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Towards elucidating the energy of the first excited singlet state of xanthophyll cycle pigments by X-ray absorption spectroscopy

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Abstract

The first excited singlet state (S_1) of carotenoids (also termed $2A_g^-$) plays a key role in photosynthetic excitation energy transfer due to its close proximity to the S_1 (Q_y) level of chlorophylls. The determination of carotenoid $2A_g^-$ energies by optical techniques is difficult; transitions from the ground state (S_0, $1A_g^-$) to the $2A_g^-$ state are forbidden ("optically dark") due to parity (g ← //→ g) as well as pseudo-parity selection rules (− ← //→ −). Of particular interest are S_1 energies of the so-called xanthophyll-cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) due to their involvement in photoprotection in plants. Previous determinations of S_1 energies of violaxanthin and zeaxanthin by different spectroscopic techniques vary considerably. Here we present an alternative approach towards elucidation of the optically dark states of xanthophylls by near-edge X-ray absorption fine structure spectroscopy (NEXAFS). The indication of at least one π* energy level (about 0.5 eV below the lowest $1B_u^+$ vibronic sublevel) has been found for zeaxanthin. Present limitations and future improvements of NEXAFS to study optically dark states of carotenoids are discussed. NEXAFS combined with simultaneous optical pumping will further aid the investigation of these otherwise hardly accessible states.

Keywords: Carotenoid; Xanthophyll cycle; NEXAFS; Violaxanthin; Zeaxanthin; X-ray absorption spectroscopy

1. Introduction

Carotenoids are a widespread class of natural polyenic pigments which have a vast variety of physiological functions [1]. Oxygen-containing derivatives of carotenes are called xanthophylls. Carotenoids are of particular importance in photosynthetic organisms: as structural components of photosynthetic pigment–protein complexes (accessory) light-harvesting pigments and major photoprotective agents [2]. The latter two functions are linked to singlet–singlet excitation energy transfer (EET) from carotenoids to chlorophylls (Chls) and singlet–singlet as well as triplet–triplet EET in the opposite direction [3]. It is assumed that the proximity of the first singlet excited state

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Abbreviations: Chl, chlorophyll; EET, excitation energy transfer; IP, ionization potential; LHC II, light-harvesting complex II; NEXAFS, near-edge X-ray absorption fine structure spectroscopy; XAS, X-ray absorption spectroscopy; XUV, extreme ultraviolet

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of relevant carotenoids (S_1, $2A_g^-$)[5] and the Q_y (S_1) state of Chls provides favorable conditions for EET [3]. However, such a close proximity of the energy levels may also enable back-transfer from Chls to carotenoids [4]. Chl-to-carotenoid EET is possibly also involved in photoprotective mechanisms operative under high-light conditions. These mechanisms are triggered in higher plants by the (dark-) reversible conversion of violaxanthin (via antheraxanthin) to zeaxanthin (the so-called "xanthophyll cycle"). The direction of carotenoid-to-Chl EET is assumed to depend on the S_1 energy of the respective carotenoid relative to S_1 of Chl *a*. The determination of the S_1 energy of carotenoids by absorption measurements is hindered by the A_g^- character of both S_0 and S_1. Thus, parity and pseudo-parity selection rules for one-photon transitions (g ← //→ g, and − ← //→ −) render a radiative transition between S_0 and S_1 forbidden [5–7]; S_1 ($2A_g^-$) is called "optically dark".

