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THE EFFECTS OF DITHIOTHREITOL ON VIOLAXANTHIN DE-EPOXIDATION AND ABSORBANCE CHANGES IN THE 500-nm REGION*

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SUMMARY

The effects of dithiothreitol on absorbance changes at 505 and 515 nm in isolated lettuce chloroplasts were investigated. Dithiothreitol inhibited the ascorbate-dependent 505-nm change that is due to the de-epoxidation of violaxanthin to zeaxanthin. Dithiothreitol was effective for both light-induced de-epoxidation at pH 7 and dark de-epoxidation at pH 5. Titration of de-epoxidase activity with dithiothreitol resulted in complete inhibition at about 5 μmoles dithiothreitol per mg chlorophyll. Removal of dithiothreitol restored de-epoxidase activity. These results are consistent with the view that dithiothreitol inhibits violaxanthin de-epoxidation and the corresponding 505-nm change by reducing a disulfide that is required for de-epoxidase activity.

Dithiothreitol was effective in resolving absorbance changes due to violaxanthin de-epoxidation and other changes that were superimposed under some conditions. At 515 nm and in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), phenazine methosulfate, and ascorbate, dithiothreitol inhibited the large, slow and irreversible change which was due to de-epoxidation but not the fast and reversible so-called 515-nm change. At 505 nm and under similar conditions, dithiothreitol revealed the presence of a slow reversible change in addition to the one from de-epoxidation. Results with dithiothreitol showed that the absorbance change at 505 nm in the presence of DCMU, 2,6-dichlorophenolindophenol and ascorbate was due entirely to de-epoxidation. Similarly, absorbance changes at 515 nm also appeared to be mainly from de-epoxidation but with the presence of a small transient change due to some other components. It is suggested that dithiothreitol may be useful in resolving complex light-induced absorbance changes in other photosynthetic systems as well as in enabling new studies on reversible absorbance changes in the 500-nm region.

INTRODUCTION

Recently, it was shown that in the presence of ascorbate, isolated chloroplasts exhibit a large and irreversible light-induced absorbance change that has the characteristics of a carotenoid shift[1]. This shift is due to the stimulation of enzymic conversion

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate.

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of violaxanthin to zeaxanthin as a result of the acidic conditions produced in chloroplast by hydrogen-ion transport[2]. Accordingly, at pH 5 and upon addition of ascorbate, de-epoxidation of violaxanthin[2,3] and the corresponding absorbance shift[2] occur in the dark. Since the absorbance change from de-epoxidation is in the spectral region where various changes have been observed in different photosynthetic organisms[4–8] it was of interest to seek additional distinguishing characteristics. We report herein that dithiothreitol inhibits the absorbance change that is due to violaxanthin de-epoxidation but not several other superimposed changes that are present under certain conditions.

METHODS

Chloroplasts were isolated from market lettuce (*Lactuca sativa* var. Romaine) in a mixture of 0.4 M sorbitol, 0.1 M NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 7.0) and 0.4% (w/w) polyvinylpyrrolidone (Type NP-K60) according to procedures described previously[2] and then suspended in a small volume of HEPES (pH 7.0) or citrate (pH 5.0) buffer. Aliquots of these suspensions were tested for absorbance changes with various reagents. Absorbance changes, chlorophyll concentrations, and actinic light intensities were measured as described previously[2]. Actinic light was obtained from a Unitron Model LKR illuminator and was filtered through Corning CS 2-58 and two heat-absorbing glass filters.

