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Photooxidative reactions in chloroplast thylakoids. Evidence for a Fenton-type reaction promoted by superoxide or ascorbate[1]

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Abstract. A methyl viologen (MV)* mediated Mehler reaction was studied using Type C and D chloroplasts (thylakoids) from spinach. The extent of photooxidative reactions were measured as (a) rate of ethylene formation from methional oxidation indicating the production of oxygen radicals, and (b) rate of malondialdehyde (MDA) formation as a measure of lipid peroxidation. Without added ascorbate, 1 μM FerricEDTA increased ethylene formation by greater than 4-fold, but had no effect on MDA production. Ascorbate (1 mM) produced a tripling of ethylene while it reduced MDA formation in the presence of iron. Radical scavengers diethyldithiocarbamate (DDTC), formate, 1,4-diazabicyclo(2.2.2octane) (DABCO), inhibited ethylene formation. Using 0.4 M mannitol to scavenge hydroxyl radicals, the rates of ethylene formation were reduced 40 to 60% with or without 1 μM Fe(III)EDTA. The strong oxidant(s) not scavenged by mannitol are hypothesized to be either alkoxyl radicals from lipid peroxidation, or 'site specific' formation of hydroxyl radicals in a lipophillic environment not exposed to mannitol. Singlet oxygen does not appear to be a significant factor in this system. Catalase strongly inhibited both ethylene and MDA synthesis under all conditions; 1 mM ascorbate did not reverse this inhibition. However, the strong superoxide dismutase (SOD) inhibition of ethylene and MDA formation was completely reversed by 1 mM ascorbate. This suggests that superoxide was functioning as an iron reducing agent and that in its absence, ascorbate was similarly promoting oxidations. Therefore, these oxidative processes were dependent on the presence of H_2O_2 and a reducing agent, suggesting the involvement of a Fenton-type reaction.

Abbreviations

DABCO, 1,4-diazabicyclo(2.2.2.octane); DCMU: 3-(3′,4 Dichlorophenyl)-1,1′-dimethyl urea; DDTC, diethyldithiocarbamate; EDTA, ethylenediaminetetraacetic acid; MDA, malondialdehyde; MV, methyl viologen; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid.

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Introduction

Despite the presence of the ascorbate-glutathione-NAD(P)H scavenging system for H_2O_2, photoinhibition of photosynthetic systems can occur under conditions unfavorable for photorespiration [33, 36]. Although the quantity of H_2O_2 produced in vivo is uncertain, there is increasing evidence that at least minute quantities are formed by the Mehler reaction [4, 20, 31]. Even if H_2O_2 is present in very small quantities and unable to cause any biological damage itself, it could react with reduced transition metals to form hydroxyl radicals ($^{\cdot}OH$). This reaction is commonly referred to as the Fenton reaction [12, 45] and is expressed as:

$$M^n + H_2O_2 \rightarrow M^{(n+1)} + {}^{\cdot}OH + OH^- \quad (1)$$

Hydroxyl radicals are highly reactive and among the strongest oxidizing reagents known. Therefore, H_2O_2 may indirectly play an important role in photoinhibition. Hydroxyl radical dependent decarboxylation of α-keto acids or oxidation of methional have been measured in chloroplasts [14, 15, 23]. These workers found that catalase and/or superoxide dismutase (SOD) inhibits the above reactions. This suggests that O_2^-, as well as H_2O_2, is important in $^{\cdot}OH$ radical formation. It was first explained as a Haber-Weiss reaction:

$$H_2O_2 + O_2^- \rightarrow {}^{\cdot}OH + OH^- + O_2 \quad (2)$$

The rate of this reaction has been found to be negligible in the absence of transition metal catalysts [7, 39, 46]. This reaction probably does not occur in biological systems. However, an iron-catalyzed Haber-Weiss cycle does occur at significant rates [12, 14, 15, 24, 32] and is represented by the following equations:

$$O_2^- + Fe^{+3} \rightarrow O_2 + Fe^{+2} \quad (3)$$

$$\underline{H_2O_2 + Fe^{+2} \rightarrow {}^{\cdot}OH + OH^- + Fe^{+3} \text{ (Fenton reaction)}} \quad (1)$$

$$O_2^- + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + O_2 \text{ (Haber-Weiss reaction)} \quad (4)$$

