Biochimica et Biophysica Acta, 357 (1974) 144–150

BBA 46780

LIGHT-INDUCED DE-EPOXIDATION OF VIOLAXANTHIN IN LETTUCE CHLOROPLASTS
III. REACTION KINETICS AND EFFECT OF LIGHT INTENSITY ON DE-EPOXIDASE ACTIVITY AND SUBSTRATE AVAILABILITY*

D. SIEFERMANN and H. Y. YAMAMOTO

Department of Food Science and Technology, University of Hawaii, Honolulu, Hawaii 96822 (U.S.A.)

(Received February 4th, 1974)

SUMMARY

The 505 nm change in isolated chloroplasts of *Lactuca sativa* var. *Manoa* showed a linear correlation with violaxanthin decrease. The corresponding difference extinction coefficient was established as 27.3 $mM^{-1} \cdot cm^{-1}$.

The 505 nm change followed first-order kinetics. The first-order rate constant, a measure of de-epoxidase activity, and the final extent of the 505 nm change, a measure of the extent of de-epoxidation, varied with light intensity. In 670 nm light the activity remained about half-maximal at intensities from 1 to 4 kerg $\cdot cm^{-2} \cdot s^{-1}$ before reaching saturation at 9 kergs $\cdot cm^{-2} \cdot s^{-1}$. In contrast, the extent showed a simpler response and reached saturation already at 4 kergs $\cdot cm^{-2} \cdot s^{-1}$. These results suggest that different photosynthetic factors determine de-epoxidase activity and de-epoxidation extent. De-epoxidase activity is known to reflect steady-state thylakoid pH whereas the de-epoxidation extent was found to reflect light-dependent variable substrate availability. The available fraction varied in size from 40 % of the total violaxanthin at 1 kerg $\cdot cm^{-2} \cdot s^{-1}$ to 67 % at saturation. The possibility that variable availability reflects conformational changes of the membrane near Photosystem II which result in variable exposure of the substrate to enzyme is discussed.

INTRODUCTION

In leaves, light induces de-epoxidation of violaxanthin to zeaxanthin through the intermediate antheraxanthin [1]; when O_2 is present re-epoxidation occurs in a light-independent backreaction [1, 2]. Together these reactions form a cycle whose turnover rate is controlled by light [3].

Abbreviations: DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

* Journal Series Publication No. 1716 of the Hawaii Agricultural Experiment Station.

Part of this cycle can be studied in isolated chloroplasts which carry out de-epoxidation but not epoxidation [4]. Hager [5] showed that in the presence of ascorbate, de-epoxidation occurs under conditions where a proton gradient develops across the thylakoid membrane. As the de-epoxidase has an optimum near pH 5, he concluded that light produces within the thylakoid the pH condition necessary to activate the enzyme.

Isolated chloroplasts in the presence of ascorbate show a large absorbance change which has a maximum at 505 nm and the overall characteristics of a carotenoid shift [6]. The first paper of this series correlated this absorbance change with de-epoxidation based on its behavior towards proton concentration inside the thylakoid [7]; and the second paper described the inhibitory effect of dithiothreitol which distinguished the 505-nm change from the 515 nm change [8].

We now describe the kinetics of de-epoxidation and provide evidence that light, in addition to affecting de-epoxidase activity through proton pumping, affects substrate availability.

MATERIALS AND METHODS

Lettuce, *Lactuca sativa* var. *Manoa* obtained directly from the producer was washed in tap water and stored in the dark at 2–4 °C until used, but no longer than 3 days. Green parts of the outer leaves (about 8 g) were homogenized in 50 ml sorbitol–NaCl–HEPES–ascorbate medium (400 mM sorbitol, 10 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 16 mM sodium ascorbate; adjusted to pH 7.0 with NaOH) for 5 s in a semimicro Waring Blendor, filtered through 1 layer of 37 μm mesh nylon monofilament bolting cloth and centrifuged by accelerating the rotor to $1500 \times g$ and stopping it quickly by hand. The pellet was washed in 50 ml sorbitol–NaCl–HEPES–ascorbate medium and suspended in 1–2 ml sorbitol–NaCl–HEPES–ascorbate medium. The isolation was completed within 3–4 min and was carried out at 2–4 °C under green safety light. Washed isolated chloroplasts showed no significant change in activity for about 3 h when stored in sorbitol–NaCl–HEPES–ascorbate solution in the dark at 0 °C. Chlorophyll concentration was determined according to Vernon [9]. Carotenoid concentrations were determined after separation by reverse-phase thin-layer chromatography [10].

