Paraquat-Catalyzed Photodestructions in Subchloroplast Particles are Independent on Photosynthetic Electron Transport

E. F. Elstner, E. Lengfelder *, and G. Kwiatkowski

Institut für Botanik und Mikrobiologie, Technische Universität München, Arcisstr. 21,
8000 München 2

* Institut für Strahlenbiologie der Universität München, Bavariaring 21, 8000 München 2

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Light dependent ethane formation and chlorophyll bleaching in isolated chloroplast lamellae are enhanced by either methylviologen or α-linolenic acid. Both ethane formation and chlorophyll bleaching are also enhanced in chloroplast particles deficient in photosynthetic electron transport, *e. g.* after aging, heat treatment or digitonin fragmentation. Ethane formation by subchloroplast particles from endogenous substrates in the presence of methylviologen is inhibited by superoxide dismutase or by a penicillamine copper complex exhibiting superoxide dismutase activity whereas chlorophyll bleaching is enhanced by superoxide dismutase – active substances.

Maximal rates of ethane formation in subchloroplast particles are observed when more than 50% of the chlorophyll is bleached and continues after 98% chlorophyll bleaching. This result indicated that methylviologen – stimulated ethane production in subchloroplast particles is not dependent on photosynthetic electron transport but involves "activated oxygen" – species like the superoxide radical ion, generated by a light receptor derived from the pigmentsystem of photosystem I or activated after its destruction.

Introduction

Ethane formation and Chl bleaching in intact leaves and algae have recently been described as closely connected reactions stimulated by both electron transport blockers like DCMU or autoxidizable electron acceptors like MV [1–3]. Both PS I and PS II seem to play a role in the above degradative processes [4–6] where in the case of DCMU lack of energy dissipation in PS II [7–9], in the case of MV deviation of reducing power and oxygen radicals [5, 10], produced at the reducing side of PS I seem to yield similar effects.

In the present paper we describe experiments undertaken in order to investigate why low potential herbicides of the bipyridylium type like MV (paraquat) can bleach and necrotize green plants knowing that, a) their activity is dependent on an electron donor for PS I and b) PS II and PET are apparently the first and most sensitive targets of MV activity [11]. Thus, the light dependent deleterious activities of MV can not fully be explained by the present hypotheses [1–11]. From the presented findings, however, we can conclude that, after a certain loss of integrity of the thylakoid structure MV stimulates Chl photobleaching and unsaturated fatty acid peroxidation (measured as ethane formation *cf.* refs [12–14]) independently of PS II and intact PET, rendering Chl itself and/or unsaturated fatty acids of the thylakoids the endogenous electron donors for PS I after prolonged illumination.

Materials and Methods

Chloroplast lamellae were prepared from spinach [15] and aged by incubation in 25 mM phosphate buffer pH 7.5 at 4 °C for 48 h. Subchloroplast particles were prepared from chloroplast lamellae by stirring chloroplast preparations in 0.5% digitonin (Merck) solutions (in 25 mM phosphate buffer pH 8.0) at room temperature for 1 h. Subchloroplast particles were harvested by centrifugation of an 1 000 × *g* supernatant at 30 000 × *g* for 30 min. Chlorophyll was determined according to Arnon [16].

Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl-urea; α-lin., α-linolenic acid; MV, methylviologen, paraquat (1,1′-dimethyl-4,4′-dipyridylium dichloride); PACu, penicillamine – copper complex; PET, photosynthetic electron transport; PS I, PS II, photosystem I, photosystem II; SOD, superoxide dismutase.

Reprint requests to Dr. E. F. Elstner.

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Incubations of chloroplast lamellae or subchloroplast particles were done in Fernbach flasks sealed with serum rubber stoppers at 20 – 22 °C in a photothermostate with illumination from the bottom (35 Klux). Superoxide dismutase (copper-zinc type) was isolated from dried green peas [17, 18]. Ethane formation was determined as described in ref. [13], the function of PS II and PS I was determined with *p*-nitrosodimethylaniline [19]. PACu was prepared as described in ref. [20]. α-lin was from Roth-chemicals (Karlsruhe) and catalase was from Boehringer (Mannheim).

