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CAROTENOID TRANSFORMATIONS UNDERLYING THE BLUE ABSORBANCE CHANGE IN FLASHED LEAVES DURING THE INDUCTION OF OXYGEN EVOLUTION

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Summary

The blue absorbance change occurring in flashed bean (*Phaseolus vulgaris* L.) leaves when exposed to continuous light (first observed by Strasser; Strasser, R.J. (1973) Arch. Int. Physiol. Biochem. 81, 935—955) is caused by the conversion of the following xanthophylls: violaxanthine → antheraxanthine → zeaxanthine. This conclusion is derived from the simultaneous occurrence of both reactions: (a) In flashed leaves, blue absorbance change and xanthophyll conversion take place under strong (2 mW · cm^{-2}) but not under weak (0.02 mW · cm^{-2}) white light. (b) In chloroplasts isolated from flashed leaves, the blue absorbance change occurs in the dark under conditions that also induce the xanthophyll conversion. (c) Blue absorbance change and xanthophyll conversion are both inhibited by dithiothreitol. In addition, the light-induced blue absorbance change is reversed in the dark if aerobic conditions are maintained, i.e. under conditions that in normal leaves favor the reversal of the above reaction sequence.

The significance of the xanthophyll conversion is discussed in relation to other phenomena occurring in flashed leaves after exposure to continuous illumination.

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2 ethanesulfonic acid.

Introduction

Chloroplasts of dark-grown leaves that have been partially greened under a light regime of brief flashes [1] differ significantly from normal chloroplasts. Although they contain well-developed thylakoids, these membranes do not form grana but are arranged in distinct arrays, the so-called primary thylakoids [2]. In primary thylakoids the chlorophyll *b* level is low compared to the level of chlorophyll *a* [2,3], indicating the lack of a light-harvesting chlorophyll *a*/*b*-protein complex [4,5]. Primary thylakoids can oxidise artificial electron donors to Photosystem II and transfer electrons through both photosystems [6] but they are not able to evolve oxygen, which indicates that their electron transport chains are defective between water and Photosystem II [6].

When flashed leaves are exposed to continuous light they develop the capacity to evolve oxygen during an induction period of less than 10 min [7], which shows that their electron transport chains are completed within this interval. However, continuous light is not only involved in the completion process but it also induces an absorbance change of the flashed leaves in the blue spectral region whose difference spectrum is characterized by maxima at 505 nm, 467 nm and 435 nm and minima at 481 nm, 450 nm and 420 nm [8], and in addition it initiates the fusion of individual thylakoids to pairs [8].

The difference spectrum of this blue absorbance change suggests that it is due to some carotenoid transformation. Indeed, a light-induced absorbance change with an almost identical difference spectrum has been observed in chloroplasts isolated from normal leaves [9], and this absorbance change has been shown to result from de-epoxidation of violaxanthine (5,6,5′,6′-diepoxizeaxanthine) to zeaxanthine through the intermediate antheraxanthine(5,6-monoepoxizeaxanthine). In normal chloroplasts this reaction sequence is reversed in the dark via an independent pathway, the oxygen and NADPH-requiring epoxidation of zeaxanthine to violaxanthine, both reactions together forming the so-called xanthophyll or violaxanthine cycle (see Ref. 10 for review). Contrary to these reactions, the blue absorbance change in flashed leaves seemed to be irreversible [8], which may be a reason why it has not been considered to result from violaxanthine de-epoxidation.

The purpose of this paper is to identify the process that causes the described blue absorbance change in flashed leaves when transfered to continuous illumination. It will be shown that the blue absorbance change results from violaxanthine de-epoxidation and that it can be reversed under appropriate conditions.

Materials and Methods

Bean seeds (*Phaseolus vulgaris* L. 'commodore' LSBR, Warsage) were planted in a mixture of earth and styropor pearls and grown for 7 days in the dark. The seedlings were then irradiated with one 4 ms flash (Revuetron a 24, Nürnberg-Langwasser) every 15 min for 9—15 days.

All experiments were carried out with primary leaves. Chloroplasts were isolated at 4° C under a green safety light. Primary leaves (approx. 15 g) were homogenized in 150 ml of isolation medium (300 mM sorbitol, 50 mM sodium

Hepes buffer, pH 7.5, 1 mM EDTA, 10 mM NaCl, 1 mM $MgCl_2$, 0.1% defatted bovine serum albumin) for 10 s in a 1 l blendor, filtered through monofilament nylon cloth (36 μm mesh width) and centrifuged for 5 min at 1500 $\times g$. The supernatant was centrifuged for 10 min at 25 000 $\times g$ and the pellet was collected.

