OCCURRENCE AND BIOSYNTHESIS OF VIOLAXANTHIN IN ISOLATED SPINACH CHLOROPLAST ENVELOPE

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1. Introduction

The spinach chloroplast envelope is a deep yellow membrane system with a unique carotenoid composition [1–5]. When leaves [2] or intact chloroplasts [5] are illuminated, the xanthophyll composition of the yellow chloroplast envelope is changed. Envelope membranes prepared from dark-treated material have a violaxanthin content up to 3 times higher than the lutein plus zeaxanthin content whereas in chloroplast envelopes from illuminated material this ratio is much lower. Based on these data, it was suggested [5] that the light-induced violaxanthin changes in envelope membranes were probably due to an exchange of pigments (violaxanthin) between the envelope and the thylakoids. Since efficient synthesis of carotenoids, especially violaxanthin, takes place in chloroplasts [6–8] and in chromoplasts [9,10] which lack thylakoids, it seems reasonable to suppose that the envelope membranes of chloroplasts may be active in carotenoid biosynthesis. In the same way, the chloroplast envelope has come under increasingly close scrutiny as a possible major site of lipid metabolism in spinach leaf cells [11].

Here, we show that violaxanthin is a genuine constituent of the chloroplast envelope which catalyzes the final step of violaxanthin biosynthesis.

2. Material and methods

2.1. *Chloroplast and envelope isolation*

Chloroplasts were prepared from 2 kg spinach (*Spinacia oleracea*) leaves. Deveined leaves were cut into 6 l chilled medium containing 300 mM sucrose, 30 mM Tricine–NaOH (pH 7.5) and 0.1% bovine serum albumin. The leaves were disrupted at low speed for 2 s in a 3.8 l Waring blendor. Unpurified chloroplasts were isolated from the homogenate as in [12] and were washed in isolation medium without albumin. Isolation of envelope membranes and thylakoids included separation of intact chloroplasts on sucrose [12] or percoll TM (silica coated with polyvinylpyrrolidone) [13] gradients, followed by gentle osmotic shock and separation of envelope membranes and thylakoids on a second sucrose gradient. From 2 kg spinach leaves the yield of envelope membranes can reach 8–10 mg protein. For good separation of envelope membranes vesicles from small pieces of thylakoids the presence of Mg^{2+} in the swelling medium and in the sucrose gradient is critical [11]: low levels (< 0.5 mM) lead to massive contamination of the envelope fraction with thylakoid fragments, whereas high levels (> 5 mM) cause the envelopes to sediment towards denser regions of the gradient. Furthermore, when too large an amount of material is layered on top of the sucrose gradient the yield falls dramatically, because the envelope vesicles are trapped in the network of thylakoid membranes and sediment towards the denser region of the gradient. Electron micrographs of the obtained envelope membranes showed clearly that no plastoglobuli were trapped in the network of the envelope membranes. Further, the chlorophyll content was < 0.02 μg/mg envelope protein.

2.2. *Chloroplast extract*

Intact, purified spinach chloroplasts (10 mg

chlorophyll, soluble protein/chlorophyll = 8.9) were suspended in ~10 ml 2 mM Tricine/NaOH buffer (pH 7.6) and allowed to stand at 4°C for 2 min. The suspension was then centrifuged at 130 000 × *g* for 2 h. The straw-coloured supernatant solution containing the soluble components of the chloroplasts was concentrated 4 times by lyophilisation. These preparations were used immediately.

2.3. *Incubation mixture*

The complete reaction mixture, if not otherwise defined, contained: 10 mM Tricine/NaOH buffer (pH 7.3); 0.1% bovine serum albumin; 0.1 mM NADPH; known amounts of [^{14}C]zeaxanthin (452 dpm .μg^{-1}), and chloroplast envelope membranes in a final volume of 4 ml. The reaction was initiated by addition of the enzyme. The reaction system was incubated at 24°C under white light (10 000 lux) for 60 min. In order to stop the reaction, and to simultaneously extract the carotenoids, 2 ml of acetone were added and the pigments were saponified overnight with KOH (15% in water, w/v). Carotenoids were transferred in light petroleum ether and determined. The separation of violaxanthin was performed in the dark by repeated chromatography on cellulose (Whatman CFII) column [6], in order to determine its specific radioactivity.

