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LHCII, the major light-harvesting pigment-protein complex is a zeaxanthin epoxidase

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Several experiments are presented indicating that LHCII, the major light-harvesting pigment-protein complex of Photosystem II isolated from rye leaves, exhibits properties of an enzyme catalyzing the epoxidation of zeaxanthin to violaxanthin. On the basis of this as well as other recent findings, a new model of a spatial organization of the xanthophyll cycle within a thylakoid membrane is proposed.

Introduction

Sapozhnikov et al. first observed in 1957 [1] a light-dependent mechanism in the photosynthetic apparatus changing an epoxidation state of xanthophyll pigments. In light of later studies by the research groups of Yamamoto, Siefermann Harms and Hager (review papers Refs. 2, 3 and 4, respectively) this mechanism, now called the violaxanthin or the xanthophyll cycle, was established to be composed of a two enzymatic reactions: light-independent epoxidation of zeaxanthin (β,β-carotene-3,3′-diol) via antheraxanthin (5,6-epoxy-zeaxanthin) to violaxanthin (5,6,5′,6′-diepoxyzeaxanthin) and the light-dependent de-epoxidation of violaxanthin via antheraxanthin to zeaxanthin. The de-epoxidase enzyme isolated by Hager and Perz [5] appeared to be a water-soluble, 54 kDa protein. The optimum pH of this enzyme found to be close to 5 indicated its localization on the lumen side of the thylakoid membrane. An epoxidase enzyme has not been isolated up to now, but washing experiments suggest that it is a membrane-bound protein [6]. In this paper we present results of several experiments indicating that LHCII, the major light-harvesting pigment-protein plays a role as an epoxidase enzyme.

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Materials and Methods

Winter rye (*Secale cereale* L., cv. Pastar) was cultivated in a greenhouse with supplemented light and a relative humidity of 60%. LHCII was isolated from 10-day-old leaves illuminated directly before treatment for 30 min with white light of 500 μmol m^{-2} s^{-1} ('light' LHCII). LHCII was isolated and purified by successive cation precipitation as described in detail previously [7,8]. Winter rye LHCII preparation isolated according to this method was found to contain mainly two polypeptides 26 and 27 kDa (electrophoretic purity > 95%) [7,8]. In order to minimize the cation concentration in purified LHCII, the preparation was resuspended and washed three times in 100 mM EDTA (pH 7.8) and at a chlorophyll concentration of 0.1 mg/ml. Purified LHCII was then resuspended in 50 mM Tricine-NaOH buffer (pH 7.8) containing 50% (by volume) glycerol and stored at −40°C until further use. Experiments were performed with three different batches of LHCII preparation. Synthetic zeaxanthin was purchased from Hoffmann La Roche and repurified chromatographically directly before use on silica gel plates with a solvent system: benzene/ethyl acetate/methanol (75 : 20 : 5, v/v) as a moving phase [9]. Absorption spectra and time course of absorbance changes were registered without stirring with a Shimadzu 160A spectrophotometer. Polarographic measurements were performed with a Hansatech oxygen electrode DW1 connected to an IBM compatible com-

puter via Hansatech IF/1 interface board. Zeaxanthin dispersion in a Tricine-NaOH buffer (50 mM, pH 7.8) containing 0.1 M sorbitol was prepared by sonication for 5 min of a buffer in a tube containing the thin film of the pigment deposited by evaporation in nitrogen. Sonication was performed with an automatic ultrasonic disintegrator type UD-20 (Techpan, Poland) at a frequency 22 kHz and amplitude 1–8 μm. Carotenoids were analyzed by means of a thin-layer chromatography as described above for zeaxanthin purification. Concentration of pigments extracted from the chromatographic plate was determined spectrophotometrically on the basis of the extinction coefficients reported in the literature [9]. Chlorophyll analyses were performed in ethanol according to Lichtenthaler [10].