Attempts to determine the S_1 energies of relevant carotenoids have been made using different techniques such as ultra-sensitive steady-state fluorescence [4], time-resolved S_1–S_2 transient absorption [9,10], two-photon [11] and resonance Raman excitation [12,13] spectroscopy (see also Refs. [8,14] for reviews). Of particular interest in this respect is the so-called xanthophyll cycle pigments (violaxanthin, antheraxanthin and zeaxanthin) due to their involvement in plant photoprotection. It was proposed that–due to the extension (*n*) of the conjugated double-bond system–S_1 of zeaxanthin ($n = 11$) is located below Q_y of Chl *a* and therefore may quench excited Chl *a*. In contrast, violaxanthin ($n = 9$) is assumed to be energetically suited as an excitation donor to Chl *a* [15]. However, S_1 energies of the individual pigments as determined by different techniques vary by as much as 1000 cm^{-1}: Frank and coworkers obtained 14.550 and 14.880 ($\pm$ 90 cm^{-1}) for zeaxanthin and violaxanthin, respectively, by fluorescence spectroscopy [4]. Polivka and coworkers estimated a value of 13.900 ($\pm$ 300 cm^{-1}) for both, zeaxanthin and violaxanthin attached to recombinant light-harvesting complex II (LHC II), from S_1–S_2 transient absorption measurements [10]. Predictions on the basis of the energy gap law resulted in 14.170 and 15.290 cm^{-1} for zeaxanthin and violaxanthin, respectively [15]. Thus, the hypothesis regarding participation of xanthophyll cycle pigments in direct Chl excitation quenching is still a matter of debate.

An alternative approach to estimate the energies of "optically dark" states based on near edge X-ray absorption fine structure (NEXAFS) spectroscopy has recently been proposed [16]. X-ray absorption spectroscopy (XAS) is a valuable tool to study the bonding of a selected atomic species to their nearest intra, as well as intermolecular environment [17]. For molecules with extended π-electronic systems (such as polyenes) the transitions from the inner atomic shell 1s to unoccupied molecular orbitals appear in XAS as intense, sharp peaks located a few eV below the ionization potential (IP) on the carbon K-edge. Both intensity and spectral position of these transitions reflect the electronic structure of the molecule including its "optically dark" state(s).

In this report we attempt to elucidate the energies of the "optically dark" S_1 states of violaxanthin and zeaxanthin by NEXAFS. The xanthophyll-cycle pigments, in particular violaxanthin, undergo light-induced, reversible *trans–cis* isomerization in leaves [18] and also in isolated LHC II [19,20]. It was demonstrated that this isomerization alters molecular organization of LHC II considerably [19,20]. Moreover, the singlet energy levels of the pigments are also affected by isomerization as indicated by a shift of the principal ($1A_g^- \rightarrow 1B_u^+$) absorption band as well as an enhanced intensity of a band corresponding to the $1A_g^- \rightarrow 1A_g^+$ transition (also called the "*cis*" band). In order to study whether isomerization affects the $2A_g^-$ energy level, too, different isomers of both, violaxanthin and zeaxanthin have been examined.

2. Materials and methods

All-*trans* zeaxanthin was a generous gift of Hoffmann-La Roche (Basel, Switzerland). 13-*cis* and 9-*cis* zeaxanthin were obtained by iodine-catalyzed photoconversion of the all-*trans* form following the procedure of Molnar et al. [21]. Violaxanthin (all-*trans*, 9-*cis*, and 13-*cis*) was isolated from blossoms of *Viola tricolor* [22]. Isomeric forms of xanthophylls were separated chromatographically on a 25 cm, 4.6 mm diameter C-30-coated high performance liquid chromatography (HPLC) column (YMC GmbH, Germany). A solvent mixture of acetonitrile:methanol:water (72:8:3, v/v) was the mobile phase. Further details of xanthophyll isolation, purification and identification have been described previously [19,20]. Amorphous xanthophyll films were deposited onto a Si_3N_4 support of 100 nm thickness by evaporation of acetone (violaxanthin) or an ethanol–hexane (1:1, v/v; in case of zeaxanthin) solutions at 40 °C. Samples were prepared in dim light and kept under a vacuum (better than 5×10^{-5} mbar). UV/VIS absorption spectra of the samples were recorded with Lambda 900 spectrophotometer (Perkin-Elmer). NEXAFS experiments were carried out with the experimental set-up at the Max-Born-Institut (MBI) as described in detail elsewhere [16,23,24]. Briefly, pulses of a Nd:YAG laser (Brilliant Quantel, $\lambda = 1064$ nm, $\tau = 3$ ns, $f = 10$ Hz, $E = 200$ mJ) were focused onto a rotating Cu-cylinder target to create a plasma that emits soft X-ray pulses with about the same duration as the laser pulse. The

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[5] Note that the usually employed carotenoid excited state designations (A_g^-, B_u^+, etc.) are strictly valid only for C_{2h}-symmetric molecules, e.g. all-*trans* polyenes. However, highly (and even unsymmetrically) substituted carotenoids are apparently well described, too. The notation of the carotenoids higher excited states will follow a recent review [8]. The terminal state of the allowed transition, $1B_u^+$, is called S_2, whereas the "optically dark" state $1B_u^-$ is not numbered, since it may be located below or above S_2—depending on conjugated chain length.