2.4. *Radioactive zeaxanthin*

[^{14}C]Zeaxanthin (β,β-carotene-3,3′-diol) was obtained from the ripening fruit of *Physalis alkekengi* which had been incubated for 10 days at 20°C in water with sodium [1-^{14}C]acetate (1 mCi; 42 mCi .$mmol^{-1}$) and exposed to continuous white light (8000 lux) until the fruit got an orange colour. Total carotenoids were extracted and saponified according to [14]. Pure, labelled zeaxanthin was obtained by repeated chromatography on cellulose (Whatmann CFII) column and crystallized as in [14]. The purified zeaxanthin was dissolved in the following medium: 2 ml diethyl ether poured into 3 ml Tween 80 (0.5%, v/v). Final concentration was 250 μg .ml^{-1} and specific radioactivity was, respectively, 452 and 470 dpm .μg^{-1} depending on the batch.

2.5. *Chemicals*

Crystallized bovine serum albumin (Sigma Chemical Co.) was defatted by acetone extraction. Violaxanthin (5,6,5′,6′-diepoxy-β,β-carotene-3,3′-diol) was obtained from a saponified acetone extract of thylakoids and purified by thin-layer chromatography on cellulose.

2.6. *Assays*

Protein content was determined by the Lowry method [15] with bovine serum albumin as standard. Total chlorophyll content was determined as in [2]. Absorption spectra of envelope and thylakoid suspensions were measured using an Aminco DW-2 spectrophotometer. Radioactivity was measured by liquid scintillation counting with quenching correction (Nuclear Chicago, Mark II).

3. Results and discussion

Figure 1 shows the absorption spectra of the envelope and thylakoid membranes, obtained at room temperature. The spectrum of the envelope obtained from dark-treated leaves shows the strong carotenoid absorption, with a typical three-banded spectrum in the blue region at 480, 448 and 426 nm mainly due to the absorption peaks of violaxanthin. The chlorophyll A_{676} is barely detectable. The presence of chlorophyll in the envelope fraction is considered to be a contamination from the light stroma lamellae. Low temperature (liquid nitrogen, 77 K) gives a higher resolution as well as an enhancement of the absorption peaks located at 487, 453, 428, 406, 384 and 367 nm. Under these conditions, the major absorption peaks are shifted a few nm towards the red end of the spectrum. By scanning the same sample, first at room temperature and then at low temperature, it is easy to measure the magnitude of the low temperature enhancement. On the average, the intensification factor is rather uniform at 5–5.2.

It is clear that the carotenoids of the envelope, where violaxanthin is dominating as was shown [1,2,5], are genuine constituents of these membranes for the following reasons:

(i) The pigments of the envelope were not contaminated by plastoglobule clusters which contain mainly β-carotene and only small amounts of violaxanthin [16];

(ii) The violaxanthin content in envelope membranes is not a contamination by carotenoids randomly separated from the thylakoids (fig.1) during

osmotic shock and redissolved in the highly lipophilic envelope membranes because violaxanthin is not a major pigment in the thylakoids;

(iii) The very low level of chlorophyll present in the *Spinacia oleracea* envelope fraction is also inconsistent with a significant contribution of pigments from fragments of the thylakoidal system (fig.1).

Table 1 clearly indicates that the envelope membrane fraction participates in violaxanthin biosynthesis: it catalyzes the epoxidation of zeaxanthin into violaxanthin. In 60 min, the specific radioactivity of violaxanthin increased if envelope protein was added to the incubation medium. The introduction of epoxide groups did not seem to require the presence of large amounts of NADPH or a high concentration of molecular oxygen in the medium (table 1).

