifted compared to the absorption and weaker than the first component. The precipitous drop-off of the fluorescence intensity at long wavelengths is due to the lack of sensitivity of the PMT beyond 850 nm. This part of the emission can be assigned to the $S_1 \rightarrow S_0$ ($2^1A_g \rightarrow 1^1A_g$) transition. In contrast to previous spectra taken at room temperature,[18] the spectra taken here at 77 K show a significant improvement in the resolution of the vibronic features. Also, unlike the $S_0 \rightarrow S_2$ transitions that shift substantially with temperature (Figure 4), very little, if any, shifts of the $S_1 \rightarrow S_0$ emission bands occur with changing the temperature. This is consistent with the idea that the transition dipole moment associated with transitions to and from the S_1 state is vanishingly small and thus not expected to be significantly affected by temperature-induced changes in the polarizability of the solvent. The enhanced vibronic resolution of the $S_1 \rightarrow S_0$ line shapes at low temperatures facilitates the analysis of the spectra by Gaussian deconvolution (see below).

Fluorescence Excitation Spectra. The fluorescence excitation spectra of the xanthophyll molecules obtained at 293 K while monitoring the $S_2 \rightarrow S_0$ emission are in good, although not perfect, agreement with the absorption spectra (Figure 6). Fluorescence excitation spectra associated with the $S_1 \rightarrow S_0$ emissions were precluded because a monochromatic laser was required to obtain sufficient excitation in those experiments.

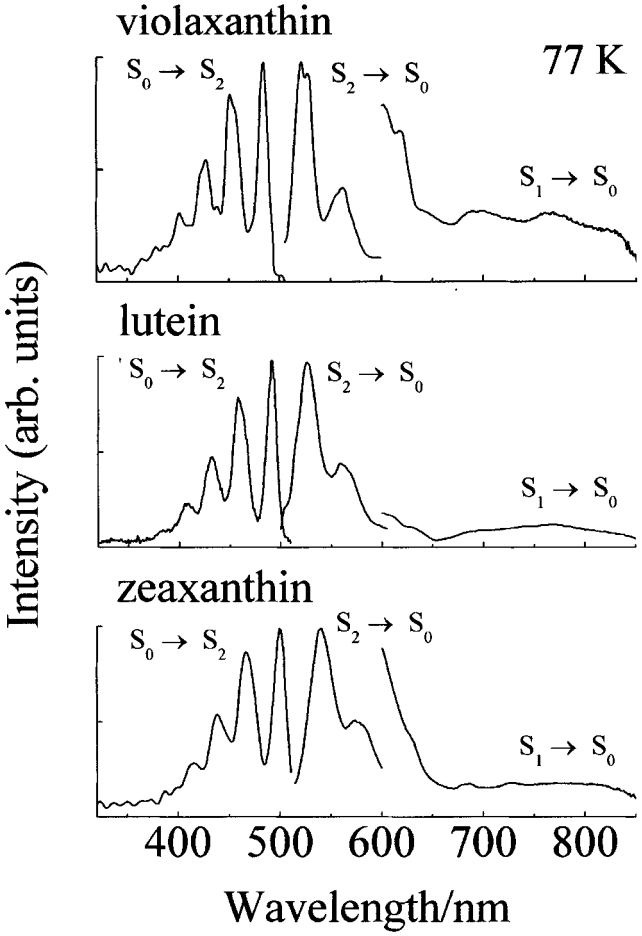


Figure 5. Absorption ($S_0 \rightarrow S_2$) and fluorescence ($S_2 \rightarrow S_0$ and $S_1 \rightarrow S_0$) spectra of violaxanthin, lutein, and zeaxanthin in EPA at 77 K. To maximize the signal-to-noise ratio of the relatively weak $S_1 \rightarrow S_0$ emissions of the three xanthophylls, the fluorescence spectrometer acquisition mode was set to photon counting for the emission scans started at 600 nm. The same mode applied to the relatively intense $S_2 \rightarrow S_0$ emission would have rendered it off-scale. The 77 K absorption spectra are the same as those presented in Figure 4.

