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Does the xanthophyll cycle take part in the regulation of fluidity of the thylakoid membrane?

Wiesław I. Gruszecki [1] and Kazimierz Strzałka [2]

[1] *Institute of Physics, Maria Curie-Skłodowska University, Lublin (Poland) and* [2] *Institute of Molecular Biology, Jagiellonian University, Kraków (Poland)*

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Upon strong illumination (30 min, 1400 W m^{-2}) of intact pea leaves the spin label-monitored fluidity of the isolated thylakoid membranes decreases in the peripheral region of the hydrophobic core (as measured with the 5-doxylstearic acid spin label) but remains unchanged in the membrane interior (as measured with the 16-doxylstearic acid spin label). At the same time, as a consequence of the light driven de-epoxidation of violaxanthin to zeaxanthin, the molar ratio of these two pigments in the photosynthetic apparatus decreases from 6.25 to 0.64. The light-induced alterations of thylakoid fluidity have been attributed to the membrane-modifying effect of zeaxanthin. Such concept is supported by the results of analogous experiments in which the xanthophyll cycle-related zeaxanthin accumulation was partially blocked by the de-epoxidase inhibitor, dithiothreitol.

Introduction

The xanthophyll cycle is a process resulting in the interconversion of two photosynthetic carotenoids [1–3]. Two enzymatic reactions are involved in this process: the light-dependent de-epoxidation of violaxanthin to zeaxanthin via antheraxanthin and the opposite reaction of light-independent epoxidation. The reactions of the xanthophyll cycle have been explored in detail and a model of its functioning is commonly accepted. The physiological importance of the cycle remains, however, elusive. There are several hypotheses concerning the significance of the xanthophyll cycle which have been proposed in recent years. One of these assumes that the xanthophyll cycle is a part of a regulatory system functioning by influencing thylakoid membrane properties [1,2]. The finding that light-stress-related chlorophyll fluorescence quenching in vivo is correlated with the zeaxanthin formation has led Demmig-Adams et al. [4,5] to the conclusion that the xanthophyll cycle might be involved in the dissipation of an excesss of absorbed light energy – a protective function. Therefore, in the present paper we provide evidence that the presence of zeaxanthin within thylakoid membranes changes their fluidity. Such a result seems to be in agreement with the both hypotheses cited above. Variable fluidity of a membrane can play a regulatory role. The process is, in our opinion, also of photoprotective significance, as will be discussed in detail below.

Correspondence: W.I. Gruszecki, Institute of Physics, Maria Curie-Skłodowska University, 20–031 Lublin, Poland.

Materials and Methods

Intact pea (*Lathyrus odoratus* L.) leaves grown under controlled environmental conditions (photoperiod 14 h, temperature 21 ± 2 °C, relative humidity 55–60%, light intensity 50 W m^{-2}) were kept in darkness for 6 h and then infiltrated for 1.5 h with 2 mM Hepes buffer (pH 7.8) containing 0.4 mM KCl, 0.04 mM EDTA and 0.3 M mannitol or the same buffer containing additionally 2 mM dithiothreitol (DTT). Infiltrated leaves were illuminated for 30 min with a white light (1400 W m^{-2} at the sample). The lamp was combined with a 4.5 cm water filter in order to eliminate heat. Leaf samples were kept on wet filter paper during light treatment in order to protect them against dehydratation. Thylakoid membranes were isolated from leaves immediately after illumination according to a standard procedure described previously [6]. Violaxanthin and zeaxanthin interconversion was quantitatively monitored by the

