BBA 46222

THE EFFECTS OF LIPASE ON SPINACH AND CHLAMYDOMONAS CHLOROPLASTS

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(Received June 14th, 1971)

SUMMARY

Light-induced fluorescence yield changes and low-temperature changes were measured in spinach and *Chlamydomonas reinhardi* chloroplasts before and after treatment with pancreatic lipase. Lipase treatment destroyed the C-550 absorption band and eliminated the fluorescence of variable yield. In spinach chloroplasts the invariant fluorescence yield, after lipase treatment, was low whereas in Chlamydomonas chloroplasts it was high. Lipase treatment modified the cytochrome b_{559} in spinach chloroplasts so that it was no longer ascorbate reducible but was dithionite reducible. In Chlamydomonas chloroplasts some of the cytochrome b_{559} was destroyed by lipase treatment. After lipase treatment, cytochrome b_{559} could be reduced by Photosystem I activity if ascorbate was present as an electron donor and benzyl viologen present as an electron acceptor for Photosystem I. Lipase also eliminated the EPR Signal II.

INTRODUCTION

Treatment of chloroplasts with pancreatic lipase inhibits electron transport in the vicinity of Photosystem II. OKAYAMA[1] showed that lipase digestion of spinach chloroplasts inhibited O_2 evolution but not the DCIP–ascorbate supported photoreduction of $NADP^+$ and caused a decrease in the fluorescence yield of chlorophyll. GRESSEL AND AVRON[2] found that pancreatic lipase and phospholipase A inhibited noncyclic photophosphorylation more than phenazine methosulfate-mediated cyclic photophosphorylation. MANTAI[3] concluded that pancreatic lipase inhibited Photosystem II activity by disrupting lamellar membranes. The structural integrity of the photosynthetic apparatus is disrupted by lipase. GREENBLATT *et al.*[4] demonstrated with phase contrast microscopy that the lamellae of Euglena chloroplasts separated under the action of lipase and BAMBERGER AND PARK[5] reported that the inner surface of thylakoids (the B face in electron micrographs of freeze–etched chloroplasts) was removed by lipase. It is apparent that lipase digestion degrades the structure of

Abbreviation: DCIP, dichlorophenolindophenol.

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chloroplasts to some extent and that Photosystem II is affected more than Photosystem I.

Following the work of KNAFF AND ARNON[6] showing that a new component, C-550, was reduced by the action of Photosystem II at liquid nitrogen temperature, it became possible to examine the primary photochemical reaction of Photosystem II. ERIXON AND BUTLER[7] showed that C-550 acted as if it were the primary electron acceptor of Photosystem II. The effect of lipase is superficially similar to the effects of treatments, such as heat or washing with high concentrations of Tris or chaotropic agents, which block electron transport between water and Photosystem II in that Photosystem II activity is inhibited and the fluorescence yield of the chloroplasts is decreased. The latter treatments do not affect the primary photochemical reaction of Photosystem II, *i.e.* the photoreduction of C-550, at −196°. We reported in a previous note[8], however, that lipase reacted directly at the Photosystem II reaction center by destroying C-550.

In the present paper we compare the action of lipase on spinach chloroplasts and chloroplast fragments of Chlamydomonas from the standpoint of fluorescence yield and components, C-550 and cytochrome b_{559} which are involved with the light-induced reactions mediated by Photosystem II at −196°. C-550 is destroyed and the fluorescence of variable yield is eliminated by lipase in both cases but, whereas in spinach chloroplasts the fluorescence yield remains low after lipase treatment, in Chlamydomonas chloroplasts the yield remains high.

METHODS

The preparation of chloroplasts from spinach leaves[9], the measurement of fluorescence yield[10] and the absorption spectroscopy at low temperature[11] were carried out by methods reported previously from this laboratory. Chloroplast fragments from Chlamydomonas cells were prepared by a modification of the method of LEVINE AND GORMAN[12]. Light-*minus*-dark difference spectra at room temperature were obtained by measuring single-beam absorption spectra in the presence or absence of a 726-nm actinic beam ($2 \cdot 10^4$ ergs/cm^2 per sec^{-1}). The S11 phototube (EMI 6255) was protected with two 600-nm short-pass interference filters (Optics Technology) and a Corning 9780 glass filter to block the actinic light. Differences between single-beam spectra were taken with the computer. EPR spectra were measured with a Varian E-3 spectrometer. Chloroplasts (1.5 mg chlorophyll per ml) were placed in a 0.3-mm-thick flat quartz cell at room temperature.

