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Formation and decay of monodehydroascorbate radicals in illuminated thylakoids as determined by EPR spectroscopy

Stephen Grace [a,b,*,1], Ron Pace [c], Tom Wydrzynski [b]

[a] *Department of Botany, Duke University, Durham NC 27708, USA*
[b] *Research School of Biological Sciences, Institute of Advanced Studies, Australian National University, Canberra, ACT 2601, Australia*
[c] *Department of Chemistry, Faculties, Australian National University, Canberra, ACT 2601, Australia*

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Abstract

H_2O_2 is reduced by an ascorbate-specific peroxidase (APX) in chloroplasts, generating the monodehydroascorbate (MDA) radical as the primary oxidation product. Using EPR spectroscopy we have measured the light-driven formation and decay of this species in thylakoids containing active APX. Illumination caused a rapid exponential rise in the steady-state MDA radical concentration in the absence of added electron acceptors other than O_2. This increase was sensitive to KCN and catalase and was prevented by anaerobic conditions, demonstrating the requirement for APX activity and endogenously generated H_2O_2, i.e., the Mehler reaction. When the illumination was removed, a second, transient increase in the radical signal was observed, indicating that photoreduction of the MDA radical and O_2 were occurring simultaneously in the light. This interpretation is also supported by the sigmoidal behavior of the chlorophyll dependence of MDA radical formation in illuminated thylakoids. Ferredoxin lowered the light-induced, steady-state MDA radical concentration, and is thus implicated as the physiological photoreductant for this Hill acceptor. In the absence of uncoupler, $NADP^+$ prevented formation of the MDA radical by lowering the flux to molecular O_2. However, in the presence of uncoupler (5 mM NH_4Cl) this constraint was apparently overcome, i.e., net formation of the radical occurred. The EPR method represents a novel approach to investigating the interaction of O_2 and ascorbate metabolism in chloroplasts under a variety of physiologically relevant conditions, to be applied in future studies of plant response to environmental stress.

Keywords: Ascorbate; Monodehydroascorbate; Ascorbate peroxidase; Oxygen photoreduction; EPR

1. Introduction

Based on the classical Z-scheme of photosynthetic electron transport, light is converted to chemical energy in the form of NADPH and ATP through the concerted action of two photosystems linked by a series of intermediate electron carriers. Photogenerated NADPH, in turn, constitutes the primary form of reducing power used to drive the CO_2-fixing reactions of photosynthesis. However, under conditions where the capacity of carbon fixation is limited, for example during periods of stomatal closure, the pool of available $NADP^+$ declines and molecular O_2 becomes the preferred acceptor on the reducing side of Photosystem I (PS I) [1,2]. O_2 photoreduction (i.e., the Mehler reaction) leads to the formation of H_2O_2 [3], which can exacerbate oxidative stress by inactivating several key photosynthetic enzymes, thus further impairing energy dissipation through the biochemical reactions of the Calvin cycle [4].

Ascorbic acid is the primary hydrophilic antioxidant in plant cells. It also serves as the reductant for ascorbate peroxidase (APX), a heme-containing enzyme which is responsible for scavenging H_2O_2 in the chloroplasts and cytosol of plant cells [5]. Recently, it has been observed that two forms of APX exist in the chloroplast, one located in the soluble stromal phase and the other embedded in the thylakoid membranes [6]. Both forms have been purified to homogeneity [7,8], and found to be similar in enzymatic

Abbreviations: APX, ascorbate peroxidase; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, 2,4-dinitrophenyl ether of iodonitrothymol; EPR, electron paramagnetic resonance; Fd, ferredoxin; MDA, monodehydroascorbate; PFD, photon flux density; PS, Photosystem; Q_A, primary quinone acceptor of Photosystem II; SOD, superoxide dismutase.

* Corresponding author. Fax: +1 (303) 4928699. See current address.
[1] Current address: Department of Environmental, Population and Organismic Biology, Campus Box 334, University of Colorado at Boulder, Boulder, CO 80309-0334, USA. Fax: +1 (303) 4928699.

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properties. However, there are some indications that these distinct enzymes serve separate functions with regard to H_2O_2 metabolism. In particular, the thylakoid-bound form of APX has been shown to couple H_2O_2 reduction to photosynthetic electron transport by generating the monodehydroascorbate (MDA) radical, which can itself serve as a Hill oxidant in thylakoids ([6], [9], Grace and Osmond, unpublished data).

In this communication we report the use of the EPR spectroscopic technique for detecting MDA radicals [6,10] to study the coupling of O_2 photoreduction and ascorbate redox cycling in illuminated thylakoid membranes containing active APX. We demonstrate the light-induced formation and decay of the MDA radical and describe the reactions which give rise to the unusual kinetic behavior observed. We also show the effects of various inhibitors, light intensity and the physiological electron acceptors ferredoxin and $NADP^+$ on the formation and decay of the MDA radical signal.

2. Materials and methods

2.1. Isolation of thylakoids

Peas (*Pisum sativum* L. cv. Greenfeast) were grown in trays in controlled environment chambers at 530 μmol quanta m^{-2} s^{-1} and a day/night temperature regime of 22/18° C. Chloroplasts were isolated from whole shoots of 2–3 week plants by the following procedure: 20 g of leaves were homogenized in 200 ml of semi-frozen grinding buffer consisting of 330 mM sorbitol, 50 mM Mes, 10 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM KH_2PO_4, 5 mM ascorbate, 0.1% BSA, pH adjusted to 6.1 with KOH. The homogenate was quickly filtered through two layers of Miracloth (Calbiochem, USA) separated by a thin layer of cotton wool and centrifuged at 1900 × *g* for 10 s in a swinging bucket rotor (Hettich model 30F/RF centrifuge). The supernatant was poured off and the crude chloroplast pellet was gently resuspended in a medium consisting of 0.33 M sorbitol, 50 mM Hepes, 2 mM $MgCl_2$, 10 mM NaCl, 5 mM ascorbate (pH 7.8) (hereafter referred to as RS1), supplemented with 0.1% BSA. Intact chloroplasts were purified by centrifugation through Percoll (44% v/v) and resuspended in RS1 buffer. Thylakoids were obtained by diluting chloroplasts from the first centrifugation step 20-fold in hypotonic buffer consisting of 50 mM Hepes and 5 mM $MgCl_2$ (pH 7.8) and allowed to sit on ice for 1 min. The preparation was then centrifuged at 1900 × *g* for 2 min and the pellet was resuspended in RS1. The thylakoids were washed twice to remove as much stromal SOD and unbound ferredoxin as possible. The entire isolation procedure was carried out at 2° C. Chlorophyll was determined spectrophotometrically using the equations of Porra et al. [11]. Following isolation thylakoids were immediately frozen in liquid N_2, stored at −80° C and quickly thawed prior to use.