extraction of the pigments from leaves and the chromatographic separation on silica gel plates (Merck). A benzene/ethyl acetate/methanol (75:20:5, v/v) solvent system was used as a developing phase [7]. Pigment concentration was evaluated in ethanol according to the extinction coefficients reported in the literature [7]. Samples of thylakoid membranes were added to a glass tube with a film of 5-doxylstearic acid spin label (5-SASL) or 16-doxylstearic acid spin label (16-SASL) (Sigma). The concentration of the label applied was 1 μmol per 1 mg of the total amount of chlorophyll in the sample. EPR spectra from non-illuminated samples were recorded with a Varian E-3 spectrometer working in the X band and equipped with Varian variable temperature-controlling accesories. Samples to be measured were placed in a 0.7 mm diameter capillary and sealed with wax. The EPR measurements were carried out under the following conditions: microwave power 10 mW, modulation amplitude 2 G in the case of spectra scanning and 5 G or 10 G for determining the accurate position of the maxima; time constant, 0.3–1.0 s; scan range, 3200–3300 G; scan time, 4 min.

Spectrophotometric measurements were carried out with a Beckman DU-7 spectrophotometer.

Electrophoretic separation of the thylakoid proteins was carried out by PAGE according to the general procedure described elsewhere [8].

Results

Effect of light

Table I presents values of the concentration ratio between two main pigments of the xanthophyll cycle: violaxanthin and zeaxanthin (V/Z). A significant rate of zeaxanthin formation by the cycle can be achieved only when light intensity is comparable with that saturating photosynthesis or higher [1–3]. The light treatment applied in the present study caused the determined value of V/Z to decrease from 6.25 (after infiltration, before light treatment) to 0.64 (after light treatment, see Table I). This is an indication of the activity of the cycle. The pool of violaxanthin was converted to a large extent into zeaxanthin, since the ratio V/Z reached a value lower than 1. The inset of

TABLE I

Effect of light treatment (30 min at 1400 W m^{-2}) on the concentration ratio between the two main pigments of the xanthophyll cycle: violaxanthin and zeaxanthin (V/Z) in pea leaves infiltrated (90 min) with a Hepes buffer without DTT or containing 2 mM DTT

	V/Z	
	before illumination	after illumination
No DTT	6.25	0.64
2 mM DTT	9.26	3.63

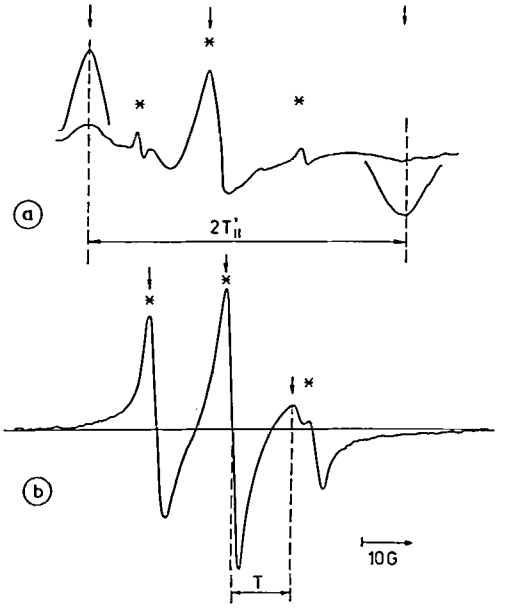

Fig. 1. Typical EPR spectrum of 5-doxylstearic acid spin label (a) and 16-doxylstearic acid spin label (b) incorporated into thylakoid membranes. The discussed in the text empirical parameters $2T'_{\|}$ and T are indicated. The maxima related with the spin label present in lipid phase are marked with arrows (↓), present in the buffer are marked with asterisks (*). Spectrum scanned at modulation amplitude 2 G, maxima in Fig. 1a at 10 G.