Pancreatic lipase (Tokyo Kasei-Kogyo Co.) was dissolved in buffer and centrifuged to remove insoluble particles. Chloroplasts (0.2 mg chlorophyll per ml) were incubated for 1 h at 20° with the lipase (0.25 mg/ml). 50 mM Tricine–KOH buffer (pH 7.5) was used for spinach chloroplasts and 10 mM phosphate buffer (pH 7.0), containing 2.5 mM $MgCl_2$ and 20 mM KCl was used for Chlamydomonas chloroplast fragments. After the digestion period the incubation mixture was centrifuged at 15000 $\times$ *g* for 10 min and the pellet was resuspended in the buffer.

RESULTS

The fluorescence-yield changes of the chloroplast preparations from spinach and from Chlamydomonas before and after lipase treatment are compared in Fig. 1.

The fluorescence yield of the untreated (control) spinach chloroplasts in the absence of an electron donor increases about 4-fold in the actinic light. This increase of yield has been related to the photoreduction of C-550 (ref. 7). Lipase treatment eliminates the fluorescence of variable yield and the fluorescence yield of the lipase-treated spinach chloroplasts (Fig. 1A) remains near minimum level of the untreated chloroplasts. A similar pattern is seen in the results with the Chlamydomonas chloroplast fragments (Fig. 1B) except that the invariant fluorescence yield after the lipase treatment is near the maximal level of the untreated chloroplasts.

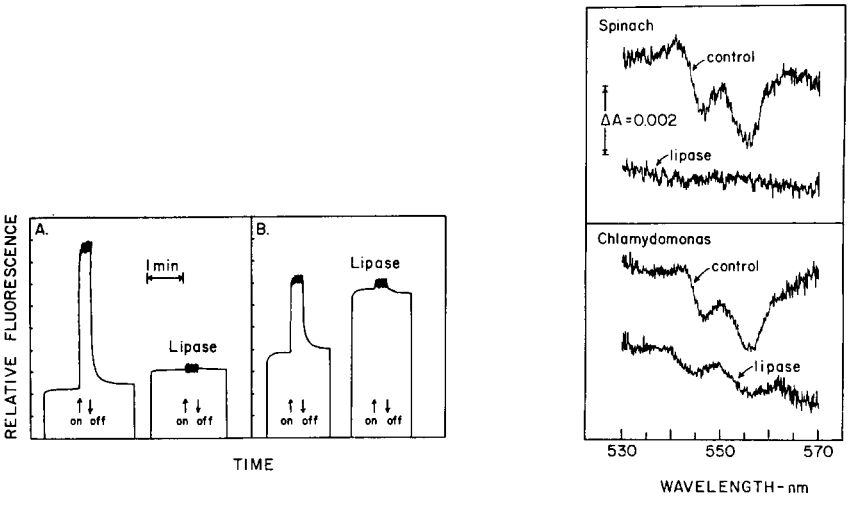


Fig. 1. Fluorescence yield of chloroplasts before and after lipase treatment. A. Spinach chloroplasts (10 μg chlorophyll per ml). B. Chlamydomonas chloroplast fragments (11 μg chlorophyll per ml). Red actinic light ($5 \cdot 10^4$ ergs/cm^2 per sec) on at upward arrow; off at downward arrow.

Fig. 2. Light-*minus*-dark difference spectra at $-196°$ obtained by irradiating chloroplasts with red light (10^4 ergs/cm^2 per sec) for 30 sec at $-196°$. A. Spinach chloroplasts (50 μg chlorophyll in 0.5 ml). B. Chlamydomonas chloroplast fragments (100 μg chlorophyll in 0.5 ml).

Electron donors to Photosystem II such as hydroquinone *plus* ascorbate were not able to restore the fluorescence of variable yield to the lipase-treated spinach chloroplasts and these chloroplasts were not capable of carrying out the Photosystem II-mediated photoreduction of dichlorophenolindophenol with hydroxylamine or semicarbazide as the electron donor. The high fluorescence yield of the lipase-treated Chlamydomonas chloroplast fragments was not affected by $Fe(CN)_6^{3-}$. In other cases where the fluorescence yield is high because C-550 is reduced, *e.g.* after irradiation in the presence of dichlorophenylmethylurea and hydroxylamine[7], $Fe(CN)_6^{3-}$ will oxidize the C-550 and restore the fluorescence yield to its minimum level.