Difference spectra of whole leaves were calculated from their absorbance spectra monitored after various treatments. Absorbance spectra were measured with a Perkin-Elmer Model 356 two-wavelength double-beam spectrophotometer. Leaves were taped with their upper surface on an opal-glass plate (Schott, Mainz) while their petioles were dipped in water, and the plate was placed directly in front of the photomultiplier. Actinic light from a 150 W halogen lamp (radiator KL 150 B, Schott, Mainz) was guided to the lower surface of the leaf by means of a fiber optics cable; light intensity was adjusted with grey filters. If required, oxygen or nitrogen were blown over the leaf. During all treatments the leaf remained undisturbed. (Difference spectra determined directly by monitoring illuminated versus dark-treated leaves, agreed with the calculated spectra; however, the selection of leaves with approximately identical initial absorbance was difficult due to heterogeneity of the plant material.)

Difference spectra of chloroplast suspensions were measured in a computerized single-beam spectrophotometer as developed by Butler [11].

Oxygen exchange in whole leaves was measured according to Strasser and Sironval [7] using a Yellow Spring oxygen electrode.

For pigment determination, 1 g leaves were homogenized under a green safety light in a cold mortar together with 1 g of $CaCO_3$, and the homogenate was extracted with pure acetone. Chloroplast suspensions were adjusted to pH 7.5 and pelleted before acetone extraction. Chlorophyll *a* plus *b* content of the acetone extracts was determined according to Jeffrey et al. [12]. Aliquots of the acetone extracts were further analysed using the gentle reverse-phase TLC system of Egger [13] that does not separate carotenoid isomers like luteine and zeaxanthine, and the alkaline thin-layer plate developed by Hager and Meyer-Bertenrath [14] that allows recovery of pure zeaxanthine while destroying the chlorophylls. Chlorophylls *a* and *b* were determined in acetone, carotene in light petroleum (b.p. 40—60° C) and the xanthophylls in ethanol using the extinction coefficients published by Hager and Meyer-Bertenrath [14].

Results

Flashed bean leaves contain the same plastid pigments as leaves grown under normal day and night periods but, on a fresh weight basis, their pigment levels are low and chlorophyll *b* occurs only in traces [15]. While confirming these earlier observations Table I shows that 10 min of weak illumination, which was enough to complete the induction period for oxygen evolution [16], did not significantly affect the pigment levels. After 10 min of strong light, however, violaxanthine concentration was decreased by approximately 40%, while the concentrations of zeaxanthine and antheraxanthine were strongly increased. Within experimental error, the decrease in violaxanthine concentration was compensated for by the increase in antheraxanthine plus zeaxanthine

TABLE I

EFFECT OF CONTINUOUS LIGHT ON THE PIGMENT CONTENT IN FLASHED BEAN LEAVES

Leaves were harvested from 16-day-old plants that had been grown in darkness for 7 days followed by flash treatment for 9 days. Aliquots were kept in the dark or were illuminated with continuous weak (0.02 mW · cm^{-2}) or strong (2 mW · cm^{-2}) white light for 10 min. Then their pigment content was analysed as described in Materials and Methods. The data presented in lines 2—7 were obtained after pigment separation [13]; pure zeaxanthine (line 8) was determined after separation [14]. Variations in the pigment content presented in columns 1 and 2 were partially due to heterogeneity of the leaf material; the experimental error from TLC analysis being in the range of ± 3%.

Pigment content	Dark	10 min weak light	10 min strong light
Total chlorophyll content (mg/g fresh weight)	0.32	0.31	0.34
Relative pigment content (mol/100 mol chlorophyll *a*)			
Chlorophyll *b*	3.9	4.5	4.3
Carotene	15.6	14.5	17.0
Neoxanthine	6.6	7.2	6.9
Violaxanthine	29.8	32.0	17.9
Antheraxanthine + luteine-epoxide	3.8	4.1	9.1
Luteine + zeaxanthine	38.0	40.1	50.5
Zeaxanthine	2.0	2.1	10.4

* A more detailed study on the occurrence of luteine-epoxide in flashed bean leaves is in preparation (F. Collard and C. Sironval).

concentration, indicating that violaxanthine was de-epoxidised to these compounds. Fig. 1B, shows that the light-induced xanthophyll transformations occurred together with an absorbance change in the blue spectral region whose difference spectrum agreed with the difference spectrum observed by Strasser and Butler for similar plant material [8]. Like the xanthophyll transformations (Table I) the absorbance change did not occur under weak illumination (Fig. 1A), nor did it occur under strong light when the leaves were infiltrated with dithiothreitol (Fig. 1C), an inhibitor of violaxanthine de-epoxidation [17].

