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ASCORBATE-INDEPENDENT CAROTENOID DE-EPOXIDATION IN INTACT SPINACH CHLOROPLASTS

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SUMMARY

Slow (> 1 s) light-induced absorbance changes in the 475–530 nm spectral region were examined in Type A chloroplasts from spinach. The most prominent absorption change occurred at 505 nm. The difference spectrum for this light-induced increase, its absence in osmotically shocked chloroplasts and restoration by ascorbate, and its sensitivity to dithiothreitol indicate that the absorption change is due to carotenoid de-epoxidation. The reaction in intact chloroplasts is characterized by its independence of exogenous ascorbate and a rate constant 3- to 8-fold higher than that reported previously for chloroplasts supplemented with ascorbate.

The relevance of carotenoid de-epoxidation to other photosynthetic processes was examined by comparing their sensitivities to dithiothreitol. Levels of dithiothreitol that eliminate the 505 nm shift are without effect on saturated rates of CO_2 fixation and do not appreciably inhibit fluorescence quenching. We conclude that carotenoid de-epoxidation is not directly involved in the reactions of photosynthesis or in the regulation of excitation allocation between the photosystems.

INTRODUCTION

Slow ($\geqslant$ 1 s) light-induced absorption changes in the spectral region from $\sim$ 500 to 520 nm have been reported for a number of photosynthetic systems. These absorption shifts have been attributed to de-epoxidation of carotenoids [1, 2], to changes in the electrochemical potential across the thylakoid membrane [3], to changes in light scattering [4] and, under certain conditions, to the photobleaching of carotenoids [5]. The relationship or significance of these slow events to the overall photosynthetic process is still unresolved. One problem has been the inability to demonstrate many of these events in isolated chloroplasts under physiological conditions. For example, carotenoid de-epoxidation, measurable either directly or via a slow, light-induced increase in absorbance at 505 nm, has been observed in broken chloroplasts (presumably Type B or C) isolated from lettuce and spinach. In such chloroplasts de-epoxidation is dependent upon the presence of substrate levels of

ascorbate [1, 2, 6, 7], whereas de-epoxidation in vivo is not [7]. Similarly, we recently reported the occurrence of a series of slow 514 nm absorption phases in the green alga *Ulva*, which we interpreted as a reflection of slow changes in electrochemical potential across the photosynthetic membranes [3]. These slow transient absorption phases, however, were never observed in isolated Type C [8] chloroplasts.

In the present experiments we examined the slow, light-induced absorption shifts in the 475–530 nm spectral region obtained with intact (Type A) chloroplasts [8]. We have characterized the most prominent slow absorption change in these chloroplasts, namely an absorbance increase at 505 nm and we have determined the relationship of this spectral shift to other photosynthetic processes.

MATERIALS AND METHODS

Chloroplasts were isolated from greenhouse spinach by the method of Cockburn et al. [9] as modified by Walker [10] and were more than 60 % intact as assayed by the ferricyanide reduction method [11].

Absorbance changes were measured with a dual-wavelength spectrophotometer using a 1-cm light path and 540 nm as the reference wavelength. The photomultiplier was protected by Corning 4-76 and Kodak Wrattan 64 filters. Chloroplasts were diluted to a chlorophyll concentration of 25 μg/ml.

For measurements of slow fluorescence quenching, Baird atomic B9-7400 and Scott RG-715 filters were inserted between sample and photomultiplier. Actinic illumination was at right angles to the photomultiplier. Chlorophyll concentration was 7.5 μg/ml.

A reaction medium containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 0.05 mM K_2HPO_4 and 50 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), pH 7.6, was used for all experiments. Red actinic light (16.3 kerg $\cdot$ cm^{-2} $\cdot$ s^{-1}) was provided by a 1000-W projector lamp and Schott KG-3, Baird Atomic B9-6500 filter combination. Temperature was thermoregulated at 20 °C.

RESULTS AND DISCUSSION

Intact spinach chloroplasts capable of substantial rates of CO_2 fixation (40–60 μmol/h per mg chlorophyll) were tested for light-induced absorption changes in the blue-green spectral region. Fig. 1 shows the kinetics of the absorption changes measured at 505 nm in saturating red light. Two distinct kinetic components can be seen with unamended, intact chloroplasts (traces a and e). A rapid ($\leqslant$ 1 s) increase in absorption can be resolved easily from a second, much slower increase requiring several minutes for completion. The rapid absorption phase was reversible; the slower phase was not. Intervening dark periods of up to 20 min failed to restore the slow absorption increase in a subsequent exposure to red light. It should be noted that the relative magnitudes of the rapid and slow absorption changes measured at 505 and 515 nm were different. Likewise, the characteristic, transient 515 nm absorption phases observed in *Ulva* [3] were conspicuously absent in intact chloroplasts.

The wavelength dependence of the rapid ($\sim$ 1 s) and slow ($\sim$ 3 min) absorption increases is shown in Fig. 2A. The spectrum for the slow absorbance change could be determined simply only for wavelengths greater than 500 nm (see below).

