Ethylene Formation by Isolated Chloroplast Lamellae in the Dark

Erich F. Elstner and Jörg R. Konze

Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum

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Isolated chloroplast lamellae from spinach produce ethylene in the dark from methylmercaptopropanal (MMP) or from 2-keto-4-methyl-mercaptobutyrate (KMB) only in the presence of both NADPH and ferredoxin. Anthraquinone-2-sulfonic acid can substitute for ferredoxin. Catalase, superoxide dismutase, ethanol and ascorbate are inhibitors of NADPH-dependent ethylene formation. Isolated NADP-ferredoxin reductase in the presence of NADPH, ferredoxin and an oxygen reducing factor (ORF, isolated by heat-treatment of chloroplast lamellae) catalyzes ethylene formation from the above substrates in the dark without chloroplast lamellae. From the results it is concluded that chloroplast lamellae in the dark can reduce oxygen monovalently at the expense of NADPH, with the production of the OH-radical as the reactive species responsible for ethylene formation from MMP of KMB.

Introduction

Formation of ethylene by plants and influence of ethylene on higher plants has been studied by numerous groups [1, 2]. It is well established by now that methionine (or its derivatives KMB and MMP) [1, 2] and/or α-linolenic acid [3] are the most likely precursors for ethylene production in higher plants and that ethylene as a plant hormone is involved in the regulation of plant-growth and -development. The observation that the enzymes involved in ethylene biosynthesis seem to be located in subcellular particles not identical to mitochondria [4, 5] led us to investigate the possibility of ethylene formation by isolated chloroplasts.

Chloroplast lamellae, upon illumination, reduce oxygen monovalently yielding in the formation of both the superoxide free radical ion [6, 7] and hydrogen peroxide [8–10]. These two species, $O_2^{\cdot -}$ and H_2O_2 are active in the formation of the OH-radical ($OH^{\cdot}$), according to the Haber-Weiss-reaction (Eqn (1))

$$O_2^{\cdot -} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + O_2 \text{ [11]}. \quad (1)$$

Since the formation of ethylene from MMP seems to depend on both H_2O_2 and $O_2^{\cdot -}$, Beauchamp and Fridovich [12] proposed that the OH-radical is the reactive species responsible for ethylene formation from MMP in the presence of xanthine and xanthine-oxidase.

We recently described the formation of ethylene from both MMP and KMB by illuminated chloroplast lamellae [13, 14]. This reaction is also dependent on both H_2O_2 and $O_2^{\cdot -}$. These and other findings provided evidence, that illuminated chloroplasts under certain conditions can produce the OH-radical [13, 14] in addition to the superoxide free radical ion [6, 7, 15–18] and H_2O_2 [8–10].

The question arises, however, whether ethylene formation by isolated chloroplast lamellae is of physiological importance. The facts that,

1. the most likely substrates for ethylene formation (methionine and/or α-linolenic acid) are synthesized in the chloroplasts [19, 20], and
2. ethylene production by chloroplasts upon ionizing radiation without exogenous substrates added has been observed [21] present evidence that chloroplasts are indeed potential sites for ethylene biosynthesis.

In addition to the previously reported light-reactions, ethylene production from methionine derivatives by isolated chloroplast lamellae can also be observed in the dark. Experiments on the conditions for this reaction are reported in the present communication.

Materials and Methods

Chloroplast lamellae, ferredoxin and NADP-ferredoxin reductase (ammonium sulfate fraction from 35% to 60% saturation) were isolated from spinach [22–24]. MMP and KMB were synthesized as

Requests for reprints should be sent to Dr. E. F. Elstner, Ruhr-Universität Bochum, *D-4630 Bochum*, Lehrstuhl für Biochemie der Pflanzen.

