Mechanism of Linolenic Acid-induced Inhibition of Photosynthetic Electron Transport$^1, 2$

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ABSTRACT

The effect of linolenic acid on photosynthetic electron transport reactions in chloroplasts has been localized at a site on the donor side of photosystem I and at two functionally distinct sites in photosystem II. In photosystem I, an increase in the electron transport rate occurs in the presence of 10 to 100 micromolar linolenic acid, followed by a decline in rate from 100 to 200 micromolar linolenic acid. The increase may result from an alteration of membrane structure that allows greater reactivity of the artificial donors 2,6-dichlorophenolindophenol (DPIP) and N,N,N',N'-tetramethyl-p-phenylenediamine with plastocyanin. The decrease is due to loss of plastocyanin from the membrane since addition of purified plastocyanin to treated and washed chloroplasts leads to the reestablishment of photosystem I rates.

In photosystem II, a reversible site and an irreversible site of inhibition have been located. At the irreversible site, there is a time-dependent loss of the loosely bound pool of Mn implicated in the water-splitting mechanism. At the reversible site, the photochemical charge separation is rapidly inhibited as evidenced by the high initial fluorescence yield upon illumination and the inhibition of artificial donor reactions in NH$_4$OH-washed chloroplasts. When chloroplasts are washed after treatment with linolenic acid, the fluorescence returns to its original low value and there is a resumption of artificial donor activity from diphenylcarbazide $\rightarrow$ DPIP. This reversible inhibition of the photosystem II is a unique characteristic of linolenic acid and suggests evidence for a new mode of inhibition of photosystem II.

Inhibition of photosynthetic electron transport reactions by unsaturated C$_{18}$ fatty acids was reported by Krogmann and Jagendorf in 1959 (14). The topic has received considerable attention because damage to photosynthetic membranes observed during stress and aging has been correlated with the presence of free fatty acids.

McCarty and Jagendorf (15) and Molotkovsky and Zheskova (16) showed that addition of linolenic acid to freshly isolated chloroplasts caused damage resembling that which occurs in chloroplasts isolated at pH 6.0 or after inactivation by gentle heating. The latter authors suggested that heat-activated lipases hydrolyze membrane lipids and release free fatty acids. Katoch and San Pietro (13) reported inhibition of NADP$^+$ reduction by linolenic acid, but noted also a stimulation of the PSI-mediated DPIP$^3$ to NADP$^+$ reaction at intermediate concentrations of fatty acids. Since this reaction eventually was inhibited in chloroplasts lacking Hill reaction activity, it was suggested that high concentrations of linolenic acid inhibit PSI as well as PSII. Cohen et al. (8) divided the concentration-dependent effects of linolenic acid into two classes. (a) At fatty acid to Chl ratios of 2–3, the variable part of fluorescense induction was inhibited 50%, but the PSI-dependent DPIP to NADP$^+$ pathway was stimulated. (b) At fatty acid to Chl ratios of 10, low temperature fluorescence at 735 nm was reduced 50% and the DPIP to NADP$^+$ pathway was inhibited.

Other effects of linolenic acid on chloroplasts which are relevant to this study include: (a) uncoupling of photophosphorylation (15); (b) chloroplast swelling and large changes in the appearance of the grana lamellae (8, 17, 19); and (c) a decline in the population of PSII reaction centers (6).

Although these investigations describe the effects of linolenic acid, a satisfactory biochemical explanation has not yet been offered. Also, the reversibility of the PSI inhibition is controversial (18, 19, 22, 24, 25) and is one of the major topics of this paper.

We investigated the action of linolenic acid on spinach chloroplasts and found an effect on the Mn enzyme in PSII and on PC in PSI. We also provide evidence for a new type of PSII inhibition, based on fluorescence studies and artificial donor reactions in isolated chloroplasts. In particular, linolenic acid was found to block charge separation reversibly within the PSII trap.

MATERIALS AND METHODS

Chloroplast lamellar membranes were isolated from greenhouse-grown spinach in a medium consisting of 0.4 m sucrose, 0.05 m Tris (pH 7.40), and 0.01 m NaCl (STN buffer). Chl concentrations were determined in 80% acetone (1).

