Correlation of Absorbance Changes and Thylakoid Fusion with the Induction of Oxygen Evolution in Bean Leaves Greened by Brief Flashes

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ABSTRACT

Dark-grown bean leaves (Phaseolus vulgaris) which had been greened for several days in a repetitive series of brief xenon flashes were studied during the initial induction period when O₂ evolution first appears. The induction of O₂ evolution requires actinic irradiation (e.g. 2 mw/cm² of red light) and goes to completion in about 8 minutes with a half-time just under 3 minutes. Absorbance measurements on the intact leaves showed that a change of a carotenoid pigment, monitored at 505 nm, was closely correlated with the rate of O₂ evolution during the induction period. Inhibitor studies, however, showed that the absorbance change persisted in the presence of a number of inhibitors which blocked O₂ evolution. Electron microscopy revealed that the primary thylakoids which were unfused in the flashed leaves before induction became fused in pairs or groups of three during the 8-minute induction period. It is postulated that the 505-nm absorbance change of the carotenoid pigment is correlated more directly with the fusion process than with O₂ evolution. Heat treatment (45°C for 5 min) or infiltration with 0.8 M tris, which prevented the fusion process, also prevented the absorbance change.

If the leaves were preilluminated for 8 minutes with very weak red light (20 µw/cm²) which induced no O₂ evolution, absorbance change, or thylakoid fusion, there was an immediate burst of O₂ evolution at the onset of actinic irradiation and the induction period, as noted by O₂ evolution or by the 505-nm absorbance change, was reduced to 2 minutes (half-time of 40 seconds). It is concluded that the electron transport system in the flashed leaves is blocked at the Mn site between water and photosystem II and that the photoreduction of Mn into the thylakoid membranes occurs during the low light, photosynthesis process. After the electron transport chain is thus repaired, ion-pumping mechanisms driven by actinic light may lead to steady-state photosynthesis as well as to thylakoid fusion.

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2 Abbreviations: DPIP: 2,6-dichlorophenolindophenol; EPR: electron paramagnetic resonance; DBMIB: dibromothymoquinone; CCCP: carbonylcyanide-m-chlorophenylhydrazone.
were of illumination at a spectrum and derivative Fourth FLI-FL, spectrum, 8-min of FL, was plotted out at a 4-fold increase of sensitivity. Fourth derivative curves of the absorption spectra are also presented, FLIV and FLV.

leaf had a platinum electrode in the center and a silver ring as a reference electrode at the edge and served as a Clark-type O2 electrode (18, 19). A 1-cm leaf disc was placed on a Teflon membrane which covered the end of the electrode and a second membrane held down by an O-ring was placed on top of the leaf. The second light pipe combination was placed on the top surface. In this manner, absorbance changes and O2 exchange could be measured simultaneously while the leaf disc was being irradiated with red actinic light delivered by one of the light pipes. Time course changes of absorbance were measured as the difference in transmission between 505 nm and a reference wavelength at 555 nm with an Amino-Chance Duochromator (dual wavelength monochromator).

Red actinic light, used to irradiate the leaf and to stimulate O2 evolution, was obtained from a He-Ne laser which delivered 2 mw/cm² of 633 nm light through the light pipe system to the surface of the leaf. For photosensitization experiments, the intensity of this same source was decreased by a 1% neutral density filter to 20 μw/cm². The far red light (a maximum at 734 nm with 50% points at 720 and 760 nm) was obtained with a xenon lamp and glass filters (one 2030, two 5850, and one 2600 Corning filter, and a Calflex heat-reflecting filter) which gave 5 mw/cm² at the leaf.

The inhibitor studies were carried out on 1-cm leaf discs which were vacuum-infiltrated in buffer (0.4 mM sucrose, 5 mM tris-HCl, 10 mM NaCl) solutions for 30 min before the measurements. For comparisons of the effects of inhibitors on absorbance changes and structural changes, the same samples that were measured spectrophotometrically were fixed in glutaraldehyde and prepared for electron microscopy as described previously (6).

