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INTERACTION OF LINOLENIC ACID WITH BOUND QUINONE MOLECULES IN PHOTOSYSTEM II

TIME-RESOLVED OPTICAL AND ELECTRON SPIN RESONANCE STUDIES

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Time-resolved spectroscopic techniques, including optical flash photolysis and electron spin resonance spectroscopy, have been utilized to monitor electron-transport activity in Photosystem II subchloroplast particles. These studies have indicated that in the presence of 100 μM linolenic acid (1) a high initial fluorescence yield (F_i) is observed upon steady-state illumination of the dark-adapted sample; (2) flash-induced absorption transients ($t > 10$ μs) in the region of 820 nm, attributed to P-680$^+$, are first slowed, then abolished; and (3) electron spin resonance Signal II_s and Signal II_f (Z^+) are not detectable. Upon reversal of linolenic acid inhibition by washing with bovine serum albumin, optical and electron spin resonance transients originating from the photooxidation of P-680 are restored. Similarly, the variable component of fluorescence is recovered with an accompanying restoration of Signal II_s and Signal II_f. The data indicate that linolenic acid affects two inhibition sites in Photosystem II: one located between pheophytin and Q_A on the reducing side, and the other between electron donor Z and P-680 on the oxidizing side. Since both sites are associated with bound quinone molecules, we suggest that linolenic acid interacts at the level of quinone binding proteins in Photosystem II.

Introduction

There are two well-characterized regions of inhibition in the photosynthetic electron-transport chain from water to lipophilic electron acceptors in green plants. The first occurs on the oxidizing side of Photosystem II in the water-splitting mechanism and is affected by agents such as NH_2OH and Tris (see Refs. 1 and 2 for a review). These inhibitors cause loss of bound manganese, and hence, oxygen evolution, but do not affect artificial donor activity [3]. A second site exists on the reducing side of Photosystem II between Q_A and Q_B and is affected by agents such as DCMU and atrazine [1,2]. These inhibitors prevent electron flow to most artificial acceptors [3] and function by occupying the plastoquinone binding site on the 32 kDa protein [4,5].

There is yet another class of inhibitor, which includes fatty acids such as linolenic acid, that functions at several undisclosed sites in Photosystem II. The inhibition by linolenic acid is unusual

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Abbreviations: F_i, initial fluorescence yield; F_{max}, maximum fluorescence yield; F_{var}, variable fluorescence yield; Ph, pheophytin intermediate electron acceptor; Q_A, primary quinone acceptor; Q_B, secondary quinone acceptor; Z, secondary electron donor; Mes, 4-morpholineethanesulfonic acid; DCMU, 3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea, Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TSF-II, Triton Photosystem-II particles.

in that it (i) cannot be overcome by artificial electron donors [6,7,8], (ii) is at least partially reversed by washing with Mn^{2+} [8] or bovine serum albumin [9], (iii) shows a loss of the 690 nm absorption change attributed to P-680 [10], and (iv) shows effects on picosecond fluorescence yield and lifetimes quite unlike other inhibitors [11]. Some of the discrepancies in the literature involving the degree [9,12] and mechanism [8,13] of reversal of inhibition were eventually explained on the basis of dual sites of inhibition in Photosystem II [14]. Linolenic acid was shown to induce (i) a slow but irreversible loss of bound manganese from the water-splitting mechanism and (ii) a fast but reversible inhibition of artificial donor reactions in Photosystem II. Accordingly, the inhibition of the Hill reaction at both short and long incubation times occurs at the reversible site that affects artificial donor activity. The reversibility of the Hill reaction depends on the degree of manganese loss incurred before washing the chloroplasts free of the inhibitor.

In addition to these effects, linolenic acid was found to induce an initial level of fluorescence (F_i) equal to the maximum level of fluorescence (F_{max}) in control chloroplasts concomitant with a complete loss in the variable yield of fluorescence (F_{var}). On washing or on incubation with bovine serum albumin, the initial level of fluorescence returned to its original, low value and artificial donor activity resumed. This unique characteristic of linolenic acid, along with the reversibility of artificial donor reactions, provided evidence for a new mode of inhibition in Photosystem II.