2.2. Ascorbate peroxidase measurements

Ascorbate peroxidase activity was determined spectrophotometrically based on the method of Nakano and Asada [12] as the loss in 290 nm absorbance due to oxidation of ascorbate by H_2O_2. Chloroplasts were diluted directly into a buffer containing 50 mM Hepes-KOH, 0.1 mM EDTA and 0.5 mM ascorbate (pH 7.8) in a final volume of 3 ml. Chl concentration varied between 3 and 6 μg ml^{-1}, and the solution was continuously stirred. The reaction was started by addition of 0.27 mM H_2O_2. The loss in absorbance in the first 20 s after H_2O_2 addition was used to calculate enzyme activity, based on an extinction coefficient of 2800 M^{-1} cm^{-1}.

2.3. EPR measurements

EPR measurements were made on a Bruker ESP 300E spectrometer (X band, 9.5 GHz). The cavity was continuously flushed with N_2. All spectra were recorded at room temperature and, unless otherwise noted in the text, the operating conditions were: microwave power 6.32 mW, modulation frequency 100 kHz, modulation amplitude 0.803 gauss, gain $4 \cdot 10^5$, time constant 328 ms. Measurements were made in RS1 buffer (pH 7.8) using a flat cell at a sample concentration of 30 μg Chl ml^{-1}, unless otherwise noted.

Spinach ferredoxin, *E. coli* manganese-containing superoxide dismutase, β-$NADP^+$ and methyl viologen were purchased from Sigma (St. Louis, MO, USA). Glucose oxidase was obtained from Boehringer Mannheim (Germany) and bovine liver catalase from Fluka (Switzerland). DNP-INT was kindly provided by Prof. A. Trebst (Bochum, Germany).

3. Results

3.1. Ascorbate peroxidase activity of chloroplasts

Chloroplast APX activity was measured spectrophotometrically as the loss of ascorbate induced by H_2O_2 (Fig. 1). In our preparations the thylakoid membranes retained approximately 70% of total chloroplast APX activity when isolated in the presence of ascorbate, the remainder apparently associated with the stroma (compare B with A). However, thylakoids isolated in the absence of ascorbate lose virtually all activity (C), confirming the observations of Miyake and Asada [6] that ascorbate is required to preserve APX activity in broken chloroplasts. KCN (0.5 mM) completely inhibited thylakoid APX activity (D), as previously reported [6]. Since we were interested in the properties of the native system, thylakoids isolated in the

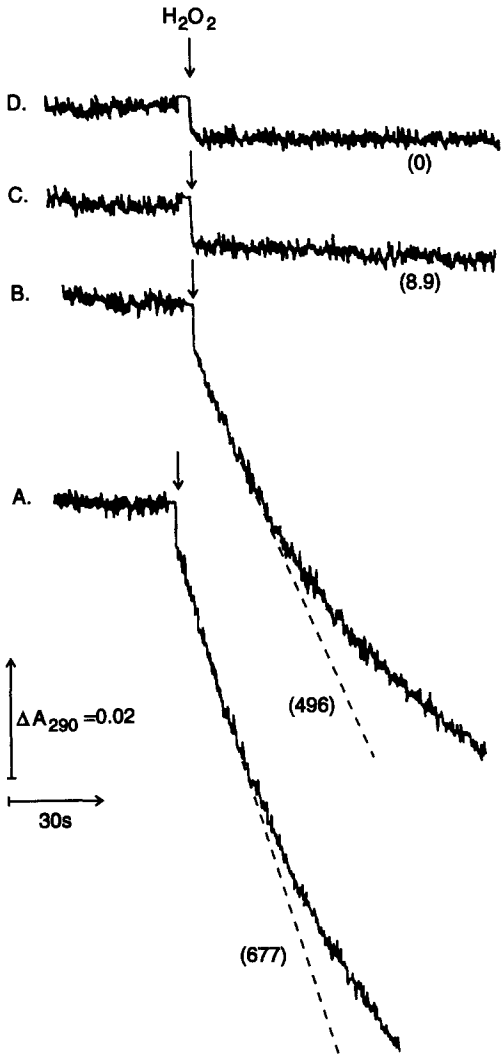
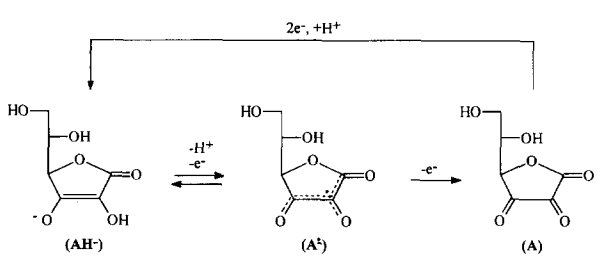


Fig. 1. Comparison of ascorbate peroxidase activity in intact and broken chloroplasts from the same preparation. APX activity was determined spectrophotometrically as described in Materials and Methods. (A) Percoll-purified intact chloroplasts; (B) thylakoids obtained by osmotic shock and centrifugation and maintained in buffer containing 5 mM ascorbate; (C) thylakoids washed twice and resuspended in ascorbate-depleted buffer; (D) thylakoids isolated in the presence of ascorbate + 0.5 mM KCN. Numbers in parentheses refer to rates in μmol ascorbate oxidized (mg Chl)$^{-1}$ h^{-1}.

presence of ascorbate were used in all the experiments reported here.

Ascorbate (AH^-) can undergo a sequential 2-electron oxidation to dehydroascorbate (A), forming the paramagnetic MDA radical ($A^{\cdot -}$) as an intermediate [13].