Fluorescence induction was measured at room temperature. The chloroplasts were suspended in STN buffer containing 5 mM MgCl$_2$ to a Chl concentration of 20 µg/ml. During studies of the kinetics of variable fluorescence yield, there was a 10-min dark equilibration period before addition of the inhibitor.

The effect of linolenic acid on electron transport rates was determined at 14 µg or 20 µg Chl/ml. Inhibition over a suitable linolenic acid concentration range could not be obtained at Chl concentrations greater than 20 µg/ml. Incubations were carried out at room temperature because of the limited solubility of linolenic acid in aqueous solution.

Kinetics of linolenic acid inhibition of electron transport activity were investigated polarographically. Linolenic acid was added to

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2 The authors wish to dedicate this paper to our counselor and friend, Dr. Bessel Kok. The work represents one of the last projects completed in his laboratory during his lifetime.

3 Abbreviations: DPIP: 2,6-dichlorophenolindophenol; PC: plastocyanin; TSF-2: Triton subchloroplast fraction 2; MV: methylviologen; DPC: diphenylcarbazide; PD$_{ox}$: oxidized form of phenylenediamine; IMPD: N,N,N',N'-tetramethyl-p-phenylenediamine; F$_{var}$: variable fluorescence; F$_i$: initial level of fluorescence; F$_{max}$: maximum level of fluorescence.
the MV reaction mixture through the vessel cap with a syringe, and illumination was begun at specified times.

Experiments to study reversal of the inhibitory effect were conducted in test tubes. After incubation with linolenic acid, Mn$^{2+}$ or BSA was added to the reaction mixture which was transferred to the polarograph for assay. Once the additions were made, there was no further decline in $O_2$ rates.

Mn and Cu were determined by atomic absorption spectroscopy (Perkin-Elmer, model 303). Chloroplast suspensions were washed prior to analysis with 0.25 ml-concentrated H$_2$SO$_4$ and 5 ml-concentrated HNO$_3$. Standards were prepared from Fisher Certified Atomic Absorption Standards.

PC was released from the membrane by sonication and was determined by the method of Pleniscar and Bendall (20). The supernatant, after centrifugation at 144,000 $g$ for 2 h, was used for analysis. Purified PC was prepared from spinach leaves.

Mn-deficient chloroplasts were prepared by washing with 5 mM NH$_4$OH as described by Cheniae and Martin (7), or with 0.8 mM Tris according to the method of Yamashita and Butler (27). Enriched PSII particles (TSF-2) were prepared according to Vernon and Shaw (26). All reagents were of the highest commercial grade and were used without further purification. BSA-fraction V, α-linolenic acid (9, 12, 25; octadecatetraenoic acid), Trizma base, and EDTA-disodium salt were obtained from Sigma. Hydroxylamine-HCl and manganous chloride were analyzed reagent grade from J. T. Baker Chemical Co. Baker was the supplier for $p$-phenylenediamine dihydrochloride (Baker grade). DPC and MV (dimethylpyrroldyl chloride) were purchased from K & K Laboratories, and DPIP from Fisher Scientific Co.

RESULTS

In this study, we characterized the site(s) of linolenic acid-induced inhibition of photosynthetic electron transport and examined the conditions for the reversal of inhibition. As previously shown (8, 13, 23), the Hill reaction ($H_2O \rightarrow PDH$) is more sensitive to linolenic acid than is PSI-dependent, MV-mediated oxygen uptake by DPIP (Fig. 1). At concentrations approaching 100 μM, linolenic acid nearly doubles the PSI reaction while this concentration completely inhibits $O_2$ evolution.

PSII activity did not recover when the chloroplasts were washed following a 10-min treatment (Fig. 1). In contrast, PSI activity was greatly affected by washing with linolenate treatment. The rate returned to control values after exposure to 40 μM linolenic acid, followed by greater decreases after 50-80 μM. Although not shown in the figure, less than 10% of the PSI rate remained at concentrations of linolenic acid near its solubility limit.