Abbreviations: AQ, anthraquinone-2-sulfonic acid; Fd, ferredoxin; SOD, superoxide dismutase; MMP, methylmercaptopropanal; KMB, 2-keto-4-methylmercaptobutyrate.

described [13]. Superoxide dismutase was prepared from dried green peas [25, 7] and catalase was purchased from Boehringer, Mannheim. ORF (oxygen reducing factor) was prepared from isolated chloroplast lamellae (spinach) by heating (4 mg chlorophyll/2 ml) for 5 min at 80 °C. The supernatant at centrifugation at 48 000 × *g* for 10 min contained the ORF-activity. The SOD used had no catalase activity and the catalase had no SOD activity at the applied concentrations.

The reactions were carried out with Fernbach flasks (with a side arm) in a Warburg thermostate at 20 °C. The test system for ethylene production contained in 1 ml: Chloroplast lamellae with 5 μg chlorophyll; 25 μmol phosphate buffer pH 7.6, 1 μmol NADP, 30 nmol Fd, 10 μmol glucose-6-phosphate, 20 μg glucose-6-phosphate dehydrogenase (Boehringer, Mannheim). 1 μmol MMP or 2 μmol KMB were used as substrates as indicated in the tables and figures. The reactions were conducted for 40 min in the dark. Ethylene was determined in a Varian Aerograph model 1400 as described [13, 14].

Results

Conditions for ethylene formation by isolated chloroplasts in the dark

Isolated chloroplast lamellae produce ethylene from MMP or KMB in the dark, but not from L-methionine or from α-ketoglutarate [26] (Table I); NADPH is an absolute requirement for ethylene formation in the dark (Table II). Glucose-6-phosphate dehydrogenase in the presence of NADP and glucose-6-phosphate stimulates the reaction. There is little, but significant ethylene production without added glucose-6-phosphate dehydrogenase.

The reaction is saturated with 5 μg chlorophyll/ml reaction mixture; higher concentrations of chlorophyll result in a decrease of ethylene formation. In the absence of ferredoxin (Fig. 1) or AQ (Fig. 2) NADPH-dependent ethylene formation by chloroplast lamellae is not observed. 60 nmol of Fd or 0.5 μmol AQ per ml reaction mixtures are saturating for the reaction.

Table I. Substrates for ethylene formation by chloroplast lamellae in the dark. For experimental conditions see Material and Methods.

Substrate	Ethylene formed [pmol/40 min]
2 μmol KMB	560
4 μmol MMP	1380
5 μmol L-methionine	0
5 μmol α-ketoglutarate	0

Table II. Dependence of ethylene formation by chloroplast lamellae in the dark on reduced NADP. For experimental conditions see Material and Methods; 4 μmol MMP were used as substrate.

	Ethylene formed [pmol/40 min]
complete system	1600
minus glucose-6-phosphate	0
minus glucose-6-phosphate dehydrogenase	46

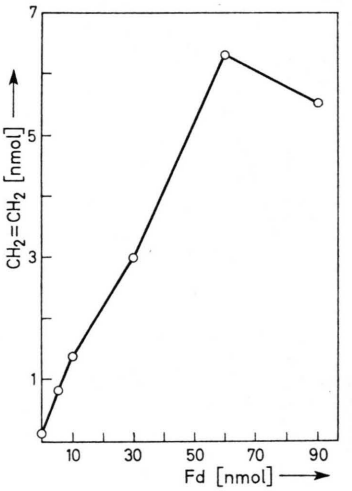

Fig. 1. Stimulation by ferredoxin of ethylene formation by chloroplast lamellae from methylmercaptopropanal (MMP) in the dark. For experimental conditions see Material and Methods.

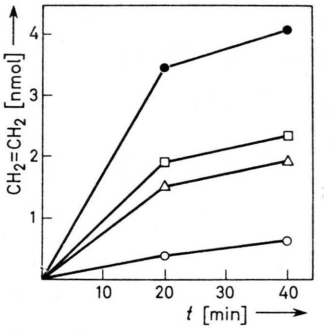

Fig. 2. Stimulation by anthraquinone-2-sulfonic acid (AQ) of ethylene formation by chloroplast lamellae from MMP in the absence of ferredoxin. For experimental conditions see Material and Methods. ○—○, 0.02 μmol AQ added; △—△, 0.1 μmol AQ added; □—□, 0.2 μmol AQadded; ●—●, 0.5 μmol AQ added.