Fig. 2 presents additionally the diagram corresponding to each curve, representing the proportion between violaxanthin and zeaxanthin (the ratio of an area of white and dark sector gives a value of V/Z). The effect of light-treatment on EPR spectra of 5-SASL and 16-SASL were measured parallel to the xanthophyll pigment composition. The n-SASL spin labels applied in the present study are commonly used to monitor the fluidity of model as well as biological membranes [9,10,11]. The maximum splitting value $2T'_{\|}$ (see Fig. 1a), an empirical parameter related to an order parameter of the alkyl chain and the rate of the alkyl chain motion of the spin label in a lipid core, is accepted as reflecting the fluidity of a membrane [9–11] and was also employed to examine the system investigated. An increase in maximum splitting is an indication of decreased fluidity of a membrane. The increase of maximum splitting value as an effect of the light treatment may be observed by comparison of the temperature profiles presented in Fig. 2a. This increase, which in temperatures higher than 10°C reaches a value of about 2 G, is significant (maximum error in determining $2T'_{\|}$ is less than 0.25 G) and reflects pronounced decrease of the fluidity in the peripheral region of the membrane hydrophobic core close to lipid headgroup zone. Such a membrane depth corresponds to the localization of the free-radical segment of the spin-label molecule (5th carbon atom of the stearic acid). The fluidity of the thylakoid membrane

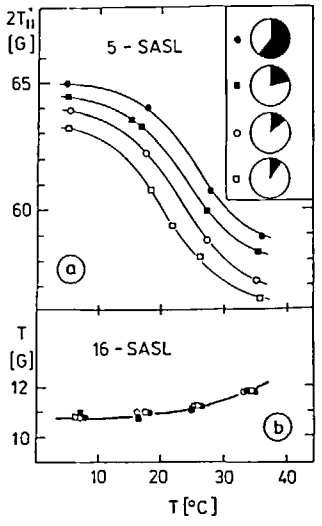

Fig. 2. Temperature dependencies of a maximum splitting $2T'_{II}$ of a 5-doxylstearic acid spin label (a) and the empirical parameter T of a 16-doxylstearic acid spin label (b) incorporated into thylakoid membranes isolated from leaves after the following treatment: ○, infiltrated with buffer; ●, infiltrated with buffer and illuminated; □, infiltrated with DTT-containing buffer; ■, infiltrated with DTT-containing buffer and illuminated. Inset: schematic representation of the proportion between violaxanthin (white sector) and zeaxanthin (dark sector) corresponding to the values of parameter V/Z determined in the samples. V/Z = ○, 6.25; ●, 0.64; □, 9.26; ■, 3.63. Composition of the buffer and the conditions of light treatment – see Materials and Methods.

more towards its interior could be monitored with the 16-SASL probe. Fig. 1b presents the typical EPR spectrum of this spin label incorporated into the thylakoid membranes [11]. The relatively high rotational freedom of the free radical-segment placed within a hydrophobic core at a depth corresponding to the 16th carbon atom of the stearic acid is manifested by the decreased width of the EPR spectrum of the label on the magnetic field scale, as compared to 5-SASL. This phenomenon is, however, related to the new spectral feature consisting of superposition of the resonance maxima of the spin label incorporated to the membrane (arrows, Fig. 1) and those present within a polar phase of the buffer (asterisks, Fig. 1) [11]. This phenomenon, combined with the lack of any evident high-field maximum, makes it impossible to evaluate the accurate value of the 16-SASL $2T'_{II}$ parameter in the present study. To monitor the fluidity changes inside the thylakoid membranes by means of 16-SASL, we decided to apply an empirical parameter, T, expressing the distance between the centre of the central line (h_o) and the maximum (h_-) related directly to the motional freedom within a lipid phase and not overlapped by other maxima. The value of T increases as motional freedom of the label (the membrane fluidity) increases. As can be seen from Fig. 2b, the increase of the temperature has some effect on the increase of the thylakoid membrane fluidity. However, in contrast to the peripheral region of the hydrophobic core (as demonstrated with 5-SASL), the fluidity of the thylakoid interior probed with the 16-SASL does not depend on zeaxanthin accumulation (Fig. 2b).