PSI. Experiments were performed to see if the inhibition of PSI by fatty acids might be due to the loss of PC. Following treatment with various concentrations of linolenic acid for 10 min, chloroplasts were centrifuged, resuspended in STN buffer containing 2 mM EDTA, and washed a second time in STN before assay of PSI activity. Figure 2 shows that a linear relationship exists between the PSI rate and the remaining lamellae copper content. Of the 3-4 Cu atoms per 400 Chl, 2 to 3 were removed by this treatment with linolenic acid. In agreement with previous reports (2, 20), we had found 2 PC molecules per 400 Chl (data not shown). Since PC contains 1 atom of Cu per molecule (9), the loss of Cu atoms in Figure 2 represents the loss of up to all of the PC present and hence is sufficient to account for the loss of PSI activity.

We confirmed this conclusion by reconstitution with purified plastocyanin. Chloroplasts were treated with 200 μM linolenic acid for 10 min and washed. The chloroplasts retained only 6% of the DPIP → MV rate of untreated chloroplasts (Table I). Although the addition of PC to untreated chloroplasts caused only a slight (~5%) increase in rate, its addition to the linolenic acid-treated chloroplasts stimulated the rate commensurate with the amount added. A double reciprocal plot (not shown) indicated that at saturating concentrations of PC, the PSI rate becomes ~ 620 μmol $O_2$/mg Chl·h, or twice the control rate. These data indicate that except for the loss of PC, linolenate would be expected to stimulate PSI electron transport even at concentrations as high as 200 μM.

The stimulation of PSI activity from 10 to 100 μM linolenate is more difficult to explain. Since the increase in the PSI rate occurred in the presence of methylamine (Fig. 1) and gramicidin (not shown), an uncoupling of photophosphorylation by linolenic acid, suggested as a cause of similar stimulation of over-all electron flow (15), is not relevant here. This conclusion is strengthened by comparing DPIP and TMPD-mediated rates. Even if DPIP supported a short proton cycle across the membrane, TMPD, a nonprotonated donor, would show none of these effects if the stimulation by linolenic acid were due solely to uncoupling. As shown in Table II, TMPD-mediated rates were stimulated to the same degree as DPIP rates, albeit with a slight offset in the concentration of linolenic acid necessary to produce the maximum effect.

The stimulation of PSI occurs even at saturating concentrations of DPIP. At 0.1 mM DPIP (concentration used in Fig. 1), the rate is within 89% of $V_{max}$ in the absence of, and within 93% of $V_{max}$ in the presence of linolenic acid. The addition of superoxide dismutase to the treated chloroplasts did not remove the stimulation.

PSII—Inhibition. Failure of the linolenic acid-treated chloroplasts to recover PSI activity after washing led to the possibility that the loosely bound pool of Mn (7) was affected by linolenic acid. Chloroplasts (20 μg/ml Chl) were incubated with various concentrations of linolenic acid for 10 min and washed by centrifugation. The linear relationship between Mn content and Hill activity with an intercept at 2 Mn/400 Chl (Fig. 2) suggests that
LINOLENIC ACID AND CHLOROPLAST REACTIONS

FIG. 2. PSI and PSII Rates as a Function of Cu and Mn Content. Spinach chloroplasts at 20 μg/ml were incubated in various concentrations of linolenic acid at room temperature for 10 min and washed free of the inhibitor as described in Figure 1. The Cu and Mn remaining in the membrane after washing are plotted against PSI (DPIP to MV) and PSII (H₂O to PD₆₅) rates, respectively.