Experiments on the mechanism of ethylene formation by chloroplast lamellae in the dark

1. The influence of catalase and superoxide dismutase

Addition of 50 units of catalase results in an almost complete inhibition of ethylene formation by isolated chloroplast lamellae in the dark from MMP (Fig. 3), although it has been shown that H_2O_2 alone is not active in the production of ethylene from the above substrates [12, 14]. Since superoxide dismutase also inhibits the reaction, the superoxide free radical ion seems to be necessary in addition to H_2O_2 in order to produce ethylene from MMP (Fig. 4).

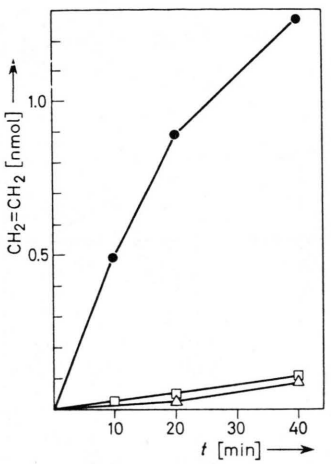

Fig. 3. Inhibition by catalase of ethylene formation by chloroplast lamellae from MMP. For experimental conditions see Material and Methods. ●—●, Without addition; □—□, with 50 units catalase; △—△, with 500 units catalase.

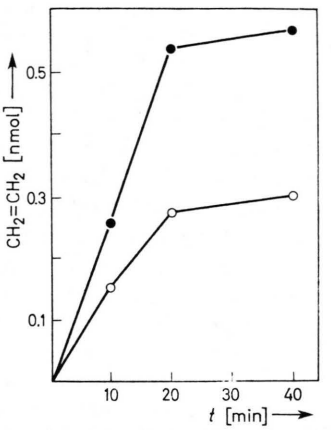

Fig. 4. Inhibition by superoxide dismutase (SOD) of ethylene formation by chloroplast lamellae from MMP. For experimental conditions see Material and Methods. ●—●, Without addition; ○—○, 10 units superoxide dismtuase added.

2. The influence of ethanol and ascorbate

Ethanol as a radical trap has been shown to strongly inhibit ethylene formation from MMP [12] in the presence of xanthine and xanthine-oxidase as a source for OH-radicals. An influence of ethanol on ethylene formation from MMP [27] or KMB [14] by other systems has also been reported, although an inhibition of more than 30% was not observed. Ethylene formation by isolated chloroplasts in the

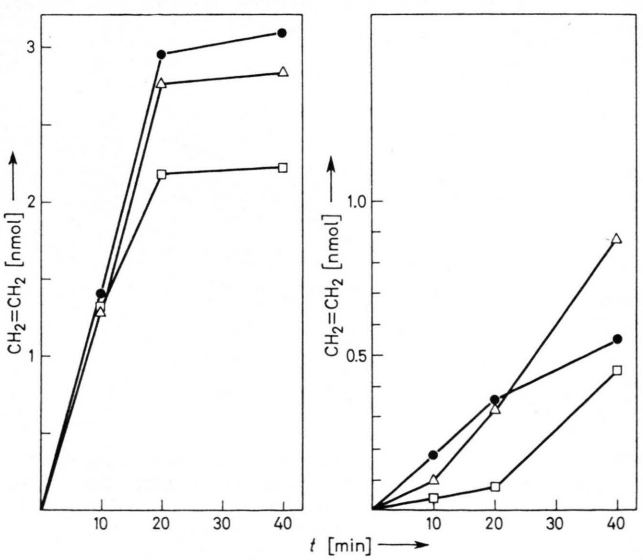

Fig. 5. Influence of ethanol on ethylene formation by chloroplast lamellae from either MMP (a) or KMB (b). For experimental conditions see Material and Methods. ●—●, Without addition; △—△, in the presence of 10^{-2} M ethanol; □—□, in the presence of 5×10^{-2} M ethanol.