Results and Discussion

Changes in absorption of the photosynthetic apparatus registered at 505 nm reported first by Strasser and Butler [11] were directly correlated with the de-epoxidation (A_{505} increases, [12]) and epoxidation (A_{505} decreases, [6]) in the xanthophyll cycle. At present these changes are commonly used to follow epoxidation and de-epoxidation processes in chloroplasts as well as in intact leaves [13]. Fig. 1 presents time-courses of the absorbance changes registered at 505 nm (ΔA_{505}) in the dispersion of zeaxanthin in a buffer mixed at $t = 0$ with the suspension of the isolated LHCII. Very distinctive decrease of ΔA_{505} during incubation indicates that one of the possible processes taking place in the sample is a zeaxanthin epoxidation to violaxanthin in the presence of LHCII. The simultaneous increase of a concentration of violaxanthin in the samples, monitored by thin-layer chromatography, proves that the

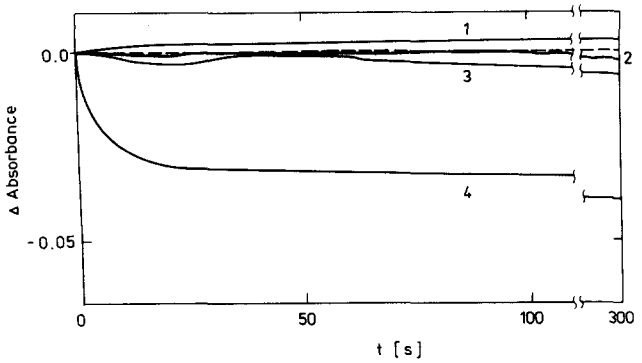

Fig. 1. Traces of time-courses of absorbance changes registered at 505 nm from the dispersion of zeaxanthin in a Tricine buffer pH 7.8 mixed at $t = 0$ with suspension of isolated LHCII. Final concentration of exogenous zeaxanthin was $4.3 \cdot 10^{-6}$ M (sample Nos. 1, 3, 4) or 0 M (sample No. 2). Final concentration of total chlorophyll was $3.9 \cdot 10^{-5}$ M. Concentration of NADPH was $5 \cdot 10^{-4}$ M (sample Nos. 1, 2, 4) or 0 M (sample No. 3). In one case (sample No. 1) dispersion of zeaxanthin was deoxygenated before the experiment by bubbling with nitrogen for 10 min. Interrupted line indicates relative absorbance (ΔA) equal to zero.

TABLE I

Concentration of violaxanthin (V) and neoxanthin (N) (mmol xanthophyll per mol total chlorophyll) determined in preparation of LHCII (before incubation) and in preparation of LHCII incubated for 5 min with exogenous zeaxanthin and NADPH (after incubation)

V/N: molar ratio of violaxanthin to neoxanthin, $\overline{V/N}$: mean value of $V/N \pm$S.D. For conditions of incubation, see legend to Fig. 1.

	Determination No.	V	N	V/N	$\overline{V/N}$
Before incubation	1	12.5	48.7	0.26	
	2	16.4	41.0	0.40	
					0.34 ± 0.03
	3	14.2	39.4	0.36	
	4	14.0	40.1	0.35	
After incubation	5	18.9	41.2	0.46	
	6	23.8	37.4	0.64	
					0.57 ± 0.06
	7	18.6	36.9	0.50	
	8	26.8	38.9	0.69	

process we are dealing with is in fact zeaxanthin epoxidation to violaxanthin followed by a ΔA_{505} decrease (see Table I). In the sample No. 4 presented in Fig. 1 violaxanthin to neoxanthin ratio (V/N) determined before experiment (in pure 'light' LHCII) and after 5 min of incubation of LHCII with exogenous zeaxanthin changed from a value of $V/N(t = 0) = 0.35$ to $V/N(t = 5) = 0.69$. Results of some other determinations of pigment concentration and a ratio V/N are presented in Table I.

During enzymatic epoxidation of zeaxanthin to violaxanthin NADPH is required as a reducing agent and molecular oxygen is another important co-substrate (see reviews [2–4]). As can be observed from Fig. 1 the molecular processes followed by a decrease of ΔA_{505} are evidently stopped (trace 1) or slowed down (trace 3) in the absence of molecular oxygen or NADPH, respectively. ΔA_{505} does not decrease in the case of the dispersion of zeaxanthin without LHCII (results not shown) or in the case of LHCII without zeaxanthin (trace No. 2, Fig. 1). This is an additional control to prove that nonenzymatic epoxidation of zeaxanthin or LHCII sedimentation is not responsible for a pronounced absorbance decrease, observed at 505 nm. The difference spectra of the samples (after incubation minus before incubation, not shown) demonstrated characteristic negative band in the region of 505 nm, typical for the activity of the xanthophyll cycle [11]. Fig. 2 presents polarographically-monitored consumption of molecular oxygen in the LHCII and zeaxanthin mixture (trace 3), additionally supporting our view about an enzymatic activity of the pigment-protein. Fig. 2 presents also results of the two control experiments: zeaxanthin dispersion without LHCII (trace 1) and LHCII suspension without exogenous zeaxanthin (trace 2). As