Chloroplast absorbance changes were determined at 26 °C with a Perkin–Elmer Model 365 two wavelength double-beam spectrophotometer. Chloroplast suspensions (10–14 μg total chlorophyll per ml) were illuminated with light filtered through a 670 nm interference filter. Opal-glass plates were placed directly before and behind the cuvette and the photomultiplier cell was shielded from actinic light with Corning filter CS-96. Actinic light energy was measured with a YSI Model 65 radiometer. To prevent settling of chloroplasts during long-term experiments, the reaction mixture was stirred continuously during measurement with a specially constructed magnetic stirrer which had shielding to protect the photomultiplier from the oscillating magnetic field.

RESULTS

Difference extinction coefficient of the 505 nm change

The quantitative relationship between 505 nm change and the conversion of

violaxanthin to antheraxanthin and zeaxanthin had not been established previously. Comparison of 505 nm change and carotenoid changes as determined by chemical analysis revealed a linear correlation between absorbance increase at 505 nm and decrease of violaxanthin concentration. The difference extinction coefficient for the absorbance change at 505 nm in terms of violaxanthin was 27.3 $mM^{-1} \cdot cm^{-1}$ (S.E. 0.66 $mM^{-1} \cdot cm^{-1}$, 12 samples). Thus the 505 nm change can be used as a precise quantitative measure of violaxanthin change.

Since the 505 nm change results from changes in the concentrations of violaxanthin, antheraxanthin and zeaxanthin, all of which absorb at 505 nm, the fact that absorbance increase is correlated only with the decrease of violaxanthin suggests that the absorbance of antheraxanthin and zeaxanthin at 505 nm in isolated chloroplasts is similar.

Kinetics and extent of de-epoxidation

Chloroplasts undergo de-epoxidation in the presence of light plus ascorbate. Fig. 1 shows the semilogarithmic plot of the absorbance change at 505 nm in chloroplasts when de-epoxidation was initiated with light or with the addition of ascorbate. The ascorbate-initiated change was first-order throughout whereas the light-initiated change showed two phases; a fast phase which lasted for about 2 min followed by a slower phase which was first order. Both phases of the light-initiated change resulted from de-epoxidation and not from interference from other absorbance changes since the quantitative relationship established earlier applied to both ascorbate and light-initiated changes. Furthermore the final extents of the 505 nm change as well as the first-order constants from the two methods of initiation were identical.

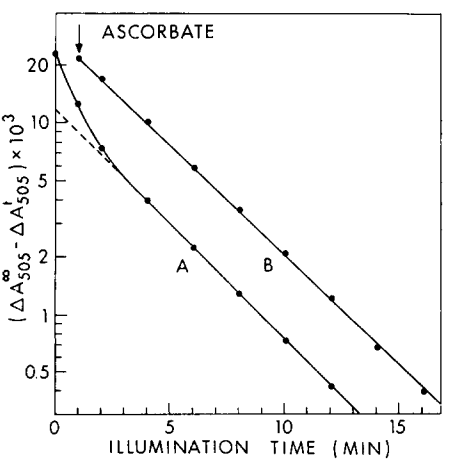

Fig. 1. First-order plot of the 505 nm absorbance change in chloroplasts initiated with light (A) or with ascorbate (B). The absorbance difference $\Delta A'_{505\,nm}$ at time t was subtracted from the final absorbance difference $\Delta A^{\infty}_{505\,nm}$ with 540 nm as reference. Reaction Mixture A (3 ml) contained 400 mM sorbitol, 10 mM NaCl, 50 mM HEPES buffer (pH 7.0), isolated chloroplasts equivalent to 11.8 μg chlorophyll per ml and 16 mM sodium-ascorbate. Reaction Mixture B was similar except that the suspension was preilluminated in the presence of 0.5 mM sodium ascorbate prior to the addition of 15.5 mM sodium ascorbate. The 670 nm actinic intensity was 36 kerg $\cdot cm^{-2} \cdot s^{-1}$.