dark is inhibited by ethanol to various extents, depending on the substrate (Fig. 5): ethylene formation from MMP is inhibited by 5×10^{-2} M ethanol to about 25% after 20 min dark reaction, while ethylene formation from KMB by the same ethanol-concentration is inhibited to more than 75% after 20 min dark reaction. After 20 min reaction, however, the influence of ethanol seems to decrease indicating that ethanol does not inhibit the formation of the oxidizing species, but inhibits the reaction of the oxidant with the substrate. Apparently ethanol is oxidized after a certain time of reaction, as indicated by the comparism of the kinetics of inhibition of 5×10^{-2} M ethanole with the one of 10^{-2} M ethanol. The reaction seems to be accelerated after the period of inhibition, assuming the initial kinetics of the non-inhibited reaction.

A similar observation as reported above for the influence of ethanol can be made by studying the influence of ascorbate on ethylene formation by chloroplast lamellae in the dark. As previously reported, ascorbate does not inhibit ethylene formation from KMB by illuminated chloroplast lamellae in the presence of artificial electron acceptors like triquat ($E'_0 = -550$ mV) or methylviologen ($E'_0 = -440$ mV), but does inhibit the reaction in the presence of ferredoxin. As shown by Fig. 6, ascorbate inhibits ethylene formation by chloroplast lamellae in the dark from both MMP and KMB. With the lowest concentration of ascorbate applied there is an inhibition of the reaction in the first 20 min, but later an acceleration of ethylene formation is observed. The rate of reaction after this inhibition period is (as in the case of ethanol) indentical with the initial rate of the non-inhibited reaction.

Experiments with isolated NADP-ferredoxin reductase

Isolated NADP-ferredoxin reductase in the presence of NADPH and either ferredoxin or an artificial electron acceptor like methylviologen or AQ presents a convenient system for monovalent oxygen reduction [7, 16]. This system may reflect the reactions involved in monovalent oxygen reduction by isolated chloroplast lamellae in the dark, except that there seems to be yet another factor involved (ORF = oxygen reducing factor) [28], participating in the production of H_2O_2 by chloroplast lamellae at the expense of NADPH. This factor seems to be acticated by autooxidation of reduced ferredoxin [29], a reaction which has been shown to represent a one electron step, yielding $O_2^{\cdot -}$ [30].

Table III. Ethylene production by isolated NADP-ferredoxin reductase in the presence of NADPH and ferredoxin. The reaction mixture contained in 1 ml: 25 μmol phosphate buffer pH 7.6; 10 μmol glucose-6-phosphate; 1 μmol NADP; 20 μg glucose-6-phosphate dehydrogenase; 4 μmol MMP. The reaction was carried out for 40 min at 20 °C.

Additions	Ethylene formed [pmol]
none	0
NADP-Fd-reductase *	0
20 nmol Fd	305 **
0.1 ml ORF ***	0
20 nmol Fd + 0.1 ml ORF	935
NADP-Fd-reductase + 20 nmol Fd	2200
NADP-Fd-reductase + 0.1 ml ORF	0
NADP-Fd-reductase + 0.1 ml ORF + 20 nmol Fd	3500

* Ammoniumsulfate-fraction with 75 μg protein.
** The Fd-preparation still contained NADP-Fd-reductase.
*** See Materials and Methods.

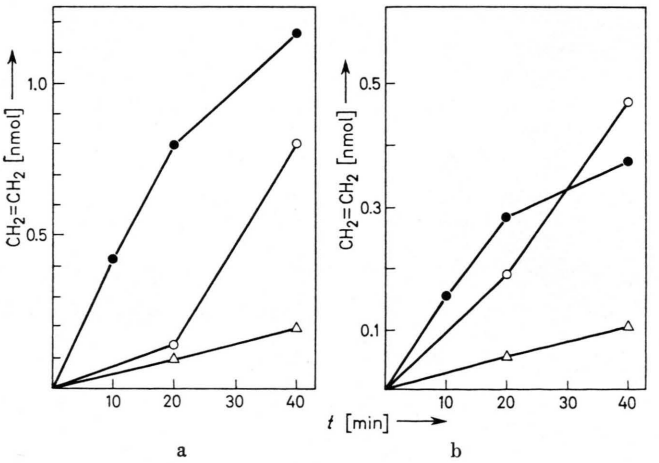

Fig. 6. Influence of ascorbate on ethylene formation by chloroplast lamellae from either MMP (a) or KMB (b). For experimental conditions see Material and Methods. ●—●, Without addition; ○—○, in the presence of 10^{-4} M ascorbate; △—△, in the presence of 10^{-2} M ascorbate.