Light-induced absorbance changes in the spinach and Chlamydomonas chloroplasts are shown in Fig. 2. Both show the bleaching at 556 nm due to the photooxidation of cytochrome b_{559} and the bleaching at 547 nm as well as the absorbance increase at 542 nm due to photoreduction of C-550. Lipase treatment eliminates these light-induced absorbance changes.

Spectral differences of the chloroplast preparations resulting from the lipase treatment were examined to determine what light-absorbing components (in the 530–

570-nm region) were affected. We showed previously[8] that the absorption band of C-550 in spinach chloroplasts was eliminated by the lipase digestion and that cytochrome b_{559} was either destroyed or oxidized. Difference spectra between lipase-treated and untreated spinach chloroplasts with both samples measured at the same redox condition are presented in Fig. 3A. The difference spectrum of treated-*minus*-untreated spinach chloroplasts, both oxidized with $Fe(CN)_6^{3-}$, shows that the lipase-treated sample lacked the absorption band at 546 nm due to the oxidized form of C-550. When both samples were reduced with ascorbate (Fig. 3A, Curve 2), the treated sample lacked C-550 *plus* cytochrome b_{559}. However, when the two samples were reduced with dithionite only the reduced band of C-550 was missing from the spectrum of the treated sample; both dithionite-reduced samples contained equal amounts of reduced cytochromes. Thus, the lipase treatment of spinach chloroplasts destroyed C-550 and modified the cytochrome b_{559} so that it was no longer ascorbate reducible but was reduced by dithionite. In untreated chloroplasts the normal state of cytochrome b_{559} in the dark is fully reduced whereas after lipase treatment the cytochrome b_{559} is fully oxidized in the dark.

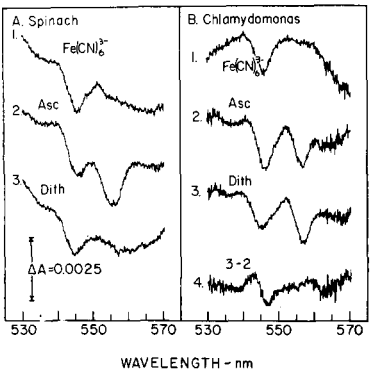
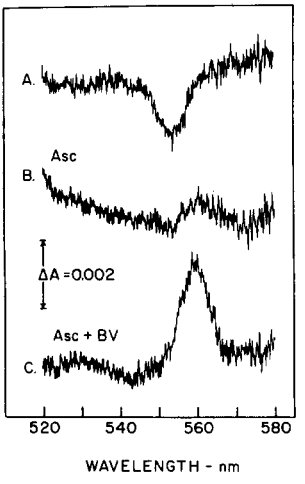


Fig. 3 Difference spectra between lipase-treated and untreated chloroplasts at −196°. A. Spinach chloroplasts. B. Chlamydomonas chloroplast fragments. Both samples in each difference spectrum had 2 mM $Fe(CN)_6^{-3}$, 20 mM ascorbate or a few grains of dithionite added as indicated. Curve 4 is the difference spectrum between curves 3 and 2 in Part B.

The same type of difference spectra (treated-*minus*-untreated) for the Chlamydomonas chloroplasts (Fig. 3B) also show that C-550 was destroyed. In this case, however, some of the cytochrome b_{559} was destroyed or denatured by the lipase treatment so that it was not reducible by dithionite (Fig. 3B, Curve 3). The difference between the dithionite-reduced difference spectrum and the ascorbate-reduced difference spectrum shows the typical biphasic absorbance change which results from the slight shift of the absorption band of C-550 to shorter wavelengths when it is reduced[8].

Steady-state light-*minus*-dark difference spectra can also be obtained at room temperature in the 520–580-nm region by irradiation with far-red light (726 nm) during the measurement and using filters to block the actinic light from the phototube. Fig. 4 shows such spectra measured with the lipase-treated spinach chloroplasts. Cytochrome *f* is fully reduced and cytochrome b_{559} fully oxidized in the lipase-treated chloroplasts in the dark. With no addition to the chloroplasts, light oxidized cyto-

chrome *f* so that the light-*minus*-dark difference spectrum shows a bleaching at 554 nm. This same spectrum would have been obtained with untreated chloroplasts[13]. Adding ascorbate to the chloroplasts prevents the light-induced oxidation of cytochrome *f* presumably because cytochrome *f* is not coupled as closely to Photosystem I after the lipase treatment (untreated chloroplasts would have photooxidized cytochrome *f* in the presence as well as in the absence of ascorbate). Possibly ascorbate donates electrons directly to P_{700} more effectively after lipase treatment as well as keeping the cytochrome *f* reduced. In the presence of ascorbate *plus* benzyl viologen far-red light causes the reduction of cytochrome b_{559}; this reaction has not been observed with normal chloroplasts. Presumably ascorbate, which is necessary for the reaction, serves as the electron donor for the photoreduction of benzyl viologen by Photosystem I and the reduced benzyl viologen reduces cytochrome b_{559}.



