*Biochimica et Biophysica Acta* 890 (1987) 215–223
Elsevier



BBA 42200

# On the mechanism of linolenic acid inhibition in Photosystem II

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(Received 30 September 1986)

Key words: Linolenic acid; Photosystem II; Quinone; ESR; (Spinach chloroplast)

**Recent studies in our laboratory have reexamined the interaction of the unsaturated fatty acid, linolenic acid, with Photosystem II and have documented two principal regions of inhibition: one associated with the donor complex (Signal $2_f$ or $D_1$) to the reaction center, and the other located on the reducing side between pheophytin and $Q_a$ (Golbeck, J.H. and Warden, J.T. (1984) Biochim. Biophys. Acta 767, 263–271). A further characterization of fatty acid inhibition of secondary electron transport in Photosystem II at room and cryogenic temperatures is presented in this paper. These studies demonstrate that linolenic acid, and related fatty acid analogs, (1) eliminate the transient absorption increase at 320 nm, attributed to $Q_a^-$; (2) abolish the production, either chemically or photochemically, of the ESR signal ($Q^-Fe$) associated with the bound quinone acceptor, $Q_a^-$; and (3) prevent the photooxidation of Signal $2_{lf}(D_1)$ at cryogenic temperature. Linolenic-acid-treated samples are characterized by a high initial fluorescence yield ($F_i$) equivalent to the maximum level of fluorescence ($F_{max}$); however, the spin-polarized triplet, associated with the reaction-center electron donor, P-680, is observed only in inhibited samples that have been prereduced with sodium dithionite. These results suggest the presence of an additional acceptor intermediate between pheophytin and $Q_a$. The donor-assisted photoaccumulation of pheophytin anion in Photosystem II particles, as monitored by the decline of fluorescence yield, is inhibited by linolenic acid. Redox titrations of the fluorescence yield in control and inhibited preparations demonstrate that the midpoint potential for the primary acceptor for Photosystem II is insensitive to the fatty acid ($E_m \approx -583$ mV) and thus indicate that primary photochemistry is functional during linolenic-acid inhibition. These data are consistent with the hypothesis that unsaturated fatty acids inhibit secondary electron transport in Photosystem II via displacement of endogenous quinone from quinone-binding peptides.**

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Abbreviations: Chl, chlorophyll; $D_1$, the primary quinoidal donor to $P\text{-}680^+$; D-10, digitonin PS II particles; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; $F_i$, initial fluorescence yield; $F_{max}$, maximum fluorescence yield; $F_v$, variable fluorescence yield; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; P-680, primary electron donor of Photosystem II; Pheo, pheophytin; PS I, Photosystem I; PS II, Photosystem II; $Q_a$, primary quinone acceptor; $Q_b$, secondary quinone acceptor; $Q^-Fe$, iron-perturbed quinone ESR resonance associated with $Q_a^-$; TSF-2, triton Photosystem II particles.

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## Introduction

The mechanism(s) associated with fatty acid inhibition of Photosystem II (PS II) has been the subject of a number of investigations since the pioneering study of Krogmann and Jagendorf [1]. As capsulized by Golbeck et al. [2] this topic is of considerable import, since membrane damage attributed to aging or stress has been associated with the release of free fatty acids, presumably induced by endogenous lipases. Unsaturated fatty acids, especially linolenic acid, have been linked causally to the following effects in thylakoid membranes: (1) modification of the membrane

surface-charge density [3], uncoupling of photophosphorylation [4], release of manganese from the water-oxidation complex of Photosystem II [2] and inhibition of artificial donor-assisted electron transport in PS I and PS II [2,5]. Precise biochemical descriptions for all of these effects have not been proffered; however, recent investigations by Vernotte et al. [7] and Warden et al. [6,8] have focussed on the origin and mechanism of the fatty-acid-induced inhibition of PS II electron transport.