Catalase would inhibit the Fenton reaction part of the cycle (eqn. 1) by removing H_2O_2. SOD would remove the O_2^- and prevent the metal from being recycled to an activated (i.e., reduced) Fenton catalyst (eqn. 5). This could also explain why SOD is present in the chloroplasts, since O_2^- itself is generally not very reactive towards biological molecules [17].

$$O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

Ascorbate, although widely thought of an as antioxidant, can function to promote oxidations [22, 47, 49]. As a pro-oxidant, ascorbate could function as a metal ion reductant, replacing superoxide in eqn. (3):

$$A_{red} + M^{(n+1)} \rightarrow A_{ox} + M^n \quad (6)$$

In this report we examine the roles of both superoxide and ascorbate in promoting photooxidative damage during the methyl viologen (MV) mediated Mehler reaction.

Materials and methods

Chloroplast Isolation

Chloroplast thylakoids (type C and D [38]) were prepared from market spinach (*spinacea oleracea*) and/or 6–8 week old leaves of *S. oleracea L* (var. Bloomsdale) greenhouse grown on Jiffy mix. Type C chloroplasts were isolated by grinding deribbed spinach leaves in an Omni-Mixer for 5 s. The buffer contained: 50 mM phosphate, 20 mM NaCl, 2 mM $MgCl_2$ and 2.5 mM NH_4Cl, pH 7.6. The ground spinach was centrifuged at 500 x g for 90 s; the supernate was recentrifuged at 1000 x g for 5 min. The pellet was resuspended in buffer for immediate use. Alternatively, chloroplasts were isolated after the method of Nakatani and Barber [34]. Before each assay, a small aliquot of the chloroplast suspension was osmotically shocked for 90 s in 1 ml of de-ionized distilled water. Following the osmotic shock, one ml of assay medium (2 x concentrated) was added to yield Type D broken chloroplasts [38]. Similar results were obtained with either isolation procedure.

Chlorophyll was assayed after the method of Arnon [2]. Saturating white light (250–300 $\mu E\, m^{-1}\, s^{-1}$) was used in all experiments. Milli-Q purified water was used for all solutions. The phosphate buffer and mannitol solutions were Chelex treated to minimize iron contamination.

Ethylene determination from methional

The method for ethylene determination was based on Beauchamp and Fridovich [5]. Methional (2 mM) was added to the assay mixture which consisted of the phosphate buffer described above. Methional oxidation to ethylene was carried out in a total volume of 1 ml in 10 ml gas tight vials. Vials were exposed to saturating light intensities between duplicate sets of two Sylvania 20 W fluorescent lamps (cool white). Reactions usually were run for 20 minutes at 25°C; lights were turned off and 100 μl sulfuric acid (0.6 M) was added to each tube by Hamilton syringe to terminate the reaction. Gas-tight syringes were used to take 1 ml gas samples from the head space of each vial. These samples were analyzed for ethylene with a Perkin-Elmer model 990 gas chromatograph equipped with 1.22 m x 3.2 mm stainless steel columns of 80/100 mesh Poropak N (Supelco, Inc.) and flame ionization detectors. The carrier was pre-purified nitrogen at a flow rate of 40 $ml\, min^{-1}$. Detector gasses were hydrocarbon free air and ultra-high purity hydrogen (Airco, Inc.). Operating temperatures for the injection block and manifold were 50 and 120°C, respectively. Signals were processed by a C-R1B-Chromatopac (Shimadzu, Inc., Columbia, MD). Minimum sensitivity

was to 5 pl of ethylene. An external standard of 1 μl l^{-1} ethylene in nitrogen was prepared by Matheson Gas Products, Inc.

Malondialdehyde determination

This method was based on that of Buege and Aust [11]. Malondialdehyde (MDA), a radical-induced breakdown product of lipids was complexed with thiobarbituric acid (TBA) for spectrophotometric measurements. One ml of the biological sample was added to the TBA solution (15 g TCA, 0.375 g TBA and 100 ml 0.25 N HCl) and heated for 15 min at 100°C. The solution was cooled and the resulting TBA adduct was measured at 535 nm. MDA concentrations were estimated using the molar extinction coefficient $E = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$. The sample reaction conditions were the same is in the methional experiments, except for the absence of methional and reaction times of 30 min.

