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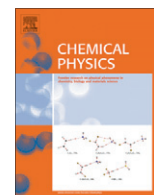

Ultrafast time-resolved absorption spectroscopy of geometric isomers of xanthophylls

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ABSTRACT

This paper presents an ultrafast optical spectroscopic investigation of the excited state energies, lifetimes and spectra of specific geometric isomers of neoxanthin, violaxanthin, lutein, and zeaxanthin. All-*trans*- and 15,15′-*cis*-β-carotene were also examined. The spectroscopy was done on molecules purified by HPLC frozen immediately to inhibit isomerization. The spectra were taken at 77 K to maintain the configurations and to provide better spectral resolution than seen at room temperature. The kinetics reveal that for all of the molecules except neoxanthin, the S_1 state lifetime of the *cis* isomers is shorter than that of the all-*trans* isomers. The S_1 excited state energies of all the isomers were determined by recording $S_1 \rightarrow S_2$ transient absorption spectra. The results obtained in this manner at cryogenic temperatures provide an unprecedented level of precision in the measurement of the S_1 energies of these xanthophylls, which are critical components in light-harvesting pigment-protein complexes of green plants.

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1. Introduction

Carotenoids consist of two groups of molecules: carotenes, which are hydrocarbons, and xanthophylls, which are the oxygenated derivatives of carotenes [1]. Members of both groups are characterized structurally by a long, conjugated, π-electron chain of carbon–carbon double bonds and spectrally, by strong visible absorption in the 400–500 nm region [2,3]. This absorption is due to an intense, allowed $S_0(1^1A_g^-) \rightarrow S_2(1^1B_u^+)$ transition. This notation considers carotenoids as belonging to the idealized C_{2h} point group [4,5]. Strictly speaking however, carotenoids do not possess this high degree of symmetry, but because many carotenoids exhibit the spectral characteristics of shorter polyenes that do have C_{2h} symmetry, it is customary to use the same electronic state notation [6]. Transitions between $S_0(1^1A_g^-)$ and the lowest excited singlet state, $S_1(2^1A_g^-)$, are forbidden by symmetry.

All-*trans* configurations of carotenes and xanthophylls can be induced thermally, photochemically or catalytically to undergo rotations about carbon–carbon double bonds to form *cis* geometric isomers [7]. The occurrence of *cis* geometric isomers of carotenoids can be confirmed through the presence of an additional absorption band in the ultraviolet region. This band is termed the "*cis*-peak," and it is associated with an $S_0(1^1A_g^-) \rightarrow S_3(1^1A_g^+)$ transition that becomes more allowed upon isomerization from *trans*-to-*cis* [1,7–9]. A number of *cis* geometric configurations as well as all-*trans* carotenoids have been resolved in three-dimensional X-ray crystal structures of pigment–protein complexes from photosynthetic organisms [10–13]. These findings have raised the question of whether nature has selected different geometric isomers in photosynthetic pigment–protein complexes for specific functional roles. Studies on different geometric isomers of carotenoids in solution and bound in photosynthetic pigment-protein complexes have addressed this issue using steady-state and time-resolved optical spectroscopic methods [14–18]. For example, the natural selection of the 15,15′-*cis* isomer in photosynthetic bacterial reaction centers has been postulated to be involved in the deactivation of harmful excited triplet states of bacteriochlorophyll [14,15,19]. In higher plants, xanthophylls are important components in a complex defense mechanism known as the xanthophyll cycle, which is thought to be a means by which excess excited states of chlorophyll (Chl) are quenched, thereby protecting the photosynthetic apparatus from photo-damage caused by excess light absorption [20–30]. The enzymatic de-epoxidation of the xanthophyll, violaxanthin, to zeaxanthin is one component of the xanthophyll cycle protection mechanism which also involves the formation of a *trans*-membrane pH gradient [31,32]. The mechanism is also facilitated in some thus far undetermined manner by a protein subunit known as PsbS [25,33–35]. Moreover, it has been suggested that the differences in molecular conformations of the xanthophylls which include not only violaxanthin and zeaxanthin, but also lutein and neoxanthin (Fig. 1), may affect the assembly or structure

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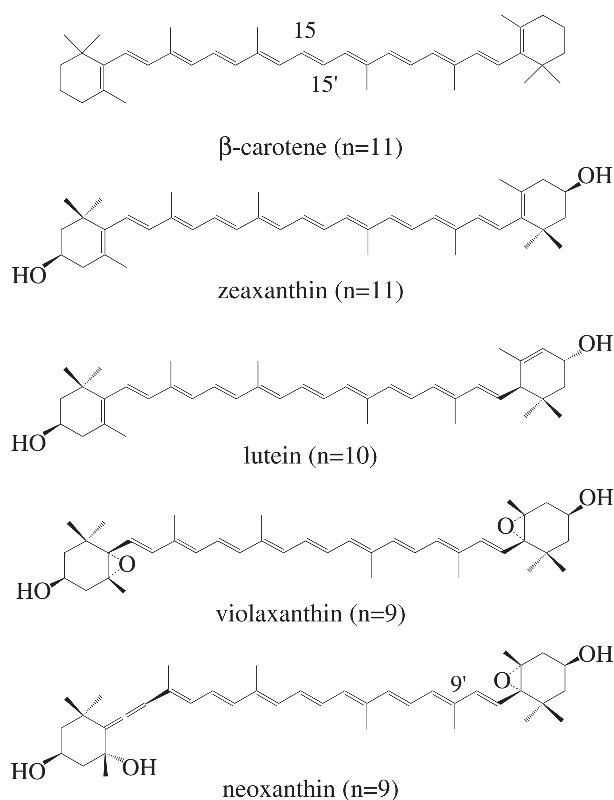