XUV-spectrometer consists of a free-standing transmission grating (10.000 lines mm^{-1}) with an entrance slit of 50 μm and a 16-bit slow scan back-illuminated XUV-CCD camera (Photometrics). The spectral resolution of the equipment was about 0.9 eV at 280 eV. All spectra displayed are raw data without smoothing. Gaussian deconvolution was performed using the software Origin (version 6.0, MicroCal Inc.).

The quality of the NEXAFS spectra critically depends on the thickness and homogeneity of the film formed upon evaporation, therefore great care was taken to obtain thin ($d \ll 1$ μm) non-aggregated layers. Attempts were made to prepare films from different isomeric forms of both xanthophylls which are known to form different aggregates [25]. We were, however, unable to obtain good quality films from 13-*cis* violaxanthin.

3. Results and discussion

The UV/VIS absorption spectrum of dissolved 13-*cis* violaxanthin is presented in Fig. 1. The assignment of the three vibronically substructured transitions from S_0 to higher excited singlet states is also indicated. In this scheme, the "optically dark" $2A_g^-$ state is marked by a dashed line (not to scale). This lowest π* state is characterized by configuration interaction with the inclusion of doubly excited configurations [26,27]. Between $1B_u^+$ and $2A_g^-$, at least one more dark state, $1B_u^-$, is located. The latter state has been originally proposed based on theoretical calculations [26,28] and has been recently verified experimentally in different carotenoids [27].

The NEXAFS spectrum (in the vicinity of the carbon, C, K-edge) of an all-*trans* violaxanthin film deposited onto a Si_3N_4 support is shown Fig. 2. The absorption in the pre-edge region (around 285 eV) represents transitions from C1s to π* states, where π* denotes molecular orbitals belonging to excited states. Adjacent to the pre-edge region is the most prominent spectral feature corresponding to the 1s ionization potential (IP). Similar overall shapes of the NEXAFS spectra have been obtained for all investigated violaxanthin and zeaxanthin isomers.

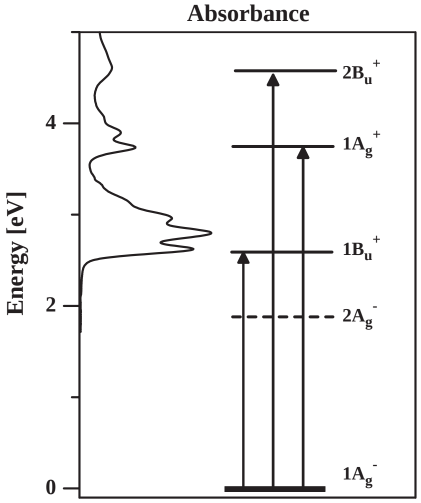

Fig. 1. Absorption spectrum of 13-*cis* violaxanthin in acetonitrile:methanol:water (72:8:3 v/v). An energy level scheme comprising the relevant electronic states (vibrational sublevels are not indicated) is given. Arrows indicate electronic transitions from the ground state. The $2A_g^-$ level (not accessible by one-photon absorption) is indicated by a dashed line.

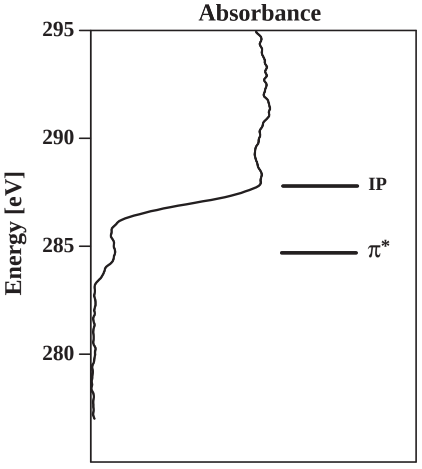

Fig. 2. NEXAFS spectrum of all-*trans* violaxanthin deposited onto a Si_3N_4 support.