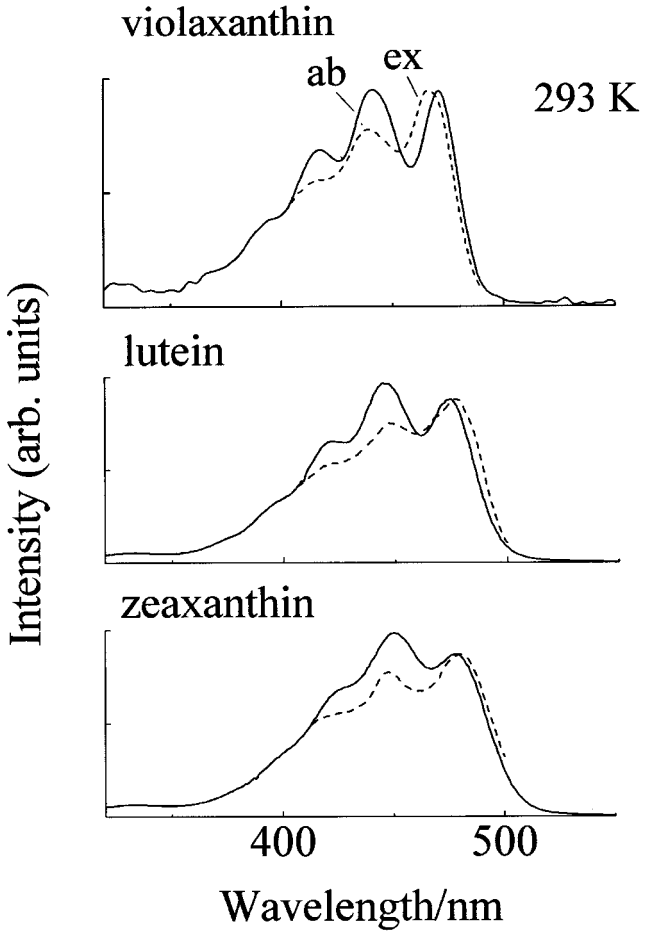


Figure 6. Overlay of the absorption (ab, solid line) and fluorescence excitation (ex, dashed line) spectra of violaxanthin, lutein, and zeaxanthin in EPA at 293 K. The instrumental conditions are described in detail in the text.

Figure 6 overlays the instrument-corrected $S_2 \rightarrow S_0$ fluorescence excitation spectra of the xanthophylls at 293 K with the $S_0 \rightarrow S_2$ absorption spectra. Besides the fluorescence excitation spectra exhibiting small shifts and relatively broader vibronic features than the absorption spectra, effects we have traced to the larger detection slit width required to obtain adequate sensitivity in the fluorescence experiments compared to the absorption experiments, there is a significant difference in the distribution of vibronic band intensities in the spectra that cannot be attributed to instrumental factors. The fluorescence excitation spectra have their maxima corresponding to the lowest energy vibronic feature, whereas, as noted above, the absorption spectra have their most significant intensity associated with the second vibronic component. This observation is highly reproducible and has been seen in the spectra of carotenoids and polyenes previously.[26,52] One interpretation of the effect is that upon $S_0 \rightarrow S_2$ excitation, there exist alternate routes of nonradiative relaxation for the higher vibronic states that are unavailable to the lowest vibronic state. This would have the effect of lowering the $S_2 \rightarrow S_0$ fluorescence yield that is observed upon excitation into higher vibronic bands. The alternate paths of deactivation may involve selective vibronic coupling to the S_1 state or other electronic states thought to be in proximity or between S_1 and S_2.[53,54] Ultrafast time-resolved spectroscopic studies as a function of excitation wavelength would be useful in examining this issue further.

Quantum Yields. The quantum yields of the $S_1 \rightarrow S_0$ and $S_2 \rightarrow S_0$ emissions measured at 77 and 293 K for the xanthophylls are given in Table 1. The values decrease, although not drastically, with increasing extent of π-electron conjugation. The changes in S_1 quantum yields correspond well with changes in the lifetimes of the S_1 states measured previously by us[17,55] and given in Table 1. These values can be used to determine the radiative rate constants associated with $S_1 \rightarrow S_0$ deactivation. The relevant equation is

$$k_r = \frac{\phi_{S_1 \rightarrow S_0}}{\tau_{S_1 \rightarrow S_0}} \qquad (2)$$

where k_r is the radiative rate constant, τ is the lifetime of the S_1 state and ϕ is the quantum yield of the $S_1 \rightarrow S_0$ fluorescence emission. The values for the radiative rate constants computed from the quantum yields measured at 293 K and the lifetimes measured previously[17,55] are given in Table 1 and are very similar for the three xanthophylls spanning a narrow range from 1.1×10^6 to 2.1×10^6 s^{-1}. The facts that the S_1 radiative rate constants are small ($\sim 10^6$ s^{-1} compared to the overall rate constant for the decay of the S_1 state, which is on the order of 10^{11} s^{-1}) and reasonably constant are consistent with the idea that nonradiative processes dominate the decay of xanthophylls from their S_1 excited states.