Under saturating light intensity and optimal ascorbate concentration (16 mM), the final extent of the absorbance change was 7.8 % (S.E. 0.94 %, 21 samples) of the total absorbance of isolated chloroplasts at 505 nm with 550 nm as reference. The first-order constant had a value of 0.29 min^{-1} (S.E. 0.04 min^{-1}, 21 samples).

Effect of light intensity on de-epoxidase activity and extent of de-epoxidation

In the past, de-epoxidase activity was evaluated by the rate of de-epoxidation over relatively long time intervals. In view of its kinetics as well as of the variability in substrate availability shown below, the first-order rate constant would appear to be a more suitable measure of de-epoxidase activity in isolated chloroplasts. Fig. 2 shows that light intensity did influence the rate constant as well as the final extent of the absorbance change. In 670 nm actinic light, de-epoxidase activity (Fig. 2a) seemed to remain near half-maximal over intensities from 1 to 4 kerg · cm^{-2} · s^{-1} before reaching saturation at 9 kergs · cm^{-2} · s^{-1}. In contrast the extent of the 505 nm change (Fig. 2b) and thus the extent of violaxanthin de-epoxidation showed a simpler response to increasing light intensity and reached saturation at a lower intensity of 4 kerg · cm^{-2} · s^{-1}. Fig. 3 shows that chloroplasts which had achieved the maximum possible absorbance at low light intensity still had full activity when illuminated with saturating light. The rate constant and final extent of the 505-nm change under saturating light were not influenced by the preceding low-intensity illumination.

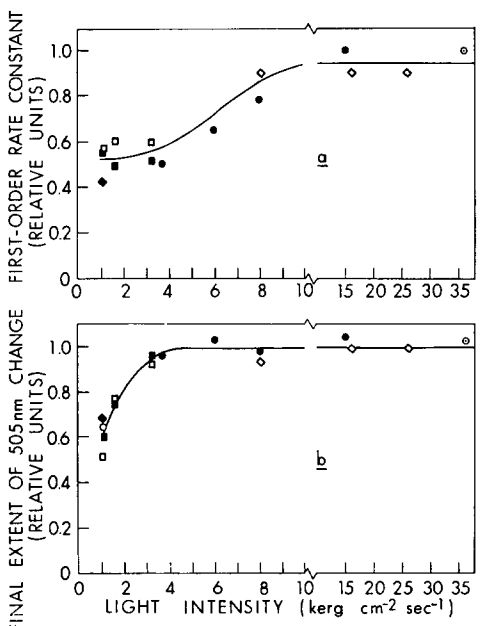

Fig. 2. Effect of light intensity on the light-initiated 505 nm absorbance change in lettuce chloroplasts. (a) Rate constant of the 505 nm change as a function of light intensity. (b) Final extent of the 505 nm change as a function of light intensity. (Note that the intensity scale changes at 10 kerg · cm^{-2} · s^{-1}.) Experimental conditions were as described in Fig. 1, Reaction Mixture A. The various symbols represent different chloroplast preparations. For each preparation the rate constant and final extent are shown relative to the control of 36 kerg · cm^{-2} · s^{-1}.

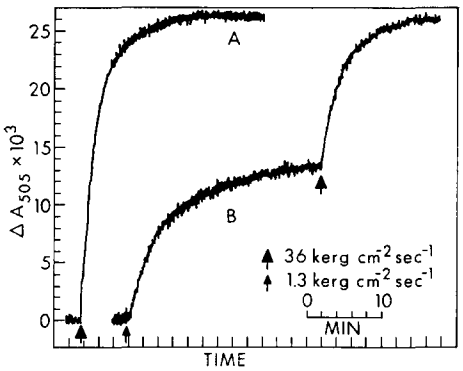

Fig. 3. Light-initiated absorbance change at 505 nm in isolated chloroplasts under saturating light intensity (A) and during consecutive phases of increasing light intensity (B). Experimental conditions were as described in Fig. 1, Reaction Mixture A. The chloroplast concentration was equivalent to 12.3 μg chlorophyll per ml.