Effect of DTT

DTT-infiltration of leaves has been shown to be an effective procedure preventing zeaxanthin accumulation [12,13], since the drug is a known inhibitor of the enzyme de-epoxidase [14]. DTT itself does not influence photosynthesis [15–17] and is also unlikely that it interacts directly with a lipid core, affecting its fluidity. Such a conclusion drawn on a basis of a chemical structure of dithiothreitol is highly supported by a comparison of the temperature profiles of $2T'_{II}$ (Fig. 2). The curves corresponding to DTT-infiltrated leaves are located below or above the nonilluminated control one, depending only on light intensity. This is an indication of a lack of any specific effect of the drug on the physical properties of a membrane. On the other hand, the difference in membrane fluidity between thylakoids from control and DTT-treated leaves not subjected to light stress argues against any involvement of such light-dependent phenomena as photoinhibition, degradation of some thylakoid proteins, lateral migration of phosphorylated LHCII, etc. in the modification of membrane physical properties.

One of the important factors governing membrane fluidity is the ratio of lipids and membrane proteins [11,18,19]. However, as we found that neither DTT treatment nor illumination in our experimental system had any apparent effect on the electrophoretic pattern of thylakoid proteins (data not shown), this rules out the possibility that these factors may influence membrane physical properties indirectly by changing thylakoid protein content and composition. Even if one assumes that there occurs some light stress or DTT-induced degradation of polypeptides indistinguishable by PAGE, this should result not in a decrease of membrane fluidity, as we found, but on the contrary in its increse [11,18,19].

Zeaxanthin formation in DTT-poisoned leaves was partially blocked, as monitored by a value of V/Z (Table I). Similarly to the previous studies [12], there is a difference between a value of V/Z determined for nonilluminated leaves infiltrated with a buffer containing and not containing DTT. This may be explained in terms of some low activity of de-epoxidation driven by the weak light (<5 W m^{-2}) during infiltration in absence of DTT and the complete blocking of this process in the presence of the inhibitor.

Discussion

The effect of carotenoid pigments on biological membranes has been reported [20] and considered as a

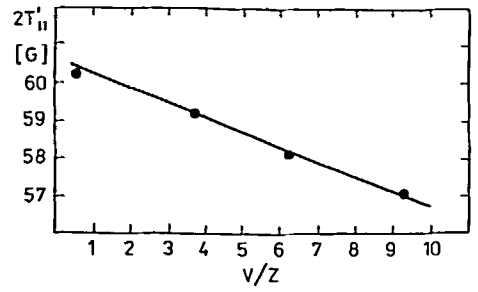

Fig. 3. Dependence of maximum splitting $2T'_{II}$ on violaxanthin to zeaxanthin ratio (V/Z) at 30°C produced on the basis of results presented in Fig. 2a.

mechanism in *Prokaryota* playing a role similar to that of cholesterol in animal cell membranes [21,22]. It has been demonstrated by means of light-scattering observations of liposome suspension [22] that xanthophyll pigments influence the mechanical properties of lipid membranes. It has been also recently shown by means of a spin-label technique that zeaxanthin decreases fluidity of dimyristoylphosphatidylcholine membranes about twice as strongly as cholesterol at the same concentration (Subczynski et al., unpublished data). As shown here, light-induced zeaxanthin formation by the xanthophyll cycle affected thylakoid membrane fluidity in the peripheral region of the hydrophobic core but had no effect on the membrane interior. Fig. 3 presents dependence of $2T'_{II}$ on V/Z at 30°C produced on basis of the temperature profiles depicted in Fig. 2a. The linearity of the dependence indicates a correlation between these two parameters. The fact that such a correlation is produced by experimental points obtained for the light-treated samples as well as those not exposed to the light-stress conditions argues against the possibility of artefacts connected with the changes of thylakoid membrane fluidity by some other light-dependent processes such as thylakoid protein phosphorylation or lipid peroxidation. The specific modifying effect only with respect to the region of the hydrophobic core close to lipid headgroup zone seems to be related to the postulated orientation of zeaxanthin within the lipid membrane [23]. The pigment molecule being anchored in the two opposite polar regions of the bilayer is able to form three hydrogen bonds with lipids (with their keto groups or with polar heads) by each peripheral hydroxyl group located at the position 3 and 3′ of the pigment molecule. Such a mechanism seems to be the main reason for the zeaxanthin-induced reinforcement of the thylakoid membrane surface region.