Golbeck et al. [2] proposed initially that the high initial fluorescence yield ($F_i$) observed in the presence of linolenic acid was either the result of a fatty-acid-mediated inhibition of the primary photoact or alternatively a decoupling of the PS II reaction center from the light-harvesting chlorophylls. In contrast to these suggestions, we have proposed recently [8], based on spectroscopic and kinetic analysis of electron transport components associated with the PS II reaction center, that linolenic acid affects multiple inhibition sites in PS II, in particular, those sites associated with bound quinones (e.g., the donor species Signal $2_s$ and Signal $2_f$ ($D_1$) on the oxidizing side of PS II and $Q_a$ the primary quinone acceptor, on the reducing locus of the reaction center). Furthermore, invoking the hypothesis of Klimov et al. [9] that variable fluorescence in PS II is a monitor of the backreaction between P-680$^+$ (the primary electron donor) and pheophytin anion (Pheo$^-$, the transient, primary electron acceptor), the development of the high $F_i$ in the presence of the inhibitor is considered to be the consequence of the absence of a functional acceptor $Q_a$ [8]. Similar conclusions have been presented by Vernotte et al. [7], who established that identical lifetimes for the slow component of PS II fluorescence (1.5 ns) exist in linolenic acid-treated and untreated chloroplasts. Hence, they have concluded that the initial charge-separation act in PS II is unaffected by fatty acid treatment; instead linolenic acid is proposed to prevent charge stabilization on $Q_a$. Additionally Vernotte et al. demonstrated that linolenic acid effects a 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) type inhibition at the secondary quinone acceptor ($Q_b$) site in PS II.

The results presented in this paper are an extension of our previous study [8] and address specifically the nature of linolenic acid inhibition at the reaction center. Utilizing time-resolved optical spectroscopy in conjunction with electron spin resonance (ESR) spectroscopy at cryogenic temperatures, we have examined the effect of linolenic acid on the photoreduction of the primary quinone acceptor, $Q_a$. Additionally we have examined the capability of fatty acid inhibited PS II preparations to perform charge separation, triplet state production and secondary electron transfer at low temperature. Finally we have achieved a redox titration of the fluorescence yield in control and inhibited samples. Our results demonstrate that linolenic acid inhibition in Photosystem II does not suppress primary photochemistry. We propose, instead, that unsaturated fatty acids inhibit secondary electron transfers in PS II via competitive displacement of bound quinoidal electron transport intermediates from their protein binding sites [10].

## Materials and Methods

Digitonin Photosystem II particles (D-10) were isolated from spinach thylakoid membranes according to the procedure of Boardman [11]. Triton particles (TSF-2) were prepared by the procedure reported previously [12]. All preparations were stored frozen in 15% glycerol prior to use.

Linolenic (*cis,cis,cis*-9,12,15-octadecatrienoic) acid was obtained from Sigma Chemical Co. (St. Louis, MO). For transient optical absorption and fluorescence studies, the fatty acid 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 [8]. During incubation of the Photosystem II particles with the fatty acid the concentration of ethanol was maintained at concentrations below 1%.

Absorption transients at 320 nm were acquired with a purpose-constructed flash-photolysis spectrometer interfaced to a Digital Equipment Corp. PDP 11/23 computer. Actinic illumination was provided by a Phase-R DL1100 flashlamp-pumped dye laser (200 ns FWHM, 660 nm, 40 mJ per flash). These ultraviolet flash-photolysis studies employed a cuvette with a 2 mm pathlength and the chlorophyll concentration, as determined by

the method of Arnon [13], was typically 100 $\mu g \cdot ml^{-1}$. Kinetics of $P\text{-}680^+$ reduction were monitored as detailed previously [8] in 3 ml samples containing PS II particles at 20 $\mu$g per ml Chl. Potassium ferricyanide (1 mM) was added to the sample 30 s prior to flash photolysis.

Chlorophyll *a* fluorescence rise-curves were obtained with a lab-constructed fluorimeter. PS II particles at 10 $\mu$g per ml Chl were contained in an anaerobic 3 ml cuvette and illuminated by blue light (Schott BG-12, $\lambda_{max} = 400$ nm, 2760 $W \cdot m^{-2}$). Fluorescence was selected utilizing a red filter combination (Melles-Griot 03 FIV 136 (700 nm, 40 nm bandpass) and Schott OG-590 and was detected at 90° to the excitation beam, utilizing a PIN 10D/SB photodiode (United Detector Technology) coupled to a low-noise, transimpedance preamp which was interfaced to a Nicolet 1073 signal averager. Redox titrations of fluorescence yield were performed similarly utilizing a custom designed titration vessel and actinic light selected by Corning 5-56 and Melles Griot SWP 013 filters (560 $mW \cdot m^{-2}$, duration of excitation, 20 ms).