Analysis of the final xanthophyll composition by thin-layer chromatography showed that neither violaxanthin nor antheraxanthin had been de-epoxidized completely under conditions which gave maximal 505 nm change. Approx. 3.6 moles of violaxanthin per 100 moles chlorophyll *a* or about 33 % of the dark level remained unchanged during both light and ascorbate-initiated de-epoxidation. The final concentration of violaxanthin was independent of ascorbate concentration which indicated that it did not represent equilibrium concentration of a reversible reaction. As the extent of absorbance change is correlated directly with the amount of violaxanthin de-epoxidized, Fig. 2b indicates that the size of the available violaxanthin fraction was dependent on light intensity and that the available violaxanthin increased from about 40 % to a maximum of about 67 % of the total violaxanthin over the actinic light range from 1 to 4 kerg $\cdot$ cm^{-2} $\cdot$ s^{-1}.

DISCUSSION

A few years ago studies on de-epoxidation kinetics required analysis of samples by chromatographic methods. Not only did such methods require much experimental effort to produce a limited amount of data but the precision of the data itself was limited. The discovery of the 505 nm absorbance change [6] and the development of a stirring arrangement which prevents settling of the chloroplasts during measurement has enabled the continuous and long term monitoring of de-epoxidation. The present finding of a direct quantitative relationship between absorbance increase and violaxanthin decrease opened the possibility for detailed kinetic studies on violaxanthin de-epoxidation.

Ascorbate initiated de-epoxidation followed first-order kinetics throughout the course of the reaction whereas the kinetics of light-initiated de-epoxidation were more complex, with a fast initial change preceding the first-order reaction (Fig. 1). This fast initial change does not appear to be caused by increased substrate availability: if more violaxanthin was available during the first minutes of illumination, the total amount of de-epoxidized violaxanthin under the light-initiated condition

should be larger than under the ascorbate-initiated reaction. The results of Fig. 1 as well as those from direct pigment analysis show instead that equal amounts of violaxanthin were de-epoxidized under both conditions. A more detailed treatment of the fast initial change is in preparation.

De-epoxidase activity as evaluated by first-order rate constant was light-dependent. It remained about half-maximal in an intensity range from 1 to 4 kerg · $cm^{-2} \cdot s^{-1}$ before increasing to a maximum at 9 kerg · $cm^{-2} \cdot s^{-1}$ (Fig. 2a). That light intensity affects de-epoxidase activity is not surprising since increasing light intensity increases proton pumping across the thylakoid membrane [11] and hence de-epoxidase activity [5]. However, the insensitivity of de-epoxidase activity towards increasing intensity at low light intensities was surprising. This intensity lag could reflect the buffering capacity of chloroplasts which according to Avron [12] is optimal around pH 6. At low intensities there should be a range where light, while stimulating proton uptake, causes only minimal pH change. The reported pH at which optimal buffering capacity is observed fits with the pH value at which activity of the purified enzyme is half maximal: Hager [5] reported half-maximal zeaxanthin formation after a reaction time of 30 min at pH 5.9 whereas Chenchin, E. and Yamamoto, H. Y. (unpublished) observed half-maximal initial velocity of de-epoxidation near pH 6.

Heath [11] found the steady state extent of external pH saturated at about 4 nEinstein · $cm^{-2} \cdot s^{-1}$ (645 nm light), which suggests that internal pH is also saturated under this condition. We obtained a saturating level of de-epoxidase activity at about 9 kerg · $cm^{-2} \cdot s^{-1}$ (670 nm light) equivalent to 5.1 nEinstein · $cm^{-2} \cdot s^{-1}$ which is in good agreement with the data of Heath and suggests that de-epoxidase activity when expressed as the first-order rate constant can be used as an endogenous probe of the steady-state thylakoid pH.