RESULTS

A flashed leaf incapable of evolving O2 becomes fully induced to evolve O2 during an 8-min irradiation with moderate intensities (2 mw/cm²) of red light. In our search for other parameters which might be correlated with the induction of O2 evolution, we examined the leaves for absorbance changes which occur during this induction process. The absorption spectrum of a flashed leaf was measured before and after an inductive irradiation: these spectra are presented as the curves marked FL and FLI, respectively, in Figure 1. The difference spectrum, FLI-FL, shows that absorbance changes occurred during the irradiation which are characteristic of a carotenoid pigment change. Fourth derivative spectra indicate the peak positions of absorption bands with much greater precision than can be deduced directly from the absolute absorption spectra (2). The second derivative curves of the absorption spectra in Figure 1, FLIV and FLV, show that the four bands apparent in the difference spectrum were each generated by a hypsochromic band shift of an absorption band during the induction process. Absorption spectra were also measured on a leaf after various cumulative periods of irradiation (Fig. 2). These absorption spectra and their corresponding irradiated-minus-dark (L-D) difference spectra show a progressive change and well defined isosbestic points, at least for the three longest wavelength difference bands. We take these isosbestic points to indicate the change of a particular carotenoid pigment from one form to another.

The absorbance change at 505 nm and the O2 exchange from a single flashed leaf were measured simultaneously as a function of time during the induction period (Fig. 3). A moderate intensity of light (2 mw/cm² at 633 nm) produced a maximal absorbance change at 505 nm and a maximal rate of O2 evolution (Fig. 3, curves marked b). The kinetic data of these two changes were essentially the same, both reaching their maximum levels in about 8 min with half-times of about 160 sec. If the leaf was illuminated with a limiting intensity of light (e.g. an intensity sufficient to stimulate O2 evolution to just above the compensation point [curve c]), the extent of the absorbance change was

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several of the treatments inhibitory inductive Light-minus-dark effect. leaves indicated inhibitors half-times versus examined by Vacuum with infiltration and two these higher intensity. irradiation a and induction after preillumination maximum shown rapid increase reaching period (curves b). 02 increased essentially the leaf indicated with arrow. The leaf was preilluminated with 2 mw/cm² of 633-nm light starting at the upward arrow. Curves b: same as curves a but no preilluminatio. Curves c: no preillumination, irradiation with 1 mw/cm² of 633-nm light at first upward arrow, increased to 2 mw/cm² at second upward arrow.

less than maximal but the half-time for the induction process indicated by either the O₂ curve or the absorbance curve was essentially the same. When the intensity of light was then increased to a saturating level (latter half of curves marked c), the rate of O₂ evolution and the extent of the absorbance change increased to their maximum levels but with more rapid kinetics (half-time of about 40 sec).

It was reported previously (21) that preillumination with a very weak intensity of light, which induces no measurable rate of O₂ evolution itself, markedly shortens the induction period for a subsequent irradiation at higher intensities. The effect of such a preillumination period for 8 min with 20 μw/cm² of red light is shown in Figure 3a. After the preillumination, irradiation with a higher intensity (2 mw/cm²) caused an immediate burst of O₂ and a further rise to the maximum rate of evolution. The concomitant absorbance measurement at 505 nm shows a relatively rapid increase reaching a maximum at about the same time that the maximum rate of O₂ evolution is reached. The half-time for induction after preillumination was about 40 sec (curves a) versus half-times of about 160 sec without preillumination (curves b). With the intermediate intensity of light (curves c), the irradiation for 8 min partially induced O₂ evolution and also served as a preillumination period so that the more rapid induction response accompanied the subsequent irradiation at the higher intensity.

The close correlation between the induction of O₂ evolution and the absorbance change raised the question as to how closely these two phenomena are coupled to one another. This question was examined by measuring the absorbance change with flashed leaves in which the O₂-evolving processes had been inhibited. Vacuum infiltration of fully induced leaves with the various inhibitors indicated in Figure 4 blocked O₂ evolution; vacuum infiltration with buffer alone (the control) had no inhibitory effect. Light-minus-dark difference spectra due to an 8-min inductive irradiation for flashed leaves subjected to the various inhibitory treatments are shown in Figure 4. It is apparent from several of the treatments, including infiltration with 10⁻³ M DBMIB, up to 10⁻⁴ M DCMU, 10⁻³ M CCCP, 0.6 M KCl, or 10⁻² M NH₂OH, that the light-induced carotenoid change can occur in the absence of O₂ evolution. On the other hand, higher concentrations of DCMU (5 × 10⁻⁴ M, not shown in Fig. 4) or CCCP (10⁻⁴ M), 0.8 M tris or heat treatment (45 C for 5 min) blocked the absorbance change as well as O₂ evolution. Figure 5 shows the sensitivity of O₂ evolution in previously induced flashed leaves to vacuum infiltration with various concentrations of DCMU and CCCP as well as the sensitivity of the light-induced absorbance change at 505 nm in uninduced leaves to these inhibitors. These data indicate that there is a concentration range somewhat over two orders of magnitude with DCMU and a range somewhat under two orders with CCCP where O₂ evolution is blocked but the absorbance change still occurs. Thus, the absorbance change can occur in the presence of inhibitors which block O₂ evolution.