In addition to the blue absorbance change described above, continuous illumination caused an absorbance increase of the leaf in the red spectral region (Fig. 1B) that was maximal at 676 nm. This absorbance increase (approximately 5% of the total absorbance at 676 nm during 10 min light) was not related to the blue absorbance change discussed here, since it also occurred after dithiothreitol infiltration (Fig. 1C). The dithiothreitol-insensitive absorbance change yielded a difference spectrum similar to the absolute spectrum of flashed leaves, therefore it might reflect de novo synthesis of plastid pigments during continuous illumination.

In chloroplasts isolated from flashed leaves violaxanthine could be converted to zeaxanthine without light (Table II). The reaction occurred in the presence of ascorbate at pH 5.0, i.e. under conditions known for normal chloroplasts to induce violaxanthine de-epoxidation in the dark [18]. Also in accordance with normal chloroplasts [17], dithiothreitol inhibited the reaction in chloroplasts from flashed leaves (Table II). Acid-induced de-epoxidation occurred together with an absorbance change in the blue spectral region (Fig. 2) whose difference spectrum, with maxima at 505 nm, 470 nm and 432 nm and minima

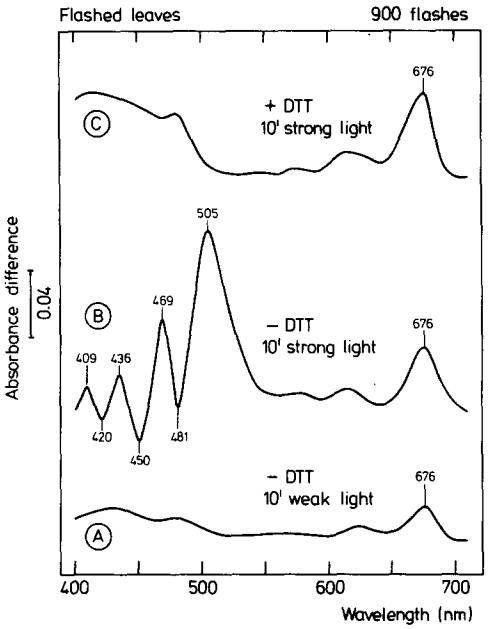

Fig. 1. The light-induced absorbance change in flashed bean leaves and its prevention by dithiothreitol (DTT). Absorbance spectra of leaves grown as described in Table I were monitored before exposure of the leaf to continuous light and after 10 min of illumination with white light at 0.02 mW · cm^{-2} (weak light) or 2 mW · cm^{-2} (strong light). Curves A—C: light minus dark difference spectra of three leaves exposed to the indicated experimental conditions. For DTT treatment, the leaf was vacuum infiltrated with 0.2 mM DTT for 30 min just before the experiment; controls infiltrated with distilled water behaved like untreated leaves.

at 485 nm and 450 nm, was similar to the difference spectra of the light-induced violaxanthine de-epoxidation in normal chloroplasts [9] and of the light-induced events in flashed leaves [8]. This absorbance change was completely inhibited in the presence of 0.2 mM dithiothreitol.

TABLE II

EFFECT OF ASCORBATE (ASC) AND DITHIOTHREITOL (DTT) ON THE PIGMENT CONTENT OF ACID-INCUBATED CHLOROPLASTS FROM FLASHED LEAVES

Chloroplasts from bean plants grown as described in Table I were isolated as described in Materials and Methods. The reaction mixtures contained in final volumes of 6 ml: 300 mM sorbitol, 50 mM sodium citrate buffer, pH 5.0, chloroplasts equivalent to 30 μg chlorophyll/ml and, where indicated, 30 mM ascorbate and 0.1 mM DTT. The samples were incubated for 45 min at 20°C, then their pigment content was analysed as described in Materials and Methods (see also Table I).