Electron spin resonance spectra were acquired with a Varian E-9 spectrometer equipped with an Air Products LTD-3-100 cryostat [14]. Sample temperatures were monitored with a calibrated carbon resistor situated directly below the 3-mm inner-diameter quartz sample tube. Data acquisition and analysis were performed via a Unix™-based software and hardware interface to a PDP 11/23 computer. Triplet state measurements were made by the actinic light modulation technique [15]. A Princeton Applied Research 5204 Lock-in Analyzer in combination with a Rolfin programmable chopper was utilized for phase-sensitive detection of the photo-excited triplet. Actinic light for ESR experiments was provided by a 1000 W tungsten-halogen source (Oriel) or by a Varian Eimac 300 W xenon arc lamp. Illuminations at 200 K were performed for 15 min in an unsilvered dewar containing a solid $CO_2$/ethanol slurry.

Redox titrations of the fluorescence yield were performed anaerobically in a lab-constructed titration vessel, fitted with an Ingold combination platinum/silver–silver chloride electrode [12]. The electrode was calibrated utilizing saturated quinhydrone. Potentials were adjusted with aliquots of 0.1 M potassium ferricyanide or 0.2 M sodium dithionite (prepared in 0.5 M glycine, pH 10.5). Titrations were executed commonly at pH 10.5 (100 mM glycine/KOH, 20 mM KCl) in both reductive and oxidative directions to confirm reversibility. A selection of redox mediators (in assorted combinations) were used to establish the independence of the titrations on mediator type. Special care was exercised in the selection of mediators and their concentrations to avoid fluorescence quenching and to minimize spurious contributions of these compounds to absorption or fluorescence (at either the excitation or emission wavelengths). Mediators utilized included: indigotetrasulfonate (−46 mV), alloxazine (−170 mV), phenosafranine (−252 mV), acridine (−313 mV), neutral red (−325 mV), methyl viologen (−454 mV), 1,1′-trimethylene-2,2′-dipyridilium dibromide (−548 mV), 1,1′-trimethylene-5,5′-dimethyl-2,2′-dipyridilium dibromide (−670 mV), 1,1′-tetramethylene-2,2′-dipyridilium dibromide (−700 mV) and 1-methyl-4-(2-pyrimidyl) pyridinium bromide (−850 mV). Mediator sets were prepared as 10 mM stock solutions in ethanol. Titrations in replicate were performed using a range of mediator concentrations (1–30 $\mu$M).

Synthesis of the 2,2′-bipyridyl and the 2-(4-pyridyl)pyrimidine salts were accomplished by literature procedures [16,17].

## Results

*The primary quinone acceptor*

The primary stable electron acceptor of Photosystem II, $Q_a$, has been proposed to be displaced competitively during fatty-acid mediated inhibition of PS II electron transport [8,10]. To test this hypothesis we examined the absorption change at 320 nm, a wavelength diagnostic for the Photosystem II acceptor quinones. When TSF-2 particles are examined at 320 nm during microsecond flash photolysis (Fig. 1a), a characteristic absorption increase is observed which has been assigned to the formation of a plastoquinone radical anion [18,19] and is presumed to arise predominantly from the reduction of $Q_a$. The total absorption change corresponds to one $Q_a$ reduced per 264 chlorophylls, as determined using a differential molar absorptivity of the semiquinone anion of 13000 $M^{-1} \cdot cm^{-1}$ [18]. This triton preparation

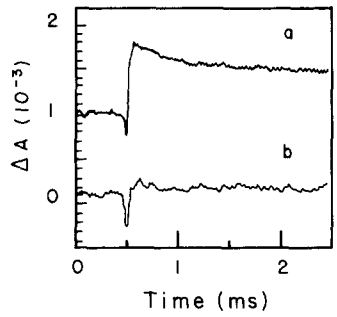


Fig. 1. Inhibition of the photoreduction of $Q_a$ in TSF-2 particles incubated with 100 $\mu$M linolenic acid. Chlorophyll concentration was 100 $\mu g \cdot ml^{-1}$ (in 0.1 M Mes-NaOH, pH 6.0) and 100 $\mu$M potassium ferricyanide was added to the 2 mm pathlength cuvette prior to photolysis. Each kinetic trace represents the average of 16 flashes given at a 0.5 Hz frequency. (a) Control particles; (b) particles incubated with linolenic acid for 15 min.

