# NADPH AND OXYGEN-DEPENDENT EPOXIDATION OF ZEAXANTHIN IN ISOLATED CHLOROPLASTS*

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Summary. NADPH and molecular oxygen were required for epoxidation of zeaxanthin to violaxanthin in isolated lettuce chloroplasts (Lactuca sativa var. Manoa). Accordingly, the epoxidase is classifed as an "external monooxygenase." Epoxidation in the dark was optimal at pH 7.3 and showed no activity below pH 5.5; it also occurred in the light where the thylakoid loculus is known to be acidified. These results suggest that the epoxidase is located in a chloroplast compartment that remains near neutrality under illumination. This may indicate that the epoxidase and de-epoxidase are separated by the thylakoid membrane; and to maintain cyclic turnover of violaxanthin under such conditions, a violaxanthin-zeaxanthin shuttle across the membrane is postulated.

Whereas information on the light-induced de-epoxidation reactions of the violaxanthin cycle has accumulated over the past eighteen years (1-4), little is known on its light-independent epoxidation reactions. Sapoznikov et al. (5) first described an increase of violaxanthin in leaves which were kept in dark after illumination, and showed that this reaction required aerobic conditions. Incorporation of $^{18}O$ into antheraxanthin and violaxanthin (6,7) gave direct evidence that it is molecular oxygen which reacts with zeaxanthin and antheraxanthin. The $^{18}O$ experiments further showed that epoxidation takes place in light as well as in dark which was confirmed by kinetic studies (8).

Until now epoxidation has been observed only in undisturbed leaves and not in isolated chloroplasts. The latter indicated that isolated chloroplasts lacked some cofactor of epoxidation which is present in chloroplasts of the

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Abbreviations: BSA, bovine serum albumin; HEPES, N-2-hydroxyethylpiperazine-N'-2 ethane sulfonic acid

whole leaf. We report herein that epoxidation can be induced in isolated chloroplasts by addition of NADPH if the thylakoid membrane is protected with BSA. We further provide evidence that the epoxidase, unlike the de-epoxidase, is not located in the thylakoid loculus but is in a chloroplast compartment which remains neutral upon illumination.

Materials and Methods

Washed whole chloroplasts were isolated from Lactuca sativa var. Manoa as described previously (9). Reactions were run at 26°. Absorbance changes at 505 nm with 540 nm as reference were monitored as reported (9). Crystalline bovine serum albumin was from Armour Pharmaceutical Company, Chicago, Ill. and glucose oxidase - catalase mixture (DeeO) from Miles Chemical Company, Elkhart, Ind.

Results

Recently we reported that the absorbance increase at 505 nm in isolated chloroplasts is directly related to violaxanthin decrease (9). Fig. 1 shows that this absorbance change can be reversed in the dark, if NADPH is present. The difference spectrum for the reversal (Fig. 2) was complementary to the difference spectrum for de-epoxidation (10). This fact as well as chemical analysis of the chloroplast pigments (Table 1) indicate that the 505 nm absorbance decrease was due to epoxidation of zeaxanthin to violaxanthin.

BSA accelerated epoxidation (Fig. 1) and influenced the amount of zeaxanthin which was finally converted to violaxanthin. Without BSA less than 50% of the zeaxanthin could be epoxidized (data not shown), whereas with BSA complete reversal of the light reaction was obtained (Table 1). Preliminary data suggest that the ratio of BSA to chlorophyll rather than the BSA concentration affects epoxidation of isolated chloroplasts. Maximal epoxidation velocity was obtained when the BSA : chlorophyll ratio (w/w) was greater than 25. Since BSA is known to bind fatty acids which are released during storage of isolated chloroplasts (11-13), the significant enhancement of epoxidation upon addition of BSA seems to indicate that epoxidation is more sensitive to released fatty acids than de-epoxidation.