In this paper, we used time-resolved optical and electron spin resonance techniques to study this unusual mode of inhibition. We monitored the effect of linolenic acid on fluorescence yield, P-680 absorption transients, and ESR Signal II_f and Signal II_s in enriched Photosystem II particles. Our results indicate that linolenic acid blocks electron flow between pheophytin (Ph) and Q on the reducing side, and between Z and P-680 on the oxidizing side of Photosystem II.

Materials and Methods

Digitonin Photosystem II particles (D-10) were isolated from market spinach according to published procedures [15]. Triton Photosystem II particles (TSF-II) were isolated according to the following modification of the published procedure [16]. Spinach leaves (1 kg) were ground for 30 s in a Waring blender with 1200 ml of 0.4 M sucrose, 0.05 M Tris (pH 7.8) 0.01 M KCl and (buffer 1) and filtered through three layers of cheesecloth. The filtrate was centrifuged at $200 \times g$ for 2 min to remove large fragments and the supernatant was recentrifuged at $4000 \times g$ for 10 min to sediment the chloroplasts. The pellet was resuspended in EDTA-containing buffer (0.02 M Tricine (pH 7.5), 0.015 M NaCl, 0.001 M EDTA), incubated for 30 min at 4°C, and centrifuged at $4000 \times g$ for 12 min. The pellet was resuspended to 800 μg/ml chlorophyll in Tricine-$MgCl_2$ buffer (0.02 M Tricine (pH 7.5), 0.01 M $MgCl_2$), and Triton X-100 was added to 0.75%. After stirring for 30 min at 4°C, the suspension was centrifuged at $14\,500 \times g$ for 20 min and the pellet was resuspended to 400 μg/ml chlorophyll. Triton X-100 was added to 0.6% and the suspension was stirred at 4°C for 30 min. The suspension was centrifuged at $14\,500 \times g$ for 15 min, and the TSF-II particles were suspended to 2.5 mg/ml chlorophyll in Tricine buffer (0.02 M, pH 7.5). The TSF-II particles were stored frozen in 20% glycerol.

For fluorescence and flash photolysis experiments, linolenic acid (Sigma) was prepared as a 10 mM stock solution in ethanol. For electron spin resonance studies, linolenic acid was prepared as a 1 M solution in ethanol. Bovine serum albumin (Sigma) was prepared as 20% stock solution in water.

P-680 kinetics were monitored at 820 nm with a flash-photolysis spectrophotometer interfaced to a PDP11/23 processor. The measuring beam was detected with a PIN-10 Schottky barrier photodiode (United Detector Technology) and amplified with a Model 113 preamplifier (EG&G PARC). The time response of the spectrometer was limited by the electrical bandwidth of the amplifier, 300 kHz. Actinic flashes were provided by a Photochemical Research Associates 610B xenon flash system (2.5 μs FWHM). Flash photolysis was carrier out on a 3 ml cuvette containing Photosystem II particles at 20 μg/ml of chlorophyll in the buffer and pH specified in the text. Potassium ferricyanide (1 mM) was added to the cuvette after

the incubation period with the inhibitor and 30 s prior to flash photolysis. Unless otherwise specified, each trace is the average of eight repetitive flashes.

Electron spin resonance studies were performed using a Varian E-9 spectrometer. Illumination was provided by a 1000 W tungsten-halogen lamp (Oriel). Specific ESR operating conditions are provided in the figure legends. Fluorescence rise curves were measured as described in Ref. 14.

Results

Variable and fixed fluorescence yields

Linolenic acid is unique in that it is the only physiological compound known to induce a high initial level of fluorescence (F_i) accompanying an absence of variable fluorescence (F_{var}) in dark-adapted chloroplasts [14]. We investigated the effect of linolenic acid on the fluorescence yield of spinach D-10 subchloroplast particles; this preparation lacks the plastoquinone pool and the rise curve covers an area corresponding to one or, at most, two electron equivalents (Q_A and/or Q_B). Fig. 1a shows a typical fluorescence rise curve of a sample containing D-10 particles at pH 7.5. Since this preparation lacks a functional water-splitting mechanism, 0.5 mM diphenylcarbazide was added to serve as electron donor. The rise curve shows a relatively low initial level of fluorescence (F_i) and a rise to F_{max} that obeys an intensity × time relationship. When DCMU is added, F_i is nearly the same level as the control particle, and the area under the rise curve is only slightly (approx. 25%) smaller.