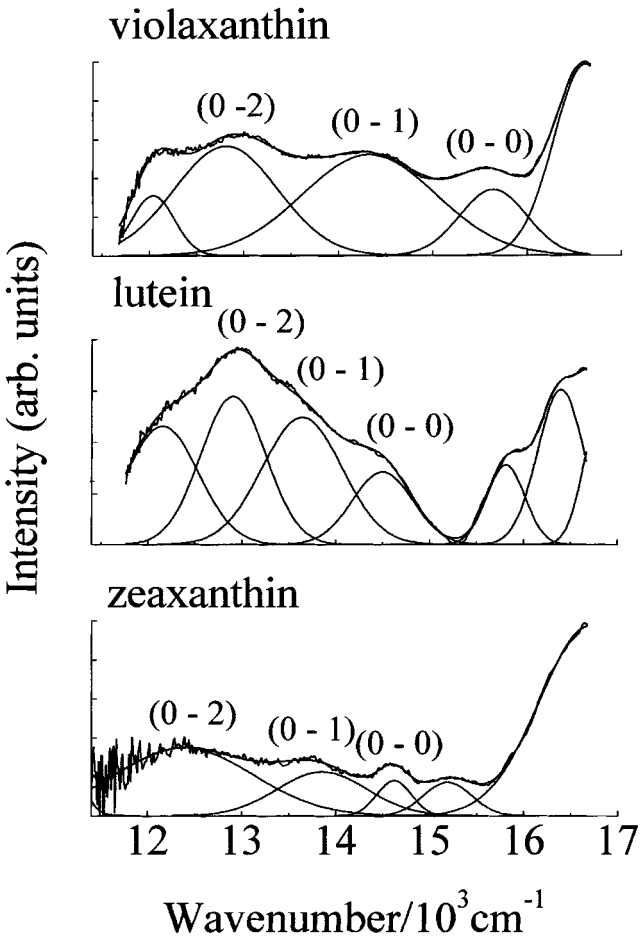


Figure 7. Gaussian deconvolution of the $S_1 \rightarrow S_0$ emission spectra of violaxanthin, lutein, and zeaxanthin in EPA at 77 K plotted on a wavenumber scale as described in the text. The experimental spectra are expanded traces of those shown in Figure 5. The parameters corresponding to the fits are given in Table 2. Note that the lack of sensitivity of the photomultiplier beyond 12 000 cm^{-1} precludes assigning a specific width to the (0−2) vibronic band of zeaxanthin in Table 2.

Spectral Origins of the $S_1 \rightarrow S_0$ Transitions. The vibronic bands and spectral origins for the $S_1 \rightarrow S_0$ transitions were determined from the fluorescence line shapes using the fitting procedures described above. The results are shown in Figure 7 and summarized in Table 2. Gaussian deconvolutions of the $S_1 \rightarrow S_0$ fluorescence spectra revealed a regular pattern of vibronic features that correspond to normal progressions of carbon−carbon bond stretching modes.[27,28] It is important to emphasize that, although the vibronic feature associated with the spectral origin (0−0) for symmetry-forbidden $S_1 \rightarrow S_0$ transitions is thought to be small, in fact this is strictly true only for symmetric polyenes under conditions where they retain their centers of inversion (e.g., in the gas phase).[56−58] Because the most prominent promoting modes in long polyenes are in-plane bending modes having frequencies <100 cm^{-1},[28] the values reported here for the spectral origins of the xanthophylls are likely to be very close to the pure electronic-state transition energies. The (0−0) bandwidths and intensities observed here should be thought of as being derived from a collection of vibronic bands associated with a distribution of slightly twisted conformers in the condensed phase solution. The difference between the true (0−0) bands and "false origins" built on the nontotally symmetric modes will not be resolved in the spectra shown in Figures 5 and 7, which have vibronic line widths >650 cm^{-1}.