Data analysis

Results from the methional and MDA experiments showed considerable variation. Therefore, test for a significant main-effect F in an analysis of variance using Student-Newman-Keuls for multi-mean comparisons on the Dec-10 SPSS program was used in the statistical analysis of these experiments.

Results

Studies of a Fenton type reaction were conducted using sensitive techniques to indirectly detect oxy radicals. One method was the determination of ethylene from the ·OH radical-dependent oxidation of methional. The other method detected MDA, a break-down product of lipid peroxidation.

The MV-mediated Mehler reaction was used as the control in the above methods. In the methional technique, all treatments were compared as a percent of the control. Iron (1 μM Ferric-EDTA) caused a considerable increase (> 4-fold) in the oxidation of methional (Table 1A). Radical scavengers DDTC, DABCO, and formate effectively reduced ethylene formation, with DDTC being the most effective. In the presence of 1 mM ascorbate (Table 1B), ethylene formation was nearly tripled in the minus iron thylakoids, and increased 15-fold in the plus iron treatment compared with the minus iron, minus ascorbate control (Table 1A). DDTC again strongly reduced the rate of ethylene formation compared to the ascorbate controls (Table 1B). Similar experiments with ascorbate were conducted in the dark. Negligible amounts of ethylene were formed without ascorbate, but significant ethylene production was observed in the presence of ascorbate. In the absence of MV, there was a reduced, although significant, amount of methional oxidation (Table 1A). Without methional, there was no detectable ethylene. DCMU severely limited ethylene formation.

Table 1. Oxidation of methional to ethylene during the methyl viologen Mehler reaction. Effects of ferric-EDTA, radical scavengers, catalase, SOD,† DCMU and ascorbate

Treatment	% Control ethylene formation rate*	
	− FeEDTA	+ Fe(III)EDTA (1 μM)
Part A (no added ascorbate)		
Control	100a‡	420g
DDTC (10 mM)	14b	37d
DABCO (10 mM)	70c	278f
Formate (100 mM)	41d	81ac
Catalase (50 units)	46d	97a
SOD (70 units)	46d	25bd
Dark treatment	6be	15b
Boiled catalase	100a	386g
Boiled SOD	105a	340fg
DCMU (10 μM)	9b	29d
No methyl viologen	44d	109a
No methional	0e	0e
EDTA (1.2 μM)	103a	ND
Part B (all treatments include 1 mM ascorbate)		
Control	294f	1589h
DDTC (10 mM)	6b	179i
Catalase (50 U)	21b	192i
SOD (70 U)	276f	1406h
Dark	ND	281f
DCMU (10 μM)	37d	ND

Results are compared to the minus iron, minus ascorbate control on a percentage basis. Experiments were conducted using market spinach chloroplasts (type C) with an average *control rate of 950 pl $C_2H_4 \cdot min^{-1} \cdot mgChl^{-1}$. Assay medium: 50 mM phosphate, 20 mM NaCl, 2 mM $MgCl_2$, 2.5 mM NH_4Cl, 100 μM MV, 2 mM methional, pH 7.6. 1 μM iron was added as ferric-EDTA with a 20% molar excess of EDTA. Units of catalase and SOD are as described by Sigma Chemical Co. Each value represents the average of three replicates. All reactions carried out in saturing white light intensities at 25°C for 20 minutes.

‡Values with the same letters were not found to be significantly different at the $p \leq 0.05$ level of the Log transformed data. ND: not determined.

†See list of abbreviations

When catalase was added, there was a 50 to 80% inhibition of methional oxidation, and this inhibition was not reversed by 1 mM ascorbate (Table 1). SOD inhibited methional oxidation by more than 50% without ascorbate (Table 1A); this SOD effect was completely reversed by 1 mM ascorbate (Table 1B). Boiled catalase or SOD did not alter ethylene formation rates.