The fluorescence rise curve in the presence of 100 μM linolenic acid is shown in Fig. 1b. Even though the inhibitor was added in complete darkness, the initial level of fluorescence is at the level of F_{max} shown in the control D-10 particle (Fig. 1a) and F_{var} is entirely absent. Illumination of the same sample after a 5 min dark period produced the same response (Fig. 1b). The effect of linolenic acid on F_i was not immediate, but required an incubation period of about 30–60 s before the rise in F_i was complete. A pH profile showed that the effect of linolenic acid on F_i was independent of pH in the tested range 5.5–7.5.

The linolenic acid-induced rise in F_i is reversible after short incubation periods in spinach D-10 particles. The addition of 100 μM bovine serum albumin to a sample that has been incubated with linolenic acid for 2 min results in restoration of the low initial level of fluorescence. In the presence of 0.5 mM diphenylcarbazide, the variable fluorescence yield is restored to near-normal characteristics (Fig. 1c). In both instances the reversal was achieved within 60 s of the addition of bovine serum albumin.

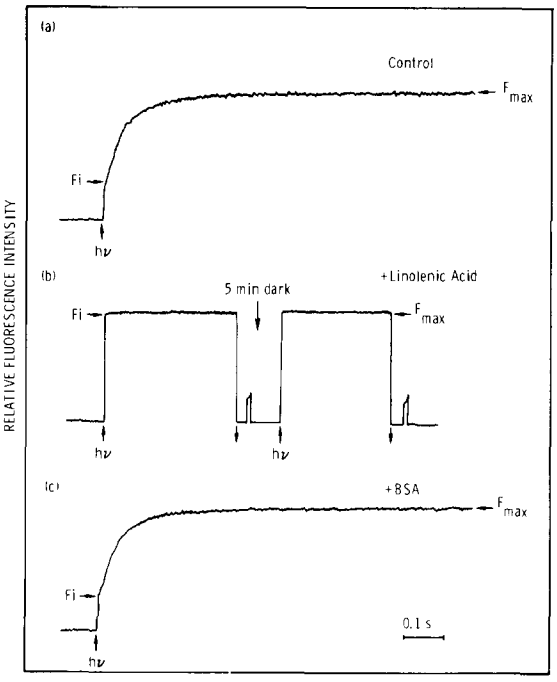

Fig. 1. Fluorescence rise curve of spinach D-10 particles in the presence of linolenic acid. (a) Control sample containing D-10 particles at a chlorophyll concentration of 10 μg/ml in Tricine-$MgCl_2$ buffer (0.050 M Tricine (pH 7.5); 0.005 M $MgCl_2$). The sample was dark adapted for 5 min prior to illumination. (b) Same as (a) except that 100 μM linolenic acid was added 2 min prior to illumination. After a 5 min dark period, the sample was illuminated a second time. (c) D-10 particles after incubation for 2 min with 100 μM linolenic acid and subsequent addition of 100 μM bovine serum albumin (BSA). In all cases, 0.5 mM diphenylcarbazide was added to the sample 30 s prior to analysis.

Klimov et al. [17] have suggested that Photosystem II variable fluorescence arises from recombination luminescence when reaction centers are in the state Z P-680$^+$ Ph$^-$ Q_A^-. If the linolenic acid inhibition site were located between the inter-

mediate electron acceptor, Ph, and the primary quinone acceptor, Q_A, an artificially induced high fluorescence state Z P-680$^+$ Ph$^-$||Q_A would arise, where || represents a site of linolenic acid inhibition. According to this model, the electron on Ph$^-$ would be forced to backreact with P-680$^+$ in the absence of a functional electron acceptor.

P-680 absorption transients

The P-680$^+$ Ph$^-$ charge recombination has a reported lifetime of 2–4 ns [18]. If linolenic acid were to block electron flow between Ph and Q_A, we would expect that absorption transients ($t > 10$ μs) in the region of 820 nm would be abolished [19,20]. The re-reduction kinetics of photooxidized P-680$^+$ in control D-10 particles are shown in Fig. 2a. Since the half-life of P-680 re-reduction has been reported to be pH sensitive [22], we performed the flash photolysis experiments between pH 3.5 and 6.0. At pH 4.5 and in the presence of 1 mM potassium ferricyanide, the fast phase of P-680$^+$ recovery has half-time of about 75 μs and