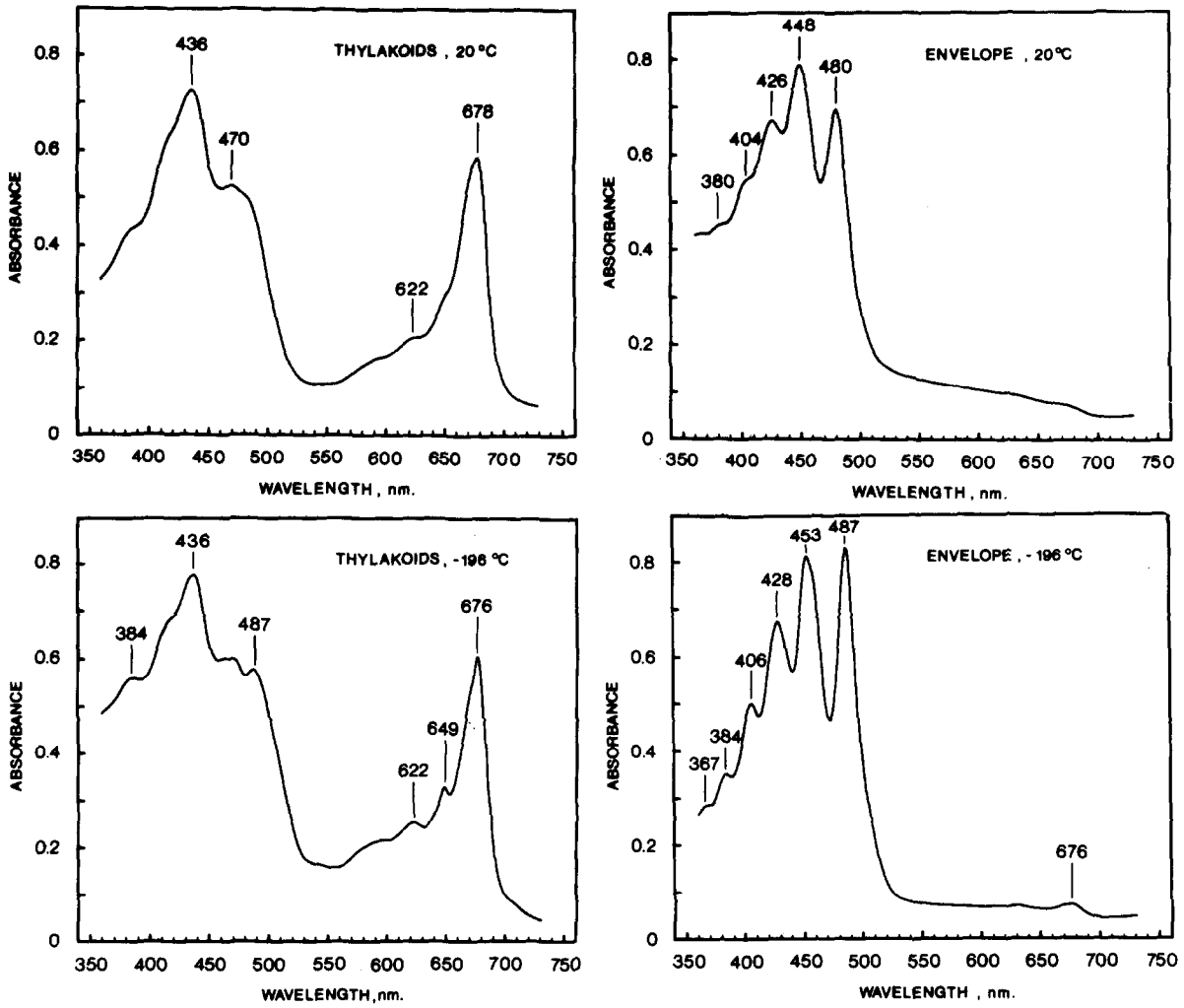

Fig.1. Absorption spectra of spinach chloroplast thylakoid and envelope membranes obtained at room temperature (20°C) and at liquid nitrogen temperature (−196°C). The membranes were suspended in the following medium: 10 mM Tricine/NaOH buffer, (pH 7.3) and 300 mM sucrose. Membrane protein concentration: 0.07 mg/ml (thylakoids, 20°C); 0.065 mg/ml (thylakoids, −196°C); 0.51 mg/ml (envelope 20°C); 0.58 mg/ml (envelope −196°C). The low temperature samples were placed in a 2 mm light-path plexiglass cuvette and cooled in a Dewar flask filled with liquid nitrogen during the scanning of the spectrum; at room temperature a 10 mm light-path quartz cuvette was used.