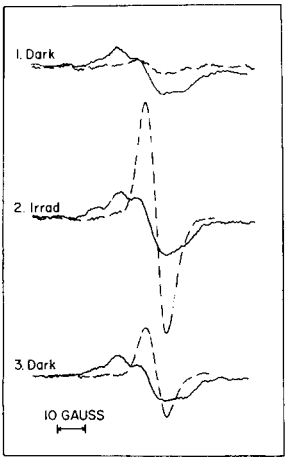


Fig. 4. Light-*minus*-dark differences spectra at room temperature of lipase-treated spinach chloroplasts (150 μg chlorophyll in 0.5 ml). Single-beam spectra were recorded in the absence and presence of far-red (726 nm) actinic light ($2 \cdot 10^4$ ergs/cm^2 per sec) and the difference is plotted A, with no additions; B, in the presence of 3 mM ascorbate; C, in the presence of 3 mM ascorbate and 120 μM benzyl viologen(BV).

Fig. 5. EPR spectra of untreated (solid curves) and lipase-treated (dashed curves) spinach chloroplasts. 1, in darkness; 2, in white actinic light; 3, in darkness 5 min after the irradiation.

Lipase also destroys the slow decaying EPR Signal II which has been associated with Photosystem II. Fig. 5 shows EPR spectra of untreated (solid curves) and lipase-treated (dashed curves) spinach chloroplasts (1) after a long period of darkness (2) during irradiation with white light and (3) 5 min after the irradiation. Signal II gives the broad low spectrum shown by the solid curve in Curve 1. Signal I (associated with the oxidized form of P_{700}) appears as an intense narrow EPR spectrum in the lipase-treated chloroplasts during irradiation. Only a very small Signal I EPR signal is observed with normal untreated chloroplasts because P_{700} remains largely reduced under the action of Photosystem II during irradiation when an electron acceptor is not present[14]. At no time, however, is Signal II observed with the lipase-treated chloroplasts.

DISCUSSION

The effects of lipase digestion of the spinach and Chlamydomonas chloroplast preparations are similar in that C-550 is lost in both cases but the differences between the two preparations are interesting also. On the assumption that C-550 is analogous to Q, the fluorescence quencher of Photosystem II, we would expect that the loss of C-550 would result in a high invariant fluorescence yield. Such a condition is found in mutants of Scenedesmus[15] and Chlamydomonas[16] which lack C-550. The expected result was obtained after lipase treatment of the Chlamydomonas chloroplast fragments but not with the spinach chloroplasts. In the latter case the invariant fluorescence yield was low, near the minimum level of the untreated chloroplasts. The other difference between the lipase-treated spinach and Chlamydomonas chloroplast preparations was that part of the cytochrome b_{559} appeared to be denatured or destroyed in the Chlamydomonas while none was destroyed but some was modified so that is was no longer ascorbate reducible in the spinach chloroplasts. When we learn more about mechanisms of fluorescence quenching in chloroplasts the differences between spinach and Chlamydomonas may provide significant information.

It was pointed out in the previous note on the action of lipase that the primary photochemical reaction of Photosystem II resulted in a shift of the absorption band of C-550 from 546 to 544 nm. Disruption of the structure of the photosynthetic apparatus by lipase not only inactivates C-550 but destroys the absorption band of C-550. Extraction of lyophilized chloroplasts by hexane or heptane has the same effect[17] but in the latter case C-550 can be restored by reconstituting the extracted chloroplasts with synthetic β-carotene. Taken together these experiments suggest that membrane structure may be important to the realization of C-550.

The absence of the EPR Signal II from the lipase-treated chloroplasts does not mean that the Signal II generating compound is destroyed by lipase. We tend to associate Signal II with the oxidizing side of Photosystem II, not with the primary electron donor but with a long-lived intermediate such as S_1 in the O_2-evolving scheme proposed by KOK *et al*[18]. Destruction of C-550 which serves as the primary electron acceptor of Photosystem II, would mean that no oxidizing power could be generated by light because no electron acceptor was available. The absence of Signal II could reflect the absence of oxidized intermediates in the O_2-evolving machinery.

ACKNOWLEDGEMENT

This work was supported by a National Institutes of Health Grant GM-15048.

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