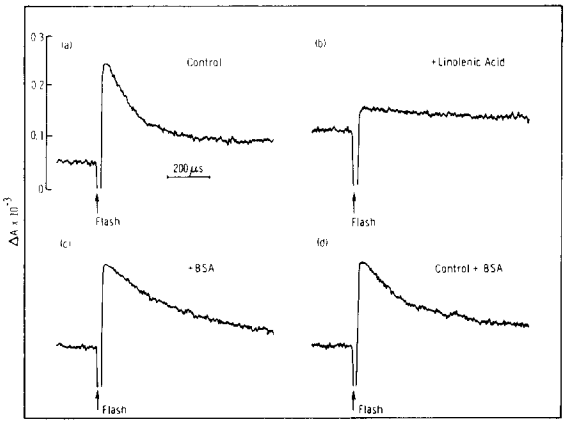

Fig. 2. Re-reduction kinetics of photooxidized P-680$^+$ in D-10 particles in the presence of linolenic acid. (a) Control sample containing D-10 particles at a chlorophyll concentration of 20 μg/ml in Mes buffer (0.1 M, pH 4.5). The sample was dark adapted for 2 min prior to illumination. (b) Same as (a) except that the sample was incubated with 100 μM linolenic acid 2 min prior to illumination. (c) D-10 particles after incubation for 2 min with 100 μM linolenic acid and addition of 0.5% bovine serum albumin (BSA). (d) Control sample containing 0.5% bovine serum albumin. In all cases, 1 mM potassium ferricyanide was added to the cuvette 30 s prior to flash photolysis. The spectra were obtained by signal averaging eight flashes spaced 5 s apart.

accounts for over 80% of the absorption change. DCMU has practically no effect on the magnitude or re-reduction kinetics of the 820 nm transient.

When 100 μM linolenic acid was added to the particles and allowed to incubate for 2 min, the fast absorption transients at 820 nm were abolished (Fig. 2b). The effect was independent of pH in the tested range 3.5–6.0. As indicated above, the loss of the absorption change does not necessarily imply that charge separation is inhibited; the high F_i indicates that charge separation still occurs. With P-680$^+$ Ph$^-$ recombination times of the order of nanoseconds, kinetic transients are outside the range of our spectrometer.

The effect of linolenic acid on the 820 nm absorption change is reversible in D-10 particles. Fig. 2c shows the recovered absorption transient at 820 nm after the addition of 0.5% bovine serum albumin to D-10 particles that have been treated with 100 μM linolenic acid for 2 min. When the amplitude of the absorption change is extrapolated to the onset of the flash, the recovery is about 75% complete, but the fast phase of the absorption change is somewhat slower than the control (175 vs. 75 μs). However, when bovine serum albumin is added to control D-10 particles, the fast phase is slowed from 75 to about 120 μs and the amplitude is 30% lower than the control (Fig. 2d). We do not yet have an adequate explanation for the variability in the half-times of the fast phase. Nevertheless, given the range of half-times reported in the literature and out experience with Tris-washed chloroplasts, D-10 and TSF-II particles, we will assign this kinetic transient to electron transfer between D1 and P-680$^+$.

The data shown in Fig. 2 were obtained by signal averaging eight repetitive flashes spaced 5 s apart. To be certain that the first flash and subsequent flashes produced the same result, we repeated the experiment in D-10 particles by adding linolenic acid in total darkness and analyzing with one flash. We found that the same result was obtained, albeit with a lower signal-to-noise ratio.

Spinach TSF-II particles behaved similarly, except that the half-time for P-680$^+$ reduction in the control sample was about 35 μs at pH 4.5 (Fig. 3a). Reversibility with bovine serum albumin was limited to 10–20% that of D-10 particles; however, unlike the D-10 particles, the recovered 820 nm