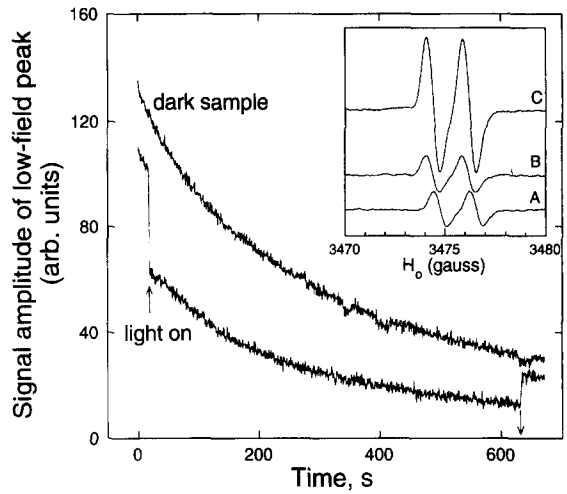


Fig. 2. H_2O_2-induced formation of the MDA radicals by the thylakoid-bound APX and subsequent light-stimulated MDA radical decay. EPR spectra (*inset*) were measured at room temperature in RS1 buffer containing 1 mM ascorbate and the following additions: (A) none, (B) $5 \cdot 10^{-4}$ M H_2O_2, (C) $5 \cdot 10^{-4}$ M H_2O_2 + thylakoids, 20 μg ml^{-1} Chl. The decay kinetics of MDA radical were monitored by the change in amplitude of the low-field EPR peak in thylakoid samples with added H_2O_2 (i.e., as in spectrum C). The upper trace is a sample kept in darkness while the lower trace is a sample illuminated with white light (1500 μmol quanta m^{-1} s^{-1}) where indicated (downward arrow indicates light off). Traces are vertically displaced for clarity. Note there was an approximate 3 min delay between addition of H_2O_2 to the sample and beginning the measurement. Further details of experimental conditions for the EPR measurements are given in Materials and Methods.

This radical occurs normally in aqueous solutions of ascorbate at ambient O_2 and neutral to alkaline pH due to the univalent autoxidation of the ascorbate anion [14]. Fig. 2 (inset, spectrum A) shows the characteristic MDA radical EPR signal observed in a 1 mM ascorbate solution at pH 7.8. Quantitation of the signal against a copper standard gave a concentration of 0.186 μM MDA radical for this solution. H_2O_2 (0.5 mM) did not affect the steady-state level of MDA radical (spectrum B), indicating that at this concentration and pH, H_2O_2 does not spontaneously oxidize ascorbate by the univalent pathway to any significant degree. The divalent reaction is also minimal, based on the measurements of ascorbate oxidation in APX-inhibited thylakoids (Fig. 1D).

When thylakoids isolated in an ascorbate-containing medium were added to a solution containing 1 mM ascorbate and 0.5 mM H_2O_2, a dramatic increase in MDA radical concentration was observed (Fig. 2, spectrum C). This is further evidence for the association of APX activity with the thylakoids and confirms that the MDA radical is a primary product of the reaction, consistent with earlier reports which supported a univalent oxidation mechanism for the stromal form of the enzyme [15].

The intensity of the low-field EPR peak was studied in dark-adapted thylakoids in which the MDA radical was generated by prior addition of H_2O_2. The signal decayed in the dark, indicating that the MDA radical is not stable

under these experimental conditions (Fig. 2, upper trace). This decay is due to the spontaneous dismutation reaction, which at pH 7.8 exhibits a second-order rate constant of approximately $8 \cdot 10^4$ M^{-1} s^{-1} [13]. Illumination of the sample caused an immediate decline in the MDA radical concentration (lower trace), consistent with previous reports that the MDA radical can be directly photoreduced by the thylakoids, as determined either by EPR [6] or O_2 exchange methods [9]. The sudden increase in the signal amplitude when the sample is returned to darkness suggests that a light-dependent formation of the MDA radical is occurring concomitantly with its reduction (see below).

3.2. Production and decay of the MDA radical in illuminated thylakoids

Since the MDA radical can apparently serve as a Hill oxidant for photosynthetic electron transport, we were interested to know what effect endogenous H_2O_2 production, i.e., the Mehler reaction, would have on MDA radical levels in illuminated thylakoids. Fig. 3 shows that thylakoids illuminated in the absence of external acceptors other than O_2 generate the MDA radical. As shown in the upper panel (spectrum B) addition of thylakoids to a 1 mM ascorbate solution in the dark does not significantly affect the background concentration of MDA radicals. Upon illumination, however, there is an approximate 4-fold increase in the signal amplitude, indicating net production of the MDA radical (spectrum C). When the light is removed, the signal decays to the background level due to the disproportionation reaction (spectrum D).

The kinetics of light-dependent formation of the MDA radical were followed directly by measuring the change in amplitude of the low-field EPR signal during a dark-light transient (Fig. 3, lower panel). The increase in the MDA radical concentration upon illumination is exponential, characterized by apparent first-order kinetics ($k_{obs} \approx 0.24$ s^{-1}). The signal reaches a quasi steady-state level within the first 20 s, followed by a period of slow decay. This slow decay was not evident in all samples. When the light was turned off there was an immediate transient increase in the signal, a feature not observed in measurements of spectra. Following this transient increase, the signal decays to the pre-illumination baseline level by disproportionation. From the reciprocal concentration vs. time plot, we calculated a second-order rate constant of $1.2 \cdot 10^5$ M^{-1} s^{-1} for this decay process (Fig. 3, inset). This is in good agreement with the rate constant for disproportionation of the MDA radical based on pulse radiolysis experiments [13].

The existence of the post-illumination burst indicates that both the MDA radical and molecular O_2 are serving as acceptors for photosynthetic electron transport. H_2O_2 formed by the Mehler reaction reacts with ascorbate to form the MDA radical in a reaction catalyzed by thylakoid-bound APX. The MDA radical is subsequently reduced back to ascorbate in a light-dependent process. When the light is removed, MDA photoreduction ceases and there is a transient increase in the MDA radical signal due to unreacted H_2O_2 in the medium. The amplitude of the post-illumination signal is interpreted to reflect the steady-state pool of H_2O_2 generated by autoxidation of reduced electron carriers in the thylakoids.