Table I. Effect of Plastocyanin on PSI Rates in Control and Linolenic Acid-treated Chloroplasts

<table>
<thead>
<tr>
<th>Conditions</th>
<th>PC</th>
<th>O₂ Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μmol/mg Chl-h</td>
</tr>
<tr>
<td>Control</td>
<td>4.85</td>
<td>307</td>
</tr>
<tr>
<td>Treated (200 μM linolenic acid)</td>
<td>1.21</td>
<td>36.2</td>
</tr>
<tr>
<td></td>
<td>2.42</td>
<td>71.5</td>
</tr>
<tr>
<td></td>
<td>4.85</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>12.12</td>
<td>214.5</td>
</tr>
<tr>
<td></td>
<td>30.30</td>
<td>357.1</td>
</tr>
</tbody>
</table>

removal of the loosely bound pool of Mn (~3 Mn/400 Chl) is responsible for the irreversible loss of PSII Hill reaction. Linolenic acid thus resembles the effect of 0.8 M Tris (27) or 5 M NH₄OH (7) on isolated chloroplasts.

Figure 3 shows the effect of 200 μM linolenic acid (added in the dark) on the fluorescence rise curve of dark-adapted chloroplasts following a 30-s incubation period. This concentration of linolenic acid was chosen because it completely inhibits O₂ evolution (Fig. 1). Fₚₚ was missing and F₁ matched Fₚₚ. BSA (0.5%) added prior to linolenic acid prevented the increase in Fₚₚ.

The effect of linolenic acid (80 μM) on the fluorescence yield was rapid (Fig. 4) and may have been within the mixing time of the reagents. Complete loss of Fₚₚ and an increase in F₁ to match Fₚₚ was achieved in less than 20 s. Following the initial, rapid increase in Fₚₚ there was a slower decline in the fluorescence yield.

To examine the site of inhibition further, we studied the PSII-mediated reduction of DPIP by DPC as a function of linolenic acid concentration in chloroplasts which were NH₄OH-washed prior to assay. The inhibition of DPC reduction by DPC depended on linolenic acid concentration to nearly the same degree as the Hill reaction with PD₆₅ using untreated chloroplasts. (Data not shown, see Fig. 8 for a comparable experiment.) Concentrations of linolenic acid similar to those effective with chloroplasts inhibited the light-dependent reduction of DPIP by DPC in the TSF-2 particle (Fig. 5).

Thus, linolenic acid affects two sites in PSII: (a) it induces a loss of the loosely bound pool of Mn from the water-splitting site; (b) it rapidly (<20 s) inhibits the photochemical charge separation in PSII.

PSII—Prevention and Reversal of Inhibition. Reports that inhibition of the Hill reaction by linolenic acid may be partially reversible (18, 19, 22) led us to investigate the conditions under which BSA and inorganic metal ions prevent or reverse damage to either PSII inhibition site. In agreement with other studies (8, 15, 19), we found that 0.5 to 2% BSA in the chloroplast reaction mixture prior to addition of linolenic acid prevented inhibition of the Hill reaction. Similarly, 10 μM Mn⁺², Ni²⁺, or Ca²⁺ in the reaction mixture prior to addition of linolenic acid preserved 78% (Mn), 75% (Ni), and 93% (Ca) of the pathway from H₂O to MV; without the ions, the rate after 2 min was nearly zero. Ten μM Mg²⁺ did not protect against linolenic acid-induced damage. Monovalent cations, even at high concentrations (0.2 M), were ineffective.

An important consideration is whether BSA or Mn⁺² is effective in reversing the inhibition to either PSII site once inhibition has occurred. We attempted to reestablish the pathway from H₂O to MV by adding BSA after incubating chloroplasts for various time intervals with 80 μM linolenic acid. The lower curve of Figure 6a shows the rates of MV-mediated O₂ uptake (H₂O as donor) as a function of incubation time in the presence of 80 μM linolenic acid. After an initial rapid decline to 30% of the control rate within 5 s, the rate slowly reached zero at 2 min. The upper curve shows the recovery of electron transport rates at specified times following the addition of 0.5% BSA. It is clear that the addition of BSA to linolenic acid-treated chloroplasts reverses inhibition of the electron transport chain, and that the degree of recovery depends on exposure time to the inhibitor. These data clarify the observation of Shaw et al. (22) that the effect of BSA on fatty acid inhibition of O₂ evolution appeared to be a time-dependent process.