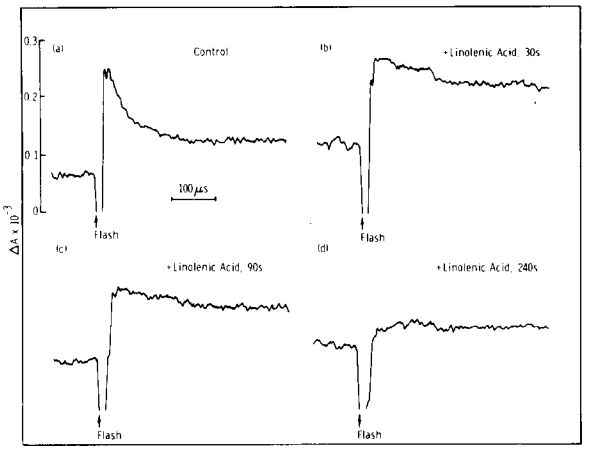

Fig. 3. Re-reduction kinetics of photooxidized P-680$^+$ in TSF-II particles in the presence of linolenic acid. (a) Control sample containing TSF-II particles at a chlorophyll concentration of 20 μg/ml in Mes buffer (0.1 M, pH 4.5). (b–d) Same as (a) except that 100 μM linolenic acid was added to the cuvette 30 s (b), 90 s (c) and 240 s (d) prior to flash photolysis. In all cases, 1 mM potassium ferricyanide was added to the cuvette after the incubation period with inhibitor. The spectra were obtained by signal averaging two flashes spaced 5 s apart.

transient in the TSF-II particles had the same half-time as the control.

We noted earlier that the linolenic acid-induced rise in F_i in both chloroplasts [14] and subchloroplast particles (see above) required a brief incubation period. We found the same to be true for the loss of absorption transients at 820 nm. Spinach D-10 and TSF-II particles were treated with linolenic acid and analyzed by flash photolysis (two flashes spaced 5 s apart) after 30, 90, and 240 s of incubation. The TSF-II particles showed the most dramatic effect. As shown in Fig. 3b, the P-680$^+$ re-reduction time lengthened to over 1 ms with only a small change in the amplitude after a 30 s incubation period with 100 μM linolenic acid. The decline in the amplitude of the absorption change required 240 s for completion (Fig. 3c and d). Presumably, at zero time, only the re-reduction kinetics and not the amplitude of the 820 nm absorption change would be affected. The D-10 particles showed the same behavior but the decline in the amplitude of the 820 nm absorption transient was complete in a shorter period of time (less than 60 s).

Warden and Lyford [23] reported a similar modification of the P-680$^+$ re-reduction kinetics using the hydrophilic electron donor, KI. In the presence of 30 mM KI, the P-680$^+$ rereduction kinetics in TSF-II particles were slowed to over 1 ms but the amplitude of the P-680 absorption change was not affected. The modification of P-680$^+$ reduction kinetics was explained on the basis of either a modification or inhibition of electron flow between D1 and P-680.

We suggest that the 1 ms P-680 re-reduction kinetics observed in the TSF-II particles with linolenic acid can be explained by invoking a second site of inhibition on the oxidizing side of Photosystem II. According to this model, the 1 ms transient would result from a slow backreaction from Q_A to P-680$^+$ when reaction centers are in the state Z||P-680$^+$ Ph Q_A^-. Large variations have been seen in the half-time of the slow phase of P-680$^+$ reduction; as noted by Mathis et al. [21,22], the slow phase is as long as 800 μs in Photosystem II particles from *Phormidium laminosum* and nearly 1 ms in the Photosystem II chlorophyll-*a*-protein complex from spinach. The subsequent decline in amplitude of the 820 nm absorption transient would result from a time-dependent block in electron flow from Ph to Q_A on the reducing side of Photosystem II. This block would be responsible for the high F_i noted earlier. At these extended incubation times, illumination of the reaction center would induce the short-lived state Z||P-680$^+$ Ph^-||Q_A.

ESR Signal II_f and Signal II_s

Because the properties of D_1 correlate with those of Signal II_f [24], we studied the effect of linolenic acid on ESR Signal II in D-10 and TSF-II particles. ESR experiments require a high concentration of chlorophyll (1–3 mg/ml) to observe Signal II in both chloroplasts and subchloroplast particles. Since linolenic acid inhibition in chloroplasts depends on the ratio of fatty acid to chlorophyll as well as on the molar concentration of fatty acid (Ref. 9, see also Ref. 12) the minimum effective concentration was determined experimentally. We found that 3–5 mM linolenic acid caused the loss of Signal II_f in spinach D-10 particles (Fig. 4). This loss would not be unexpected if there were a site of inhibition between Z and P-680 or between Ph and Q_A in Photosystem II. We also