The UV/VIS absorption spectrum of a 9-*cis* zeaxanthin film deposited onto a Si_3N_4 support is displayed Fig. 3. Compared to the absorption spectrum of monomeric 9-*cis* zeaxanthin in solution (not shown), a partial lack of the vibronic substructure as well as broadening of the band are obvious. Similar observations have been made previously with β-carotene [23]. The behavior appears to be typical for the formation of pigment aggregates [29–32]. A Gaussian deconvolution of the spectrum results in a minimum of three subbands (cp. also the figure legend). The simulated

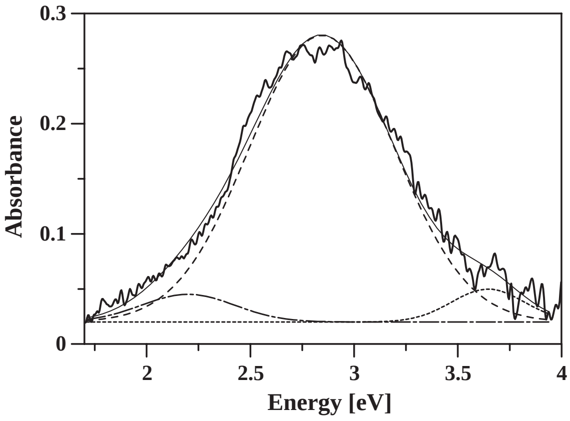

Fig. 3. Absorption spectrum of 9-*cis* zeaxanthin deposited onto a Si_3N_4 support (thick solid line). Gaussian deconvolution components (dashed lines) and the fit to the data (thin solid line) are shown. Parameters: center energies and band widths (full width at the half-maximum, FWHM) of the components, $E_1=2.20$ eV, $\Delta E_1=0.45$ eV; $E_2=2.84$ eV, $\Delta E_2=0.70$ eV; and $E_3=3.65$ eV, $\Delta E_3=0.35$ eV.

spectrum (composed of the Gaussian components) is also shown in Fig. 3.

A magnified representation of the pre-edge spectral region of the NEXAFS spectrum of 9-*cis* zeaxanthin is shown in Fig. 4 (lower panel). NEXAFS spectra of the two other zeaxanthin isomers showed no significant differences to the one given in Fig. 4. On the other hand, there is no indication of isomerization processes during the experiments: absorption spectra of the samples, recorded before and after the NEXAFS measurement, with particular focus on the short-wavelength region of the $1A_g^- \rightarrow 1A_g^+$ transition indicative of the respective isomer showed no alterations.

Besides the experimental NEXAFS spectrum and its second derivative (on top), the result of a Gaussian deconvolution into five subbands is shown in Fig. 4 (cp. also the legend). The deconvolution has been performed with the following assumptions: (i) the shape of the spectrum at higher photon energies is mainly that of the atomic K-edge absorption, (ii) the initial peak positions of the subbands correspond to the minima in the second derivative of the spectrum, (iii) the main spectral feature in the pre-edge region corresponds to the transition to $1B_u^+$ (with the second, third and fourth components from the low-energy side of the pre-edge spectrum, the distance between which roughly corresponds to the distance between the Gaussian components of the UV/VIS absorption spectrum shown in Fig. 3). The initial spectral widths of the three subbands were set to values obtained from the deconvolution of the UV/VIS spectrum; (iv) the remaining two Gaussian components (the low-energy and the high-energy