The effect of DCMU on the ability of a preillumination period with weak red light to sensitize the flashed leaf so that it re-

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sponds more rapidly to a subsequent irradiation with an actinic intensity was examined by measuring the kinetics of the absorbance change at 505 nm. The data in the top part of Figure 6 show that preillumination of an untreated (control) leaf for 10 min with 20 μW/cm^2 of 633-nm light markedly decreased the induction period for the absorbance change. The preillumination had an effect on a leaf which had been treated with DCMU (lower part of Fig. 6). We conclude that DCMU has no effect on the sensitization processes which occur in very weak light.

The submicroscopic morphology of the flashed leaves was examined by electron microscopy to determine if any structural changes accompany the induction process. Figure 7A shows a typical morphological arrangement of thylakoids in flashed leaves. The thylakoids align in parallel arrays separated by a characteristic distance of about a thylakoid thickness. There are always small regions which appear to be fused but the predominant characteristic of chloroplasts of the flashed leaves is long regions of parallel, evenly separated thylakoids. After an induction irradiation of 2 mw/cm^2 for 8 min (Fig. 7B), the overlapping thylakoids all fused in pairs or groups of three and occasionally in larger groups. The fusion process occurred in the presence of 10^{-4} M DCMU (Fig. 7C), or lower concentrations, but not in the presence of 5 × 10^{-4} M DCMU (Fig. 7D); likewise, it occurred in the presence of 10^{-4} M CCCP (Fig. 7E) but not with 10^{-4} M CCCP (Fig. 7F). Infiltration with 0.8 M tris (Fig. 7G) and heat treatment (45 C for 5 min [Fig. 7H]) also blocked the fusion process. In all cases we have investigated, the carotenoid absorbance change accompanied fusion of the primary thylakoids during the induction process. The preillumination treatment with very weak light (20 μW/cm^2 for 8 min) caused no appreciable thylakoid fusion (data not shown).

One final experiment is presented in this series which hopefully provides some clues as to the nature of the induction process and the mechanisms which lead to thylakoid fusion. Figure 8 shows the 505-nm absorbance change induced by red (2 mw/cm^2 at 633 nm) and far red (5 mw/cm^2 at 734 nm) actinic light. With a normal flashed leaf, red light caused the absorbance change but far red light did not. With a flashed leaf which had been infiltrated with 0.1 M KCl, however, both red and far red light were active. We take these measurements to indicate that PSI is active in producing the absorbance change in the presence of KCl.

![Graph](image)

**FIG. 6. Light-induced absorbance changes at 505 nm. Upper curves: normal flashed leaves with, FLS, and without, FL, a 10-min preillumination with 20 μW/cm^2 of 633-nm light; lower curves: same after infiltration with 5 × 10^{-4} M DCMU.**

**DISCUSSION**

The induction of O_2 evolution in flashed leaves involves two light-dependent processes which occur at different light intensities. We will use the term photoactivation to refer specifically to the low intensity process which is saturated at about 20 μW/cm^2 of red light and goes to completion in about 8 min. After a flashed leaf has been sensitized by such a preillumination, it is capable of O_2 evolution, as shown by the immediate burst of O_2 on irradiation with actinic light (Fig. 3, curve a). Inoue et al. (8) also showed that the photoreduction of DPIP (measured with plastid preparations from flashed leaves) was activated by a similar preillumination period. However, even after the flashed leaves have been photoactivated, complete induction, as noted by the steady-state rate of O_2 evolution or by the extent of the 505-nm absorbance change, requires an additional irradiation with actinic intensities (2 mw/cm^2) for approximately 2 min. When a flashed leaf is irradiated with actinic light without preillumination, the maximal rate of O_2 evolution is reached in about 8 min with a half-time just under 3 min. We infer from these observations that the induction of O_2 evolution in flashed leaves without preillumination is rate-limited by the photoactivation process and that photoactivation proceeds to completion with a half-time of about 3 min. This is essentially the same half-time that Cheniae and Martin (5) reported for the photoactivation of Mn in dark-grown Anacystis cells. The low light requirement for photoactivation reported by Cheniae and Martin (5) for the photoactivation of Mn is also consistent with the low light requirement for the photoactivation of the flashed leaves. Thus, we propose that the low light photoactivation process involves the activation of Mn in the thylakoid membranes. However, Cheniae and Martin (4) reported that low concentrations of DCMU inhibited the photoactivation of Mn-depleted cells of Anacystis, whereas we inferred from the absorbance measurements at 505 nm that DCMU did not inhibit the photoactivation of flashed leaves. This point needs further clarification.