Zeaxanthin – the xanthophyll pigment appearing only transiently, in response to irradiation, is synthesized at the inner side of the thylakoid membrane and epoxidized to violaxanthin at the opposite side [1–3]. Such a transmembrane organization of the xanthophyll cycle implies the direct presence of zeaxanthin within the lipid environment of the thylakoid membrane, in contrast to violaxanthin, reported to be an accessory pigment [24] attached to chloroplast proteins [25]. Such a different localization of the pigments of the xanthophyll cycle can be also concluded from the fact that violaxanthin has been found to be a better protector of antenna pigments, in contrast to zeaxanthin protecting better the thylakoid membrane lipids when subjected to photoinhibitory treatment [12]. An excess of light has strong deletorious effects related to the blocking of the reaction centers and singlet – singlet energy transfer in the antenna system, chlorophyll triplet formation, photosensitization and photodestruction [26–29]. Protective action by zeaxanthin with respect to thylakoid membranes seems to be a mechanism resulting directly from its fluidity-modifying effect reported in the present study. Highly unsaturated lipid membranes are a very sensitive target for the destructive action of singlet oxygens and free radicals. Penetration of these species deeply into membrane hydrophobic interior, where majority of the susceptible fatty-acid double bonds are located, should obviously be limited with decreased fluidity of the membrane regions adjacent to polar lipid headgroup zone. This photoprotective aspect of the presence of zeaxanthin within thylakoid membranes may be considered as an additional to the well-known ability of carotenoid pigments to act as a singlet oxygen quencher and scavenger of free radicals [30].

The presence of large amounts of unsaturated lipids in thylakoid membranes [31] indicates the importance of their highly fluid state for photosynthetic activity. Any decrease in a fluidity of thylakoids would affect their physiological state. Therefore the question arises as to whether the light-stress-protective action of zeaxanthin has at the same time some deletorious effects on photosynthetic processes due to decreased fluidity of the thylakoid membrane? Two points should be discussed when answering this question. First of all it must be stressed that a zeaxanthin-induced decrease in fluidity occurs only in the membrane region adjacent to the polar lipid headgroup zone, while the mobility of fatty acid chains within the hydrophobic interior of the membrane remains unaffected. Secondly, the commonly accepted view about the high degree of thylakoid membrane fluidity due to its unique composition of lipids possessing a high content of polyunsaturated fatty acids should be clarified more precisely. When comparing the fluidity ($2T'_{II}$ parameter) of the region of the thylakoid membrane close to lipid headgroup zone using the 5-SASL spin probe at room temperature (23–25°C), it turns out that in spite of the unusually high proportion of unsaturated fatty acids it is still more rigid than the corresponding region of Golgi membrane from rat liver [32] and liposomes made of egg phosphatidylcholine (Strzałka, K. unpublished data)

dimyristoylphosphatidylcholine [33] or dipalmitoylphosphatidylcholine [34], even if the latter two lipids contain only saturated fatty acids. In the light of these findings it can be concluded that it is not the polar headgroup region but rather the hydrophobic interior of the thylakoid membrane which requires a highly fluid state for photosynthetic processes to occur efficiently. Thus, we may assume that zeaxanthin, decreasing the fluidity only in the region adjacent to the headgroup zone without affecting the fatty acid chain mobility in the membrane hydrophobic interior, could perform its light-stress-protective action without interfering with essential photosynthetic processes such as, for example, turnover rate of the quinone pool [35] or lateral diffusion of other thylakoid membrane components [36].

As a conclusion of the results and discussion presented above a hypothesis is proposed according to which one of the possible physiological functions of the light-induced zeaxanthin accumulation via the xanthophyll cycle is to provide the thylakoid membrane with the specific structure-modifying agent involved in a complex protective mechanism against light stress. Such hypothesis is consistent with the recent finding [37] that the xanthophyll cycle pigment level is considerably higher in plants growing under strong light conditions.

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