Fig. 1. Structures of the all-*trans* isomers of β-carotene, zeaxanthin, lutein, violaxanthin and neoxanthin.

of the light-harvesting pigment-protein complexes and lead to different extents of Chl excited state quenching [36,37]. Recent reports have implicated conformational twisting of neoxanthin, which exists in a 9′-*cis*-configuration in the LHCII antenna complex, as a critical component signaling aggregation and quenching [38,39]. Yet, despite the common occurrence, broad distribution, and physiological importance of xanthophylls in photosynthetic organisms, no detailed systematic study of the photophysical properties of the geometric isomers of these molecules has been carried out. In this work we present a steady-state and ultrafast time-resolved optical spectroscopic investigation of the excited state energies, lifetimes and spectra of purified all-*trans* and *cis* geometric isomers of the xanthophylls, neoxanthin ($n = 9$), violaxanthin ($n = 9$), lutein ($n = 10$), and zeaxanthin ($n = 11$), where n is the number of conjugated π-electron double bonds (Fig. 1). β-Carotene ($n = 11$) was also examined.

The spectroscopic experiments were carried out on the molecules purified by high performance liquid chromatography (HPLC) and immediately frozen to prevent isomerization. Another advantage to carrying out the experiments at low temperatures is that the spectral features are better resolved than those observed at room temperature which leads to higher precision in the determination of the excited state energy levels of the isomers. The overall goal of this work is to examine the relationship between the stereochemistry of xanthophylls and their photophysics which determine their biological functions in photosynthetic organisms.

2. Materials and methods

Lutein, violaxanthin and neoxanthin were obtained using methods described previously [40]. Briefly, approximately 10 g of spinach leaves were ground in 50 mL acetone/methanol (50/50 v/v technical grade), filtered, dried, then redissolved in 87/10/3 v/v/v acetonitrile (Fisher)/methanol (Fisher)/water (Sigma), filtered, and injected to a Millipore Waters 600E high performance liquid chromatography (HPLC) system. The HPLC was equipped with a Model 996 single diode array detector and used a 3.9 mm × 300 mm Nova-Pak C_{18} reverse phase column, a gradient mobile phase of 100% A to 100% B in 40 min (A, 87/10/3 v/v/v acetonitrile (Fisher)/methanol (Fisher)/water (Sigma); B, ethyl acetate (Fisher)), and a flow rate of 1 mL/min. Zeaxanthin and 15,15′-*cis*-β-carotene were obtained as gifts from F. Hoffman LaRoche. Geometric isomers of β-carotene were obtained using a YMC C_{30} carotenoid column and an isocratic mobile phase of acetone (Fisher) with a flow rate of 1 mL/min. Isomers of zeaxanthin and lutein were obtained using the same column and an isocratic mobile phase of 97/3 v/v methanol (Fisher)/MTBE (Fisher) with a flow rate of 1 mL/min. Isomers of violaxanthin and neoxanthin were obtained using same column and an isocratic mobile phase of 87/10/3 v/v/v acetonitrile (Fisher)/methanol (Fisher)/water (Sigma) with a flow rate of 1.5 mL/min. The purified geometric isomers were obtained by collecting the peaks from the HPLC as they emerged. Subsequently the samples were dried with a gentle stream of nitrogen gas in the dark at room temperature and stored at −80 °C until ready for use.

All of the spectroscopic experiments carried out at 77 K used 2-methyltetrahydrofuran (2-MTHF) as a solvent which forms a clear glass at that temperature. Steady-state absorption spectra were recorded using a Varian Cary-50 spectrophotometer and a custom-made (Kontes) liquid nitrogen cryostat. Transient absorption spectra were taken using a femtosecond time-resolved spectrometer system described previously [41,42] and an optical cryostat (Janis STVP100). The visible light and NIR continuum probe beams were generated by a 3 mm Sapphire plate obtained from Ultrafast Systems LLC. Two different types of detectors were used: an Ocean Optics Model S2000 charge-coupled detector with a 2048 pixel array for detection in the visible range, and a 512 pixel array SU-LDV high resolution InGaAs Digital Line Camera from Sensors Unlimited in the NIR region. The samples were excited at wavelengths corresponding to their spectral origin (0–0) vibronic bands and had optical densities of ~0.5 in a 2 mm path length cuvette at the excitation wavelength. The pump beam was set to 1 μJ energy focused in 1 mm spot, corresponding to an intensity of $(3.2 \pm 0.1) \times 10^{14}$ photons pulse^{-1} cm^{-2}. The integrity of the samples was checked by taking absorption spectra before and after every transient absorption experiment. Surface Explorer (v.1.0.6) was used for dispersion correction in the transient absorption datasets, and ASUFit 3.0 program provided by Dr. Evaldas Katilius at Arizona State University was used for global fitting analysis. The temporal response function of the instrument was obtained for each measurement as a parameter in the global fitting analysis (Table 1).

3. Results

3.1. High performance liquid chromatography

Room temperature (RT) absorption spectra of selected elution peaks corresponding to all-*trans* and *cis* geometric isomers of the carotenoids were obtained using the single diode array detector on the HPLC and are shown in Fig. 2. In all cases the main visible bands of the *cis* isomers of the molecules are blue-shifted relative to their corresponding all-*trans* isomers; e.g. by ~4 nm for β-carotene and ~11 nm for neoxanthin. Also, the *cis* isomers display pronounced absorption in the ultraviolet (UV) region between 300 and 350 nm. These UV absorption bands from carotenoids are referred to as "*cis*-peaks" and occur when a symmetry forbidden transition becomes allowed upon *trans*-to-*cis* isomerization. The large ampli-

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