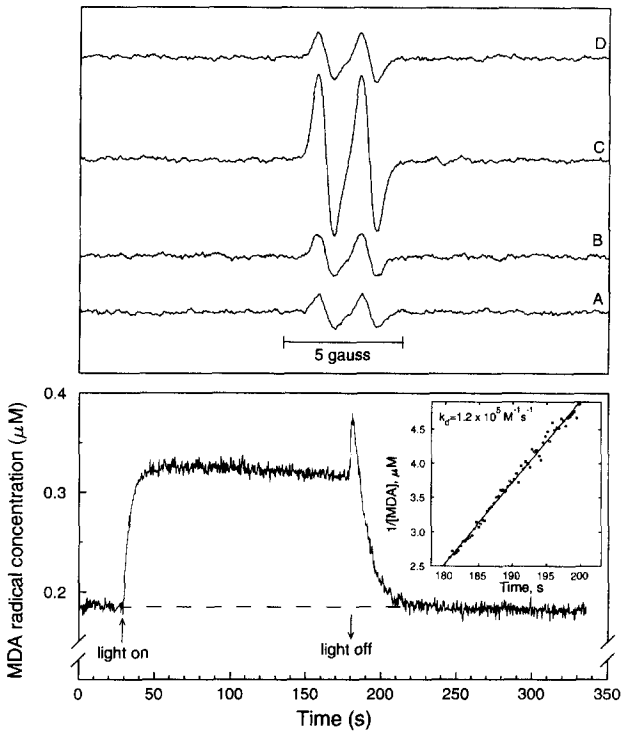


Fig. 3. Light-induced formation and decay of MDA radicals by isolated thylakoids and kinetics of the process. Upper panel: (A) Background level of MDA radical in a 1 mM ascorbate solution, (B) thylakoids (30 μg ml^{-1} chlorophyll) were added to a 1 mM ascorbate solution in darkness, (C) sample illuminated for 3 min (1500 μmol quanta m^{-1} s^{-1}), (D) 3 min darkness after illumination. Lower panel: Kinetics of MDA radical formation and decay in illuminated thylakoids by continuous monitoring of the low field EPR peak. The background signal was quantified against a copper standard using the scaled double integral. White actinic illumination (1500 μmol quanta m^{-1} s^{-1}) was administered and removed where indicated. The sample consisted of thylakoids in RS1 buffer + 1 mM ascorbate, chlorophyll concentration 30 μg ml^{-1}. Inset: A plot of the reciprocal concentration vs. time for the decay of the MDA radical in darkness. Instrument conditions are given in Materials and Methods.

The light-induced increase in the MDA radical signal was further investigated as a function of chlorophyll concentration (Fig. 4). Since this titration simultaneously varies several activities involved in ascorbate redox reactions, a simple relationship was not expected. Following an apparent initial lag, there is a linear increase in the light-induced steady-state MDA radical concentration until the signal saturates at approximately 30 μg Chl ml^{-1}. The lag observed at low chlorophyll concentrations was even more evident in the plot of the initial rate of MDA radical formation (not shown). This behavior is consistent with the simultaneous operation of second-order MDA-forming and

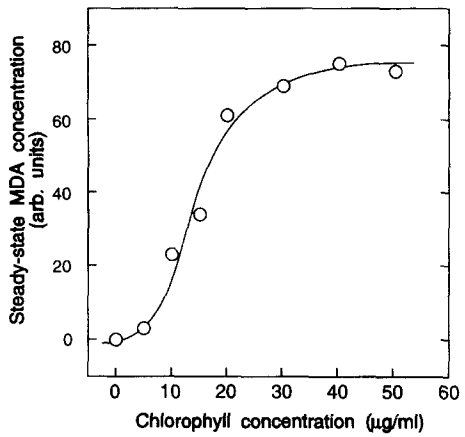


Fig. 4. Chlorophyll dependence of MDA radical formation in illuminated thylakoids. The plot shows the amplitude of the EPR signal reached at steady-state during illumination. Experiments were carried out in the presence of 1 mM ascorbate, other conditions as in Fig. 3.

MDA-consuming reactions in the illuminated samples (see Discussion). It should be noted that at chlorophyll concentrations above 40 $\mu g\,ml^{-1}$ the rate of MDA radical formation was extremely rapid, reaching steady-state within 2 s.

3.3. Inhibitors of the MDA radical formation in illuminated thylakoids

Experiments with a variety of inhibitors confirmed that formation of the MDA radical was dependent on O_2 photoreduction, followed by APX-catalyzed oxidation of ascorbate by H_2O_2 (Fig. 5). Formation of the radical is prevented under anaerobic conditions using the glucose/glucose oxidase system, demonstrating the need for molecular O_2 in the overall process. Catalase substantially diminished the light-induced increase in the signal and completely prevented the post-illumination burst, implicating H_2O_2 in this transient. The inability of catalase to fully abolish the signal was interpreted to reflect competition with ascorbate peroxidase, which has a much higher affinity for H_2O_2 ($K_m = 8 \cdot 10^{-5}$ M) [8] as compared to catalase ($K_m \approx 1$ M) [16]. APX is also embedded in the thylakoid membrane and therefore presumably closer to the site of H_2O_2 production. The APX inhibitor KCN, which does not affect electron transport at the low concentrations used here, caused virtually complete inhibition of the signal. A similar effect was also observed when the thylakoid-bound APX was inactivated by isolation in the absence of ascorbate (data not shown). The small residual signal generated in the presence of KCN may reflect the direct oxidation of ascorbate by photogenerated superoxide [17].

Unlike conventional Hill reactions, O_2 photoreduction shows a pH optimum around 5.5 [18]. However, in our experiments formation of the MDA radical was not observed at this pH (Fig. 5A). Two factors could account for this result. First, the rate of spontaneous disproportionation of the MDA radical increases dramatically under acidic conditions; the observed second-order rate constant is approximately 1000-fold greater at pH 5.5 than at 7.8 [13]. Second, ascorbate peroxidase activity, which is responsible for generating the MDA radical, is inhibited at low pH. The pH optimum of the thylakoid enzyme is around 7.0 [6].

We also examined the effects of electron transport inhibitors on the light-generated MDA signal (Fig. 5B). Interestingly, DNP-INT, an inhibitor of the cytochrome