Relative pigment content (mol/100 mol chlorophyll *a*)	No addition	ASC	ASC + DTT
Chlorophyll *b*	7.7	8.1	8.0
Carotene	10.0	9.7	n.d.
Neoxanthine	1.7	1.5	1.9
Violaxanthine	6.9	3.5	5.6
Antheraxanthine + luteine-epoxide	1.7	2.1	2.1
Luteine + zeaxanthine	9.5	13.0	10.9
Zeaxanthine	0	2.4	1.0

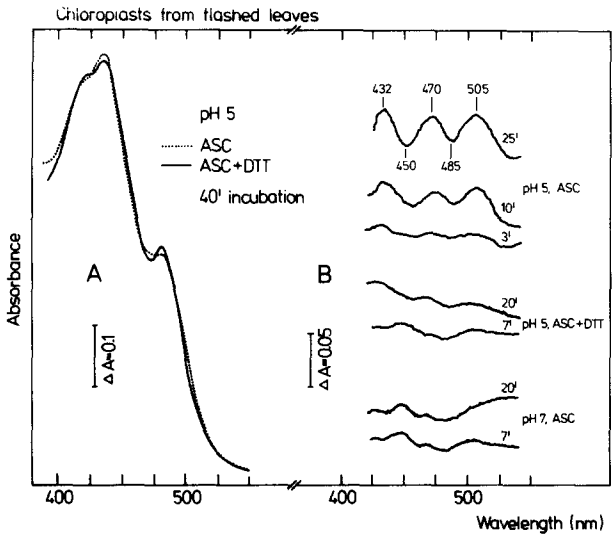

Fig. 2. Effect of pH and dithiothreitol (DTT) on the blue absorbance change of ascorbate-incubated chloroplasts from flashed bean leaves. Chloroplasts from plants grown as described in Table I were incubated in reaction mixtures containing 300 mM sorbitol, 30 mM ascorbate, 50 mM sodium citrate buffer, pH 5.0, or sodium Hepes buffer, pH 7.0, and, where indicated, 0.2 mM DTT. (A) Absorbance spectra of 3-ml samples measured in the double-beam instrument; chlorophyll content 10 μg/ml, optical path 10 mm. The spectrum of the ascorbate (ASC) + DTT sample remained unchanged throughout the incubation period. (B) Difference spectra of 0.4 ml samples measured in the single-beam instrument; chlorophyll content 60 μg/ml, optical path 3.5 mm. Spectra (S_t) of the samples were taken at the indicated incubation times, and the differences (S_t minus S_1) were plotted out directly by the computer.

The data presented suggested a correlation between blue absorbance change and de-epoxidation of violaxanthine. Therefore we asked whether the light-induced absorbance change in flashed leaves could be reversed under conditions known to reverse violaxanthine de-epoxidation in normal leaves, namely darkness plus oxygen [19,20]. Fig. 3 compares the difference spectrum of the light-induced absorbance change (curve A) with the difference spectrum of the absorbance change that occurred in preilluminated leaves during dark plus oxygen treatment (curve B). Both difference spectra agreed in the position of their extrema; they had a major maximum at 676 nm, while at wavelengths shorter than 550 nm maxima of the one difference spectrum corresponded to minima of the other and vice versa. A second light treatment (Fig. 3D) yielded a difference spectrum similar to that induced by the first illumination period. When the preilluminated leaf was exposed to darkness plus nitrogen, no significant absorbance changes occurred (Fig. 3C). These results suggest that the light-induced blue absorbance change of flashed leaves was reversed in the dark if oxygen was present. However, the difference spectrum occurring in the dark (Fig. 3B) was not simply antiparallel to the light-induced spectrum (Fig. 3A). Instead, it seems to result from at least two absorbance changes, namely the reversal of the blue absorbance change and, in addition, an absorbance increase with a peak at 676 nm and a broad maximum at wavelengths shorter than 540 nm, similar to the dithiothreitol-insensitive absorbance increase during con-