Results

Isolated chloroplast lamellae lose part of their chlorophyll (up to 20%) after incubation for longer than 60 min at 22 °C in the dark or in the light. Stimulation of Chl bleaching by α-lin is stronger in the light as compared to the dark control and is already visible after 45 min incubation. Ethane formation by chloroplast lamellae is measurable after 60 min incubation and is light-dependent; in the presence of α-lin it starts already after 15 min incubation with almost identical rates in the dark and in the light. The dark reaction is terminated after 45 min; the light reaction is terminated already after 30 min incubation, where the plateau of ethane formation in the dark is approx. 1.7 times higher than that in the light. Ethane formation in the light, however, starts again after 45 min incubation and resumes the same sigmoidal increasing rate as measured in the absence of α-lin (Fig. 1).

Since it is known that after aging of the thylakoid membranes, the loss of the structural integrity is connected with both the liberation of unsaturated fatty acids (predominantly α-lin) and concommitant loss of PET-activity [21, 22], we compared the PET activities with light dependent ethane formation in freshly prepared, aged and digitonin fragmented (see methods) chloroplasts:

In aged or digitonin fragmented chloroplasts, the rates of PET (as indicated by *p*-nitroso-dimethylaniline reduction through both PS II and PS I, *cf.* ref. [19]) are only ca. 2% of that of freshly prepared chloroplasts (data not shown). As shown in Table I, ethane formation is increased after aging and, in contrast to freshly prepared chloroplasts, not stimu-

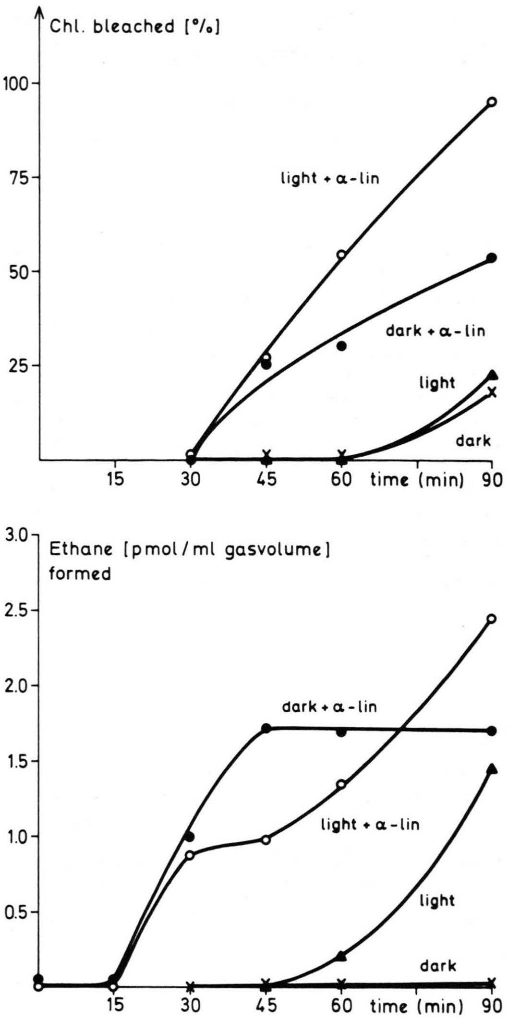

Fig. 1. Effects of light and α-linolenic acid on ethane formation and chlorophyll bleaching in isolated chloroplast lamellae. Experimental conditions were as described under Materials and Methods. 7.5 mM α-lin were present as indicated.

Table I. Ethane formation by freshly prepared and by aged chloroplast lamellae. For experimental conditions see "Materials and Methods".