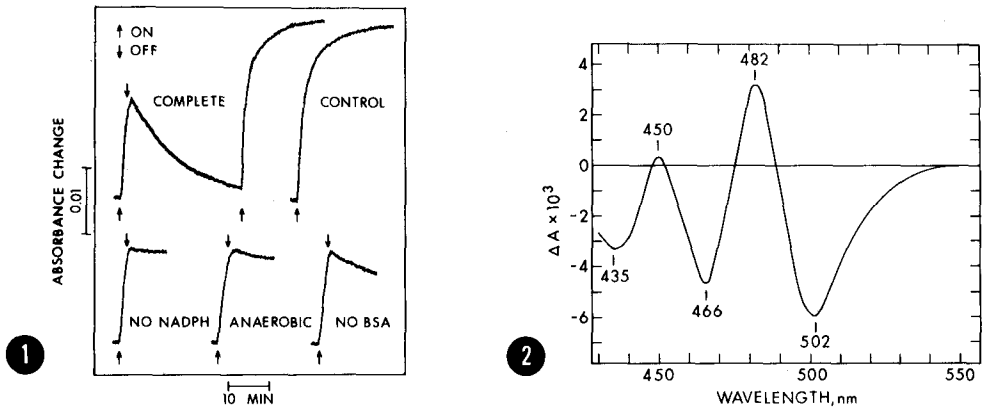


Figure 1. Reversal of the light-induced absorbance change at 505 nm in isolated chloroplasts.
Chloroplasts (0.1 ml of concentrated stock suspension) were osmotically shocked for 15 s in 1 ml water with or without 0.1% BSA, then mixed with 1.9 ml medium to give the following reaction mixture: 400 mM sorbitol, 50 mM HEPES-NaOH buffer (pH 7.2), 16 mM sodium ascorbate, chloroplasts equivalent to 12 μg chlorophyll $ml^{-1}$ and when present 0.03% BSA and 0.5 mM NADPH. For anaerobic conditions 50 mM glucose and 0.02 ml glucose oxidase-catalase mixture were present. Actinic light of 670 nm at 36 kerg $cm^{-2}s^{-1}$ was used.

Figure 2. Difference spectrum of NADPH and oxygen-dependent absorbance change in isolated chloroplasts.
Sample and reference containing 350 mM sorbitol, 50 mM glucose, 50 mM HEPES-NaOH buffer (pH 7.2), 16 mM sodium ascorbate, 0.03% BSA, 0.5 mM NADPH and swollen chloroplasts equivalent to 11.5 μg chlorophyll $ml^{-1}$ were illuminated for 3 min with 670 nm light; after illumination 0.02 ml glucose oxidase-catalase mixture was added to the reference. The difference spectrum for epoxidation was determined after 60 min and corrected for absorbance due to the glucose oxidase-catalase mixture.

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Epoxidation was half-saturated at 0.04 mM NADPH and saturated at 0.4 mM NADPH. NADPH could be replaced by NADH which at 0.5 mM yielded the same epoxidation rate. Besides pyridine nucleotides, molecular oxygen was required for epoxidation (Fig. 1), which is consistent with earlier results with whole leaves (6,7).

Dithiothreitol is known to inhibit de-epoxidation (14) but had no effect on epoxidation. The use of dithiothreitol allowed the study of epoxidation under illumination or low pH conditions where de-epoxidation otherwise would mask epoxidation. Fig. 3 shows that in illuminated as well as in dark-incubated chloroplasts, epoxidation was optimal between pH 7 and pH 8 and was

Table 1. Reversal of light-induced de-epoxidation of violaxanthin in isolated chloroplasts, determined by chromatographic analysis.

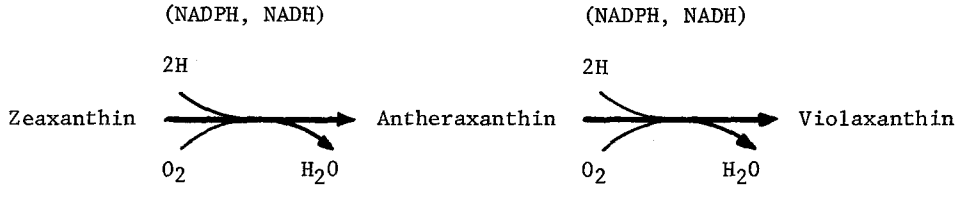
| | Violaxanthin | Antheraxanthin | Lutein plus zeaxanthin |
|---|---|---|---|
| | (Moles pigment per 100 moles chlorophyll a) | | |
| Dark | 9.8 | 1.0 | 12.5 |
| 2 min light | 6.1 | 1.8 | 15.5 |
| 2 min light plus 35 min dark | 8.9 | 1.6 | 12.5 |