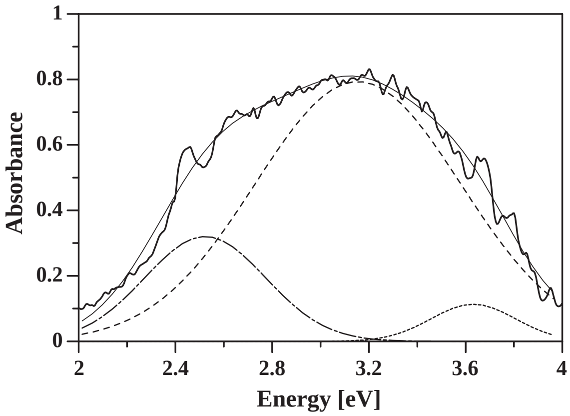

Fig. 5. Absorption spectrum of all-*trans* violaxanthin deposited to a Si_3N_4 support (thick solid line). Gaussian deconvolution components (dashed lines) and the fit to the data (thin solid line) are shown. Parameters of the components: center energies and band widths (FWHM), $E_1=2.52$ eV, $\Delta E_1=0.50$ eV; $E_2=3.15$ eV, $\Delta E_2=0.85$ eV; and $E_3=3.63$ eV, $\Delta E_3=0.35$ eV.

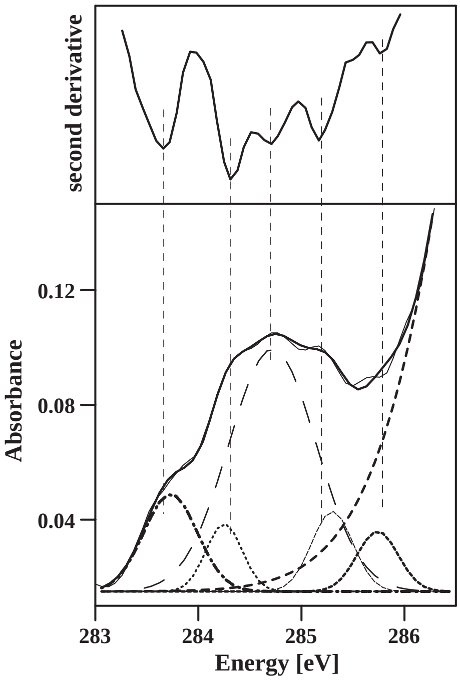

Fig. 4. NEXAFS spectrum of 9-*cis* zeaxanthin deposited onto a Si_3N_4 support (thick solid line). Gaussian deconvolution components (dashed lines) and the fit to the data (thin solid line) are shown. The K-edge is indicated by the bold dashed line. Parameters: center energies and band widths (FWHM), $E_1=283.7$ eV, $\Delta E_1=0.5$ eV; $E_2=284.2$ eV, $\Delta E_2=0.35$ eV; $E_3=284.7$ eV, $\Delta E_3=0.85$ eV; $E_4=285.3$ eV, $\Delta E_4=0.4$ eV; $E_5=285.7$ eV, $\Delta E_5=0.4$ eV; and $E_6=288.8$ eV (ionization potential, according to [2]). The top panel shows the second derivative of the original spectrum with the minima indicated.

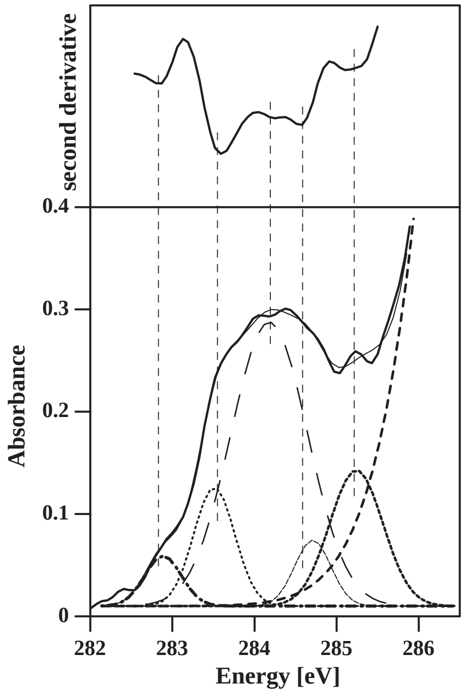

Fig. 6. NEXAFS spectrum of all-*trans* violaxanthin deposited onto a Si_3N_4 support (thick solid line). Gaussian deconvolution components (dashed lines) and the fit to the data (thin solid line) are shown. The K-edge is indicated by the bold dashed line. Parameters: center energies and band widths (FWHM), $E_1=282.9$ eV, $\Delta E_1=0.45$ eV; $E_2=283.5$ eV, $\Delta E_2=0.50$ eV; $E_3=284.2$ eV, $\Delta E_3=0.95$ eV; $E_4=284.7$ eV, $\Delta E_4=0.45$ eV; $E_5=285.2$ eV, $\Delta E_5=0.65$ eV; and $E_6=288.8$ eV (ionization potential, according to [17]). The top panel shows the second derivative of the original spectrum with the minima indicated.