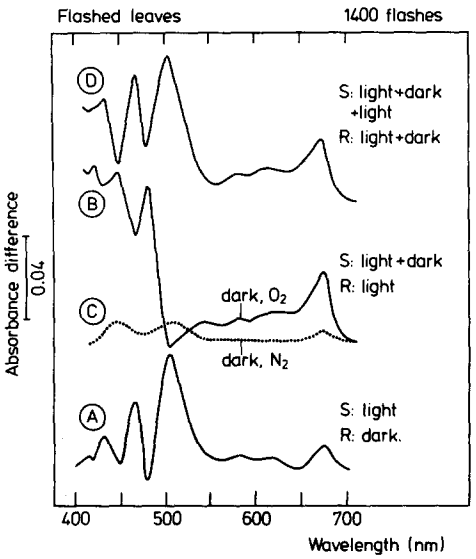

Fig. 3. The oxygen-dependent reversal of the light-induced blue absorbance change of flashed bean leaves in the dark. Leaves were from 21-day-old plants that had obtained 1400 flashes during a period of 14 days. Absorbance spectra of the leaves were taken consecutively before illumination, after 10 min of strong light (2 mW · cm^{-2}), after a subsequent dark period of 60 min and after a second light period of 10 min. (A—D) Difference spectra (S minus R) of leaves exposed to the indicated treatments. Curves A, B and D were obtained from the same leaf.

tinuous illumination (Fig. 1C). The latter absorbance change may therefore reflect processes that are initiated by continuous illumination and continue in subsequent darkness if aerobic conditions are maintained.

The described dark reversal of the light-induced blue absorbance change required approx. 1 h, as compared to the light-induced change that took place within 5—10 min. Only slight dark reversibility was observed in plants that were younger than 18 days indicating that in flashed leaves this activity was developed later than the ability for the light-induced blue absorbance change.

Discussion

In this paper we provide evidence that the light-induced blue absorbance change in flashed leaves, first reported by Strasser [21] and Strasser and Butler [8], results from the de-epoxidation of violaxanthine to zeaxanthine. Our conclusion is based on the following observations: (a) In flashed leaves, blue absorbance change and violaxanthine de-epoxidation occur under the same light conditions (Table I and Fig. 1). (b) Chloroplasts isolated from flashed leaves are able to de-epoxidise violaxanthine without light, and this reaction is accompanied by a similar blue absorbance change (Table II, Fig. 2). (c) In the presence of dithiothreitol, neither flashed leaves nor chloroplasts isolated from flashed leaves exhibit such blue absorbance change (Figs. 1 and 2), and de-epoxidation of violaxanthine is inhibited (Table II). (d) The blue absorbance change in flashed leaves is reversed under similar conditions as violaxanthine de-epoxidation in normal leaves (Fig. 3).

In normal thylakoids, de-epoxidation of violaxanthine is initiated by light or by low medium pH, which led Hager [18] to conclude that the de-epoxidase is located inside the thylakoids and is activated through light-induced acidification of the internal thylakoid space. As the de-epoxidation in primary thylakoids showed a pH dependence similar to that in normal thylakoids (Fig. 2, Table II) it seems reasonable to consider de-epoxidation activity in flashed leaves as an endogenous indicator for the acidification of the inner thylakoid space. De-epoxidation activity, monitored as blue absorbance change or 505 nm change by Strasser and Butler [8] occurred as soon as flashed leaves were exposed to strong continuous light, while oxygen evolution occurred only after an induction period of several minutes [8]. If the 505 nm change can be assumed to indicate pH variations in the inner thylakoid space, then this observation suggests that upon continuous illumination thylakoids of intact flashed leaves are able to accumulate protons while linear electron transport from water is still blocked. This light-induced acidification of the inner thylakoid space might result from cyclic electron transport through Photosystem I since 3-(3,4-dichlorophenyl)-1,1-dimethylurea at levels that completely inhibit oxygen evolution after induction, have no effect on the 505 nm change [8].

According to Strasser and Butler [8] the 505 nm change of flashed leaves occurs simultaneously with the fusion of unstacked thylakoids, and both processes are sensitive to or unaffected by the same treatment. Therefore the authors suggested that the process underlying the absorbance change might enable the thylakoids to fuse. After this process has been identified as violaxanthine de-epoxidation, one might ask whether in primary thylakoids the conversion of violaxanthine to zeaxanthine is involved in thylakoid fusion. Although this possibility is not ruled out by the fact that in fully developed chloroplasts violaxanthine de-epoxidation occurs in both, unstacked stroma lamellae and stacked grana lamellae [22], the simultaneous occurrence of violaxanthine de-epoxidation and thylakoid fusion in flashed leaves may simply indicate that proton pumping or subsequent ion translocations are required for thylakoid fusion. In this case, violaxanthine de-epoxidation that occurs in flashed leaves during the induction period is not related directly to the induction processes but, represents an independent process requiring continuous illumination.

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