Chloroplast suspensions (12.5 µg chlorophyll $ml^{-1}$ in 3 ml of the complete reaction mixture described in Fig. 1) were illuminated with 670 nm light at 36 kerg $cm^{-2}s^{-1}$ or kept in dark as indicated while changes in their absorbance at 505 nm were monitored. The chloroplasts were extracted with acetone and analyzed by reverse-phase thin-layer chromatography on fat impregnated Kieselguhr.

completely inhibited below pH 5.5; light caused only a slight pH shift towards higher pH. Furthermore, optimal epoxidation velocity was greater in light than in dark by approximately 25%. This observation may indicate an effect of light on the enzyme as well as on zeaxanthin and its environment. The latter would not be surprising since light-induced conformational changes of the thylakoid membrane are well-known.

## Discussion

Epoxidation of zeaxanthin *via* antheraxanthin to violaxanthin required molecular oxygen as well as NADPH (Fig. 1) or NADH, which suggests the following reaction scheme:



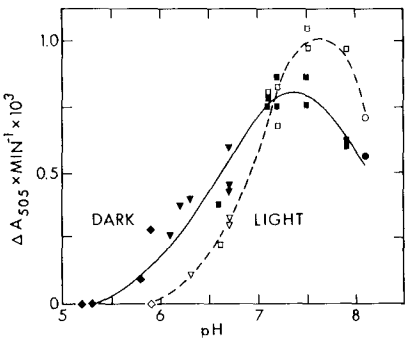



Figure 3. Effect of pH on epoxidation velocity of chloroplasts in dark and light.
Reaction mixtures were similar to the complete mixture in Fig. 1, except that different buffers (50 mM) were used: (● , ○ ) tricine, (■ , □ ) HEPES, (▲ , △) sodium phosphate, (◆ , ◇) sodium citrate. Chloroplast suspensions were illuminated with 670 nm light at 36 kerg $cm^{-2}s^{-1}$ until about half of the available violaxanthin was converted to zeaxanthin; 0.3 mM dithiothreitol was added and illumination was either terminated (closed symbols) or continued (open symbols). Epoxidation velocity was determined as the rate of absorbance decrease at 505 nm at a defined zeaxanthin concentration. The data were obtained from three different chloroplast preparations.

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In both reaction steps, one atom of the oxygen molecule is incorporated into the substrate while the other is reduced to water. Following the nomenclature of Hayaishi (15) the epoxidase can be classified as an "external monooxygenase" with pyridine nucleotides as electron donors. NADPH requirement of epoxidation is not limited to lettuce but has been discovered independently for spinach chloroplasts (A. Hager, personal communication).

Epoxidation was optimal between pH 7 and pH 8 whereas no reaction was observed near pH 5 (Fig. 3). Since epoxidation takes place in light -- as shown previously for whole leaves (6-8) and now for isolated chloroplasts (Fig. 3) -- the epoxidase does not appear to be located inside the thylakoid loculus, which is acidified under such conditions, but rather in a chloroplast compartment that remains neutral upon illumination. It should be mentioned that dithiothreitol which had to be present for these pH measurements (Fig. 3) does not uncouple photophosphorylation (16) and therefore should not decrease light-induced thylakoid acidification.

A view consistent with the low sensitivity of epoxidation towards acidic

loculus pH is that the epoxidase -- in contrast with the de-epoxidase (3) -- is located in or external to the thylakoid membrane where it is shielded against loculus pH. A consequence of this interpretation would be that violaxanthin is de-epoxidized at the internal membrane-surface and after its conversion to zeaxanthin diffuses towards the external membrane-surface where it is epoxidized. Thus the complete cycle would consist of a violaxanthin-zeaxanthin shuttle across the thylakoid membrane.

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