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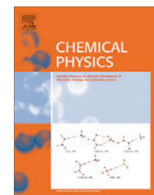

Photoprotective role of the xanthophyll cycle studied by means of modeling of xanthophyll–LHCII interactions

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ABSTRACT

The problem of photoprotection associated with the xanthophyll cycle is addressed by examination of effects of exogenous violaxanthin and zeaxanthin on isolated antenna complex LHCII. Analysis of electronic absorption spectra suggests multiple sites of binding of violaxanthin and zeaxanthin to the protein environment. Xanthophyll binding results in enhancement of excitonic bands at ca. 530 and 740 nm, as concluded on the basis of Resonance Light Scattering spectra. The energy states attributed to these excitonic bands are concluded to quench singlet excitations. Fluorescence Lifetime Imaging Microscopy shows that both xanthophylls, but in particular zeaxanthin, promote formation of supramolecular structures characterized by extremely low fluorescence yield. Photoprotective meaning of the xanthophyll cycle is concluded to be primarily based upon role of zeaxanthin in promoting formation of supramolecular structures of LHCII characterized by high rate of energy dissipation.

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1. Introduction

Carotenoids are a class of pigments widespread in living organisms and playing diverse physiological functions [1]. In the photosynthetic apparatus carotenoids act as accessory pigments in the antenna, absorbing light energy and transferring it to chlorophylls which further convey the excitation energy to the reaction centers. They also play other important physiological roles, such as structural stabilization of photosynthetic membranes [2–5] and the membrane-bound proteins [6,7] and, very importantly, photoprotection against oxidative damage associated with light stress [1]. The photoprotection by carotenoids is based on photophysical quenching of (bacterio)chlorophyll triplets and reactive oxygen species, however, there is also accumulating evidence for their involvement in chemical quenching [8]. Moreover, the protective action of carotenoids seems to strongly depend on their environment as shown in model studies [9,10]. In higher plants and some algae subjected to bright illumination exceeding capacity of the photochemical reactions of the reaction centers, the antenna xanthophyll violaxanthin is converted to antheraxanthin and eventually to zeaxanthin, via the enzymatic de-epoxidation [11,12]. Zeaxanthin, accumulated under light stress conditions, replaces violaxanthin in the xanthophyll-binding sites of the antenna pigment–protein complexes, including the largest photosynthetic antenna complex LHCII [11]. The fact that the xanthophyll cycle activity is associated with overall response of the photosynthetic apparatus to light stress conditions suggests photoprotective meaning of the cycle. From the chemical standpoint, violaxanthin, a 3,3′-dihydroxy-carotenoid with two epoxy groups located at the 5,6 and 5′,6′ positions, is exchanged in the course of the de-epoxidation to a pigment with additional double bonds in the 5,6 and 5′,6′ positions, conjugated to the double bond system (zeaxanthin). Elongation of the conjugated double bond system, from 9 (violaxanthin) to 11 (zeaxanthin), results in energy lowering of the electronic excitation energy levels, including the S1 state [13] which is roughly isoenergetic with the chlorophyll *a* Q_y state. Fine tuning of energy of the S1 state of the xanthophyll cycle pigments with respect to the energy levels of chlorophyll *a* has been proposed to exchange a carotenoid playing a role of photosynthetic antenna (violaxanthin) to a carotenoid enabled to quench excessive singlet excitations (zeaxanthin) [14]. Such a mechanism is very often referred to as the "energy gear shift model". On the other hand, the theoretical calculations and the time resolved spectroscopic studies have shown that the energy differences of the S1 state of the antenna-protein-bound violaxanthin and zeaxanthin are not large

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enough to support physiological importance of such a mechanism [15,16]. A new concept has been proposed, similar to the energy gear shift hypothesis, based on the formation of the chlorophyll–zeaxanthin charge transfer complexes having a potential of the singlet excitation quenching. However, this mechanism has also been recently questioned by presenting the results of the quantum chemical calculations [17] and the two-photon two-color experiments in combination with the chlorophyll lifetime measurements [18,19]. Physiological importance of the xanthophyll cycle was also discussed in terms of structural differences of violaxanthin and zeaxanthin, which gave rise to different supramolecular organization of the antenna complexes, e.g. xanthophyll-induced LHCII aggregation [20,21]. On the other hand, the mechanism of singlet excitation quenching in LHCII has been demonstrated, which operates independently of the protein aggregation [22]. Very recently, the xanthophyll-specific light-driven molecular mechanism operating in isolated LHCII has been reported, and the xanthophyll cycle pigment violaxanthin has been implied as being involved in this process [23,24]. Two possible light-driven mechanisms have been considered in analysis of the effects observed: a violaxanthin photo-isomerization [25] and a configurational transformation of violaxanthin assisted by photo-thermal effect [25,26]. The light-driven mechanism has been concluded to affect the organization status of the trimeric structures of LHCII [27] and to influence the singlet excitation quenching capacity within the complex [24]. Another important aspect of such a mechanism could be increasing of availability of violaxanthin to enzymatic de-epoxidation [24]. The mechanisms reported seem to be important for operation and regulation of the xanthophyll cycle but, on the other hand, the findings do not extend our understanding regarding a physiological importance of accumulation of zeaxanthin under light stress conditions in the photosynthetic apparatus. In the present work we address the problem of photoprotective activity of the xanthophyll cycle by means of model study of violaxanthin–LHCII and zeaxanthin–LHCII interactions.