As shown in Figure 6b, the addition of Mn⁺² also reversed the inhibition of the H₂O → MV pathway, and, as with BSA, recovery was

Table II. Effect of Linolenic Acid on DPIP and TMPD-mediated PSI Rates

<table>
<thead>
<tr>
<th>Linolenic Acid</th>
<th>O₂ Uptake (DPIP)</th>
<th>O₂ Uptake (TMPD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μM</td>
<td>μmol/mg Chl-h</td>
<td>μmol/mg Chl-h</td>
</tr>
<tr>
<td>0</td>
<td>363</td>
<td>368</td>
</tr>
<tr>
<td>40</td>
<td>420</td>
<td>389</td>
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<tr>
<td>60</td>
<td>471</td>
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<td>80</td>
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<td>206</td>
<td>254</td>
</tr>
<tr>
<td>200</td>
<td>116</td>
<td>138</td>
</tr>
</tbody>
</table>
Linolenic acid in STN of plants determined after 710

Plant 2-dependent order (t1/2, upon fluorescence kinetics)

Mn2+ directly, thereby doubling the control

Water as proposed by Epel and Neumann (10), Mn2+ reduces O2 to H2O2 directly, thereby preventing its disproportionation to ½ O2 and ½ H2O2. With water as donor, the uptake should triple in the presence of Mn2+; we usually found a 2-fold increase.

The addition of 10 mM Mn2+ to normal chloroplasts resulted in a doubling of the control rate of O2 uptake in the presence of MV. However, as proposed by Epel and Neumann (10), Mn2+ reduces O2 to H2O2 directly, thereby preventing its disproportionation to ½ O2 and ½ H2O2. With water as donor, the uptake should triple in the presence of Mn2+; we usually found a 2-fold increase.

FIG. 3. Fluorescence characteristics of spinach chloroplasts in presence of linolenic acid. Top: spinach chloroplasts at 20 μg/ml Chl and 200 μM linolenic acid in STN buffer containing 5 mM MgCl2. Linolenic acid was added in complete darkness to 10-min dark-adapted chloroplasts. Fmax was determined after addition of DCMU to assay mixture. Middle: control chloroplasts. Bottom: effect of 2% BSA added to assay mixture prior to addition of 200 μM linolenic acid.

FIG. 4. Effect of time of incubation of spinach chloroplasts with linolenic acid. Chloroplasts at 20 μg/ml Chl in STN buffer (pH 7.5) containing 5 mM MgCl2 were incubated in the dark at room temperature for 10 min. Linolenic acid was then added to 80 μM and the suspension was illuminated at the specified times. F0 and Fmax were determined as indicated in Figure 3 and plotted against time of incubation. A fresh sample was used for each determination. Fvar is the difference in magnitude between Fmax and F0.

FIG. 5. Artificial donor activity in an enriched system II particle as a function of linolenic acid concentration. TSF-2 particles, at 14 μg/ml Chl, were incubated for 2 min with linolenic acid and measured spectrophotometrically for DPIP reduction at 590 nm in a reaction mix containing 0.25 mM sucrose, 30 mM phosphate (pH 6.4), 64 μM DPIP, and 50 μM DPC. An identical reaction mix containing 16.7 μM DCMU was prepared; DCMU rate was subtracted from the former to obtain DCMU-dependent PSII rate.

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The decline in rate at 100–200 μM linolenic acid may be caused by either loss of PC or by the larger volume that PC would occupy if it were present within the swollen intrathylakoid space. It has been shown that 100 μM linolenic acid added to chloroplasts causes numerous vesicles to appear from grana, many of which are enclosed in a balloon-like outer membrane (20). If PC was distributed reversibly between the thylakoid membrane and the intrathylakoid space, an increase in intrathylakoid volume would result in a shift away from binding and cause a decline in PSI-mediated rate (see ref. 3).

In PSI, two inhibitory sites could be distinguished. We found a slow loss of water-splitting activity that correlated with the loss of Mn and a reversible, rapid loss of artificial donor activity that can be attributed to a suppression of primary photochemistry.