As shown by Table III, NADP-ferredoxin reductase in the presence of NADPH and Fd, is active in the formation of ethylene from MMP. ORF in the presence of Fd shows a synergistic effect on the reaction.

Discussion

Isolated chloroplast lamellae have been shown to produce ethylene from methionine derivatives in the light [13, 14]. This communication describes the conditions for the production of ethylene from methionine derivatives (MMP or KMB) by chloroplast lamellae in the dark. This reaction is dependent on NADPH as electron donor and ferredoxin as a cofactor for monovalent oxygen reduction producing both H_2O_2 [8–10] and $O_2^{\cdot-}$ [30, 7]. The dependence on both H_2O_2 and $O_2^{\cdot-}$ of ethylene formation from either MMP or KMB can be concluded from the inhibition by both catalase and SOD (*cf.* ref. 12 – 14 and ref. 27). The inhibition of ethylene production form MMP or KMB by catalyase, SOD and ethanol provides evidence that neither H_2O_2 nor $O_2^{\cdot-}$ are direct oxidants, but rather the OH-radical, derived from the dismutation of H_2O_2 and $O_2^{\cdot-}$ according to the Haber-Weiss reaction [11, 12] (*cf.* Eq. (1)).

The observation that ascorbate inhibits ethylene formation from both MMP and KMB by isolated chloroplasts in the dark is in agreement with a previously postulated mechanism for oxygen reduction by chloroplast lamellae [28, 29] which includes the function of an oxygen reducing factor (ORF). This factor is not involved in a light-dependent O_2-reduction in the presence of low potential dyes, but it is involved in a light-dependent reaction in the presence of ferredoxin and chloroplast lamellae as well as in the dark reactions in the presence of NADPH, chloroplast lamellae and ferredoxin. Ascorbate as a scavenger for the superoxide free radical ion inhibits the activation of membrane-bound ORF (*cf.* ref. 29). As soon as ascorbate is oxidized, however, ORF is activated by $O_2^{\cdot-}$ and functions as an oxygen reductant (*cf.* Fig. 5). The function of ORF can also be demonstrated by a model reaction, using isolated NADP-ferredoxin reductase, NADPH and ferredoxin as an oxygen reducing system. The results presented in Table III indicate that ORF enhances ethylene formation. ORF in the absence of Fd is inactive.

The formation of OH-radicals by chloroplast lamellae in the presence of either NADPH as electron donor or in the light (with H_2O as electron donor) may also be the explanation for the findings of Trebst and Eck [31] and Gestetner and Conn [32], who demonstrated that isolated chloroplast lamellae may be responsible for para- [31] or orthohydroxylations [32] of aromatic compounds (*cf.* ref. 33). *ortho*-hydroxylation of cinnamic acid by chloroplast lamellae was observed under similar conditions as demonstrated for ethylene formation, either in the presence of NADPH as electron donor or in the light. Furthermore, evidence has been provided that reactions similar to the ones indicated for ethylene formation in the dark by chloroplast lamellae might be operating during the desaturation of fatty acids in *Euglena* [34] or in the case of ω-hydroxylation of alkanes or fatty acids by *Pseudomonas oleovorans* [35]. The latter system has been shown to produce the superoxide free radical ion in the presence of NADH, a flavoprotein and rubredoxin [36].

Ethylene formation and hydroxylations of aromatic compounds may represent examples of the potential of chloroplasts to monovalently reduce oxygen both in the light and in the dark.

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