ones) have been fitted to the pre-edge spectrum without initial constraints.

Following the above given interpretation, the subband at 284.2 eV can be assigned to a C1s→π* transition. Consequently, the subband at 283.7 eV is due to a low-energetic π* state, which is located about 0.5 eV below the $1B_u^+$ spectral origin. According to theoretical [33] and recent experimental work with carotenoids of comparable extension of the conjugated double bond system ($n = 9$ and 11) [34], two "optically dark" states, $2A_g^-$ and $1B_u^-$, would be expected. For zeaxanthin, the former ($2A_g^-$) is located about 0.84 eV (6770 cm^{-1}) below $1B_u^+$, according to [4,10]. The energetic difference between $1B_u^+$ and $1B_u^-$ is about 0.46 eV (3750 cm^{-1}) for other carotenoids with $n = 11$ [34]. The experimental value obtained in this study (0.5 eV below $1B_u^+$) falls in-between these previously determined values. The spectral resolution of our NEXAFS spectra (>0.1 eV after deconvolution) does not allow to decide whether the band centered at 283.7 eV represents one state or a convolution of both "optically dark" states. On the other hand, it is conceivable that $1B_u^-$ of zeaxanthin is located much closer to $1B_u^+$ than expected for carotenoids with $n = 11$, because the transition $1A_g^- \rightarrow 1B_u^+$ of zeaxanthin behaves more like in carotenoids with $n = 9$. The gap between $1B_u^+$ and $1B_u^-$ is only on the order of 0.1 eV (800 cm^{-1}) for the latter [34]. Therefore, $1B_u^-$ of zeaxanthin may be obscured by the lowest vibronic level(s) of $1B_u^+$, and the subband at 283.7 eV may correspond solely to the transition to the $2A_g^-$ state.

To tackle this problem, NEXAFS investigations with violaxanthin would be useful. However, preparations with all violaxanthin isomers resulted in absorption spectra similar to that of all-*trans* violaxanthin as shown in Fig. 5. Considerably larger broadening and bathochromic shifts than with zeaxanthin films (cp. Fig. 3) and β-carotene [23] obviously indicate an aggregation of the pigment, so that a straightforward interpretation of the NEXAFS spectra of these violaxanthin films is precluded at present. However, the magnified pre-edge region (Fig. 6) of the NEXAFS spectrum of all-*trans* violaxanthin shows the same general pattern as zeaxanthin: There is an unusual overall shift to lower energies (e.g. the lowest-energy subband is located below 283 eV), which necessitates further investigations before any interpretation can be made.

In conclusion, we have shown that–despite the complex nature of C1s NEXAFS spectra of carotenoids (including, e.g., the overall response from a manifold of individual carbon atoms, a vibronic structure, several pre-edge transitions)–there is a general pattern which opens a new route of access to the "optically dark" lowest excited π* states. In particular, we have found a NEXAFS subband at 283.7 eV for 9-*cis* zeaxanthin, which can be interpreted as transition from C1s to the lowest excited π* state (possibly corresponding to the "optically dark" $2A_g^-$). Together with the NEXAFS data of β-carotene [23], peridinin in vitro as well as in the peridinin-Chl *a*-protein (PCP), Chl *a*, and other organic molecules [35], there is sufficient experimental background for future parallel theoretical studies. Moreover, recent experimental developments render NEXAFS spectroscopy with simultaneous optical pumping [16] feasible, even in a "single-shot" regime [36]. The latter development is an important step towards unambiguous interpretation of the spectra as well as non-destructive investigations of biologically relevant samples.

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