Several Gaussian components were needed to reproduce each of the $S_1 \rightarrow S_0$ fluorescence spectral traces of the xanthophylls, and it is true that the fits shown in Figure 7 are not unique. Different combinations of Gaussian functions were able to faithfully reproduce the line shapes. Yet, it is also true that no matter how many Gaussian functions of varying amplitudes and widths were used to reconstruct the line shapes, in each case the (0−0) spectral origin was clearly identified as the lowest energy vibronic component, and the uncertainty in the value for the energy of this component fell within a very narrow range. The particular fits shown in Figure 7 represented by the parameters tabulated in Table 2 were obtained using broad Gaussian functions representing the coalescence of single and double bond stretching modes into peaks having vibronic spacings on the order of $\sim$1000 cm^{-1}. This magnitude of spacing between the peaks and the fact that the (0−2) feature is the most intense feature in all the spectra are typical vibronic signatures for carotenoid and polyene spectra.[28] The analysis of the data has revealed the (0−0) spectral origins for the $S_1 \rightarrow S_0$ transitions of violaxanthin, lutein, and zeaxanthin to be 15 580 $\pm$ 60, 14 570 $\pm$ 70, and 14 610 $\pm$ 40 cm^{-1}, respectively. The uncertainties are represented by the standard deviations from the mean obtained by averaging the results of a large number of acceptable fits.

It is important to compare these values with those deduced from previous studies. The three molecules studied here are representative of different groups of xanthophylls: those having two terminal rings in the conjugated chain (zeaxanthin), those having one terminal ring (lutein) and those having no terminal rings (violaxanthin). As discussed above, a different number of rings in conjugation will introduce different amounts of configurational disorder. Thus, the most direct comparison of the present results should be done with molecules having the same number of rings in association with the π-electron conjugation.

Zeaxanthin has the same extent of π-electron conjugation and the same number of rings as β-carotene whose S_1 energy has been reported by fluorescence spectroscopy to be 14 500 cm^{-1} in *n*-hexane and in single crystals,[24,59] and 14 200 $\pm$ 500 cm^{-1} in CS_2.[22] These values are in good agreement with the value of 14 610 $\pm$ 40 cm^{-1} reported here for zeaxanthin. The present result is also in excellent agreement with the value of 14 550 $\pm$ 90 cm^{-1} determined previously by fluorescence spectroscopy at room temperature, albeit with less spectral resolution.[18] The present value differs significantly, however, from that determined by Polívka et al.[32] who used transient $S_1 \rightarrow S_2$ absorption spectroscopy and reported a value of 14 030 $\pm$ 90 cm^{-1} for the S_1 energy of zeaxanthin. These investigators have postulated that the discrepancies in their results on zeaxanthin, violaxanthin (see below) and spheroidene compared to other reports may be explained by the fact that their experimental approach may be detecting different conformational species of the molecules in the S_1 excited state.[33] This issue will be discussed in more detail below.

Christensen et al.[27] have explored the electronic spectra of a series of "apo-carotenoids" that have one ring and "diapo-carotenoids" that have no rings. In these studies the signal-to-noise ratio of the $S_1 \rightarrow S_0$ fluorescence traces was not sufficient to assign unambiguously the spectral origin of the β-apo-6′-carotene, which has ten carbon−carbon double bonds, analogous to lutein. However, extrapolating the energy from clearly resolved spectra from the diapo-molecules having five to nine carbon−carbon double bonds yielded a value of $\sim$14 700 cm^{-1}

for β-apo-6′-carotene. This is in good agreement with the value of 14 570 ± 70 cm^{-1} value reported here for lutein.

Violaxanthin can be considered a quasi-diapo-molecule because the π-electron conjugation in the terminal rings is removed by the presence of the epoxide functional groups. A value of 15 580 ± 60 cm^{-1} was deduced here for the spectral origin of the $S_1 \rightarrow S_0$ transition of violaxanthin. This is in reasonable agreement with the ∼15 300 cm^{-1} values reported by Fujii et al.[25] and DeCoster et al.[26] for the S_1 energies of the open-chain, neurosporene and methoxyneurosporene molecules that have nine conjugated carbon−carbon double bonds. Also, recent spectroscopic investigations of C_{30}-open-chain carotenoids having π-electron chain lengths from seven to nine carbon−carbon double bonds[37] revealed very well-resolved spectra, from which was deduced a value of 15 500 ± 80 cm^{-1} for the "nonaene" molecule having nine conjugated carbon−carbon double bonds. This is in excellent agreement with the present result for violaxanthin. In fact, the positions of the vibronic bands of the $S_1 \rightarrow S_0$ emission spectrum of the nonaene molecule are almost perfectly superimposable with those from the emission spectrum of violaxanthin. The present assignment of the $S_1 \rightarrow S_0$ transition energy of violaxanthin is further supported by considering the energy difference between the (0−0) spectral origin and the Franck−Condon maximum, which corresponds to the (0−2) band for virtually all diapo-carotenoids. Diapo-carotenoids exhibit a relatively constant separation of ∼2600 cm^{-1} between these features.[25,27,37] The present data for violaxanthin reveal a difference of 2700 ± 400 cm^{-1}, which is entirely consistent with this behavior and lends strong support to the present assignment.