When methional oxidation was re-examined in the presence of 0.4 M mannitol (hydroxyl radical scavenger), ethylene formation was consistently reduced (Table 2) compared to no mannitol (Table 1). The effect of added FeEDTA was significantly reduced in a mannitol environment, but was still twice its control. The general effects of the radical scavengers DDTC and

Table 2. Oxidation of methional to ethylene during the methyl viologen Mehler reaction in the presence of 0.4 M mannitol. Effects of ferric-EDTA, radical scavengers, catalase, SOD,† DCMU and ascorbate

Treatment	% Control ethylene formation rate*	
	− FeEDTA	+ Fe(III)EDTA (1 μM)
Part A (no added ascorbate)		
Control	100a‡	198b
Minus mannitol	144b	544f
DDTC (10 mM)	13c	40d
DABCO (10 mM)	91a	180b
Formate (100 mM)	56d	118a
Catalase (50 units)	31d	54d
SOD (78 units)	48d	96a
Part B (all treatments include 1 mM ascorbate)		
Control	423e	713g
DDTC (10 mM)	13c	116a
Catalase (50 U)	20c	90a
SOD (78 U)	433e	695fg
Dark	123a	111a
DCMU (10 μM)	53d	ND

Results are compared to the minus iron, minus ascorbate control on a percentage basis. Average *control rate of 591 pl $C_2H_4 \cdot min^{-1} \cdot mg\,Chl^{-1}$. Assay medium and conditions are as described in Table 1, except that chelex treated mannitol was added to the buffer to give a final concentration of 0.4 M.
‡Values with the same letters were not found to be significantly different at the $p \leq 0.05$ level. ND: not determined.
†See list of abbreviations

formate were similar to what seen without mannitol, but DABCO had no effect (Table 2). Catalase remained an effective inhibitor of ethylene formation under all conditions. But as before, SOD was effective in reducing methional oxidation only when ascorbate was not present.

The effects of iron and ascorbate on the formation of the lipid peroxidation product MDA were examined in the presence of 0..33 M sorbitol (Table 3). Neither iron nor ascorbate enhanced MDA formation rates. Catalase strongly inhibited MDA formation; but SOD was ineffective in limiting MDA synthesis when ascorbate was present.

Discussion

Oxygen can function as an electron acceptor in photosynthesis. The one electron reduction of oxygen forms superoxide which dismutes slowly (non-enzymatically) and rapidly (with superoxide dismutase) to form the 2 electron reduced state hydrogen peroxide [4, 42]. Although superoxide is a free radical, it is neither a strong reductant, nor a strong oxidant [42]. Since superoxide appears to be a relatively nontoxic radical, this has led to a

questioning of the physiological function of SOD [17]. Hydrogen peroxide levels are not allowed to build up in chloroplasts. Recent evidence suggests that an ascorbate-glutathione-NAD(P)H system scavenges H_2O_2 [4, 27]. Nevertheless, photoinhibition can still occur under conditions found unfavorable for photorespiration [33, 36]. H_2O_2 (and indirectly superoxide) may be responsible for this photoinhibition. The Fenton reaction could play an important role in a H_2O_2-dependent photoinhibition by generation of the strong oxidant, the hydroxyl radical. For a continuous Fenton reaction, the transition metal must be re-reduced. Superoxide is known to be an efficient reductant of transition metals [12, 42, 47]. With O_2^- as the reductant, the overall reaction is termed the metal catalyzed Haber-Weiss reaction (Eqn. 4) and expressed as already noted earlier as the sum of Eqn. (1) and (3), where M^n is used instead of the specific Fe^{+2}.

Superoxide promotion of photooxidation

If superoxide were functioning as a metal reducing agent, one would expect SOD to inhibit the Haber-Weiss reaction by greatly increasing the rate of dismutation of superoxide (see Eqn. 5).

This is what we found (Table 1A). Catalase inhibited ethylene formation 50–80%, while SOD gave greater than 50% inhibition (Table 1A and [15]). Fe(III)EDTA promoted ethylene formation without SOD, but was strongly inhibited with SOD (Table 1A). The radical scavengers DDTC, formate, and DABCO reduced ethylene formation, with DDTC being the most effective and DABCO the least (Table 1). Both dark treatment and DCMU blocked ethylene formation, indicating the involvement of the electron transport system.

Ascorbate promotion of photooxidation

If O_2^- was the only reductant of the oxidized transition metal, we could predict much greater inhibition by SOD (Tables 1A, 2A). We found that SOD inhibition can be reversed by ascorbate (Tables 1B and 2B). It is likely that ascorbate is replacing superoxide as a reductant for the oxidized metal ion (Eqn. 6). Similar findings for the ascorbate reversal of SOD inhibition of methional oxidation to ethylene have been found in stored human plasma [48].