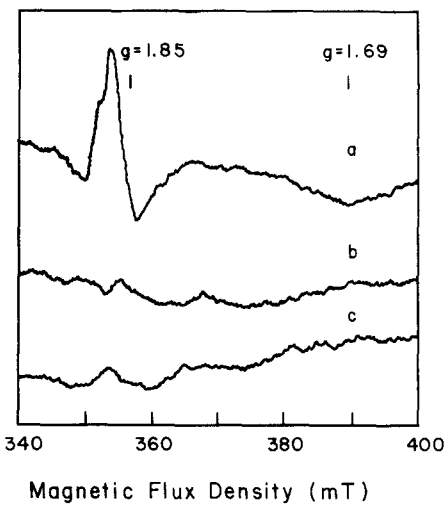


Fig. 2. Electron spin resonance spectra of the iron-quinone acceptor $Q^-$ Fe in TSF-2 particles. (a) Control particles after illumination for 15 min at 200 K; (b) same as (a), except that the sample was incubated with 7 mM linolenic acid for 15 min prior to photolysis at 200 K; (c) same as (b), except particles were incubated and frozen in the dark in the presence of 50 mM sodium dithionite. The particles were suspended at 6.8 mg per ml Chl in 0.1 M Hepes buffer (pH 7.8). Instrument parameters: scan dwell time, 3 s per point (512 points); time constant, 3 s; modulation amplitude, 2.0 mT; receiver gain, 5000; microwave power, 10 mW, microwave frequency, 9245 MHz, temperature, 6.3 K.

has been shown to contain approx. one P-680 per 245 chlorophylls [12], hence the absorption change at 320 nm represents the photoreduction of $Q_a$ in approx. 93% of the reaction centers. Incubation of the particles with 100 $\mu$M linolenic acid for 15 min (Fig. 1b) suppresses the absorption transient at 320 nm. No significant alteration of the reoxidation kinetics for $Q_a^-$ is observed during the development of fatty acid inhibition. The inhibition of $Q_a$ photoreduction in the presence of the inhibitor is complementary to the abolition of $\mu$s P-680$^+$ reduction kinetics reported previously [8].

The inhibition of the 320 nm absorption change of $Q_a^-$ is necessary, but not sufficient in itself, to verify the hypothesis that fatty acids disrupt stable PS II charge transfer by displacing $Q_a$ from its binding site. In order to establish whether $Q_a$ is still resident in the acceptor complex after fatty acid inhibition, we have utilized electron spin resonance as a probe for the $g = 1.82$ resonance ($Q^-$Fe) which signifies an interaction of the semiquinone anion of $Q_a$ and an $Fe^{2+}$ ion in close proximity [20,21]. Fig. 2a illustrates the $Q^-$Fe resonance in control TSF-2 particles; this resonance can be generated to equal extent either by photoreduction of the sample at 200 K or by chemical reduction of the sample by dithionite in the dark prior to freezing. The $Q^-$Fe signal in Fig. 2a is characterized by features at $g = 1.85$ and 1.69 and is analogous to signals for the primary quinone acceptor that have been characterized in other PS II and bacterial preparations [22]. Incubation of the PS II particles with linolenic acid induces an abolition of the $Q^-$Fe resonance as assayed after illumination at 200 K (Fig. 2b) or chemical reduction (Fig. 2c). These ESR data therefore corroborate the optical studies at 320 nm and indicate that the $Q_a$ binding site is no longer functional for photochemically or chemically mediated reduction.

*Secondary electron transport at cryogenic temperatures*

Studies of Photosystem II photochemistry at cryogenic temperatures have demonstrated that at least two components can be stably photooxidized by P-680$^+$ : cytochrome *b*-559 [23], and a form of Signal 2, attributed to the donor to P-680 and denoted as Signal $2_{lt}$ [24]. The reduction of P-680$^+$ by these components competes, albeit inefficiently, with a backreaction from $Q_a^-$, hence the

cumulative effect of low-temperature illumination of PS II is the trapping of $Q_a^-$ [20,25]. The data presented in the previous section indicate implicitly that stable electron transfers from the donor side of PS II would be eliminated in the presence of the fatty acid inhibitor, since $Q_a$ is not accessible for reduction. Fig. 3a illustrates the photoreduction of the quinoidal species, Signal $2_{lt}$, during illumination of control D-10 particles at 9 K. As reported by Nugent and Evans [24], production of Signal $2_{lt}$ is accompanied also by a 0.8–1.0 mT resonance (Fig. 3a), attributed to a chlorophyll cation radical associated with PS II. However, the fatty-acid-treated sample is devoid of the light-induced Signal 2 or chlorophyll cation resonances (Fig. 3b). The studies presented in Fig. 3 demonstrate that consistent with room temperature observations [8], fatty acid inhibition in Photosystem II prevents the development of secondary electron transport and the consequent storage of oxidizing equivalents at cryogenic temperatures.