The present value for the S_1 energy of violaxanthin is significantly different from that which we reported previously on the basis of fluorescence studies carried out at room temperature.[18] We believe the source of this inconsistency is the lack of sufficient resolution of the (0−0) vibronic component in the room-temperature spectrum to assign clearly the position of the spectral origin. The value of 15 580 ± 60 reported here is more compelling because it is based on a clear observation of the vibronic feature associated with the spectral origin in the 77 K spectrum (Figure 7) and is in excellent agreement with data from analogous diapo-molecules having nine carbon−carbon double bonds. Polívka et al.,[32] however, using $S_1 \rightarrow S_2$ fast-transient absorption techniques, reported a value of 14 470 ± 90 cm^{-1} for the $S_1 \rightarrow S_0$ transition energy of violaxanthin. This is lower by ∼1000 cm^{-1} than the present determination on violaxanthin and the values of the other diapo-carotenoids possessing nine conjugated carbon−carbon double bonds. The reason for this discrepancy and the one involving zeaxanthin (see above) is still not clear. The original interpretation alluded to above, invoked the presence of various conformers created due to the excitation of low-energy torsional modes during $S_2 \rightarrow S_1$ relaxation.[33] This could also be the case at low temperatures, however, there the conformers must be frozen in the sample initially rather than formed by the process of relaxation between S_2 and S_1. A more recent idea is that because the fluorescence experiment is monitoring a forbidden $S_1 \rightarrow S_0$ transition, and the fast-transient absorption is probing an allowed $S_1 \rightarrow S_2$ transition, it could be that these two disparate techniques are detecting two different subsets of xanthophyll molecules in the S_1 state. Clearly, these hypotheses require further investigation.

Implications for the Roles of Xanthophylls in Photosynthesis. The maximum absorption of the Chl Q_y transition at 672 nm in the light-harvesting complexes of higher plants, corresponds to an $S_0 \rightarrow S_1$ (Q_y) transition energy of Chl *a* of 14 880 cm^{-1}. This is lower by 740 cm^{-1} than the S_1 energy of violaxanthin determined here, but higher by 270 cm^{-1} than the energy of the S_1 state of zeaxanthin. It is also higher by 200 cm^{-1} than the S_1 energy of lutein. It is important to emphasize that the values of the energies of the excited states of these xanthophylls measured here in EPA solutions are only approximations to their values in the native proteins. If the protein induces conformational distortion of the molecule, or if excitonic interactions with other pigments are substantial, the energies may change. Protein-induced twisting of the terminal rings or bending the polyene chain away from planarity could result in modulation of the excited-state energies of these molecules. Spectroscopic studies of the S_1 states of xanthophylls bound in different pigment−protein complexes are underway and will ultimately reveal the importance of structural deformations and interactions in controlling the photochemical properties and function of the xanthophylls in their native environment. Yet, because the $S_1 \rightarrow S_0$ transition is symmetry forbidden for xanthophylls, it possess a small transition dipole moment and is less susceptible to solvent-induced energy shifts compared to the strongly allowed $S_0 \rightarrow S_2$ transition. Therefore, unless the protein induces a major structural deformation of the xanthophyll, which would likely be accompanied by a substantial change in the line shape and position of the $S_0 \rightarrow S_2$ transition, the $S_1 \rightarrow S_0$ transition energies measured here are probably very close to those of the molecules in the protein environment. If we assume this is the case, then the S_1 state of zeaxanthin is energetically more suitable for quenching the excess excitation energy of Chl *a* than the S_1 state of violaxanthin. Also, the ∼1000 cm^{-1} difference in the S_1 energies of violaxanthin and zeaxanthin is clearly sufficient to affect the flow of energy among Chl molecules in antenna systems containing these xanthophyll cycle pigments. However, despite numerous attempts to detect Chl fluorescence quenching by zeaxanthin directly using fast-transient spectroscopy, it has never been observed. This may be explained by the fact that the process of quenching in vivo is not 100% and thus, must be kinetically relatively slow. The measured lifetime of the S_1 state of zeaxanthin is 9.0 ps (Table 1), which would lead to an exceptionally small steady-state population of the S_1 state of zeaxanthin that would be difficult to detect by transient absorption methods. Thus, the mechanism by which energy flow among Chl excited states is regulated by the enzymatic interconversion of violaxanthin and zeaxanthin involving preferential energy transfer from Chl to zeaxanthin, although energetically feasible, is not supported by any direct experimental results.

