Interconversion of Carotenoids and Quinones after Onset of Photosynthesis in Chloroplasts of Higher Plants

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The interconversion of carotenoids and quinones was investigated in beech and spinach leaves as well as isolated intact spinach chloroplasts following a dark-light transition. It is shown that isolated intact chloroplasts which are preincubated for 2 h at pH 7.6 in the dark and re-illuminated with strong white light are capable not only of deepoxidizing violaxanthin into antheraxanthin and zeaxanthin but simultaneously change the redox state of the plastoquinone-pool in their thylakoid membrane. At the same time as violaxanthin is deepoxidized plastohydroquinone-9 is oxidized to plastoquinone-9. If the light is turned off zeaxanthin is epoxidized into antheraxanthin and violaxanthin but no significant change in the redox state of the plastoquinone-pool occurred.

It is concluded that the deepoxidation of violaxanthin is connected to the photosynthetic electron transport in that way that an acidification of the intrathylakoidal compartment by the vectorial release of protons from the water photooxidizing enzyme system and the plastoquinone-pool is required for the activation of the violaxanthin deepoxidase. This may be taken as further evidence that violaxanthin deepoxidase is located at the inner side of the thylakoid membrane. Additional evidence for this location site is given by the observation that neither deepoxidation of violaxanthin nor photooxidation of plastohydroquinone-9 occurred after onset of photosynthesis if non cyclic electron transport was inhibited by DCMU.

Introduction

If a green leaf is kept in the dark and reilluminated rapid changes in the concentration of violaxanthin, antheraxanthin and zeaxanthin occur. Since its discovery by Sapoznikov *et al.* in 1957 this phenomenon has been investigated extensively [1, 2] but particularly by Hager and Stransky [3–11] and Yamamotos group [12–17]. It is clear now that shortly after beginning of irradiation the concentration of violaxanthin decreases very rapidly whereas those of antheraxanthin and zeaxanthin increase simultaneously [4, 5]. If a leaf is transferred from strong light into dim light or complete darkness the reverse reaction can be observed; the concentration of zeaxanthin is decreasing but those of antheraxanthin and violaxanthin are increasing [5, 13].

In a very comprehensive investigation Hager and Stransky could show that this so-called xanthophyll cycle is existing in chlorophyceae and all higher plants but not in photosynthetic bacteria, cyanophyceae, cryptophyceae and rhodophyceae [9–11]. They further showed that in most of the other algae a similar cycle with diatoxanthin and diadinoxanthin as interconvertible carotenoids exists [7, 11]. An advanced approach concerning the biological importance of the xanthophyll cycle was carried out by Hager and his group who could show that deepoxidation of violaxanthin can be achieved by red light which is absorbed exclusively by the chlorophylls [6]. Hager also reported that violaxanthin was deepoxidized in DCMU-treated uncoupled spinach thylakoids in the presence of ferredoxin and ascorbate simply by changing the pH from 7 to 5 [6]. From this observation and the pH-optimum of the isolated enzyme (pH = 5.2, [7, 19]) it was proposed that violaxanthin deepoxidase is located at the inner side of the thylakoid membrane. The pH-optimum of the back-reaction (pH = 7.5) and its dependence on $NADPH+H^+$ was taken as evidence that the zeaxanthin-epoxidase is located at the outer side of the thylakoid [19–21].

In this contribution further evidence will be presented that the deepoxidation of violaxanthin into antheraxanthin and zeaxanthin takes place at the inner side of the thylakoid membrane. Data which suggest that the enzyme violaxanthinde-

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epoxidase is activated by an acidification of the intrathylakoidal compartment due to the vectorial release of protons from the water splitting enzyme system and the plastoquinone pool are also presented.

Materials and Methods

In vivo experiments

Sun leaves of beech were harvested from the tree in juli, kept in the dark for 12 h and irradiated with white light (Philips Attralux bulbs 150 W, 40000 lux, 100 W/m^2) for 30 min. After 12 h in the dark and at different times during the course of the experiment pigments and quinones were extracted from the leaves and quantitatively estimated.

Spinach was grown under natural conditions in the botanical gardens. After 4 weeks plants were transferred into darkness for 2 h and their pigment and quinone content assayed. After 2 h in the dark leaves were irradiated with white light (Osram HQI-E bulbs DV, 400 W, 25000 lux, 100 W/m^2) and their pigment and quinone content assayed during the following 30 min.