Additions	Ethane formed [nmol/mg Chl] by:	
	freshly prepared chloroplasts	aged (48 h at 4 °C) chloroplasts
none	0.2	1.4
7.5×10^{-3} M α-lin.	0.8	0.9
5×10^{-4} M MV	1.2	4.3

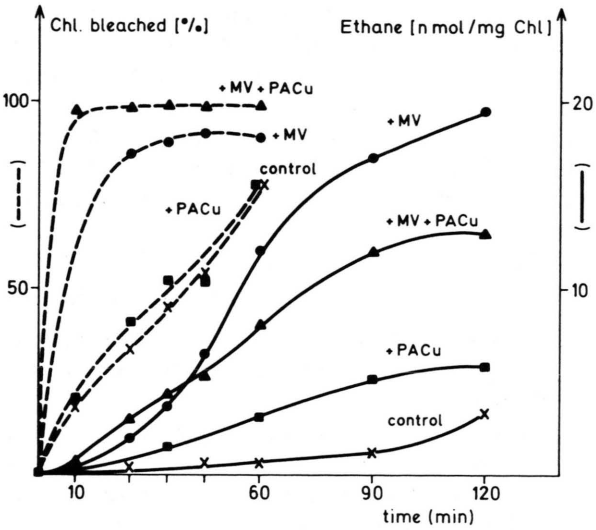

Fig. 2. Effects of methylviologen (MV) and a superoxide dismutase-active penicillamine copper complex (PACu) on ethane formation and chlorophyll bleaching by subchloroplast particles. Experimental conditions were as outlined under Materials and Methods.

lated by the addition of α-lin. After addition of MV, ethane formation is increased in both preparations, although PS II cannot supply electrons for MV reduction by PS I in aged chloroplasts. In subchloroplast particles (= digitonin fragmented chloroplasts), PET is disintegrated [23] and, surprisingly, the time course of Chl bleaching and ethane formation is reversed: while in chloroplast lamellae ethane formation is measurable before Chl bleaching, in subchloroplast particles Chl bleaching by far precedes ethane formation (see controls in Fig. 2). In subchloroplast particles, MV strongly stimulates both Chl bleaching and ethane formation. In the absence of MV, PACu initially stimulates ethane formation. In the presence of MV, PACu initially stimulates both Chl bleaching and ethane formation (from 10 to 30 min illumination), after ca. 60 min illumination PACu inhibits ethane formation, however (Fig. 2).

It is noteworthy that the maximal rate of MV-stimulated ethane formation in subchloroplast particles is measured when far more than half of the chlorophyll of these particles is bleached.

In Table II we compare the effects of copper-zinc superoxide dismutase from higher plants [17, 18] with those of ascorbate on Chl bleaching and ethane formation by subchloroplast particles in the presence and absence of MV:

Table II. Effects of ascorbate and superoxide dismutase (SOD) on ethane formation and chlorophyll bleaching by subchloroplast particles. Subchloroplast particles were centrifuged for 20 min at 10000×*g* before harvesting by 60 min centrifugation at 21000×*g*. Subchloroplast particles with approx. 0.3 mg Chl were incubated for 90 min at 20 °C in 25 mM MES buffer pH 5.5.

Additions	Ethane formed [nmol/mgChl]	Chl bleached [%]
none:	0.2	13
$+6\times10^{-3}$ M ascorbate	0.3	9
+50 units SOD	0.2	22
3×10^{-4} M MV:	0.6	28
$+6\times10^{-3}$ M ascorbate	0.1	12
+50 units SOD	0.2	32
+100 units catalase	0.6	35

The rates of Chl bleaching and ethane formation shown in Table II are lower than those shown in Fig. 2, probably due to the removal the larger particles of "sub-chloroplasts" by centrifugation for 20 min at 10000 × *g* before harvesting the smaller particles by centrifugation for 60 min at 21000 × *g*. This preparation allows the differentiation of subtle effects of additions due to the reduced rates of photooxidation. In the absence of MV, superoxide dismutase stimulates Chl bleaching (compare the PACu-effect shown in Fig. 2). In the presence of MV, both Chl bleaching as well as ethane formation are enhanced, as already demonstrated in Fig. 2. Both superoxide dismutase and ascorbate reduce ethane formation, but only ascorbate significantly inhibits Chl bleaching. Catalase, in the presence of MV has no significant effect on either Chl bleaching or ethane formation (Table II).