It should be noted that the carbon skeletal structures of violaxanthin and zeaxanthin are different. The double bonds in the rings at the terminal ends of the zeaxanthin molecule make an angle of ∼40° with respect to the extended polyene chain.[60,61] In violaxanthin, the rings are uncoupled from the π-electron conjugation owing to the presence of the epoxide functional groups, and therefore are less restricted in the conformational positions they may adopt compared to zeaxanthin. It has been suggested that the differences in molecular conformations of the xanthophylls may affect the assembly or structure of the light-harvesting pigment−protein complexes and lead to different extents of quenching.[20] In this model, either the xanthophylls or Chl *a* aggregates (or both) may be the quenchers, and the role of the xanthophylls is thought of as allosteric, effecting regulation of energy flow via modulation of the structure of the overall protein complex.

With regard to the light-harvesting role, the fact that the S_1 state of violaxanthin is significantly higher than the S_1 state of Chl *a* while the S_1 states of zeaxanthin and lutein are lower than the S_1 state of Chl *a* suggests that violaxanthin may be more adept than either zeaxanthin or lutein at energy transfer. On the sole basis of spectral overlap between the $S_1 \rightarrow S_0$ emission spectra of the xanthophylls and the absorption spectrum of Chl *a*, violaxanthin is predicted to be slightly more than a factor of 2 times more efficient at transferring energy from its S_1 state to Chl *a* than either of the other molecules.[18] Thus, while the difference in energies between the S_1 states of violaxanthin compared to zeaxanthin and lutein is significant, it is not likely to lead to a major advantage in antenna function, particularly since transfer via the S_2 states of the xanthophylls has also been shown to be contributing to the overall efficiency of energy transfer to Chl a in light-harvesting proteins.[62,63] Spectroscopic investigations of xanthophylls bound in the central protein binding sites of native and recombinant pigment−protein complexes did not show significant differences in the energy transfer efficiencies deduced either from spectral deconvolutions of the absorption and fluorescence excitation spectra or from dynamics measurements.[63,64] This supports the idea that in antenna proteins containing these xanthophylls, nature uses the diverse electronic and vibrational states of the pigments to harvest energy efficiently regardless of the precise positions of the excited-state energies of the donor molecules.

The S_1 state energies of violaxanthin, lutein, and zeaxanthin are presented here from an investigation using low-temperature fluorescence spectroscopy. The data are consistent with fluorescence studies carried out previously on analogous compounds and further emphasize the importance of fluorescence spectroscopy in determining the precise energies of the S_1 states of xanthophylls.

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References and Notes

(1) Horton, P.; Bowyer, J. R. In *Methods in Plant Biochemistry*; Harwood, J. L., Bowyer, J. R., Eds.; Academic Press: London, 1990; Vol. 4, p 259.

(2) Krause, G. H.; Weis, E. *Annu. Rev. Plant Physiol. Mol. Biol.* **1992**, *42*, 313.

(3) Horton, P.; Ruban, A. V.; Walters, R. G. *Annu. Rev. Plant Physiol. Mol. Biol.* **1996**, *47*, 655.

(4) Horton, P.; Ruban, A. V.; Young, A. J. Regulation of the structure and function of the light harvesting complexes of Photosystem II by the xanthophyll cycle. In *The Photochemistry of Carotenoids*; Frank, H. A., Young, A. J., Britton, G., Cogdell, R. J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; Vol. 8, p 271.

(5) Sapozhnikov, D. I.; Krasovskaya, T. A.; Mayevskaya, A. N. *Dokl. Akad. Nauk.* **1957**, *113*, 456.

(6) Hager, A. The reversible light-induced conversions of xanthophylls in the chloroplast. In *Pigments in Plants*; Czygan, F.-C., Ed.; Fischer: Stuttgart, 1980; p 57.

(7) Yamamoto, H. Y. *Pure Appl. Chem.* **1979**, *51*, 639.

(8) Yamamoto, H. Y.; Higashi, R. M. *Biochim. Biophys. Acta* **1972**, *267*, 538.

(9) Demmig-Adams, B.; Adams, W. W. I. *Annu. Rev. Plant Physiol. Mol. Biol.* **1992**, *43*, 599.

(10) Demmig-Adams, B.; Gilmore, A. M.; Adams, W. W., III. *FASEB J.* **1996**, *10*, 403.

