

Effects of Linolenic Acid on the Spectral Properties and Picosecond Fluorescence of Pea Chloroplasts

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Linolenic acid induces changes in the absorption spectrum and in the picosecond fluorescence of pea chloroplasts. The effects of linolenic acid are dependent on concentration and time.

Linolenic acid increases the fluorescence life time of chloroplasts at room temperature. The contribution of the slow fluorescence component relative to the fast component is increased almost 10 fold in the presence of 0.5 mM linolenic acid. The synergistic action of digitonin and linolenic acid increases the ratio of "closed" to "open" traps in the photosynthetic units.

Upon addition of 0.5 mM linolenic acid there are increases in absorbance at 676 and 436 nm, and decreases in absorbance at 705 and 496 nm. Some of the spectral changes have a biphasic character; they reach a maximum after about 30 min then start to reverse. Based on the spectral changes at 496 and 705 nm it appears that linolenic acid has at least two effects, *i.e.* it modifies the thylakoid membrane and secondarily decreases the concentration of P700, respectively.

Introduction

Linolenic acid has been shown to effect a wide range of biological processes and phenomena. For example the fluorescence spectrum is modified and the yield is decreased upon the addition of linolenic acid (or *Ricinus* leaf extract) [1, 2]. The fluorescence changes depend on the concentration of linolenic acid. Maximum effect is reached at a concentration of 100 μ M or a linolenic acid to chlorophyll ratio of 4 to 1 [3, 4]. These same concentrations were also reported in studies of linolenic acid binding [5].

It was shown that 100 μ M of linolenic acid (or extracts of *Ricinus* leaf) completely inhibits the light induced spectral changes at 705 nm which is associated with Photosystem I [3, 4]. Furthermore, the photosynthetic oxygen burst is inhibited by linolenic acid [6]. In addition, membrane permeability is increased by linolenic acids, as shown by the spectral changes at 515 nm [3, 4, 7]. Electron microscopy reveals large alterations in the lamellar ultrastructure upon addition of linolenic acid [1, 7]. The present study probes the effect of linolenic acid on

pea chloroplasts using difference absorption spectroscopy and picosecond fluorescence techniques.

Materials and Methods

Linolenic acid obtained from Sigma (St. Louis, Mo.), was used without further purification, and dissolved in absolute ethanol. Pea chloroplasts were prepared as described by Nakatani and Barber [8]. The chloroplasts were suspended in low salt buffer, LSB, (0.33 mM sorbitol and 10 mM Hepes, pH 7.6).

Difference spectra were measured using an Aminco DW-2 spectrophotometer. Two identical chloroplast samples were prepared, one was used in the reference beam, the other was used in the sample beam. The spectral difference between identical samples was used as a baseline. While the baseline drifted slightly with time, nevertheless, the shape of the baseline remained unchanged. Linolenic acid was added to the chloroplasts in the sample beam. An equal volume of LSB was added to the reference sample to avoid a change in absorbance due to dilution. To facilitate comparisons of spectral data the optical densities of all spectra were set equal at 750 nm, a wavelength where there is no significant absorption by chloroplasts.

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The apparatus used to generate, measure and record the picosecond fluorescence was described previously [9, 10]. Fluorescence was sensitized by a 6 picosecond flash (530 nm) generated with the aid of a mode-locked Nd³⁺ glass laser and frequency double. Fluorescence from chloroplasts passed through a sharp red cut-off filter into a streak camera. Nine fluorescence decay curves were summed for each experiment. A computer program was used to find the best fit to the summed fluorescence curves, using the equation $y(t) = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$, where the subscript 1 refers to the long lived fluorescence and subscript 2 refers to the fast fluorescence component.

Results and Discussion

Difference spectroscopy: Since linolenic acid is dissolved in ethanol, before the effect of linolenic acid on the spectral properties of chloroplasts can be determined, it is necessary to first determine the effects of ethanol. Fig. 1, Curve A shows the difference spectrum, 20 min after adding 5 μ l of ethanol to a 3 ml suspension of chloroplasts. The result-

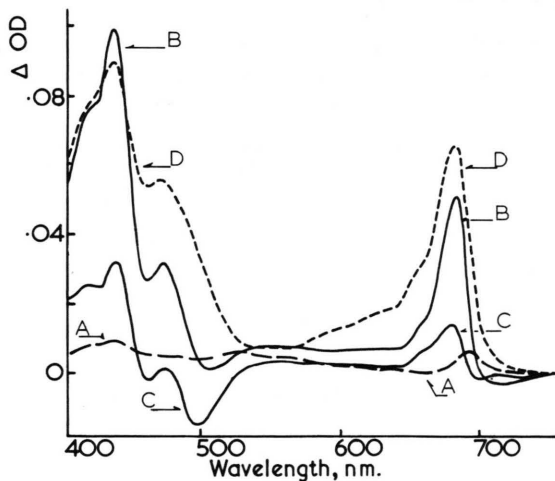


Fig. 1. The difference spectrum between pea chloroplasts without and with 0.16% ethanol is shown as curve A (dashed line). Difference spectra are measured between chloroplasts in the absence and in the presence of 0.5 mM linolenic acid. Curve B (solid line) shows the difference spectrum 22 min after treatment with linolenic acid. Curve C (solid line) shows the spectrum 104 min after treatment with linolenic acid. For comparison purposes the absorption spectrum of pea chloroplast is shown (curve D, dashed line). Chloroplasts are suspended in 0.33 M sorbital and 10 mM Hepes (pH 7.6).

ing difference spectrum is similar to the baseline in shape and magnitude.