In vitro experiments

In vitro experiments were performed by using intact chloroplasts that were isolated from 4 weeks old spinach leaves [22, 23]. In order to delay chloroplast degeneration 0.1 mM dithioerythrol was present in the isolation and incubation media. Isolated intact chloroplasts were resuspended in 300 ml incubation medium prepared according to Jensen and Bassham [24] and incubated in the dark at 20 °C for 2 h. After 2 h the plastid suspension was irradiated with white light (Attralux bulbs, 150 W, 20000 lux, 20 W/m^2) for 30 min and kept in the dark for further 30 min. At different times during the dark-light-dark transition aliquots of the chloroplast suspension were removed from the incubation vessel and their pigment and quinone composition assayed. Chlorophyll content was determined according to Ziegler and Egle [25]. Carotenoids were separated by thin-layer-chromatography, dissolved in ethanol and quantitatively determined using an absorbance coefficient of $E\,\frac{1\%}{1\text{ cm}} = 2500$ [26, 27]. Quinones were assayed using standard techniques [28, 29].

Results

Identification of carotenoids and quinones

Before investigating the interconversion of carotenoids and quinones following a dark-light transition carotenoids and quinones were isolated from beech and spinach leaves and identified by mass-spectrometry. Leaves of both, beech and spinach contained violaxanthin (M^+ at $m/z = 600$), antheraxanthin (M^+ at $m/z = 584$) and zeaxanthin (M^+ at $m/z = 568$) as xanthophylls in their chloroplasts. Among the quinones plastoquinone-9 (M^+ at $m/z = 749$) and plastohydroquinone-9 that was converted into plastoquinone-9 by oxidation with ferrichloride could be identified.

Interconversion of carotenoids and quinones in the intact leaf

If green leaves are kept in the dark and re-illuminated, with strong light rapid changes in the concentrations of violaxanthin, antheraxanthin and zeaxanthin occur although the concentrations of β-carotene and neoxanthin, are not changing at all (Table I, Fig. 1), [19]. On a chlorophyll basis nearly 30% of the violaxanthin were deepoxidized. As far as violaxanthin was deepoxidized the concentrations of its deepoxidation products antheraxanthin and zeaxanthin were increasing simultaneously.

At the same time as violaxanthin was deepoxidized into antheraxanthin and zeaxanthin, rapid changes in the concentration of plastohydroquinone- and plastoquinone-9 also occurred (Table I, Fig. 2).

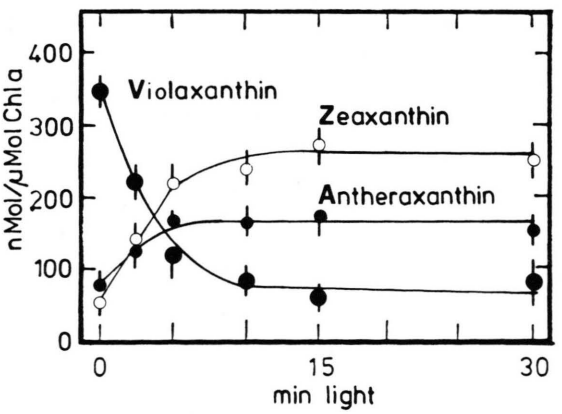

Fig. 1. Interconversion of xanthophylls in beech sun leaves preincubated in the dark for 12 h after exposure to continuous white light (40000 lux, 100 W/m^2).

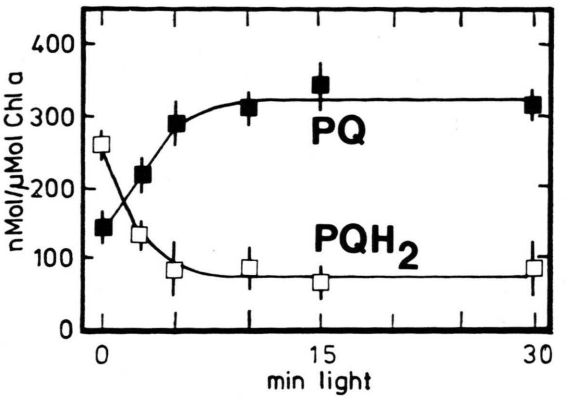

Fig. 2. Photooxidation of plastohydroquinone-9 in beech sun leaves preincubated in the dark for 12 h and exposed to continuous white light. Otherwise as for Fig. 1.