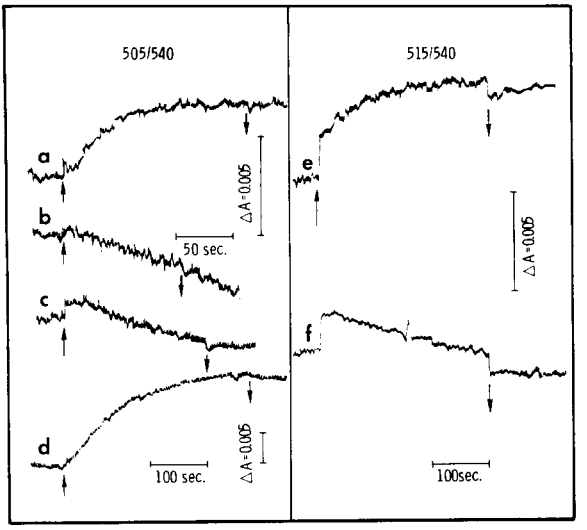

Fig. 1. Time course of light-induced absorbance changes at 505 and 515 nm in Type A spinach chloroplasts. (a, e) Untreated chloroplasts. (b) Osmotically shocked chloroplasts: chloroplasts were mixed initially with 1.0 ml distilled water; after 2 min an equal volume of twice concentrated assay medium was added. (c, f) Intact chloroplasts plus 0.25 mM dithiothreitol. (d) Osmotically shocked chloroplasts plus 16 mM ascorbate. Actinic light on (↑) and off (↓).

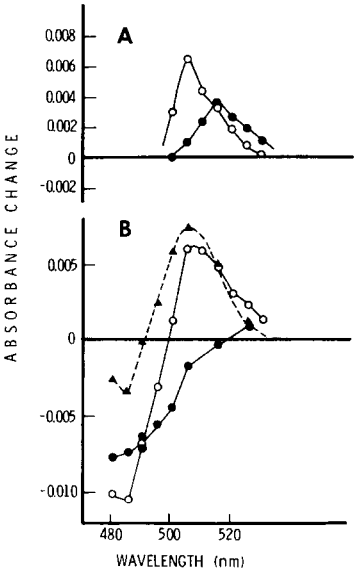

Fig. 2. Difference spectra for rapid and slow light-induced absorbance changes in Type A chloroplasts. (A) Difference spectrum for the steady-state absorbance change (○) and for the rapid initial spike (●). (B) The extent of the light-induced absorbance change 3 min after the onset of illumination in the absence (○) and the presence (●) of 0.25 mM dithiothreitol as a function of wavelength. The difference (▲) between these two difference spectra represents the difference spectrum of the slow, dithiothreitol-sensitive 505 nm change.

Clearly, the absorbance increase peaking at 505 nm represents the major contribution to slow light-induced changes in this region of the spectrum in intact chloroplasts. It is also spectrally distinct from the characteristic rapid 515 nm shift [12].

Several properties indicate that the slow light-induced absorption phase we observed in unamended intact chloroplasts is equivalent to the ascorbate-dependent changes studied by others and attributed to carotenoid de-epoxidation [1, 2]. As shown in Fig. 1 (trace b), subjecting our chloroplasts to brief osmotic shock eliminated the slow absorption increase at 505 nm. The slow change could be restored in osmotically shocked chloroplasts simply by adding ascorbate (trace d). The slow component of the 505 or 515 nm change in intact chloroplasts was also selectively inhibited by dithiothreitol (traces c and f). Yamamoto and Kamite [13] reported a similar selective inhibition in broken chloroplasts supplemented with ascorbate.

As noted above, the kinetics of the slow, red-light-induced absorption changes at wavelengths $\leqslant$ about 500 nm (using a 540 nm reference beam) were complex. Slow non-specific decreases in light scattering appear to be the major interfering factor. The effect is most apparent in intact chloroplasts treated with dithiothreitol (see Fig. 1, traces c and f). Since the light scattering effects and the characteristic 515 nm change appeared unaffected by dithiothreitol, we constructed a spectrum for the slow, light-induced absorption change by subtracting the difference spectrum obtained with intact chloroplasts plus dithiothreitol (Fig. 2B) from that obtained without dithiothreitol. The resulting difference, difference spectrum, plotted in Fig. 2B, is identical to that reported by Yamamoto et al. [1, 2] for the 505 nm change in ascorbate-supplemented broken lettuce chloroplasts.

The carotenoid de-epoxidation reaction in unamended, intact chloroplasts is less extensive, but substantially more rapid than that observed in chloroplasts supplemented with ascorbate [14]. Furthermore, in contrast to previous reports, we observed first-order kinetics from the onset of illumination. Using the difference extinction co-efficient reported by Siefermann and Yamamoto ($27.3\ mM^{-1} \cdot cm^{-1}$), we calculated (six chloroplast preparations) that between 0.45 and 0.95 mol violaxanthin per 100 mol chlorophyll were de-epoxidated by intact chloroplasts in saturating light, compared to 7.2 mol violaxanthin per 100 mol chlorophyll *a* in chloroplasts supplemented with ascorbate (calculated from the data of Siefermann and Yamamoto [14]). The rate constant varied from $0.64\ min^{-1}$ to $2.4\ min^{-1}$, but was significantly greater than that reported for chloroplasts supplemented with ascorbate ($0.29\ min^{-1}$). It should be noted that we also found a lower rate constant and larger extent for carotenoid de-epoxidation in osmotically shocked chloroplasts supplemented with ascorbate, i.e. a rate constant of $0.4–0.65\ min^{-1}$ and a total change equivalent to 1.3–2.6 mol violaxanthin per 100 mol chlorophyll.