However the catalase inhibition was not reversed by ascorbate (Table 1B) pointing to the essentiality of hydrogen peroxide in these oxidation reactions. We found that ascorbate caused a three-fold increase in ethylene formation without added iron and a 15-fold increase with 1 μM FeEDTA (Table 1B). This is likely due to a three-fold increase in the respective rates of formation of oxy-radicals.

Beauchamp and Fridovich [5] found that hydroxyl radical scavengers, such as ethanol or benzoate, inhibited methional oxidation. Pryor and Tang [37] have shown that alkoxyl radicals ($RO^{\cdot}$) can also oxidize methional to

ethylene. Since catalase effectively blocks ethylene formation in our system, the formation of hydroxyl radicals (from H_2O_2) is implicated. Our thylakoids, without methional, did not produce ethylene (Table 1). This indicates that the thylakoids were not producing endogenous ethylene which could have interfered with our measurements. Chloroplast thylakoids are capable of methional oxidation in the absence of MV (Table 1A).

The Mehler reaction in mannitol

Mannitol (0.4 M) was used to selectively scavenge hydroxyl radicals in our thylakoid system (Table 2). Mannitol (and other sugar alcohols) scavenge hydroxyl radicals rapidly (rate constant: $1 \times 10^9\ M^{-1}\ s^{-1}$) [24]. However, other forms of active oxygen are not readily scavenged by mannitol including singlet oxygen [18], and alkoxyl radicals [10].

The presence of 0.4 M mannitol reduces the rate of ethylene formation by approximately 40% (Table 2A). With exogenous 1 μM Fe(III)EDTA, the mannitol effect is more striking (compare Table 2 with Table 1). This is true both with and without ascorbate, suggesting that more hydroxyl radicals are being formed (and scavenged) when chelated iron is present. Nevertheless, it is clear that some strong oxidant(s) is(are) still present to oxidize methional to ethylene. It has been shown that neither superoxide nor hydrogen peroxide will significantly oxidize methional [5]. It is not clear whether singlet oxygen can directly oxidize methional to ethylene. Klebanoff and Rosen [28] presented evidence that this can occur. Our tests with photochemically (i.e., by the use of Rose Bengal) generated singlet oxygen (data not shown) agree with Kutsuki and Gold [29] who found no significant ethylene formation due to singlet oxygen. The radical scavengers DDTC and formate did further inhibit ethylene formation (Table 2A and 2B), but DABCO had no effect. DABCO is widely used to scavenge singlet oxygen, but is an even better scavenger of hydroxyl radicals [1]. Takahama and Nishimura [43] have presented evidence that singlet oxygen is involved in lipid peroxidation in thylakoids with benzyl viologen. They found DABCO inhibited this peroxidation. In our system, the lack of a DABCO effect with 0.4 M mannitol (Table 2A) suggests that DABCO inhibition seen in Table 1A was largely due to hydroxyl radical scavenging, not singlet oxygen scavenging.

To account for the residual oxidants not scavenged by 0.4 M mannitol, we offer two hypotheses: (a) the involvement of alkoxyl radicals; (b) the 'site specific' radical formation.

(*a*) *Alkoxyl radical formation*

The effectiveness of DDTC and formate as inhibitors of methional oxidation in a mannitol environment may be due to their ability to scavenge alkoxyl radicals ($RO^{\cdot}$). Although DDTC is a metal chelator, its effectiveness as a radical scavenger is unrelated to this property [30, 35]. Formate is often used as to scavenge hydroxyl radicals [13, 22, 24], but a report [40] suggests that

it will also scavenge alkoxyl radicals. The alkoxyl radical is a strong oxidant and could be formed during lipid peroxidation [3, 6, 25]. A Fenton-type reaction involving lipid hydroperoxide reduction would yield alkoxyl radicals (RO·) [3]. With superoxide (7) or ferrous iron (8) as the reductant we have respectively:

$$O_2^- + ROOH \longrightarrow RO\cdot + OH^- + O_2 \quad (7)$$

$$Fe^{+2} + ROOH \longrightarrow RO\cdot + OH^- + Fe^{+3} \quad (8)$$

Whether superoxide can directly reduce lipid peroxides (Eqn. 7) is controversial [8, 44]. It if could, the toxicity of superoxide to cells would be easy to explain. However it is clear that chelated ferrous iron will rapidly catalyze such a reduction (Eqn. 8) [3].