After onset of photosynthesis in strong light within 5 min the concentration of plastohydroquinone-9 was decreasing very rapidly whereas that of plastoquinone-9 was increasing stoichiometrically. Except these alterations in the redox state of the plastoquinone pool no changes in the concentration of any other quinone could be detected.

If we consider that these changes in the redox state of the plastoquinone-9 pool take place in the thylakoid membrane and not in the plastoglobuli as reported recently [30] then they may reflect the build up of a transmembrane potential across the thylakoid membrane which is produced by the vectorial translocation of protons by the plastoquinone pool [31].

The acidification of the intrathylakoidal compartment by the vectorial translocation of protons is necessary not only for photophosphorylation but also for the activation of the violaxanthin deepoxidase.

In order to confirm this assumption further the light dependent oxidation of plastohydroquinone-9 and the interconversion of violaxanthin into antheraxanthin and zeaxanthin was reinvestigated using young spinach leaves. As compared to plastids from sun leaves of beech which contain high amounts of plastoquinones in their plastoglobuli chloroplasts of young spinach leaves are devoid of plastoglobuli and the plastoquinones are contained exclusively in the thylakoid membrane [32–34].

As already presented for beech leaves similar results were obtained in spinach leaves (Table I), although the deepoxidation of violaxanthin like the oxidation of plastohydroquinone-9 proceeded much more slowly. This difference may be explained by the observation that the extent of the deepoxidation of violaxanthin in the light is increasing with increasing light intensity that is used during the course of the experiment [15]. That the amount of zeaxanthin that is formed during the deepoxidation of violaxanthin in the light is increasing with increasing light intensity has been reported [35].

On the other hand it is well known that the pigment and quinone composition of the thylakoid membrane changes due to the environmental conditions under that a plant is grown [36, 37]. This means that the actual concentrations of the xanthophylls and quinones which are involved in a light dependent interconversion and the extent of the interconversion may vary considerably.

Interconversion of carotenoids and quinones in isolated intact chloroplasts

A light dependent interconversion of violaxanthin into antheraxanthin and zeaxanthin and a partial

Table I. Interconversion of plastoquinones and xanthophylls in 4 weeks old spinach leaves after onset of photosynthesis. Presented data are given as nano mol quinone or xanthophyll per µmol chlorophyll a ($n = 3$; $\bar{n} \pm$ SE).

Quinone or Xanthophyll	min strong light					
	2 h dark	+2.5	+5.0	+10	+20	+30
Plastoquinone-9	20.7 ± 1.3	25.6 ± 1.1	32.5 ± 2.0	32.0 ± 1.9	33.8 ± 3.0	32.1 ± 4.1
Plastohydroquinone-9	35.0 ± 2.1	29.4 ± 0.9	23.5 ± 3.3	26.1 ± 2.7	24.5 ± 1.6	25.7 ± 3.0
Zeaxanthin	2.5 ± 0.8	3.0 ± 0.5	5.3 ± 0.6	8.0 ± 0.9	13.1 ± 2.1	13.6 ± 1.8
Antheraxanthin	8.8 ± 1.1	11.8 ± 2.3	12.2 ± 0.7	14.8 ± 1.0	17.1 ± 0.8	18.8 ± 3.1
Violaxanthin	74.9 ± 5.1	63.0 ± 4.6	51.7 ± 6.0	51.0 ± 2.1	55.6 ± 4.3	52.7 ± 2.9

Table II. Carotenoid content (nano mol carotenoid per 1 μmol chlorophyll a; $n = 5$, $\bar{n} \pm$ SE) of radish cotyledone and beech leaves grown under different intensities of white light. Beech sun and shade leaves were harvested from the tree by the end of July.

Carotenoid	Radish			Beech	
	0.33 W/m²	6.0 W/m²	96.5 W/m²	shade leaf	sun leaf
Zeaxanthin	2.6 ± 0.2	16.4 ± 2.0	31.3 ± 4.2	21.5 ± 2.5	47.4 ± 5.0
Antheraxanthin	59.2 ± 3.4	41.1 ± 1.9	34.6 ± 3.4	90.3 ± 6.3	77.8 ± 3.3
Violaxanthin	43.7 ± 2.9	51.7 ± 0.9	60.8 ± 5.0	195.7 ± 20.7	300.1 ± 27.8

Table III. Interconversion of plastoquinones and xanthophylls in isolated intact spinach chloroplasts after onset of photosynthesis and subsequent reincubation in the dark. Presented data are given as nano mol quinone or xanthophyll per μmol chlorophyll ($n = 2$; $\bar{n} \pm$ SE).