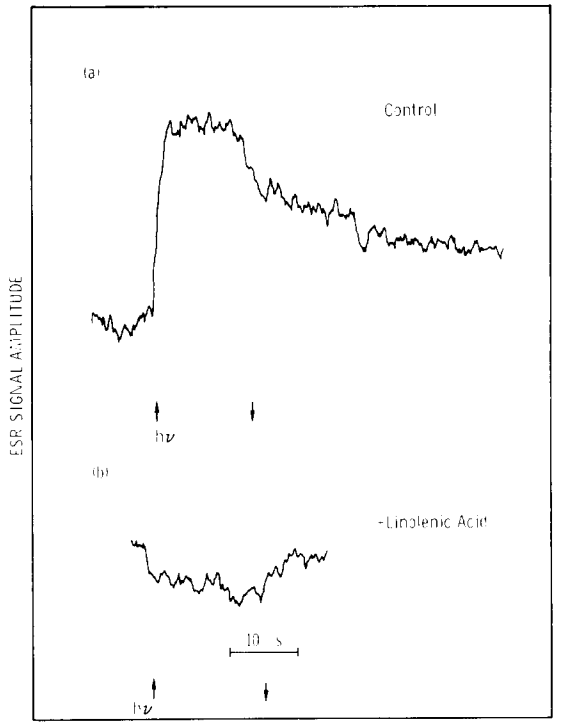

Fig. 4. ESR Signal II_f in spinach D-10 particles. (a) Control sample in Tricine buffer (0.05 M, pH 7.5) at a chlorophyll concentration of 2.7 mg/ml. (b) Same as (a) except that 5 mM linolenic acid was added to the sample in darkness and allowed to incubate 5 min prior to analysis. ESR conditions: field setting, 3830 G; scan time, 1 min; time constant, 0.3 s; modulation frequency, 100 kHz; modulation amplitude, 5 G; receiver gain, 8000; microwave frequency, 9.524 GHz; microwave power, 30 mW.

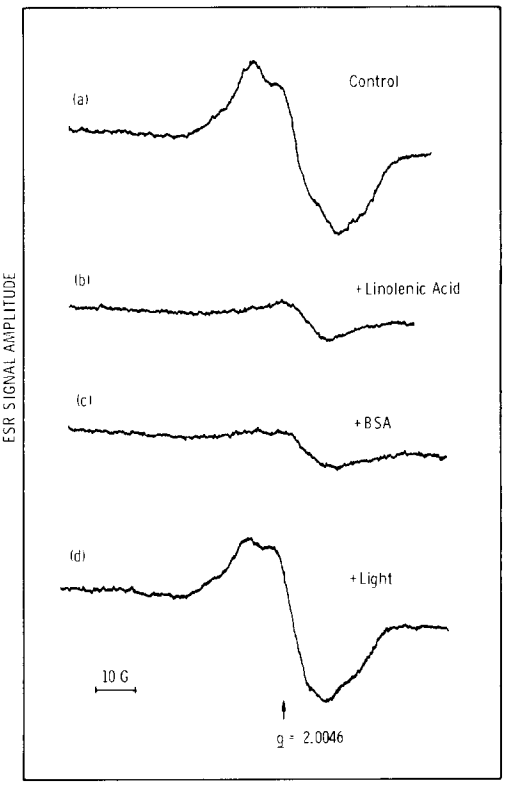

Fig. 5. ESR Signal II_s in spinach D-10 particles. (a) Control sample in Tricine buffer (0.05 M, pH 7.5) at a chlorophyll concentration of 2.7 mg/ml. (b) Same as (a) except that 5 mM linolenic acid was added to the sample and allowed to incubate 5 min. (c) D-10 particles were incubated for 10 min with 5 mM linolenic acid; the reaction was stopped by the addition of 3.8% bovine serum albumin (BSA). All manipulations were carried out in complete darkness. (d) Same as (c) except that the sample was illuminated for 2–3 s in the ESR cavity. ESR conditions: field setting, 3393 G; scan range, 100 G; scan time, 8 min; time constant, 0.3 s; modulation frequency, 100 kHz; modulation amplitude, 5 G; receiver gain, 5000; microwave frequency, 9.524 GHz; microwave power, 30 mW.

found that transient electron spin resonance signals at $g = 2.0025$ arising from $P\text{-}680^+$ were abolished by treatment with 3 mM linolenic acid.