RESULTS

The effect of dithiothreitol on ascorbate-dependent and light-induced 505-nm absorbance change at pH 7.0 is shown in Fig. 1. Dithiothreitol at 100 μM completely

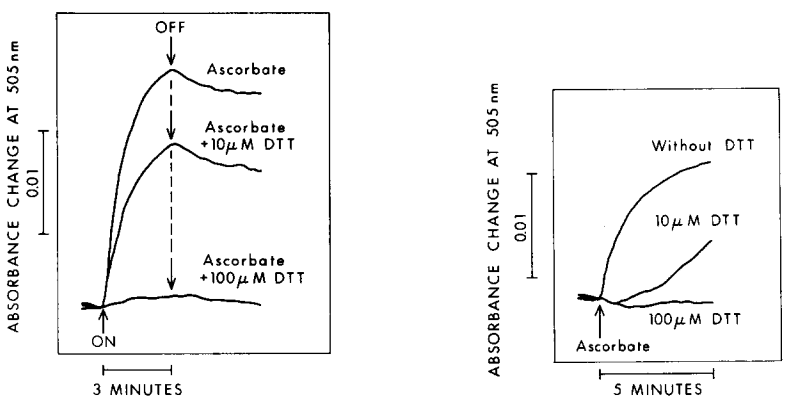

Fig. 1. The effect of dithiothreitol on light-induced absorbance changes at 505 nm in the presence of ascorbate. The reaction mixtures contained isolated chloroplasts equivalent to 23 μg chlorophyll per ml, 50 mM HEPES buffer at pH 7.0, 30 mM sodium ascorbate, and dithiothreitol (DTT) as shown. Actinic light intensity at the surface of the cuvette was $6.4 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$s^{-1}. The photomultiplier was shielded with Corning filter CS4-96. The reference wavelength was 540 nm.

Fig. 2. The effect of dithiothreitol on ascorbate-induced dark absorbance changes at 505 nm. Sodium ascorbate (10 mM final concentration) was added where indicated (↑) to mixtures containing chloroplasts equivalent to 17 μg chlorophyll per ml, 50 mM sodium citrate buffer at pH 5.0, and dithiothreitol (DTT) as shown.

inhibited the change whereas it was only partly effective at lower concentration. Inhibition by dithiothreitol was rapid, taking effect soon after addition of the reagent and was reversible when dithiothreitol was removed by washing. Activity was also restored in preparations that were incubated in the dark for 15 min or longer presumably because of oxidative depletion of dithiothreitol. The effect of dithiothreitol on ascorbate-dependent dark change at pH 5.0 is shown in Fig. 2. Dithiothreitol concentrations which inhibited the light-induced absorbance change also inhibited this change. These results are consistent with the view that dithiothreitol inhibits the ascorbate-dependent absorbance change at 505 nm by a direct effect on the de-epoxidase through reduction of a disulfide that is required for activity.

Titration of de-epoxidase activity in twice-washed chloroplasts gave complete inhibition at about 5 μmoles dithiothreitol per mg chlorophyll for both light-induced and dark reactions. This ratio, however, probably reflects total titratable disulfides of washed chloroplasts rather than of violaxanthin de-epoxidase alone. Other thio reagents tested, such as glutathione and cysteine, were not as effective as dithiothreitol.

Previously it was shown that the 505-nm change could be mediated by Photosystem I electron transport[2]. The difference spectra indicated, however, that when phenazine methosulfate (PMS) was present other changes were superimposed on the de-epoxidation change. Dithiothreitol was found effective in resolving these different changes. Fig. 3 shows the effect of dithiothreitol on absorbance changes at 505 nm with PMS present. The absorbance change appeared to consist of at least two components; one, a large irreversible change that was inhibited by dithiothreitol and thus due to de-epoxidation and the other, a smaller but reversible component that was insensitive to dithiothreitol. Fig. 4 shows absorbance changes at 515 nm under similar conditions. Again, the absorbance change was composed of two components, one a slow change which was sensitive to dithiothreitol and another small