Whereas the relationship of de-epoxidase activity with proton pumping of photosynthesis is well established, the concept of variable substrate availability has not been reported previously. Our experiments demonstrate that under optimal conditions only about 67 % of violaxanthin could be acted on by the de-epoxidase and suggest heterogenous location of violaxanthin in the thylakoid membrane. The change in the available fraction of violaxanthin at lower light intensities (Fig. 2b) may reflect intensity dependent changes in the physical arrangement of violaxanthin in the membrane which are established almost instantaneously upon illumination (Fig. 3). This light-induced aspect of de-epoxidation suggests that there could be a closer link with primary photosynthetic events than is indicated by the relatively slow kinetics of de-epoxidation or by the fact that de-epoxidation is enzymic.

Violaxanthin is known to be an accessory pigment of Photosystem II [14] and Photosystem II particles are assumed to be located on the inner side of the thylakoid membrane [15]. Murakami and Packer [16] found that the thickness of grana membranes decreased by 25 % after illumination reflecting structural changes in the organization of grana membranes upon illumination. Recently Wang and Packer [17] reported that "particles seen in the hydrophobic core of grana membranes can change their orientation within the membrane" upon illumination. They did not identify those particles as Photosystem II particles, although these particles are described as being located at the same fracture face of freeze-etched membranes as the Photosystem II particles [15].

Although not conclusive one may speculate that the variable availability of violaxanthin reflects variable exposure of violaxanthin to the de-epoxidase caused by conformational changes of the membrane; and the conformational changes may consist in changes of the orientation of Photosystem II particles in the thylakoid membrane. With this background it is of interest to investigate what photosynthetic reactions determine the size of the available violaxanthin fraction.

ACKNOWLEDGMENTS

This work was supported in part by Grant GB-32022X from the Division of Biological and Medical Sciences, National Science Foundation.

REFERENCES

1 Yamamoto, H. Y., Nakayama, T. O. M. and Chichester, C. O. (1962) Arch. Biochem. Biophys. 97, 168–173
2 Yamamoto, H. Y. and Chichester, C. O. (1965) Biochim. Biophys. Acta 190, 303–305
3 Siefermann, D. (1972) in Proc. 2nd Int. Congr. Photosyn. Res. (Forti, G., Avron, M. and Melandri, A., eds), Vol. 1, pp. 629–635, W. Junk, The Hague
4 Hager, A. (1966) Ber. Dtsch. Bot. Ges. 79, 94–112
5 Hager, A. (1969) Planta 89, 224–243
6 Yamamoto, H. Y., Wang, Y. and Kamite, L. (1971) Biochem. Biophys. Res. Commun. 42, 37–42
7 Yamamoto, H. Y., Kamite, L. and Wang, Y. (1972) Plant Physiol. 49, 224–228
8 Yamamoto, H. Y. and Kamite, L. (1972) Biochim. Biophys. Acta 267, 538–543
9 Vernon, L. P. (1960) Anal. Chem. 32, 1144–1150
10 Siefermann, D. (1971) Dissertation, University of Tuebingen
11 Heath, R. L. (1971) Biochim. Biophys. Acta 256, 645–655
12 Avron, M. (1972) in Proc. 2nd Int. Congr. Photosyn. Res. (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 861–871, W. Junk, The Hague
13 Heath, R. L. and Hind, G. (1972) J. Biol. Chem. 247, 2917–2925
14 Vernon, L. P., Shaw, E. R., Ogawa, T. and Raveed, D. (1971) Photochem. Photobiol. 14, 343–357
15 Arntzen, C. J., Dilley, R. A. and Crane, F. L. (1969) J. Cell Biol. 43, 16–31
16 Murakami, S. and Packer, L. (1970) J. Cell Biol. 47, 332–351
17 Wang, A. Y.-I. and Packer, L. (1973) Biochim. Biophys. Acta 305, 488–492