At least three factors controlling carotenoid de-epoxidation in isolated chloroplasts have been identified: presence of a suitable reductant [2, 6, 15], activation of the de-epoxidase enzyme by low pH [2, 7] and "availability" of violaxanthin [14]. The occurrence of the de-epoxidase reaction in unamended intact chloroplasts may simply reflect the presence of an endogenous reductant in these chloroplasts, possibly ascorbate. Ascorbate levels equal to chlorophyll concentration by weight have been reported for chloroplasts from field-grown summer spinach [10]. Whether the reductant is normally present in the chloroplast and retained during the isolation of Type A chloroplasts or is generated in the light [15] was not established. The larger

rate constant for de-epoxidation in intact as compared to broken chloroplasts suggests a more efficient interaction of the endogenous reductant with the de-epoxidase enzyme.

The significance of carotenoid de-epoxidation in photosynthesis has yet to be satisfactorily determined [16, 17]. It has been postulated to regulate electron or energy flow between the two photosystems [16]. Recently, the "availability" of violaxanthin for de-epoxidation has been proposed as an indicator of membrane conformational changes [14].

The slow 505 nm change reported here for Type A spinach chloroplasts resembles slow fluorescence quenching seen in similar chloroplast preparations [18–20] in three major respects: kinetics, loss upon osmotic shock, and an apparent dependence upon internal acidification of the thylakoid [6, 7]. Fluorescence quenching in intact chloroplasts has been shown to depend on Mg^{2+} efflux from the thylakoids [19, 20]. Quenching is thought to reflect alterations in energy distribution between the photosystems [21], possibly mediated by ion-induced changes in membrane conformation [22]. Fluorescence quenching was tested for the dithiothreitol sensitivity that would indicate carotenoid involvement.

The upper trace in Fig. 3 shows the typical slow fluorescence quenching observed in intact chloroplasts and its reversal in the light upon addition of the ionophore A23187 and Mg^{2+} (or of 3(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU, data not shown). As shown in the lower traces of Fig. 3, dithiothreitol, at concentrations that eliminate carotenoid de-epoxidation in these chloroplasts, only minimally affected slow fluorescence quenching. (Note that, in fact, although the concentration of dithiothreitol was the same in both experiments, the ratio of dithiothreitol to chlorophyll was several-fold higher for the fluorescence measurements.) Thus, we conclude that carotenoid de-epoxidation is not an obligate reaction in the events associated

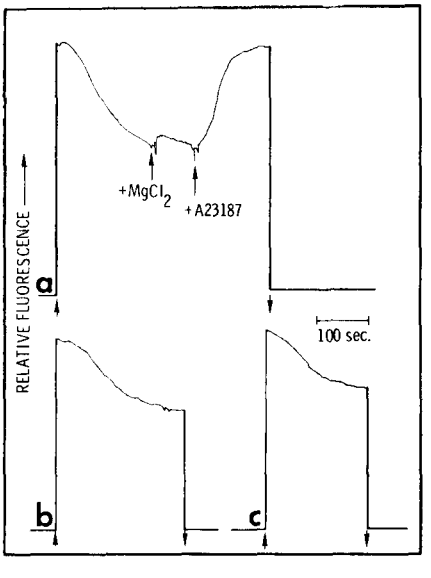

Fig. 3. The effect of dithiothreitol on fluorescence quenching in Type A spinach chloroplasts. (a) Reversal of quenching upon addition, in the light, of 7.5 mM $MgCl_2$ and A23187 (1 μg/ml). (b) Chloroplasts alone. (c) Chloroplasts plus 0.25 mM dithiothreitol. Actinic light on (↑) and off (↓).

with slow fluorescence quenching. These results do not exclude, however, the possibility that carotenoid de-epoxidation may be an "indicator" of these events. Our results are consistent with the proposal that dithiothreitol inhibits the de-epoxidase enzyme directly [13]. Dithiothreitol acts on the enzyme clearly, without affecting thylakoid acidification, a distinction that could not formerly be made.

We also determined the effect of dithiothreitol on saturated rates of O_2 evolution (measured polarographically [23]) with bicarbonate as electron acceptor in Type A chloroplasts. Dithiothreitol, again, at concentrations that eliminate carotenoid de-epoxidation, has essentially no effect on these rates. This argues against a direct involvement of carotenoid de-epoxidation in electron flow or CO_2 fixation in intact chloroplasts, a conclusion that is substantiated by the slow turnover of the carotenoid cycle (see also ref. 17).

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