Quinone or xanthophyll	Dark 2 h	min light		min dark	
		+15	+30	+15	+30
Plastoquinone-9	16.0 ± 1.7	21.0 ± 1.5	21.1 ± 1.1	18.6 ± 1.5	23.6 ± 1.2
Plastohydroquinone-9	35.6 ± 2.5	35.3 ± 1.8	32.9 ± 2.0	35.0 ± 1.8	36.8 ± 1.1
Zeaxanthin	3.3 ± 0.2	23.8 ± 1.8	28.0 ± 2.0	8.8 ± 1.0	5.0 ± 0.4
Antheraxanthin	7.1 ± 1.1	12.7 ± 2.3	19.2 ± 0.9	8.1 ± 1.4	6.7 ± 0.8
Violaxanthin	70.0 ± 6.5	43.4 ± 5.1	19.7 ± 3.0	63.4 ± 7.5	71.0 ± 5.1

photooxidation of the plastoquinone pool could also be achieved in isolated intact spinach chloroplasts (Table III), but the reactions proceeded more slowly *in vitro* as compared to the *in vivo* conditions. It should be noticed, however, that in some chloroplast preparations no changes in the redox state of the plastoquinone pool occurred although violaxanthin was deepoxidized.

If the light was turned off and the chloroplast suspension incubated in the dark for further 30 min the concentrations of zeaxanthin and antheraxanthin were decreasing but that of violaxanthin was increasing simultaneously (Table III). This so-called back reaction of the xanthophyll cycle proceeded either in the dark or at very low intensities of light [5, 19]. As compared to the biosynthesis of violaxanthin no significant changes in the redox state of the plastoquinone pool could be observed (Table III). Nevertheless these results may suggest that during a dark-light-dark transition and probably also under natural day-night rhythms or after changing the light intensity not only the xanthophyll composition of the thylakoid membrane but also the redox state of the plastoquinone pool is changing as it is the case for photosynthetic electron transport and photophosphorylation [31, 38, 39]. This adaptation mechanism may be very important for the chloroplast in terms of avoiding a photooxidative damage of the photosynthetic apparatus.

Discussion

In a green leaf chlorophylls and carotenoids are located in the thylakoid membrane as light harvesting pigments together with quinones which act as electron carriers and proton translocators (plastoquinone) [40, 41] or structural components and antioxidative agents (α-tocopherol). Chlorophylls and carotenoids are contained exclusively in the thylakoid membrane but quinones have also been detected in reasonable amounts in plastoglobuli, particularly in chloroplasts of older leaves [42–44]. Chloroplasts from young leaves are more or less devoid of plastoglobuli and the main part of the quinones is contained in the thylakoid.

Depending on the light intensity that they received during growth plants alter the pigment and quinone composition of their thylakoid membrane [36, 37]. As compared to plastids of leaves grown in dim light (shade leaves) chloroplasts of leaves grown in strong light (sun leaves) are particularly enriched in zeaxanthin, violaxanthin and plastoquinone-9 (Table II). Besides these slow adaptations