*The spin-polarized reaction center triplet*

Suppression of secondary electron transfer in green-plant Photosystem II by reduction of $Q_a$ results in the formation during illumination of a spin-polarized reaction-center triplet [26,27]. This triplet, which can be observed by ESR only at cryogenic temperatures, is proposed to originate via charge recombination between $P\text{-}680^+$ and pheophytin anion. Analogous to the observations described previously, an induction of the ESR-detectable triplet would be predicted as a consequence of fatty acid inhibition of PS II. Reduction of D-10 or TSF-2 particles with 50 mM dithionite (pH 7.8) yields the characteristic ESR spectrum (Fig. 4a) of the reaction-center triplet with a polarization pattern: aeeaae [26]. This triplet is characterized by zero-field splitting parameters of $|D| = 0.0287$ cm$^{-1}$ and $|E| = 0.0041$ cm$^{-1}$. In accord with the recent observation by Evans et al. [28] (but contrary to the report of Rutherford et al. [26]), we do not observe significant production (less than 10%) of the reaction-center triplet at

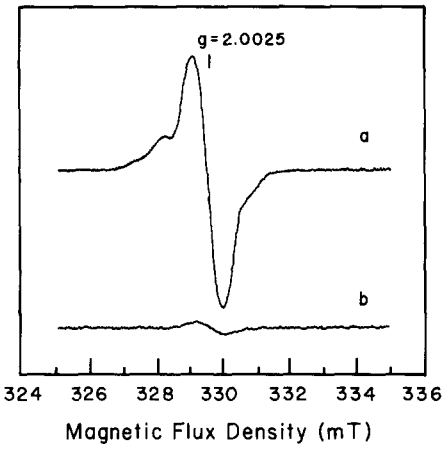


Fig. 3. Light-minus-dark ESR difference spectra in the $g = 2.00$ region of control (a) and a linolenic acid inhibited (7 mM) sample (b). Chlorophyll concentration, 4.1 mg·ml$^{-1}$. Instrument parameters: scan dwell time, 1 s per point (512 points); time constant, 0.3 s; modulation amplitude, 0.32 mT; receiver gain, 500; microwave power, 0.02 mW, microwave frequency, 9245 MHz, temperature, 9 K.

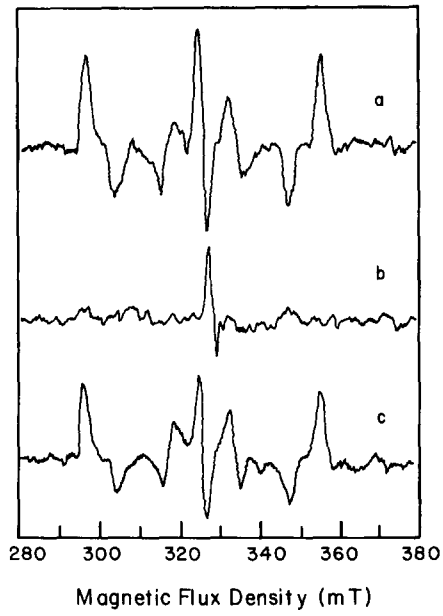


Fig. 4. The spin-polarized reaction-center triplet in TSF-2 particles monitored at 7.8 K. (a) TSF-2 particles (4.3 mg per ml chlorophyll) incubated with 50 mM sodium dithionite (0.1 M Hepes, pH 7.8) and frozen in the dark; (b) TSF-2 particles incubated with 7 mM linolenic acid for 30 min and frozen in the dark; (c) particles as per (b), but additionally treated with 50 mM dithionite 10 min prior to freezing. Instrument parameters: scan dwell time, 3 s per point; time constant, 1 s; modulation amplitude, 3.2 mT; receiver gain, 6300; microwave power, 0.02 mW; microwave frequency, 9251 MHz; actinic light modulation frequency, 41 Hz; phase, 292 degrees.

potentials above −400 mV. Incubation of the PS II particles with linolenic acid in the absence of a strong reductant (Fig. 4b), in contrast to our expectations, did not induce a marked increase in triplet yield; although these samples were determined (in parallel experiments) to exhibit maximal fluorescence yield and to lack microsecond-detectable P-680$^+$ reduction kinetics, both criteria characteristic of linolenic acid inhibition. However, reduction of the linolenic acid treated sample with dithionite (Fig. 4c) resulted in the appearance of the spin-polarized triplet at an intensity comparable to the control.