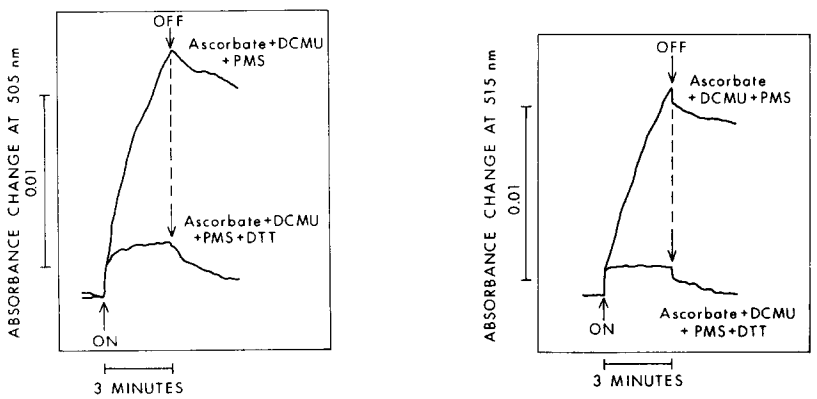

Fig. 3. The effect of dithiothreitol on light-induced absorbance changes at 505 nm in the presence of PMS. The reaction mixtures contained chloroplasts equivalent to 13 μg chlorophyll per ml, 50 mM HEPES (pH 7.0), 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), 50 μM PMS, 30 mM sodium ascorbate, and when present, 1 mM dithiothreitol (DTT). Other conditions were as described for Fig. 1.

Fig. 4. The effect of dithiothreitol on light-induced absorbance changes at 515 nm in the presence of PMS. Except for the measuring wavelength, the experimental conditions were the same as described for Fig. 3.

but fast and reversible change not sensitive to dithiothreitol. The fast change appeared to be the well-known 515-nm change[4] and the slow component the de-epoxidation change. The slow reversible component at 505 nm that is insensitive to dithiothreitol (Fig. 3) would appear not to be related to the 515-nm change based on its kinetics.

The results of absorbance changes at 505 nm in the presence of dichlorophenol-indophenol (DCIP) are shown in Fig. 5. Dithiothreitol nearly completely inhibited the change, thus showing that under this condition there was no significant absorbance contribution from other components. This is consistent with previous conclusions based on the difference spectra for de-epoxidation obtained with DCIP[2]. Studies at 515 nm revealed that the light-induced change in the presence of DCIP was also predominantly due to de-epoxidation (Fig. 6). There was, however, a small superimposed transient change which was not inhibited by dithiothreitol. The transient nature of this change would suggest it is different from the 515-nm change.

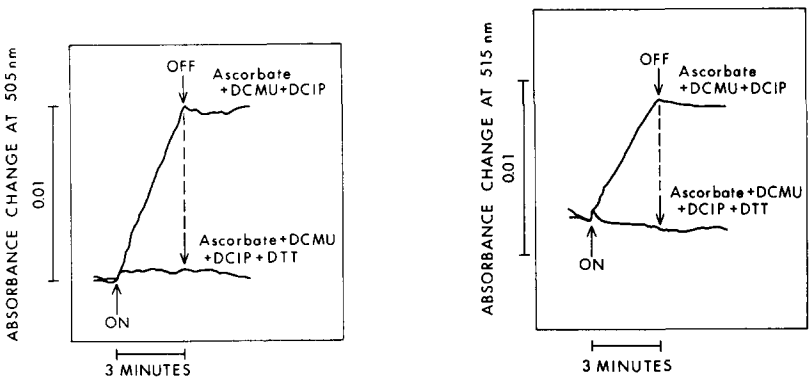

Fig. 5. The effect of dithiothreitol on light-induced absorbance changes at 505 nm in the presence of DCIP. The reaction mixtures contained chloroplasts equivalent to 13 μg chlorophyll per ml, 50 mM HEPES (pH 7.0), 10 μM DCMU, 50 μM DCIP, 30 mM sodium ascorbate, and when present, 1 mM dithiothreitol (DTT). Other conditions were as described for Fig. 1.

Fig. 6. The effect of dithiothreitol on light-induced absorbance changes at 515 nm in the presence of DCIP. Except for the measuring wavelength, the experimental conditions were as described for Fig. 5.