(11) Demmig-Adams, B. *Biochim. Biophys. Acta* **1990**, *1020*, 1.

(12) Gilmore, A. M.; Yamamoto, H. Y. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 1899.

(13) Ruban, A. V.; Horton, P. *Plant Physiol.* **1999**, *119*, 531.

(14) Gilmore, A. M. *Physiol. Plant.* **1997**, *99*, 197.

(15) Niyogi, K. K.; Björkman, O.; Grossman, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 14162.

(16) Crofts, J.; Horton, P. *Biochim. Biophys. Acta* **1991**, *1058*, 187.

(17) Frank, H. A.; Cua, A.; Chynwat, V.; Young, A.; Gosztola, D.; Wasielewski, M. R. *Photosynth. Res.* **1994**, *41*, 389.

(18) Frank, H. A.; Bautista, J. A.; Josue, J. S.; Young, A. J. *Biochemistry* **2000**, *39*, 2831.

(19) Owens, T. G. Excitation energy transfer between chlorophylls and carotenoids. A proposed mechanism for nonphotochemical quenching. In *Photoinhibition of Photosynthesis*; Baker, N. R., Bowyer, J. R., Eds.; Bios Scientific Publishers: Oxford, U.K., 1994; p 95.

(20) Ruban, A. V.; Phillip, D.; Young, A. J.; Horton, P. *Photochem. Photobiol.* **1998**, *68*, 829.

(21) *The Photochemistry of Carotenoids*; Frank, H. A., Young, A. J., Britton, G., Cogdell, R. J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; Vol. 8.

(22) Andersson, P. O.; Bachilo, S. M.; Chen, R.-L.; Gillbro, T. *J. Phys. Chem.* **1995**, *99*, 16199.

(23) Bondarev, S. L.; Knyukshto, V. N. *Chem. Phys. Lett.* **1994**, *225*, 346.

(24) Koyama, Y.; Fujii, R. Cis−trans carotenoids in photosynthesis: Configurations, excited-state properties and physiological functions. In *The Photochemistry of Carotenoids*; Frank, H. A., Young, A. J., Britton, G., Cogdell, R. J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; Vol. 8, p 161.

(25) Fujii, R.; Onaka, K.; Kuki, M.; Koyama, Y.; Watanabe, Y. *Chem. Phys. Lett.* **1998**, *288*, 847.

(26) DeCoster, B.; Christensen, R. L.; Gebhard, R.; Lugtenburg, J.; Farhoosh, R.; Frank, H. A. *Biochim. Biophys. Acta* **1992**, *1102*, 107.

(27) Christensen, R. L.; Goyette, M.; Gallagher, L.; Duncan, J.; DeCoster, B.; Lugtenburg, J.; Jansen, F. J.; Hoef, I. v. d. *J. Phys. Chem. A* **1999**, *103*, 2399.

(28) Christensen, R. L. The electronic states of carotenoids. In *The Photochemistry of Carotenoids*; Frank, H. A., Young, A. J., Britton, G., Cogdell, R. J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1999; Vol. 8, p 137.

(29) Englman, R.; Jortner, J. *Mol. Phys.* **1970**, *18*, 145.

(30) Chynwat, V.; Frank, H. A. *Chem. Phys.* **1995**, *194*, 237.

(31) Frank, H. A.; Farhoosh, R.; Gebhard, R.; Lugtenburg, J.; Gosztola, D.; Wasielewski, M. R. *Chem. Phys. Lett.* **1993**, *207*, 88.

(32) Polivka, T.; Herek, J. L.; Zigmantas, D.; Akerlund, H. E.; Sundstrom, V. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4914.

(33) Polivka, T.; Zigmantas, D.; Frank, H. A.; Bautista, J. A.; Herek, J. L.; Koyama, Y.; Fujii, R.; Sundstrom, V. *J. Phys. Chem. B* **2001**, *105*, 1072.

(34) Polivka, T.; Zigmantas, D.; Herek, J. L.; Bautista, J. A.; Frank, H. A.; Sundstrom, V. *Springer Ser. Chem. Phys.* **2001**, *66*, 668.

(35) Walla, P. J.; Yom, J.; Krueger, B. P.; Fleming, G. R. *J. Phys. Chem. B* **2000**, *104*, 4799.

(36) Walla, P. J.; Linden, P. A.; Ohta, K.; Fleming, G. R. *J. Phys. Chem. B*, in press.