The fusion of the individual primary thylakoids is a part of the initial induction process. We take the one-to-one correspondence between the 505-nm absorbance change and thylakoid fusion, established by the inhibitor studies, to indicate that the absorbance change is a consequence of thylakoid fusion and, therefore, is a reliable indicator of fusion. On that basis, the kinetics of the 505-nm absorbance change may be taken to indicate the kinetics of the fusion process. Electron microscopy of flashed leaves without preillumination showed that thylakoid fusion was complete after 8 min of actinic light which seemed reasonably fast for such a profound structural change. After preillumination, however, which causes no thylakoid fusion itself, the 505-nm absorbance change goes to completion within 2 min, from which we infer (in the absence of direct confirmation by electron microscopy) that thylakoid fusion can occur even in this time period.

The induction process, as noted by the 505-nm absorbance change or by thylakoid fusion, is insensitive to moderate concentrations of DCMU which block noncyclic electron transport. These processes could depend on cyclic electron transport driven by PSI. The high concentrations of DCMU and CCCP which do inhibit fusion and the absorbance change may also inhibit PSI (11). If PSI activity alone was sufficient, we would expect to see some activity by far red light. Figure 8 confirms that this does occur provided the flashed leaves are infiltrated with a moderate concentration of KCl. The reason for the salt dependence for the far red light effect is not known, but perhaps a high energy state or ion pumps driven by PSI are facilitated by the salt. Further electron microscopy is needed to determine if, indeed, far red light in the presence of KCl induces thylakoid fusion as well as the 505-nm absorbance change. If we accept that processes driven by PSI may be able to induce thylakoid fusion, then we must suspect that the correlation between the 505-nm absorbance change and O_2 evolution noted above may not be found
FIG. 7. Electron micrographs of flashed bean leaves. A: Control; B: after 8 min of 2 mw/cm² of 633-nm light; C: after same irradiation of leaf infiltrated with 10⁻⁴ M DCMU; D: same after infiltration with 5 × 10⁻⁴ M DCMU; E: same after infiltration with 10⁻⁴ M CCCP; F: same after infiltration with 10⁻⁴ M CCCP; G: same after infiltration with 0.8 M tris, pH 8; H: same after heat treatment, 45 C for 5 min.
The results reported here suggest a mechanism by which light may induce the fusion of primary thylakoids in the flashed leaves. Izawa and Good (9) showed that the association of thylakoids into grana in suspensions of broken chloroplasts was controlled by the ionic conditions of the medium; the grana become unstacked into individual thylakoids in low salt media, and the individual thylakoids could be reassociated into grana-like stacks by the addition of salts. Murakami and Packer (10) confirmed these observations and noted that divalent cations were much more effective than monovalent cations for the reformation of the grana. From these considerations, we propose that Mg\(^{2+}\) is concentrated in the intrathylakoid space of the primary thylakoids of flashed leaves. On irradiation, Mn\(^{2+}\) is first sequestered in an active form into the thylakoid membranes thereby turning on noncyclic electron transport. Then, as H\(^{+}\) is pumped into the thylakoids, Mg\(^{2+}\) is driven out (7) into the narrow space between adjacent thylakoids to give a concentration sufficient to induce fusion. Although such a mechanism seems attractive to account for the light-induced structural changes, concentrations of 10\(^{-5}\) M CCCP, which we would assume should short circuit the proton pump, did not prevent the fusion of the primary thylakoids of the flashed leaves. On the other hand, 10\(^{-8}\) M CCCP did block the light-induced fusion of primary thylakoids in bean leaves partially greened in far red light (3). (Leaves greened in far red light appear to be very similar to those greened with brief flashes in that they show parallel arrays of primary thylakoids which fuse together in actinic irradiation.) Further work with uncouplers and ionophores is needed to confirm or dispel this proposed role of ion pumps to induce fusion of the primary thylakoids and to address the question of whether ion pumps driven solely by PSI can be sufficient for these processes.

The requirement for actinic intensities of light, above that needed for the photoactivation of Mn, during the induction process may also be related to ion pumps. If Mg\(^{2+}\) is concentrated within the thylakoid bags, steady-state photosynthesis should require the activation of stroma enzymes. On that basis, the ion-pumping mechanisms which drive Mg\(^{2+}\) into the stroma to activate stroma enzymes could also lead to thylakoid fusion. Thus, the apparent correlation between thylakoid fusion and the capacity for O\(_2\) evolution may not be a direct correlation.

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LITERATURE CITED