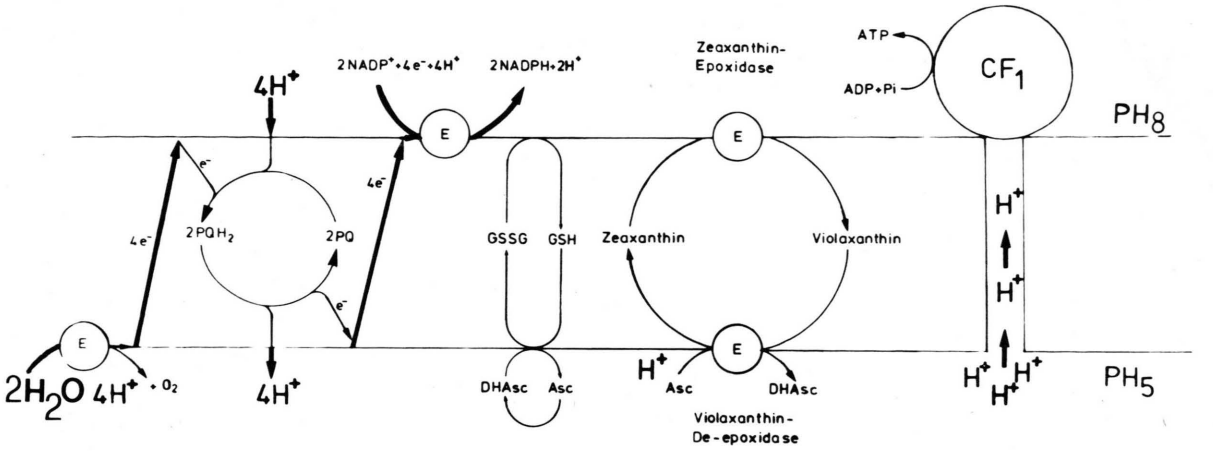

Fig. 3. Simplified model of the thylakoid membrane comprising the proton carrier-like function of the plastoquinone pool as well as the proton induced deepoxidation of violaxanthin and photophosphorylation; adopted from [19].

to prolonged irradiation rapid changes in the concentration of certain carotenoids occur if a green leaf is kept in the dark and reilluminated [1–7]. Within 5 to 7 min after onset of photosynthesis the amount of violaxanthin is decreasing very rapidly while that of antheraxanthin and zeaxanthin is increasing simultaneously. If a leaf is transferred from strong light into darkness the reverse reaction occurrs; zeaxanthin is epoxidized into antheraxanthin and violaxanthin [5, 7].

As shown in Table III deepoxidation of violaxanthin and the epoxidation of zeaxanthin could be achieved in isolated intact spinach chloroplasts at pH 7.6 without adding any cosubstrates. These alterations in the xanthophyll composition of the thylakoid which occur in chloroplasts of all higher plants were discovered in 1957 by Sapoznikov *et al.* [1] and elucidated in full detail by Hager and Stransky [3–11] and Yamamoto *et al.* [12–17]. From the observation that the deepoxidation of violaxanthin also occurred in red light and is inhibited in the presence of DCMU a strong connection between the xanthophyll cycle and photosynthetic electron transport has been suggested [7, 19]. Moreover it was found that violaxanthin was deepoxidized in uncoupled spinach thylakoids already in the dark if the pH of the incubation medium was changed from 7 to 5 [6]. This result as well as the pH-optimum (pH = 5.2) of the isolated violaxanthin-deepoxidase [18] lead to the conclusion that the enzyme is located at the inner side of the thylakoid membrane.

Additional evidence for this location site is given in this contribution (Fig. 2, Table I, III). By following the alterations in the xanthophyll composition of a green leaf during a dark-light transition it was shown that as rapidly as violaxanthin is deepoxidized in the light plastohydroquinone is photooxidized to plastoquinone-9 (Fig. 2, Table I, III). This partial photooxidation of plastohydroquinone-9 was inhibited completely by DCMU as it was the case for the deepoxidation of violaxanthin [19, 45]. Although clear evidence has been given by flash spectroscopy that in the very beginning of photosynthesis the plastoquinone pool is reduced by photosystem II [46] and that the rate limiting step in electron transport is the oxidation of the plastoquinone pool by cytochrome *f* and photosystem I [47, 48] under our experimental conditions plastohydroquinone-9 was oxidized after 2.5 min light significantly (Fig. 2, Table I). This release of protons from the plastoquinone pool after onset of photosynthesis which contributes to the formation of a transmembrane proton gradient and the acidification of the intrathylakoidal compartment is in accordance with the concept of the formation of a native energy conserving site (Fig. 3).

Since the deepoxidation of violaxanthin is depending on light intensity as it is the case for the photosynthetic electron transport and the formation of ATP, this interconversion of xanthophylls after onset of light must play an important role in photosynthesis. This is particularly important if we consider that virtually all of the xanthophylls are sup-

posed to be contained in the light-harvesting complex and that various plants are capable of adapting to alterations in the light intensity by changing the pigment and quinone composition of their thylakoid membrane more or less rapidly. An analysis of the carotenoid composition of a purified light-harvesting complex and photosystem I and II particles would be necessary in order to know more about the biochemical importance of the xanthophyll cycle.

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