The dithiothreitol concentration required to achieve complete inhibition of de-epoxidation in the presence of PMS or DCIP was higher and more variable than the inhibition of de-epoxidation in the presence of ascorbate alone. Presumably, this higher concentration was required because of the increased oxidative depletion of dithiothreitol that occurs in the presence of these compounds.

DISCUSSION

Light-induced turnover of violaxanthin in plants and algae is well established[9,10]. Turnover proceeds by interconversion of violaxanthin to zeaxanthin through the intermediate antheraxanthin[11] and results in O_2 uptake[12]. In isolated chloroplasts, only the forward de-epoxidation phase has been demonstrated. In such systems, it has been shown that de-epoxidation of violaxanthin is an enzymic reaction which has an optimum near pH 5.0 and requires the presence of reducing potential. The role

of light in de-epoxidation is to produce the acidic condition in chloroplasts necessary for activity[2,3].

This laboratory showed that in isolated chloroplasts de-epoxidation of violaxanthin could be seen as a carotenoid shift in a difference spectrum[2]. This spectrum had the expected alternating positive and negative changes with a major positive peak at 505 nm. Maslova and Meister[13] observed second derivative absorbance changes in chlorophyll *b*-deficient leaves and attributed such changes to violaxanthin de-epoxidation. Light-induced absorbance changes which appear to be due to shifts in carotenoid spectra have been observed in various photosynthetic cells[5,7,8]. Such shifts are thought to reflect a physical change that is closely associated with the primary photochemical reaction because of their fast kinetics. However, both the nature of the specific pigments involved and the significance of the changes themselves are controversial.

The carotenoid shift that is due to violaxanthin de-epoxidation is different in that it is due to a specific enzymic conversion of one carotenoid to another and a process mediated by events several steps removed from the primary photochemical act. Available evidence points to violaxanthin as a type of photosynthetic back reaction operating between the two photosystems[14]. Such a reaction could have a role in energy transfer between the two photosystems, although other proposals have been made[3,15].

In the present study it was shown that dithiothreitol inhibited the ascorbate-dependent change at 505 nm that is due to de-epoxidation under both light-induced conditions at pH 7.0 (Fig. 1) and dark conditions at pH 5.0 (Fig. 2). Inhibition of the dark reaction indicates that dithiothreitol acts directly on the de-epoxidase, inactivating it by reducing a critical disulfide group.

Dithiothreitol is presumed to inhibit the light-induced change in a similar manner although additional effects also could be involved. Inhibition of the light reaction with dithiothreitol, however, is not due to inhibition of electron transport since other activities such as the 515-nm change are retained. Furthermore, preliminary studies indicate that dithiothreitol does not inhibit the photoreduction of $NADP^+$. On the other hand, the possibility that dithiothreitol has an additional indirect effect on de-epoxidation through inhibition of hydrogen-ion transport has not been excluded.

The use of dithiothreitol confirmed previous conclusions that absorbance changes mediated by PMS were complex[2]. At 505 nm a slow, reversible change was present and at 515 nm a fast, reversible change was evident. Both changes were insensitive to dithiothreitol (Figs 3 and 4). In de-epoxidation mediated with DCIP, the effect of dithiothreitol indicated the changes at 505 and 515 nm to be predominantly due to de-epoxidation, except for a small persistent change at 505 nm (Fig. 5) and a small transient change at 515 nm (Fig. 6). The fast reversible change at 515 nm in the presence of PMS was presumed to be the well-known 515-nm change[4]. If so, the extent of the change was variable under the different de-epoxidation conditions investigated, being considerably smaller in the absence of PMS. The nature of the other reversible changes observed have not been determined but some could be due to chlorophyll[2]. Although the effect of dithiothreitol on de-epoxidation in other systems was not tested, it may be reasonable to expect dithiothreitol to have similar effects on de-epoxidases in other photosynthetic organisms. If this is the case, it would be of potential value in testing for the contribution of de-epoxidation to absorbance changes

in other systems. The apparent specific effect of dithiothreitol on de-epoxidation appears to provide a means for investigating reversible absorbance changes in the 500-nm region under new conditions.

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