The addition of linolenic acid produces much larger changes in the absorption spectrum of chloroplasts than does ethanol alone. In Fig. 1, Curve B is shown the difference spectrum 22 min after adding 0.5 mM linolenic acid to the chloroplast suspension. The spectral changes induced by linolenic acid are a function of time and concentration. At concentrations of linolenic acid less than 0.1 mM there are no significant changes in absorption. At 0.3 mM linolenic acid spectral changes similar to, but smaller than those shown in Fig. 1 are observed. The difference spectrum 104 min after adding linolenic acid is shown in Fig. 1, Curve C.

The spectral changes in absorption at 436 and 676 nm, are characteristic of chlorophyll a; the change at 652 nm is characteristic of chlorophyll b; the overlapping spectra of chlorophyll b and carotene contribute to the spectral change at 472 nm; the change at 496 nm is characteristic of carotene and the electrochromic pigment. The small change in absorption at 705 nm may be associated with the chlorophyll (P 700) of reaction centre I. For comparison purposes, the absorption spectrum of the chloroplast suspension is shown in Fig. 1, Curve D.

The small decrease in absorbance at 705 nm induced by linolenic acid could reflect a decrease in the concentration of P 700. Such a decrease of P 700 could account for the inhibition, by linolenic acid, of the light induced spectral changes at 705 nm associated with photosystem I [3, 4].

The time dependent changes in absorption induced by 0.5 mM linolenic acid at some wavelengths, are shown in Fig. 2. The absorbance at time zero corresponds to the baseline, at the wavelength indicated. The change in absorbance, at some wavelengths, has a biphasic character. After about 30 min there is a reversal of the change in absorbance at 436, 472 and 676 nm (see Fig. 2). At 496 nm after an initial increase in absorbance, during the first few minutes, there is a continuous decrease in absorbance out to 100 min. The absorbance at 652 nm increases slightly upon adding linolenic acid, then remains essentially constant.

The biphasic change in absorbance (Fig. 2) shows that linolenic acid has at least two effects on the chloroplast. A similar conclusion was reached by Brody *et al.* [4] from evidence obtained on light induced changes in absorption. They showed that

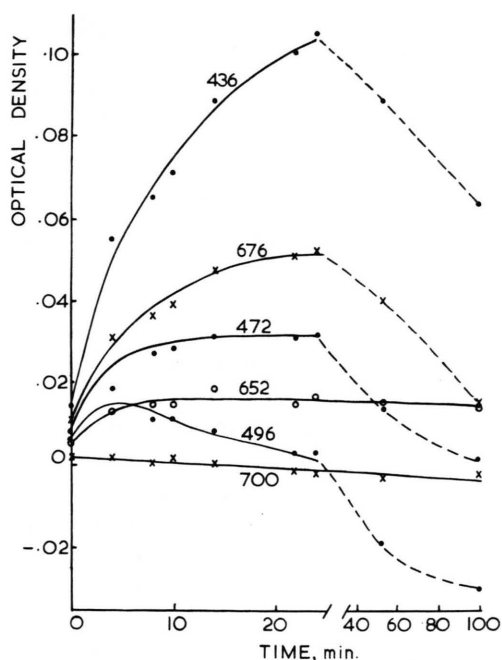


Fig. 2. Time dependent changes in absorbance of pea chloroplasts after addition of 0.5 mM linolenic acid. The wavelengths measured, in nm, are indicated on the curve. At 30 min the time scale is changed.

linolenic acid rapidly inhibited photosystem I, and at a slower rate it effected photosystem II.

Using flashing light it was shown that changes in absorption at 515 nm were modified upon adding linolenic acid [3, 4]. The endogenous electrochromic probe at 515 nm reflects changes in membrane permeability and ion gradients [11]. The spectral change at 496 nm induced by linolenic acid in the present work (see Fig. 1) could well be related to the spectral change at 515 nm. Linolenic acid produces permanent spectral changes of the electrochromic probe (or pigment at 496 nm) as shown by the decrease in absorbance at 496 nm. Such a spectral change could arise if there is a permanent modification of the thylakoid membrane (*e.g.* permeability). A permanent modification of the membrane and, therefore, of the electrochromic probe, would decrease the concentration of pigment available to register variable spectral changes at 515 nm.