2. Materials and methods

The largest photosynthetic antenna pigment–protein complex, LHCII, was isolated from fresh spinach leaves according to Krupa et al. [28] with slight modifications [23]. Preparation purity and integrity was monitored by means of electrophoresis, HPLC and mass spectrometry [23]. LHCII was suspended in a Tricine buffer (20 mM, pH 7.6) containing 10 mM KCl. Violaxanthin was isolated from *Viola tricolor* blossoms and zeaxanthin was isolated from fruits of *Lycium barbarum* and purified chromatographically as described previously [25,29]. Digalactosyldiacylglycerol (DGDG) was purchased from Lipid Products (UK) and detergent n-dodecyl-β-D-maltoside (DM) was purchased from Sigma Chem. (USA). Exogenous-xanthophyll-enriched LHCII samples were prepared according to the following protocol. LHCII was suspended in the buffer containing 0.025% DM and transferred to glass test tubes containing deposited film composed of a mixture of exogenous either violaxanthin or zeaxanthin and DGDG or DGDG without exogenous xanthophylls in the case of the control sample. A film was deposited via evaporation under stream of gaseous nitrogen from pigment and lipid mixture in ethanol. DGDG was present in the film in order to prevent xanthophyll aggregation and to facilitate xanthophyll binding to the protein. The molar fraction of LHCII:DGDG:exogenous xanthophyll was as 1:3:3. Suspension was vigorously vortexed for 40 min, diluted 10-fold to induce protein oligomerization and subjected to centrifugation for 10 min at 15,000*g*, in order to collect aggregated LHCII. Pellet was suspended in the buffer containing 0.025% DM, in order to disassemble aggregates of the complex.

Electronic absorption spectra were recorded with Shimadzu 160A-PC UV–Vis spectrophotometer (Japan) and fluorescence excitation, emission and resonance light scattering (RLS) spectra were recorded with Cary Eclipse fluorescence spectrophotometer from Varian (Australia). Spectra were corrected for the Xenon lamp characteristics and for the sensitivity of photomultiplier. More details of fluorescence measurements were described previously [30]. Circular dichroism spectra were recorded on a JASCO J-815 spectropolarimeter, equipped with a R928 photomultiplier (Hamamatsu, Japan). The spectra were recorded in 2 cm path length quartz cells, with the following data collection parameters: increment 0.2 nm, time constant 1 s, spectral bandwidth 1 nm, scanning speed 100 nm/min. Photoacoustic measurements were performed with the home-constructed spectrometer and according to the methodology described previously [31]. Photoacoustic spectra were corrected for lamp intensity by dividing original spectra by the spectrum of carbon black, recorded under the same conditions. Thermal deactivation (TD) was calculated by dividing a corrected photoacoustic signal by value of one minus transmission ($1 - T$) at each wavelength. Infrared absorption spectra were recorded with the Fourier-transform infrared (FTIR) spectrometer, model Vector 33 from Bruker (Germany). Before measurements (40 min) and during all measurements the instrument was purged with dry argon. The attenuated total reflection (ATR) configuration was used with a 10-reflections ZnSe crystal (45° cut). Typically 10 interferograms were collected, averaged and Fourier transformed. Absorption spectra in the region between 4000 and 600 cm^{-1}, at a resolution of one data point every 2 cm^{-1}, were obtained using a clean crystal as the background. ATR crystals were cleaned with organic solvents. Spectral analysis was performed with OPUS software (Bruker, Germany) and Grams/AI 8.0 spectroscopy software from Thermo Electron Corporation (USA).

Fluorescence lifetime imaging microscopy (FLIM) measurements were performed on a confocal MicroTime 200 (Picoquant, Germany) system coupled with OLYMPUS IX71 microscope. Photons were collected from different places of chosen area with 60X water immersed infinity corrected objective (NA 1.2, OLYMPUS). Samples were placed on non-fluorescent Menzel-Glaser #1 cover slips and mounted on a stage piezo-scanner. The fluorescence was excited by a solid state pulsed laser (635 nm – LDH-P-C-635B) with repetition rate of 20 MHz. The excitation light was spectrally cleaned by z636/10x bandpass filter (Chroma Technology Corp.) and delivered to the main optical unit by single mode fiber. A 30 μm pinhole and combination of emission 720/240 and 650 longpass (Semrock) filters were placed on the detection path. Fluorescence photons were collected with the Perkin–Elmer SPCM-AQR-14 single photon sensitive avalanche photodiode (APD) and processed by the PicoHarp300 time-correlated single photon counting (TCSPC) module based on detection of photons of a periodical light signal. Decay data analysis was performed using SymPhoTime (v. 5.0) software package, that controlled the data acquisition as well.

All experiments have been performed at room temperature (25 ± 1 °C). The spectroscopic data presented on all the figures below were collected from one series of preparation (modification with exogenous xanthophylls) but all the experiments have been repeated at least five times and the spectral effects presented are found to be reproducible.

3. Results and discussion

3.1. Binding of violaxanthin and zeaxanthin to LHCII

Incubation of LHCII with exogenous violaxanthin and zeaxanthin, under experimental conditions which prevent the protein

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