Table 1
Requirements for incorporation of [^{14}C]zeaxanthin into envelope membrane violaxanthin

Incubation mixture	[^{14}C]Zeaxanthin incorporated into violaxanthin (dpm)	Spec. radioact. of violaxanthin (dpm .μg^{-1})
Complete	201–240	25–35
– NADPH	188	21
– Envelope	0	0
+ Argon (with traces O_2)	266	39

Incubation mixture and carotenoid extraction were as in section 2 with omissions and addition where noted. [^{14}C]Zeaxanthin, 50 μg (452 dpm .μg^{-1}) and 2.5 mg purified envelope protein were used; assays were for 60 min. The Thunberg tube was flushed with argon gas several times to remove excess oxygen.

As shown in table 2, chloroplast stroma added to the incubation mixture increased the yield of the incorporation of zeaxanthin into envelope violaxanthin. Under these conditions it is possible that the role of the stroma is to supply a stimulating factor: in fact the stroma proteins alone were unable to catalyze the epoxidation of zeaxanthin.

According to [17] the epoxide groups are introduced stereospecifically, indicating that an enzymic reaction is involved. Though the results obtained here clearly indicate that the chloroplast envelope is a site of zeaxanthin epoxidation they explain neither the mechanism involved nor the origin of the epoxide oxygen. Experiments with $^{18}O_2$ have confirmed that the epoxide oxygen comes from molecular oxygen [18,19]. However (see table 1), the epoxidation of zeaxanthin into violaxanthin catalyzed by the envelope membranes does not seem to be sensitive to low partial pressure of oxygen. Consequently we are forced to conclude that if the epoxidase utilizes molecular oxygen as substrate it must have a very strong affinity for oxygen, like the oxygenase involved in sterol biosynthesis (Benveniste, personal communication). Furthermore, the fact that epoxidation is not strongly dependent on NADPH may indicate that the substrate of epoxidation of zeaxanthin in the chloroplast envelope could be water. Clearly further work has to be done to clarify the origin of the epoxide oxygen.

Finally, the physiological significance of the epoxidation of zeaxanthin into violaxanthin catalyzed by the envelope membranes is entirely different from that catalyzed by the thylakoids. The former is involved in violaxanthin synthesis whereas the latter is involved

Table 2
Incorporation of [^{14}C]zeaxanthin into violaxanthin by combination of various fractions from chloroplasts

Incubation mixture	[^{14}C]Zeaxanthin incorporated into violaxanthin		Spec. radioact. of violaxanthin (dpm .μg^{-1})
	(dpm)	(%)	
Complete	217	3	37
+ Stroma	345	5	57
– Envelope + stroma	0	0	0

Incubation mixture and carotenoid extraction were as in section 2 with omissions and additions where noted. [^{14}C]Zeaxanthin, (15 μg) (470 dpm .μg^{-1}), 1.7 mg purified envelope protein and/or 10 mg chloroplast stroma protein were used; assays were for 70 min

in the violaxanthin cycle; this cycle is a transmembrane system in which de-epoxidation (violaxanthin → zeaxanthin) occurs on the loculus side and epoxidation (zeaxanthin → violaxanthin) on the stroma side of the thylakoid membrane [20]. In this case zeaxanthin epoxidase (a mono-oxygenase) is tightly bound to the stroma side of the thylakoid [21]. However, it is possible that the pigments of the cycle are dynamically linked in some way with the violaxanthin of the chloroplast envelope [5].

These mechanisms are also in agreement with the structural and metabolic heterogeneity of violaxanthin described in leaves [22].

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