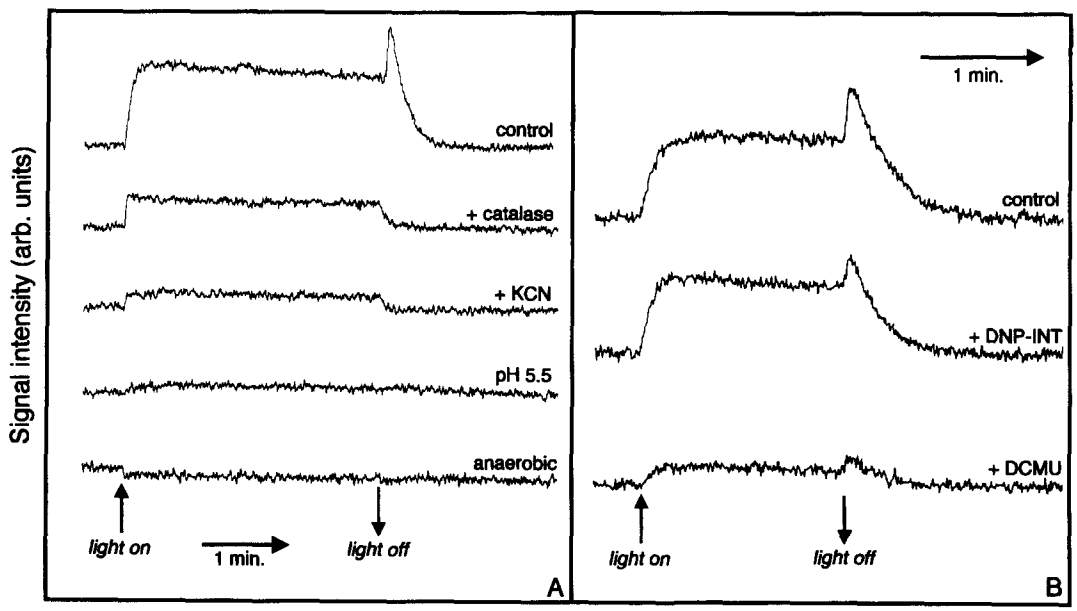


Fig. 5. Effects of inhibitors on MDA radical formation and decay in illuminated thylakoids. Measurements were carried out as in Fig. 3. Where indicated the following compounds were included in the assay: (A) 500 units catalase, 1 mM KCN pH 5.5 buffer (50 mM Hepes replaced with 50 mM Mes), 5 mM glucose + 50 units glucose oxidase. (B) 10 μM DNP-INT, 5 μM DCMU.

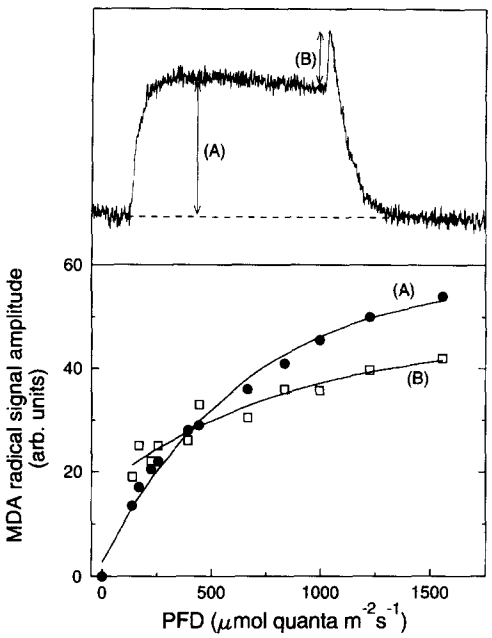


Fig. 6. Irradiance dependence of MDA radical formation in illuminated thylakoids. Conditions were as in Fig. 3 except that PFD at the cuvette surface was adjusted with neutral density filters. Curve (A) Maximum amplitude of the low-field EPR peak during illumination. Curve (B) Amplitude of the post-illumination burst.

b/f complex which prevents electron flow by binding to the Rieske Fe-S center [19], did not cause any significant changes in the light-induced steady-state MDA level and caused only a small decrease in the post-illumination H_2O_2 burst. This indicates that O_2 photoreduction can occur via plastoquinone when electron flow to PS I is blocked. We have found that the ability to generate superoxide is retained in DNP-INT-inhibited thylakoids using the cytochrome *c* detection assay (data not shown). The semiplastoquinone radical is likely to be the actual autoxidizing species in these experiments. Semiplastoquinone radicals have been directly observed by a flash-induced EPR signal in isolated thylakoids [20]. DCMU caused a much stronger inhibition of the MDA radical signal, which supports the interpretation that autoxidation of plastoquinone can account for significant H_2O_2 production in thylakoids. However, the failure of DCMU to completely abolish the light-induced formation of the MDA radical indicates that O_2 photoreduction via the primary quinone acceptor of PS II (Q_A) represents yet another pathway for H_2O_2 formation.

3.4. Effects of irradiance

The amplitude of the MDA radical EPR signal as a function of incident light intensity was also determined (Fig. 6). The light-induced maximum amplitude (curve A) exhibited a much greater dependence on irradiance than the post-illumination burst (curve B), reflecting greater overall H_2O_2 production (a shift in the equilibrium towards MDA production). Interestingly, neither parameter appears to completely saturate, despite saturation of the rate of electron transport to O_2 at much lower PFDs (data not shown). The lower dependence of the post-illumination signal suggests that reduction state of the electron transport chain at the end of the illumination period (3 min) reaches approximately the same level over the range of PFDs.

3.5. Effects of ferredoxin and $NADP^+$

The experiments presented so far were conducted on isolated thylakoids which were essentially free of bound ferredoxin, based on the inability of $NADP^+$ to stimulate O_2 evolution. A recent study by Miyake and Asada [17], however, demonstrated that ferredoxin can serve as a photoreductant for the MDA radical in isolated thylakoids or when reduced enzymatically by NADPH. This is in contrast to the earlier study of Miyake and Asada [6] and

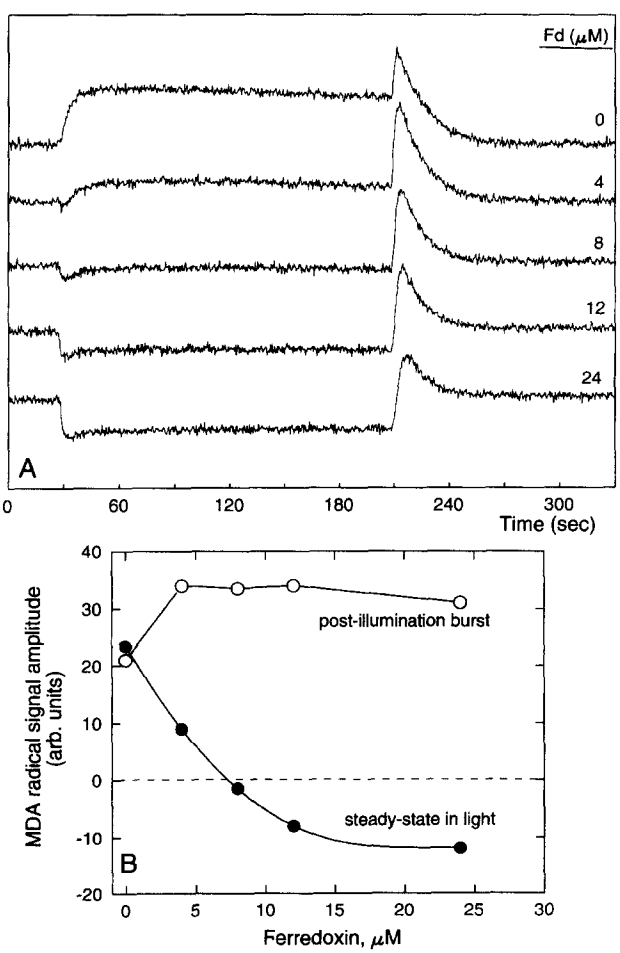


Fig. 7. Effects of ferredoxin on MDA radical formation in illuminated thylakoids. Conditions were as in Fig. 3 except that ferredoxin was included in the assay at the indicated concentrations. The top part of the figure shows the kinetic traces obtained with increasing ferredoxin while the bottom summarizes the data in terms of the steady-state amplitude of the signal in the light (●) and the magnitude of the post-illumination burst (○).