*Fluorescence yield as a monitor of primary photochemistry*

The origin of chlorophyll *a* variable fluorescence ($F_v$) in Photosystem II has been proposed by Klimov et al. [9] to reflect the recombination luminescence (i.e., delayed fluorescence) resulting from reversal of the primary photochemical process in the reaction center [29–31], namely annihilation of the P-680$^+$ · Pheo$^-$ radical pair. According to this postulate maximal fluorescence yield ($F_{max}$) would be observed when $Q_a$ is reduced either chemically or photochemically. Additionally we have proposed that inhibitors which block electron transfer between pheophytin and $Q_a$ (or displace $Q_a$ from its binding site) induce a high fluorescence yield, which results from the backreaction between P-680$^+$ and Pheo$^-$ [8,10,32].

A corollary to the recombination luminescence hypothesis for the origin of PS II variable fluorescence predicts that reduction of the primary acceptor pheophytin should result in the quenching of PS II fluorescence to a yield ($F_i$) comparable to that observed in the open reaction center (i.e., P-680 · Pheo · $Q_a$) [9]. If $Q_a$ is reduced in the presence of a strong reductant, intense actinic illumination of the PS II containing sample promotes a fluorescence decline to a level nominal to $F_i$. The decrease in fluorescence yield is accompanied by optical absorbance changes attributed to the photoaccumulation of pheophytin anion [9,33,34]. The quantum yield for this process is low [9] and presumably requires the presence of an endogenous electron donor to facilitate trapping the pheophytin anion [34]. Indeed, a PS II core preparation deficient in the donor to P-680 is incapable of photoaccumulating pheophytin anion in the presence of dithionite [35,36].

We have demonstrated previously that fatty acid inhibition is expressed both at the $Q_a$ site and at Signal $2_f$, the quinoidal donor to P-680$^+$ [8]. Thus photoaccumulation of pheophytin anion would not be expected in PS II particles inhibited with linolenic acid. Fig. 5a illustrates the fluorescence decline observed in D-10 particles incubated with 20 mM dithionite (0.05 M Hepes, pH 7.8) and illuminated with strong actinic light (2760 W · m$^{-2}$). As demonstrated by Renger et al. [33] this fluorescence decrease is largely irreversible. In contrast to the control sample, linolenic-acid-treated particles (70 μM, 10 min) exhibited negligible fluorescence decrease during actinic illumination in the presence of reductant (Fig. 5b). Subsequent addition of a PS II donor, hydroxyl-

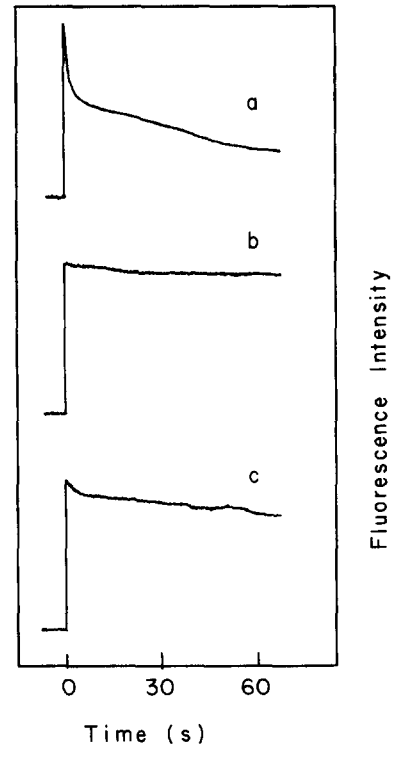


Fig. 5. Fluorescence yield kinetics in control and fatty acid inhibited D-10 particles. (a) Particles (10 μg per ml Chl) incubated for 5 min in the dark with 20 mM dithionite (0.05 M Hepes, pH 7.8); (b) same as (a), except in the presence of 70 μM linolenic acid; (c) same as (b), with the addition of 20 mM hydroxylamine. Actinic illumination was supplied at time = 0.

amine, in combination with dithionite did not accelerate significantly the diminution of $F_{max}$ during continuous illumination (Fig. 5c). Similar observations to those presented in Fig. 5 were obtained also with spinach TSF-2 particles.