it can be seen, in these two cases oxygen consumption is considerably lower than in the case of LHCII and zeaxanthin mixture. Consumption of the molecular oxygen which may be estimated on the basis of the increase of violaxanthin concentration reported in Table I under assumption that formation of one epoxide consumes one oxygen molecule (two oxygen molecules in one zeaxanthin to violaxanthin transition) is about one order of magnitude lower than the oxygen consumption presented in Fig. 2 (trace 3 minus trace 2). This discrepancy may be partially explained by simultaneous antheraxanthin formation in the sample but we think that the main reason of much higher rate of oxygen consumption combined to its different kinetics is the fact that in the case of pollarographic measurements sample was stirred but stirring was not applied during spectrophotometric measurements.

Fig. 3 presents a model of the spatial localization of pigments and proteins essential for the operation of the xanthophyll cycle. The following points were considered as a base for the hypothetical model:

1. Localization and orientation of the pigments of the xanthophyll cycle within a thylakoid membrane.
2. Localization of the de-epoxidase enzyme.
3. Localization of LHCII with respect to the thylakoid membrane.
4. The fact that LHCII may serve as an epoxidase of zeaxanthin.

The experimental evidence related to the points presented above is as follows:

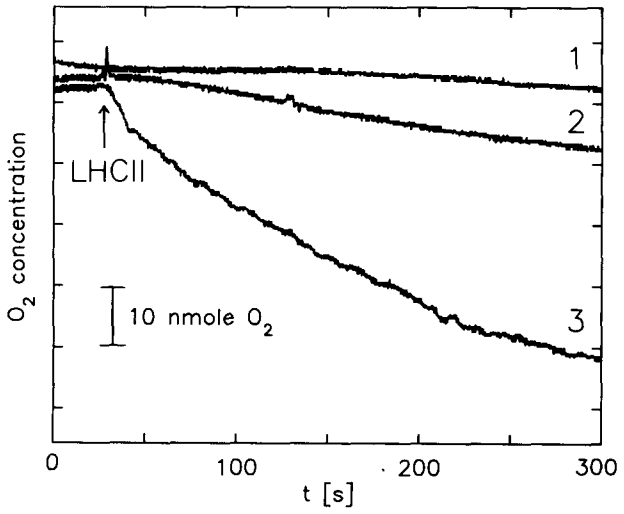

Fig. 2. Traces of polarographic measurements of zeaxanthin dispersion in a buffer (1) and after injection of suspended LHCII to the buffer (2) and to the dispersion of zeaxanthin in the buffer (3). LHCII was injected at the moment indicated with an arrow. Final concentration of zeaxanthin was $4.3 \cdot 10^{-6}$ M (sample Nos. 1 and 3), total chlorophyll was $13.9 \cdot 10^{-5}$ M (sample Nos. 2 and 3) and NADPH was $5.0 \cdot 10^{-4}$ M (sample Nos. 1, 2 and 3). Sample volume 1 ml.

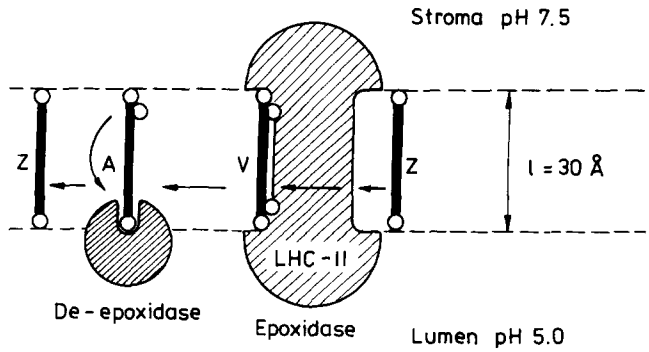

Fig. 3. Proposed model of the processes of the xanthophyll cycle located in the thylakoid membrane. Notation: Z – zeaxanthin molecule, A – antheraxanthin molecule, V – violaxanthin molecule, l – thickness of the hydrophobic core of the membrane. Open circles indicate oxygen atoms bound to the xanthophyll molecules. Arrows indicate sequence of processes essential for the xanthophyll cycle.