More surprising, however, was the complete absence of Signal II_s in preparations treated with linolenic acid. Fig. 5a shows the spectrum of ESR Signal II_s in control D-10 particles. The sample was illuminated for 90 s during the initial part of the scan; this pre-illumination caused photochemical reduction of the small amount of Signal I (P-700) still present in the preparation. When 5 mM linolenic acid was added to the sample, Signal II_s was lost. Illumination of the sample with a 1000 W lamp for 90 s in the presence of inhibitor did not result in recovery of Signal II_s (Fig. 5b). To determine if the loss of Signal II_s was reversible, D-10 particles at 2.7 mg/ml were incubated for 10 min with 5 mM linolenic acid, diluted 10-fold with buffer containing 0.5% bovine serum albumin, and spun at $35\,000 \times g$ for 30 min. When these manipulations were carried out under room illumination, Signal II_f and Signal II_s fully recovered in the resuspended sample (data not shown).

To determine if light was necessary for the recovery of Signal II_s, the experiment was re-

peated under conditions of complete darkness and with a slightly different protocol. Linolenic acid (5 mM) was added to D-10 particles in complete darkness, resulting in the loss of Signal II_f and Signal II_s (see Figs. 4b and 5b). After 10 min of incubation, 3.8% bovine serum albumin was added to the sample, also in darkness. As shown in Fig. 5c, there was no recovery of Signal II_s. However, when the sample was illuminated in the cavity for 2–3 s with a 1000 W lamp, Signal II_s fully recovered (Fig. 5d). Signal II_f also recovered under the described conditions. These results indicate that light is not required for the linolenic acid-induced inhibition of Signal II, but it is necessary for the recovery of Signal II_s upon removal of the inhibitor.

Discussion

The proposal that linolenic acid suppresses photochemical charge separation in Photosystem II [14] is reinterpreted in light of the data presented in this paper and in view of experimental evidence [25] that F_{var} arises from charge recombination when reaction centers are in the state Z P-680$^+$ Ph$^-$ Q_A^-. We conclude that the high F_i and lack of F_{var} in linolenic acid-inhibited chloroplasts and subchloroplast particles is the result of a linolenic acid-sensitive site located between Ph and Q_A in Photosystem II. Charge separation would still occur when the centers are in the state Z P-680 Ph$\|Q_A$, but charge recombination would occur from the short-lived state Z P-680$^+$ Ph$^-\|Q_A$. This dissipative charge recombination would account for the high F_i in the fluorescence measurements and for the absence of the 820 nm absorption transient. The time-course of the loss of 820 nm absorption transient and rise in F_i in TSF-II particles shows that inhibition at this site requires an incubation period of 1–2 min with linolenic acid.

The change in the P-680$^+$ re-reduction kinetics that occurs within 30 s after the addition of linolenic acid indicates that an additional site of inhibition may be operating between Z and P-680 in Photosystem II. When the water splitting mechanism in chloroplasts has been inactivated with Tris or NH_2OH, the re-reduction of photooxidized P-680$^+$ shows biphasic kinetics with half-times of about 20–30 μs and 100–1000 μs at pH 5.0. According to Mathis and co-workers [21,22], the slow phase of P-680$^+$ re-reduction has been attributed to the back reaction from Q_A^- when reaction centers are in the state Z$^+$ P-680$^+$ Ph Q_A^-. The fast phase represents electron donation from D_1, a one-electron high-potential redox donor. Under oxygen-evolving conditions, electron transfer from the immediate donor to P-680$^+$, Z, occurs in times under a microsecond [20], but it can be slowed to the microsecond range by inhibiting oxygen evolution [19]. This slower donor, D_1, can also be observed without special treatment in subchloroplast particles devoid of oxygen evolution. It is likely that Z, the species active in oxygen-evolving material, is the same moiety that gives rise to D_1, the species active after destruction of the water-splitting site. Z has been observed in oxygen-evolving material and has absorbance changes in the visible and near ultraviolet similar to those expected from a semiquinone [26].