(37) Frank, H. A.; Josue, J. S.; Bautista, J. A.; van der Hoef, I.; Jansen, F. J.; Lugtenburg, J.; Wiederrecht, G.; Christensen, R. L. *J. Phys. Chem. B* **2002**, *106*, 2083.

(38) Khachik, F.; Beecher, G. R.; Whittaker, N. F. *J. Agric. Food Chem.* **1986**, *34*, 603.

(39) Dale, J. *Acta Chem. Scand.* **1954**, *8*, 1235.

(40) *Cis−trans isomeric carotenoids, vitamin A, and arylpolyenes*; Zechmeister, L., Ed.; Academic Press: New York, 1962.

(41) Christensen, R. L.; Kohler, B. E. *Photochem. Photobiol.* **1973**, *18*, 293.

(42) Basu, S. *Adv. Quantum Chem.* **1964**, *1*, 145.

(43) Andersson, P. O.; Gillbro, T.; Ferguson, L.; Cogdell, R. J. *Curr. Res. Photosynth., Proc. Int. Conf. Photosynth., 8th* **1990**, *2*, 117.

(44) Andersson, P. O.; Gillbro, T.; Ferguson, L.; Cogdell, R. J. *Photochem. Photobiol.* **1991**, *54*, 353.

(45) Nagae, H.; Kuki, M.; Cogdell, R. J.; Koyama, Y. *J. Chem. Phys.* **1994**, *101*, 6750.

(46) Kuki, M.; Nagae, H.; Cogdell, R. J.; Shimada, K.; Koyama, Y. *Photochem. Photobiol.* **1994**, *59*, 116.

(47) Koyama, Y.; Nishizawa, E.; Kuki, M.; Nagae, H. *Springer Proc. Phys.* **1994**, *74*, 215.

(48) Bautista, J. A.; Connors, R. E.; Raju, B. B.; Hiller, R. G.; Sharples, F. P.; Gosztola, D.; Wasielewski, M. R.; Frank, H. A. *J. Phys. Chem. B* **1999**, *103*, 8751.

(49) Frank, H. A.; Bautista, J. A.; Josue, J.; Pendon, Z.; Hiller, R. G.; Sharples, F. P.; Gosztola, D.; Wasielewski, M. R. *J. Phys. Chem. B* **2000**, *104*, 4569.

(50) Meyer, B. *Low-temperature spectroscopy*; American Elsevier: New York, 1971.

(51) Turro, N. J. *Modern Molecular Photochemistry*; University Science Books: Mill Valley, CA, 1991.

(52) Cosgrove, S. A.; Guite, M. A.; Burnell, T. B.; Christensen, R. L. *J. Phys. Chem.* **1990**, *94*, 8118.
(53) Tavan, P.; Schulten, K. *J. Chem. Phys.* **1979**, *70*, 5407.
(54) Tavan, P.; Schulten, K. *J. Chem. Phys.* **1986**, *85*, 6602.
(55) Frank, H. A.; Chynwat, V.; Desamero, R. Z. B.; Farhoosh, R.; Erickson, J.; Bautista, J. *Pure Appl. Chem.* **1997**, *69*, 2117.
(56) Geldorf, P. A.; Rettschnick, R. P. H.; Hoytink, G. *Chem. Phys. Lett.* **1971**, *10*, 549.
(57) Petek, H.; Bell, A. J.; Choi, Y. S.; Yoshihara, K.; Tounge, B. A.; Christensen, R. L. *J. Chem. Phys.* **1995**, *102*, 4726.
(58) Christensen, R. L.; Kohler, B. E. *J. Chem. Phys.* **1976**, *80*, 2197.
(59) Hashimoto, H.; Koyama, Y.; Mori, Y. *Jpn. J. Appl. Phys., Part 2* **1997**, *36*, L916.
(60) Sterling, C. *Acta Crystallogr.* **1964**, *17*, 1224.
(61) *Carotenoids*; Isler, O., Ed.; Birkhauser: Basel, 1971.
(62) Gradinaru, C. C.; van Stokkum, I. H. M.; Pascal, A. A.; van Grondelle, R.; van Amerongen, H. *J. Phys. Chem. B* **2000**, *104*, 9330.
(63) Croce, R.; Müller, M. G.; Bassi, R.; Holzwarth, A. R. *Biophys. J.* **2001**, *80*, 901.
(64) Frank, H. A.; Das, S. K.; Bautista, J. A.; Bruce, D.; Vasilev, S.; Crimi, M.; Croce, R.; Bassi, R. *Biochemistry* **2001**, *40*, 1220.