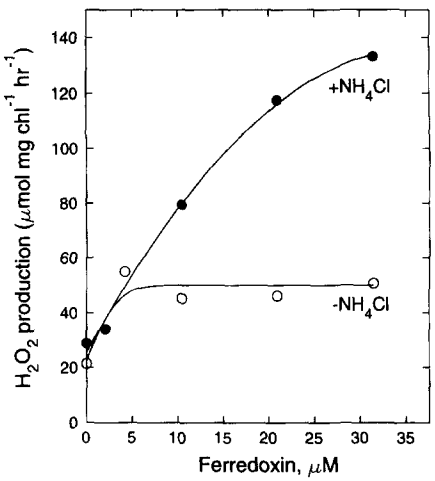


Fig. 8. Effects of uncoupler on Fd-mediated H_2O_2 production in illuminated thylakoids. H_2O_2 production was determined polarographically as the difference in the rate of O_2 uptake with and without catalase, multiplied by 2. Where shown, 5 mM NH_4Cl was included in the assay. Actinic PFD was of saturating intensity.

that of Forti and Ehrenheim [9], in which added ferredoxin had no effect on the photoreduction rate of the MDA radical. We sought to address this question by examining the effects of Fd on the kinetics of MDA radical formation and decay in thylakoids containing active APX. The results presented so far clearly demonstrate that Fd is not an absolute requirement for MDA radical photoreduction by isolated thylakoids. However, addition of increasing concentrations of Fd to illuminated thylakoids did cause a progressive decline in the light-induced steady-state level of MDA radical, ultimately below the pre-illumination baseline at concentrations above 8 μM Fd (Fig. 7). This can occur only if the rate of MDA radical photoreduction exceeds the rate of formation. Thus, ferredoxin is apparently more efficient at photoreducing the MDA radical than the thylakoid-bound redox components, consistent with the recent study of Miyake and Asada [17]. The possibility that the Fd-dependent decrease in MDA radical concentration is due to oxidation of the radical can be largely excluded based on a consideration of redox potentials. The low redox potential of Fd ($E_{m,7} = -0.42$ V) favors reduction of the MDA radical to ascorbate ($E_{m,7} = +0.33$ V) rather than oxidation to DHA ($E_{m,7} = -0.21$ V) [14]. Furthermore, it is likely that Fd is quickly converted to the reduced state under the high irradiance conditions used in Fig. 7.

Fd also increased the steady-state H_2O_2 concentration in the illuminated sample, as indicated by the increase in the post-illumination burst (Fig. 7). This is consistent with the role of Fd as an autoxidizable PS I acceptor.

Like other types of Hill reaction, Fd-mediated electron transport increases substantially in the presence of an uncoupler. Since O_2 serves as the terminal oxidant in this case, the stimulation by uncoupler is reflected as an increase in H_2O_2 production. This is demonstrated in Fig. 8, where 5 mM NH_4Cl was used to dissipate the electrochemical proton gradient. Note that in the absence of Fd, there is very little response to the uncoupler, as we have previously observed in measurements of superoxide forma-

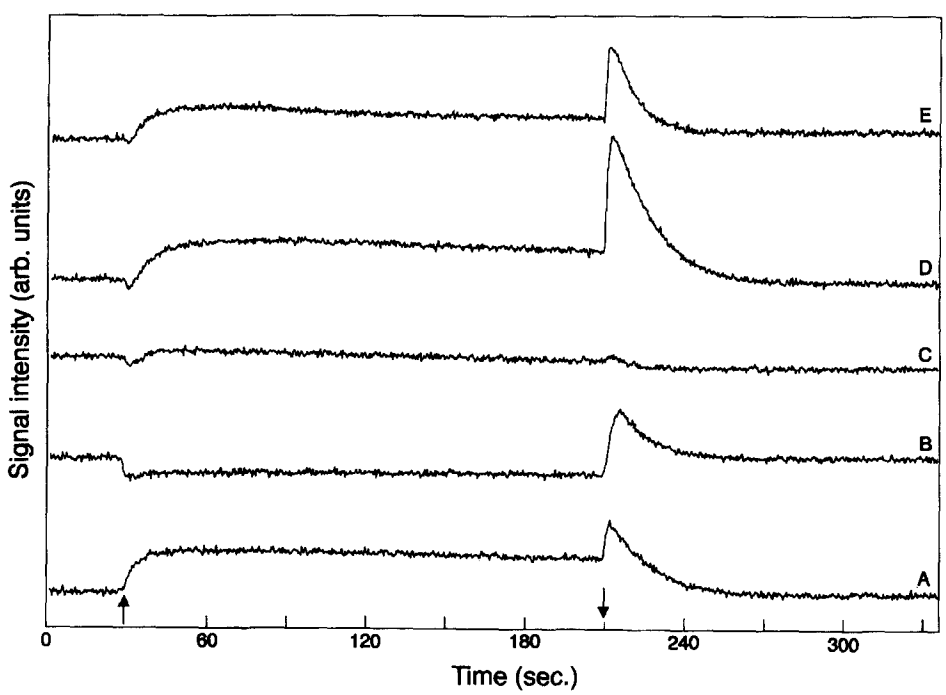


Fig. 9. Effects of uncoupler and $NADP^+$ on MDA radical formation in illuminated thylakoids. Conditions were as in Fig. 3 except the following compounds were included in the assay where indicated: (A) control (no additions), (B) 12 μM ferredoxin, (C) 12 μM ferredoxin + 0.2 mM $NADP^+$, (D) 12 μM ferredoxin + 5 mM NH_4Cl, (E) 12 μM ferredoxin, 0.2 mM $NADP^+$, 5 mM NH_4Cl.

tion (Grace and Osmond, unpublished data). Based on these results, we were interested to determine what effect uncoupler would have on the kinetics of Fd-mediated formation and decay of MDA radicals in illuminated thylakoids. In a control sample without Fd there was only a slight stimulation of MDA radical formation by uncoupler (data not shown). However, in the presence of Fd, NH_4Cl had dramatic effects, causing both a net increase in the light-induced steady-state MDA radical concentration (as opposed to a net decrease), and nearly a doubling of the post-illumination H_2O_2 burst (Fig. 9, compare trace B with D). This effect is consistent with an increased rate of O_2 photoreduction, and consequent stimulation of H_2O_2 production.