(1) All photosynthetic xanthophylls are believed to be attached, in vivo, to functional proteins. However, the group of pigments involved in the xanthophyll cycle, seems to be not so tightly bound to the complexes [14] and their relative motional freedom is related to a spatial separation of the enzymes of the cycle [2–4]. The transient but direct presence of zeaxanthin within a lipid phase of the thylakoid membrane may be additionally concluded from the effect of the xanthophyll cycle on regulation of the membrane fluidity [15] and protection of unsaturated fatty acids against photooxidation [16]. The distance between the two opposite hydroxyl groups of zeaxanthin (30.2 Å, [17]) was demonstrated to be a factor determining the pigment orientation with respect to a membrane with defined thickness of a hydrophobic core [18,19]. Since the thickness of the thylakoid membrane is approximately 30 Å [20], the orientation of hydroxycarotenoids in such a membrane can be predicted to be as presented in Fig. 3 (see also Ref. 17).

(2) On the basis of the optimum pH and the polarity of de-epoxidase this enzyme was located at the lumenal surface of the thylakoid membrane [2–4]. According to the model and assuming an orientation of the xanthophylls presented above, movement of the pigments should be rather limited to lateral diffusion. De-epoxidation of violaxanthin to antheraxanthin is possible in such a model since one of the epoxy-oxygens to be taken off can reach the de-epoxidase vicinity by the lateral diffusion of the violaxanthin molecule. De-epoxidation of antheraxanthin to zeaxanthin may be possible, however, only after a 'flip-flop' of the antheraxanthin molecule. This kind of molecular motion seems to be very probable taking into account an asymmetric structure of the mono-epoxycarotenoid.

(3) LHCII protein was crystallized and its three-dimensional polypeptide structure was determined by Kühlbrandt and Wang [21], on the basis of X-ray

crystallography. The important feature for the discussed model is that the hydrophobic part of the LHCII apoprotein (30 Å) fits very well the molecular size of zeaxanthin (see point 1). The fit of zeaxanthin is also reasonably good with the predicted folding structure of LHCII [22].

(4) The preparation of LHCII isolated by us was tested to be electrophoretically, as well as spectroscopically pure [7,8]. It is therefore rather unlikely that some other enzyme was isolated and exhibited its activity in our samples. On the other hand, on the basis of this study we can not exclude possibility that there exist some other thylakoid proteins possessing also activity of an epoxidase enzyme. The importance of chlorophyll *b*-containing LHCII in zeaxanthin epoxidation can be additionally concluded from the fact that the xanthophyll cycle in its form: zeaxanthin-antheraxanthin-violaxanthin is usually present in photosynthetic apparatus containing also chlorophyll *b* [1,2,4]. Chlorophyll *b*-lacking bean leaves demonstrated light-induced (ΔA_{505} increase (de-epoxidation) which could not be reversed (epoxidation) after turning off the light [11]. Zeaxanthin epoxidation can be achieved also in the absence of any proteins [23], but what is now of interest is a mechanism in which the activation energy for epoxidation in suspension is decreased according to the discussed model. As was recently shown by means of Fourier-transform infrared spectroscopy (Gruszecki, Tajmir-Riahi, Leblanc, submitted for publication) there is a very strong hydrophobic interaction between violaxanthin and the apoprotein of cytochrome *c*. The model suggests that immobilization of the pigment interacting with LHCII apoprotein should decrease the vibrational freedom of zeaxanthin and facilitate epoxide formation in the presence of molecular oxygen and NADPH.

The unknown physiological meaning of the xanthophyll cycle has been widely discussed in recent years [13,15,24–26]. This discussion was focused on the role of zeaxanthin formation. We would like to discuss the physiological role of the xanthophyll cycle also from the point of view of violaxanthin. Xanthophylls act not only as accessory pigments or photoprotectors but also as important factors stabilizing the native structure of pigment-proteins [27,28]. Light-dependent de-epoxidation of violaxanthin (preceded by the step of making the pigment available for de-epoxidation understood as detaching of the pigment molecules from the protein environment and migration towards the membrane where the de-epoxidase is located [29]), is a process affecting the antenna-protein native structure and in consequence should decrease the overall light-harvesting efficiency [27,28]. In excess of light such a process may play a protective role decreasing excitation flow to the reaction centers. The absence of light-dependent cyclic interconversions of xanthophyll pigments in photobacteria and simple photosynthetizing organisms [3,4] and the fact that zeaxanthin formation in higher plants starts essentially after saturation of photosynthesis [3] seems to suggest that the xanthophyll cycle may have also physiological importance for information transfer (signal transduction) from photosynthetic apparatus in order to moderate overall plant response to strong light. Such mechanism could be, for example, mediated by the xanthophyll cycle-related fluidity changes of the thylakoid membrane [15].

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