The authors wish to emphasize that the effect on the fluorescence yield is a unique characteristic of linolenic acid. This result differs from that observed in Tris- or NH₄OH-washed chloroplasts where Fm was missing, but F₁ remains low. Only under certain circumstances can a high F₁ be observed after NH₄OH treatment (4). In the presence of NH₄OH and DCMU, chloroplasts show a normal DCMU rise curve on the first illumination provided NH₄OH is added to equilibrated chloroplasts in complete darkness. Upon a second illumination (after a dark period), the fluorescence rise appears similar to that of the linolenic acid-treated chloroplasts; that is, F₁ is high, equaling Fm. Under these conditions, Q remains reduced following the first illumination since DCMU prevents electron flow to the plastoquinone pool, and the back reaction of Q₈ is slow in the absence of the S₂ state.

In linolenic acid-treated chloroplasts, dark reduction of Q cannot be the cause of the high F₁; rather it appears that the photochemical charge separation is inhibited. If charge separation were prevented, fluorescence would remain unquenched, and the system would behave as if Q₈ were present prior to illumination. It appears, therefore, that linolenic acid functions either by inhibiting the transfer of energy from antennae Chl to the trap or by inhibiting the charge separation with the PSI trap.

The reversibility of the fluorescence characteristics after washing chloroplasts with BSA supports the suggestion that the cause of the instantaneous inhibition of water-splitting activity was the suppression of photochemical charge separation rather than loss of Mn. Accordingly, the time course of water-splitting recovery (Fig. 6) shows the kinetics of removal of the loosely bound pool of Mn from its site(s) on the membrane. Inhibition of the photoact was completely reversible if chloroplasts were exposed to moderate concentrations (<100 μM) of linolenic acid. Artificial donor studies confirmed this conclusion by showing that electron flow from DCP to DPIP was restored under the same conditions that allowed F₁ to recover its low, original value. Although the recovery of F₁ and of artificial donor rates were incomplete at higher concentrations of linolenic acid, this may be understandable in light of studies with [14C]linolenic acid, which show that removal of fatty acid from the membrane is incomplete even after several washings (18).

Our results indicated a low specificity of divalent cations for prevention or reversal of linolenic acid effects. Ni²⁺ and Ca²⁺, but not Mg²⁺, were as effective as Mn²⁺. Co²⁺ has effects similar to Mn²⁺ and Ca²⁺. It seems likely that these effects are a consequence of formation of insoluble salts of linolenic acid with the various cations.

The mechanism of inhibition of the photoact may ultimately lie in the ability of linolenic acid to penetrate its hydrophobic tail into the lipid membrane and change the orientation of electron donor and acceptor complexes relative to one another. Alternatively, the hydrophobic tail might interact with the antenna Chl bed and inhibit transfer of energy to the photoactive trap. Either mechanism would explain the high initial fluorescence yield.

A potentially more interesting effect of linolenic acid would be...
its direct interaction with membrane proteins. Fragata (11) suggested that thylakoid transformations during aging result from conformational changes in membrane proteins because of their interaction with free fatty acids. Since lipids are important in the three-dimensional structure of lipoproteins (21), and since PSII and its accessory pigments are most likely bound in a membrane-protein complex, it is reasonable to suppose that alteration of the membrane structure would induce organizational changes in the associated peptides. These altered structures might be incapable of facilitating energy transfer to the trap or of initiating a charge separation between electron donor and acceptor complexes.

We are aware of only one other treatment that inhibits PSII and causes a high initial fluorescence yield upon illumination. Berry et al. (5) indicated an increase in $F_o$, in a study of thermal damage to Atriplex sabulosa after heat treatment of whole leaves. In the presence of DCMU, $F_o/F_{max}$ increased from 40 to 50% for undamaged tissue to 70–90% for severely damaged chloroplasts. In contrast to changes in $F_o$, no changes in the magnitude of $F_{max}$ occurred. Based partly on this evidence, Berry et al. suggested that the anomalous fluorescence characteristics reflect heat damage to the reaction center itself. In light of the linolenic acid-induced effects on chloroplasts discussed in this paper, we suspect that the effect of heat on A. sabulosa is due to linolenic acid acting on the reaction center of system II following its liberation from the photosynthetic membrane.

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