We also investigated the effects of $NADP^+$ on the formation of the MDA radical in the presence and absence of NH_4Cl, since $NADP^+$ serves as the physiological terminal acceptor in photosynthetic electron transport. As shown in Fig. 9 (trace C), when $NADP^+$ was added to a thylakoid sample containing Fd, there was no formation of the MDA radical upon illumination, indicating that $NADP^+$ prevents O_2 photoreduction by competing for electrons from reduced Fd. Surprisingly, however, when NH_4Cl was included in the assay, there was a net formation of the MDA radical, similar to the enhancement observed in the presence of Fd alone (trace E). This indicates that when the rate of electron transport is high due to the presence of uncoupler, O_2 can effectively compete with $NADP^+$ for thylakoid-driven reducing power, perhaps due to a greater reduction state of Fd. The stimulatory effect of uncoupler on MDA radical production was not reversed even at $NADP^+$ concentrations up to 0.8 mM, which is generally considered saturating for this Hill reagent (data not shown).

4. Discussion

Over the last decade it has been established that the scavenging of H_2O_2 in chloroplasts is coupled to photosynthetic electron transport through the action of an as ascorbate-specific peroxidase [21–24]. It is now clear that the underlying mechanism of this coupling involves the production of the MDA radical, which serves as a Hill oxidant in the thylakoids [6]. In this communication we demonstrate by EPR spectroscopy the simultaneous generation and photoreduction of MDA radicals by illuminated thylakoids. Unlike previous studies of the Hill activity of MDA, which relied on enzymatic generation of MDA radicals prior to illumination [6,9,17], here we show the kinetics of MDA radical formation and decay under conditions where H_2O_2 is formed endogenously by the Mehler reaction. This represents a more physiological approach, since O_2 constitutes a potential alternative acceptor under many conditions in vivo [1,2,25,26]. In addition, an advantage of the EPR method described here is that it allows for direct determination of low concentrations of the MDA radical without the use of external mediators (dyes, spin traps, etc.) that often complicate measurements due to problems of solubility, limited accessibility, kinetic constraints and side reactions which may interfere with other chloroplast reactions.

For purposes of the present analysis, the behavior of the light-induced changes in MDA radical concentration can be interpreted as a combination of four concurrent reactions:

$$2D_1 + O_2 + 2H^+ \xrightarrow{k_1} 2D_1^+ + H_2O_2 \text{ (O_2 photoreduction)} \quad (1)$$

$$H_2O_2 + 2AH^- \xrightarrow{k_2} 2H_2O + 2A^{\cdot -} \text{ (MDA radical formation)} \quad (2)$$

$$D_2 + A^{\cdot -} + H^+ \xrightarrow{k_3} D_2^+ + AH^- \text{ (MDA radical photoreduction)} \quad (3)$$

$$2A^{\cdot -} + H^+ \underset{k_{-4}}{\overset{k_4}{\rightleftarrows}} AH^- + A \text{ (spontaneous dismutation)} \quad (4)$$

where D_1 and D_2 represent unspecified thylakoid-bound electron donors for O_2 and MDA, respectively. The normal site of O_2 photoreduction in chloroplasts is on the acceptor side of PS I. A second-order rate constant of approximately 10^6 M^{-1} s^{-1} has been calculated for the reaction between O_2 and a bound redox component of PS I [27]. However, under conditions where electron flow to PS I is blocked by inhibitors (i.e., DNP-INT), reduction of O_2 can occur by autoxidation reactions at upstream sites in the transport chain, such as the acceptor side of PS II [28], the plastoquinone pool and possibly cytochrome *b*-563 [29]. It is emphasized that both O_2 photoreduction and APX activity are required for the generation of the MDA radical during illumination of thylakoids. This interpretation was confirmed by the inhibitor experiments shown in Fig. 5.

Reaction (2) is catalyzed by ascorbate peroxidase and is the predominant MDA forming reaction in the light. Another possible route for MDA radical formation is the non-enzymatic oxidation of ascorbate by the superoxide anion radical ($O_2^{\cdot -}$), since this species is generated as a primary product of O_2 photoreduction in illuminated thylakoids [30,31]. Evidence for this is given in Fig. 7, where a small increase in the MDA radical signal was observed in KCN-inhibited thylakoids, which do not have APX activity. Miyake and Asada [17] have also recently obtained evidence for $O_2^{\cdot -}$-dependent MDA radical formation in APX-inactivated thylakoids. However, $O_2^{\cdot -}$ can also react with the MDA radical, oxidizing it to dehydroascorbate [32]. It is thus difficult to predict the effect of $O_2^{\cdot -}$ on the steady-state MDA radical concentration. Superoxide dismutase did have a slight stimulatory effect on the amplitude of the MDA signal under the experimental conditions used here, perhaps indicating the involvement of $O_2^{\cdot -}$ in

the overall decay of the MDA radical (data not shown). However, since SOD is present at concentrations up to 50 μM in the stroma of intact chloroplasts [33] and shows one of the highest rate constants reported for any enzyme [34], it is likely that O_2^- will have only minor effects on the redox state of ascorbate in chloroplasts in vivo.

Once formed, the MDA radical is photoreduced by an electron donor in the thylakoid membranes (reaction 3). Since a steady-state concentration of the MDA radical is achieved, the formation and decay reactions must occur at constant rates in the light. This steady-state is influenced strongly by the incident light intensity (Fig. 6) and the chlorophyll concentration (Fig. 4). It is assumed that the reverse dismutation reaction, which could potentially contribute to MDA radical formation, occurs at a negligible rate under the present conditions, since the equilibrium constant of $4.85 \cdot 10^{-9}$ [13] heavily favors the forward reaction and the concentration of DHA is already presumed to be low at steady-state.

An interesting feature of the kinetics of MDA radical formation is the transient burst in the signal which occurs when illumination is removed. This represents either unreacted H_2O_2 in the sample or drainage of electrons from reduced intersystem carriers to O_2, thus forming H_2O_2 which then reacts in the ascorbate peroxidase reaction to generate MDA. The elimination of this feature the signal by catalase (Fig. 5) is further evidence for the involvement of H_2O_2. The existence of the post-illumination burst also indicates that photoreduction of the MDA radical and O_2 are occurring simultaneously in the illuminated sample. Thus, when the light is removed, MDA photoreduction ceases and a transient increase in the MDA radical concentration is observed. The subsequent dark decay of the signal to the pre-illumination steady-state is accounted for by the spontaneous dismutation reaction (reaction 4).