Picosecond Fluorescence: The effect of linolenic acid on the picosecond fluorescence kinetics is shown in Fig. 3. To facilitate comparison of the fluorescence curves the maxima are normalized to unity. Curve A is a semilogarithmic graph of the

fluorescence decay from our pea chloroplast suspension in LSB. For the control the ratio of the intensity of the slow fluorescence component, A_1 , to the fast component A_2 is $A_1/A_2 = 0.16$. The fluorescence decay upon addition of 50 μ M DCMU is shown in Fig. 3, Curve B. Adding DCMU lengthens the life time and also increases the relative contribution of the slow component so that $A_1/A_2 = 0.33$ (see Table I). The addition of 0.5 mM linolenic acid to the chloroplasts, treated with DCMU, results in minor changes in the fluorescence decay (Fig. 3, Curve C). The addition of 4% digitonin to chloroplasts treated with DCMU results in only a minor change in the fluorescence decay. On the other hand, the addition of 0.5 mM linolenic acid to chloroplasts treated with DCMU and digitonin results in a significant increase in the contribution of the slow fluorescence component; the ratio A_1/A_2 increases to 1.4 (Fig. 3, Curve D) (see Table I).

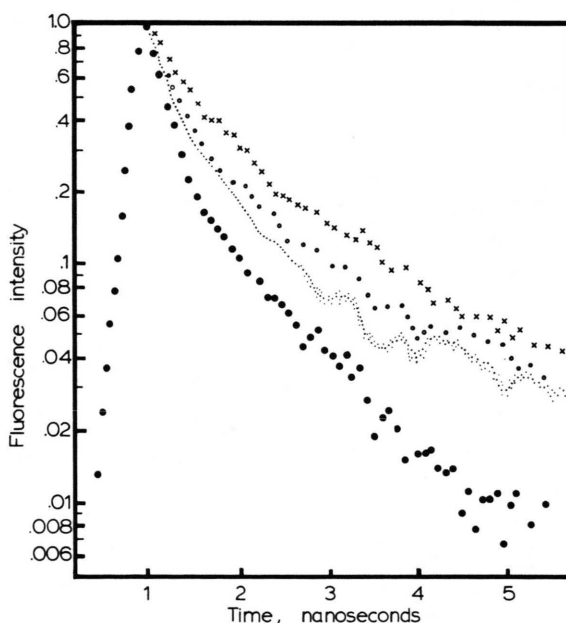


Fig. 3. Semilogarithmic plot of the picosecond fluorescence from pea chloroplasts is shown by curve A. Fluorescence is sensitized by a 6 ps laser pulse (520 nm). After treatment with 50 μ M DCMU the fluorescence decay is shown by curve B. About 30 min after the addition of 0.5 mM linolenic acid to the sample containing DCMU, the fluorescence decay becomes as shown by curve C. Curve D shows the fluorescence decay in the presence of 0.5 mM linolenic acid, 50 μ M DCMU and 4% digitonin. Only, every fourth data point is shown in curves A, C and D. To facilitate comparison the maximum of each fluorescence curve is normalized to unity. $\times$, D; $\circ$, C; $\square$, B; $\bullet$, A.

Table I. Fluorescence properties

Chloroplast	A_1/A_2	τ_1 , ps	τ_2 , ps
control	0.16	840	166
+ DCMU (50 μ M)	0.33	900	200
+ DCMU (50 μ M) + LINO (0.05 mM) + Digitonin (4%)	1.4	960	228

A_1 is the relative intensity of the slow fluorescence component.

A_2 is the relative intensity of the fast fluorescence component.

The short lived fluorescence is associated with photosynthetic units that have "open" traps. The fluorescence is from antenna chlorophyll a of photosystem I, and light harvesting photosystem II pigment combinations that are strongly coupled to photosystem I antenna chlorophyll [12]. The long lived fluorescence is associated with photosynthetic units that have "closed" traps. The fluorescence is ascribed to light harvesting photosystem II pigment combinations, not coupled with antenna of photosystem I [12]. The proportion of "closed" traps to "open" traps determines the ratio of the intensities of the long to short lived fluorescence components [13]. Closure of photosystem II centers (*e.g.*, by DCMU) is seen as an increase in contribution of the slow fluorescence component relative to the fast component.

By the synergistic action of digitonin and linolenic acid the fluorescence traps can be efficiently closed without light. The presence of digitonin enhances the effectiveness of linolenic acid in modifying the picosecond fluorescence decay from chloroplasts. Digitonin, on the other hand, does not promote the action of DCMU on the fluorescence decay. The detergent properties of digitonin may aid the passage of linolenic acid through the thylakoid membrane, so that linolenic acid may more effectively inhibit the photosystem. The inhibition of photosystem I is seen in the picosecond fluorescence measurements as an increase in the relative contribution of the slow component compared to the fast component. Linolenic acid in the presence of digitonin and DCMU, results in an increase in the ratio of closed to open traps by almost on order of magnitude. The accompanying increase in life time of the fast fluorescence component (Table I) indicates less energy transfer to a nonfluorescent pigment [13]; this could result from the decrease in concentration of nonfluorescent, aggregated chlorophyll associated with the observed decrease in absorption at 705 nm.

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