(b) Site specific radical formation

The high reactivity of hydroxyl radicals with organic molecules (k's: $10^8 - 10^9\ M^{-1}\ s^{-1}$) [13] points to a minimal opportunity for such radicals to diffuse from their site of formation before reacting [7, 9, 16]. For the initiation of lipid peroxidation (from Fenton generated hydroxyl radicals), one would expect the redox active iron to be in or on the membrane [3]. Our system of thylakoids suspended in a phosphate buffer are a lipid/aqueous heterogeneous system. Borg's group [see e.g. ref. 6] has shown that iron-EDTA complexes will be taken up by lipids to a significant extent. This would effectively compartmentalize the system and prevent some substrates and scavengers from reaching reactive sites. Lipophobic scavengers (mannitol) may find such hydroxyl radicals inaccessible [21, 41]. Because hydroxyl radicals formed in a lipid environment could initiate lipid peroxidation, alkoxyl radicals would also result [3, 9]. Due to their high reactivity (hence short lifetimes), alkoxyl radicals may not be able to migrate into the aqueous environment either. But as Bors et al. [9] have pointed out, though the site-specific mechanism is an attractive explanation, it is very difficult to verify.

Malondialdehyde formation

We also used TBA to detect MDA. MDA is a common breakdown product of radical-induced oxidation of lipids and nucleic acids [16]. They found that ·OH radical scavengers formate, mannitol and thiourea would prevent MDA formation. We used the MDA assay to supplement our methional experiments. Our system used 0.33 M sorbitol as a hydroxyl radical scavenger. Ascorbate did not enhance MDA formation with iron-EDTA, but may have acted to partially protect the membrane lipids (Table 3). Catalase gave good protection in limiting MDA formation. H_2O_2 therefore is an important component of these reactions, but superoxide appears to be an essential factor only when ascorbate is not added. Again this suggests superoxide is functioning as a reducing agent.

Table 3. Formation of malondialdehyde (MDA) during the methyl viologen Mehler reaction in the presence of 0.33 M sorbitol. Effects of ascorbate, iron-EDTA, catalase, SOD

	MDA formation ($nmoles \cdot min^{-1} \cdot mgChl^{-1}$)	
Treatment	− Ascorbate	+ Ascorbate (1 mM)
Control	0.838ab‡	0.776ab
FeEDTA (5 μM)	1.101a	0.392b
Catalase (440 units)	0c	0.163d
SOD (220 units)	0c	0.819a

Assay medium was described in Table 1 with the following changes: 0.33 M sorbitol; 2 mM EDTA; 50 mM HEPES was substituted for phosphate; 0.33 M sorbitol; 2 mM EDTA; methional was not included. Each value represents the average of three replicates.
Reactions were incubated in saturating light intensities at 25°C for 30 min.
‡Values with the same letters were not found to be significantly different at the $p \leq 0.05$ level

Conclusions

Ascorbate is generally viewed as an antioxidant [25], but from the above data, we can also consider it to be a pro-oxidant. If ascorbate does indeed re-reduce the metal as O_2^- does, one must reevaluate the physiological function of SOD [17]. It was thought that SOD removed O_2^- to prevent the activation of Fenton catalysts, but ascorbate is present in vivo at greater concentrations than is O_2^- [19]. Therefore, ascorbate would be more likely to function as a metal reductant and would make Eqn. 3 physiologically less significant. Fenton reactions occuring in biological systems probably involve chelated metals [12, 24, 32, 49]. In chloroplasts, H_2O_2 is found to reoxidize ferredoxin, with consequent $^{\cdot}OH$ radical formation, at a rate nearly ten times faster than that of Ferredoxin-dependent O_2 reduction to O_2^- [26]. Therefore, ferredoxin itself can function as a Fenton catalyst. In view of the currently accepted ascorbate-glutathione-NAD(P)H scavenging system for H_2O_2 and the stimulatory effects of ascorbate in $^{\cdot}OH$ radical production, the role of ascorbate in the chloroplasts becomes more complex. In order to get a clearer picture of the relationship of ascorbate to the Mehler reaction, more physicochemical data will be needed.

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