Although no detailed quantitative kinetic analysis of the MDA formation and consumption processes will be presented in this publication, we note that reactions (1) through (4) provide a simple rationalization for the behavior seen in Fig. 6. Under the conditions of these experiments, the concentration of AH^- is essentially fixed (1 mM) and non-limiting. Thus, the kinetics in reaction (2) are determined by the ascorbate peroxidase and H_2O_2 concentrations:

$$d[A^{\cdot -}]/dt = k_2'[APX][H_2O_2] \quad (5a)$$

$$\alpha[Chl][H_2O_2] \quad (5b)$$

since (APX) is proportional to (Chl). Initially, under illumination H_2O_2 will be generated at a constant rate from reaction (1) which will be proportional to the thylakoid concentration so that:

$$[H_2O_2] \propto [Chl] \times t \quad (6)$$

Combining (5b) with (6) gives:

$$d[A^{\cdot -}]/dt \propto [Chl]^2 \times t \quad (7)$$

This suggests that MDA formation is initially a second-order process in both time and chlorophyll concentration

$$[A^{\cdot -}] \propto [Chl]^2 \times t^2 \quad (8)$$

Reaction (1) provides a simple heuristic explanation for the dependence of these rates on chlorophyll concentration (in the region of low concentrations) where the MDA consumption reactions are not expected to influence the early maximum rate significantly. At higher chlorophyll concentration levels, the consumption reactions (3) and (4) evidently exert a significant effect on the maximum rate, resulting in the plateauing of the steady-state MDA level, as shown in Fig. 6. A detailed investigation of these effects will be presented in a future publication.

Though ferredoxin serves as a catalyst for O_2 photoreduction and thus increases H_2O_2 production when added to thylakoids, the steady-state level of MDA radical in the light actually declined with increasing Fd concentrations. Thus, in the presence of this soluble acceptor, the rates of both reactions (1) and reaction (3) are shown to substantially increase (Figs. 7 and 8). This strongly suggests that Fd is the physiological photoreductant for the MDA radical, in agreement with the recent findings of Miyake and Asada [17]. These workers reported a second-order rate constant of $2.7 \cdot 10^6$ M^{-1} s^{-1} for the reaction between photoreduced Fd and the MDA radical, which was higher than when Fd was reduced enzymatically by the NADPH-FNR system, suggesting a role of the thylakoids in electrostatic screening. In our study $NADP^+$ prevented the formation of the MDA radical by competing with O_2 for reducing equivalents from Fd. However, addition of uncoupler accelerated the rate of electron transport such that O_2 photoreduction could occur concomitantly with $NADP^+$ reduction, resulting in net production of the MDA radical (Fig. 9). Evans [35] reported that the rate of O_2 evolution in leaves closely matched the uncoupled rate of whole-chain electron transport in thylakoids, consistent with the suggestion of Foyer et al. [36] that lumen pH may exert only minimal restriction on the rate of electron transport in vivo. Our data therefore indicate that significant O_2 photoreduction may occur under physiological conditions in leaves.

The question arises as to which side of the thylakoid membranes the MDA-forming and MDA-consuming reactions are occurring. Based on the properties of the solubilized enzyme, Miyake et al. [8] suggested that the APX-catalyzed reaction between H_2O_2 and ascorbate occurs on the stromal surface of the membrane in PS I-enriched thylakoid regions. Similarly, the acceptor side of PS I is implicated as a major site for MDA photoreduction in thylakoids based on the following observations: (1) MDA is photoreduced by far-red light in the presence of DCMU provided electrons are supplied to PS I [17], and (2) MDA radical photoreduction by thylakoids shows Emerson enhancement [9].

Another candidate, however, for a thylakoid-bound photoreductant of the MDA radical is cytochrome *b*-563, as first suggested by Miyake and Asada [6]. This idea is based on analogy with other membrane-bound MDA reductases. The membranes of secretory vesicles in the adrenal medulla and outer mitochrondrial membranes both contain a *b*-type cytochrome which catalyzes the reduction of the MDA radical [14]. These systems appear to act as a shuttle for reducing equivalents between different cellular compartments. The glyoxysomal membranes of higher plants also appear to contain an MDA reductase, though it is unknown whether the activity is due to a cytochrome [37]. The possibility that thylakoid cytochrome *b*-563 might serve as a reductant for the MDA radical is supported by the results of the DNP-INT experiment (Fig. 5B). Electron flow to PS I is blocked by this inhibitor apparently without affecting MDA radical reduction. The efficacy of DNP-INT as an inhibitor was confirmed in measurements of whole-chain electron transport using methyl viologen as a terminal acceptor (data not shown). The concentration range for DNP-INT inhibition agreed well with the original study of Trebst et al. [38]. Reduction of the MDA radical by cytochrome *b*-563 could occur on the lumen side of the membrane, thus providing a mechanism to regenerate ascorbate required for lumenal reactions such as the de-epoxidation of violaxanthin. Interestingly, this reaction also generates the MDA radical, as does the reaction between ascorbate and the α-tocopheryl radical at the membrane surface [39]. While these reactions are considered to be of minor importance in the present study, a more detailed appraisal of their contribution to MDA radical formation in chloroplasts will be a subject of future reasearch.

One of the interesting implications of this work relates to the question of how plants respond to environmental conditions which cause over-reduction of the $NADP^+$ pool. Such conditions will favor the reduction of O_2 and consequently H_2O_2 formation, which can inhibit photosynthesis by inactivating several key photosynthetic enzymes, such as fructose-1,6-bisphosphatase and ribulose-5-phosphate kinase, and possibly glyceraldehyde-3-phosphate dehydrogenase [4,40]. H_2O_2 would thus exacerbate the oxidative stress by preventing energy dissipation through the Calvin cycle. To avoid such stress, not only do chloroplasts have an effective H_2O_2 scavenging enzyme, ascorbate peroxidase, but the product of the reaction serves as an alternative electron acceptor. Hence, MDA radical production both diminishes the electron flux to O_2 and also allows re-oxidation of the electron transport chain. The coupling of O_2 photoreduction to ascorbate metabolism may therefore represent a type of feed-back mechanism to minimize the production of active O_2 species under conditions of environmental stress. Since the MDA radical is readily detectable in leaves [10], these ideas could be tested at the in vivo level, a project which is currently underway.

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