The experiments presented thus far are consistent within a conceptual framework that associates the abolition of secondary electron transport in PS II with the fatty-acid mediated displacement of critical quinone intermediates from their binding sites. The generation of the maximal fluorescent state of the PS II reaction center after inhibition is then a consequence of the enhanced charge recombination between P-680$^+$ and Pheo$^-$. This latter hypothesis, although attractive, cannot be confirmed solely based on the data presented above. An alternative interpretation of fatty acid inhibition views the increased fluorescence yield as diagnostic of the destruction of the phototrap in PS II. A test to distinguish between these two extreme interpretations is based on the redox titration of the pheophytin acceptor presented by Klimov et al. [37]. These workers demonstrated that actinic light-induced absorption changes at 685 nm and 450 nm associated with the photoreduction of pheophytin in samples poised at low potentials are correlated with the diminution of fluorescence yield. Redox titrations of the absorption changes at 685 nm and 450 nm indicated that pheophytin exhibits a one-electron reduction potential of $-610$ mV [37]. This seminal experiment has been extended by a number of laboratories, which have demonstrated that the spin-polarized triplet of the PS II reaction center displays an $E_0 = -570$ to $-620$ mV [27,28]. These observations indicate that if the fatty-acid-inhibited reaction center is nonfunctional in primary photochemistry, then the fluorescence yield ($F_{max}$ would be predicted to be independent of the ambient potential; however, if the primary charge separation is functional then $F_{max}$ should titrate to a level near $F_i$ as the ambient potential is lowered below circa $-600$ mV. Fig. 6 demonstrates the composite data from a exhaustive number of titrations of the fluorescence yield in TSF-2 and D-10 PS II preparations. Both in the presence and absence of the inhibitor, $F_{max}$ exhibits a substantial decrease at potentials lower than $-550$ mV. The observed midpoint potentials for the fluorescence

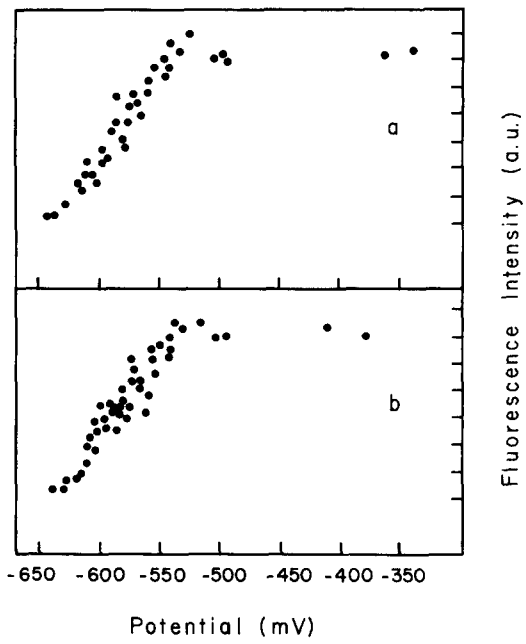


Fig. 6. Redox titration of the fluorescence yield in PS II particles in the absence (a) and the presence (b) of 100 $\mu$M linolenic acid. Each curve is a composite of a number of titrations. The fluorescence intensity at the lowest potential is approx. 20% greater than $F_i$. Experimental details are presented in the text.

yield in the absence ($-585 \pm 10$ mV) and in the presence ($-583 \pm 10$ mV) of linolenic acid are identical within experimental error. Thus if PS II variable fluorescence is a monitor of the recombination of the primary radical pair, then the results of Fig. 6 demonstrate conclusively that fatty acid inhibition of PS II does not proceed by destruction of the primary photoact.

## Discussion

Recent studies in our laboratory have defined a general class of inhibitors, designated as quinone binding site inhibitors, which function, we hypothesize, by displacement of endogenous quinone from quinone-binding peptides associated with both the reducing and oxidizing sites of Photosystem II. Structurally divergent compounds such as the fatty acid, linolenic acid [8], and salicylaldoxime [10] appear to function as reversible, quinone-binding site inhibitors. In contrast, the

covalent modifier, phenylglyoxal [32], irreversibly disrupts electron transport associated with PS II quinone-binding sites. The observations presented in this paper extend further our analysis of fatty acid inhibition in PS II and are consistent with the hypothesis that these lipoidal inhibitors target secondary electron-transport components, but do not interfere with primary charge separation in the reaction center.