Discussion

Two different mechanisms of paraquat (= MV) – toxicity in plants have recently been discussed [4, 5], namely: a) deviation of reducing power necessary for CO_2-fixation and thus starvation and b) formation of toxic oxygen species after autooxidation of the reduced herbicide and oxidative destruction of membraneous cell compartments by these oxygen species. It has early been recognized, however, [25] that starvation due to deviation of reducing power cannot primarily be responsible for the fast, light – and oxygen – dependent destructions observed in the presence of

MV. Thus oxygen activation and subsequent membrane destruction by unsaturated fatty acid peroxidation must be seen as the predominant cause of the light-dependent herbicidal action of MV [5]. One of the first measurable effects of MV-treatment of unicellular algae is inactivation of PS II and PET whereas PS I proves rather insensitive [11]. Thus, no reasonable explanation exists so far for all the successive light-dependent destructions since in isolated chloroplast lamellae MV-catalyzed oxygen reduction (yielding the superoxide free radical ion, *cf.* ref. [10]) is dependent on electrons from the reducing side of PS I which in turn depends on PET and electrons from PS II through water splitting.

The results presented in this communication may bridge this gap by suggesting that MV-enhanced unsaturated fatty acid peroxidation as well as Chl bleaching occur independently from electrons of PS II after a certain degree of damage of the thylakoid structure. In aged chloroplasts or in subchloroplast particles which lack PET through both photosystems, unsaturated fatty acid peroxidation (as indicated by ethane production, refs. [12–14]) as well as Chl bleaching are nevertheless enhanced by MV. We therefor suggest that the herbicidal activity of MV must be subdivided into two phases:

1) PET-driven oxygen activation and production of aggressive oxygen species which eventually inactivate PS II [11]; destructions may also be caused by certain products of unsaturated fatty acid peroxidation [26].

2) PS I-mediated MV-reduction and oxygen activation independent on electrons from PS II; under conditions where the electron donor pool of PS I is completely oxidized, Chl itself and/or unsaturated fatty acids (*e. g.* α-lin) seem to function as electron donors for PS I.

It is remarkable that the time courses of ethane formation and Chl bleaching are reverse in freshly prepared chloroplasts and in subchloroplast particles. This may certainly be due to vesicle formation and thus perturbation of the original thylakoid structure [23] in subchloroplast particles, rendering Chl the primary target of photooxidation instead of unsaturated fatty acids.

The chain of biochemical events induced and catalyzed by MV is therefore suggested to comprise:

a) MV-enhanced unsaturated fatty acid peroxidation [1–5] and concomitant inactivation of PET from PS II [11].
b) bleaching of photosynthetic pigments, where the pigments function as electron donor for PS I and thus MV, which in turn can produce oxygen radicals upon autooxidation.

As shown in Fig. 2 the rate of MV-stimulated fatty acid peroxidation is highest when more than 90% of the Chl of the subchloroplast particles is bleached. Since MV-stimulated Chl bleaching is increased by SOD-active compounds while ethane formation is decreased, different mechanism of Chl bleaching and unsaturated fatty acid peroxidation must be operating in this system. This observation is confirmed by the results presented in Table II: both ascorbate and SOD inhibit ethane formation, but only ascorbate inhibits Chl bleaching. Ascorbate can function as an electron donor for PS I in subchloroplast particles as well as an $O_2^{\cdot -}$ scavenger, while SOD only interacts with $O_2^{\cdot -}$. Thus, Chl bleaching may predominantly be driven by an oxidant formed at the electron donor side for PS I, while ethane formation seems to involve an $O_2^{\cdot -}$ dependent step. The nature of the reported novel light reaction of subchloroplast particles as well as the products of Chl degradation are currently under investigation.

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