Z, in its oxidized state, gives rise to ESR Signal II_{vf} [27]. When the oxygen-evolution mechanism has been destroyed, a slower ESR transient, Signal II_f, arises [28]. The properties of the spectroscopic signal, D_1, are consistent with this latter ESR signal [24,29]. On this basis, we would expect that linolenic acid should inhibit Signal II_f. While we did find an inhibition of Signal II_f, we could not be certain that this was due to inhibition on the oxidizing side of Photosystem II (the experiment required 3–5 min for completion and it is reasonable to expect the Ph$\|Q_A$ site as well as the Z$\|$P-680 site to be operating at these extended times). The rapid charge recombination between Ph$^-$ and P-680$^+$ expected when the Ph$\|Q_A$ site is operating would preclude electron transfer from Z (observed as Signal II_f) to P-680$^+$.

The effect of linolenic acid on Signal II_s was quite unexpected. The role of this component in photosynthetic electron transport is not known; however, it serves as an electron donor to Photosystem II and must be oxidized before Signal II_f is observed [30]. From extraction and reconstitution studies, it has long been appreciated that a quinone functions on the oxidizing side of Photosystem II [31]. The ESR properties of Signal II, including the line-shape and microwave saturation characteristics, are similar to those of plastosemiquinone radicals [32–34]. If linolenic acid were to perturb

the binding of the quinone to its host protein or, alternately, disturb a local, protected environment surrounding the protein, the oxidized quinone may become free to interact with a nearby reductant. On removal of the inhibitor, the quinone could reestablish orientation with its host protein, or the local environment surrounding the quinone could regain its structure. Under these conditions, the quinone would regain function with its reaction partner and a brief illumination would induce oxidation. Signal II_s as well as Signal II_f would be expected to reappear.

The findings reported in this paper and elsewhere indicate that the effect of linolenic acid on thylakoid structure and function is multivalent and not indiscriminate. Indeed, the inhibition sites on the oxidizing side and reducing side of the Photosystem II share a common feature: both are associated with bound quinone molecules. It is possible that the binding topology of the quinones at both sites in Photosystem II possess similar features to the extent that linolenic acid affects both with near-equal efficiency. We suggest that the interaction of linolenic acid with bound quinones and/or quinone binding proteins constitutes the mechanism for this unique mode of inhibition in Photosystem II.

Just prior to the submission of this manuscript, we became aware of a recent paper by Vernotte et al. [35] in which two linolenic acid inhibition sites were found on the reducing side of Photosystem II. At one site, linolenic acid prevents charge stabilization on the primary acceptor, Q_A. At the second site, linolenic acid was shown to interact with the protein carrying Q_B, the secondary electron acceptor of Photosystem II. In contrast with our finding that the dark rise in F_i in subchloroplast particles was independent of pH, Vernotte and coworkers [35] reported that the effect in pea chloroplasts was pH-dependent. At pH 7.5, and in the presence of 10 mM $MgCl_2$, linolenic acid raised F_i to F_{max}, whereas at pH 6.0, practically no rise in F_i occurred. The pH dependence was proposed due to either an effect of pH on membrane components or to the effect of pH on the linolenic acid incorporated into the membrane. We have no adequate explanation for the differences observed in pH sensitivity in the two sets of experiments except to suggest that detergent treatment may have altered the membrane to render the binding site accessible to the inhibitor at low pH values. All of our studies were performed with digitonin and Triton-treated subchloroplast particles. Nevertheless, the finding that linolenic acid interacts with two sites on the reducing side lends substantial support to the proposal that linolenic acid interacts with quinones or quinone binding proteins in Photosystem II.

Acknowledgments

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Note added in proof (August 2nd, 1984)

We recently became aware of two papers in which lipase treatment of chloroplasts resulted in many of the effects that we observed with linolenic acid. Okayama et al. [36] found that lipase treatment of *Chlamydomonas reinhardtii* chloroplasts resulted in elimination of the variable fluorescence yield an increase in the invariant fluorescence yield. Lipase treatment also eliminated ESR Signal II. Jordan et al. [37] recently showed that phospholipase A_2 treatment of pea chloroplasts resulted in the inhibition of Photosystem II activity together with an increase in the initial fluorescence yield and a subsequent loss of variable fluorescence. Phospholipase A_2 was shown to hydrolyze 75% of the phosphatidylglycerol and 60% of the phosphatidylcholine in the thylakoid membrane. We suggest that the effects on fluorescence yield and Signal II observed in these papers may be due to the action of fatty acids liberated as a result of lipase treatment rather than to lipid depletion.

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