Inhibition at the primary quinone acceptor site, $Q_a$, is documented both by the elimination of absorbance changes at 320 nm (Fig. 1) and the abolition of the ESR resonance from $Q^-Fe$ (Fig. 2). Recent studies have demonstrated that the $Q^-Fe$ resonance in PS II is responsive to changes in its environment induced by pH [22], bicarbonate depletion [38] and herbicide binding [39]. Although the absence of the $Q^-Fe$ signal in fatty acid inhibited samples might be attributed to a lipid-induced modification of the magnetic coupling between the quinone and $Fe^{2+}$, such a conjecture cannot account for the abolition of absorbance changes at 320 nm. The lack of functionality of the $Q_a$ site is explained most easily by a fatty-acid-induced expulsion of bound quinone from the binding site.

That the function of the primary reaction pair in the Photosystem II reaction center is immune to the effects of fatty acid inhibition is shown by the data of Figs. 4–6. Significantly, the fluorescence titration experiments reveal that at low potentials (less than $-400$ mV) the fluorescence yields in inhibited and control samples are comparable, and that the $E_m$ for the pheophytin anion/pheophytin redox couple is not altered by the fatty acid. These findings suggest that the structure of the reaction center core, especially the microenvironment associated with P-680 and pheophytin, is relatively resistant to modification. This conclusion is substantiated further by the observation (Fig. 4) that the zero-field splitting parameters of the reaction center triplet in the linolenic acid treated sample are similar to that observed for the reduced control sample.

The observation of the spin-polarized reaction center triplet in reduced samples inhibited by fatty acid, in conjunction with the redox titration of fluorescence yield, demonstrates incisively that charge separation in the PS II reaction center is not incapacitated in the presence of linolenic acid. Indeed, the development of the polarization pattern aeeaae can occur only via a radical pair mechanism [40]. However, the absence of the triplet signal in linolenic acid inhibited samples frozen at moderate potential ($\approx 0$ mV) is unexpected, since the samples exhibit a high fluorescence yield attributed to recombination luminescence [9]. We interpret these data as indicating the presence at cryogenic temperatures of another acceptor intermediate between pheophytin and $Q_a$. Since we do not observe significant production of the polarized triplet (less than 10%) at potentials above $-400$ mV in either control or fatty-acid-inhibited samples, we propose that this acceptor must operate with an $E_m \approx -450$ mV. Similar observations on the redox dependence of the reaction center triplet in particles from *Chlamydomonas reinhardtii* Str F54-14 have been detailed recently by Evans et al. [28]. Additional evidence for an intermediate carrier prior to $Q_a$ has been presented by Eckert and Renger [41], Joliot and Joliot [42] and the recent observation by Hoff and Proskuryakov of a spin-polarized ESR signal ($g = 2.0034–2.0038$) in PS II particles, which were depleted in iron [43]. Our data do not preclude the existence of two electron carriers intermediate between pheophytin and $Q_a$ [28]; however, the lack of a triplet signal in the presence of the fatty acid (at potentials of $\approx 0$ mV) signifies resistance of the intermediate component(s) to displacement by the inhibitor.

In this paper we have presented a variety of experiments to support our assertion that fatty acids can function as quinone-binding-site inhibitors in PS II [8]. We hypothesize that the unique, reversible inhibitory activity of the unsaturated fatty acids has its origin in a displacement of endogenous quinones via competitive binding to quinone-binding sites in PS II. In this regard, fatty acids have been proposed to inhibit quinone-binding sites in the mitochondrial respiratory chain [44]. A consequence of the competitive inhibition hypothesis for fatty acids is the possible reinsertion of exogenous quinone into the Photosystem II quinone-binding sites. We have demonstrated recently that such a reconstitution of secondary electron transport in D-10 particles is feasible [6], and studies to optimize the activity and recon-

stitution yield of these chemically-modified reaction centers are in progress.

## Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (GM 26133). Acknowledgment is given to Nathan Lacoff for development of the Unix™-based ESR data collection and analysis programs.

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