Plant Physiol. (1980) 66, 797–800
0032-0889/80/66/0797/04/$00.50/0

Copper-mediated Lipid Peroxidation Processes in Photosynthetic Membranes[1]

Received for publication February 6, 1980 and in revised form April 25, 1980

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ABSTRACT

The phytotoxic effect of Cu via the photosynthetic electron transport system was studied with isolated spinach chloroplasts. Cu(II) ions induce a light-driven peroxidation of membrane lipids leading to ethylene formation, the latter dominating over a concurrent ethane production. Seemingly, the hydroxyl radical originating from superoxide anion is the starting reactive O_2 species. Cu ions inhibit photosynthetic electron transport and apparently catalyze the formation of hydroxyl radical and Fenton-type reactions that result in destruction of unsaturated membrane fatty acids. The concept on the mode of action of Cu(II) and Cu(I) ions in lipid peroxidation as presented here suggests the influence of Cu on different reactions. Two sites are in the photosynthetic redox system; Cu participates in two Fenton-type reactions and in the conversion of ethyl radical to ethylene and ethane.

Studies of fluorescence and Cyt *f/c* photooxidation in intact cells indicated that Cu blocks the photosynthetic electron transport at both the oxidizing side of PS II and the reducing side of PS I (5). For the green alga, *Scenedesmus*, different sites of Cu attack are known, and Cu in high concentrations is toxic to most species (23). Membranes and Chl are destroyed by peroxidation reactions in the presence of toxic Cu concentrations (20). The destruction of membrane lipids became apparent from the concurrent formation of C_2H_6 and C_2H_4 that could be traced by gas chromatography. Peroxidation reactions in the photosynthetic tissues are initiated by either excited Chl or by O_2 species resulting from the superoxide anion radical (see ref. 10 for review). Spinach chloroplast lamellae provide a system to test the deleterious effect of Cu on lipid peroxidation in conjunction with the treatment of chloroplasts with herbicides like MV[2], DCMU, or diphenyl ethers (K. J. Kunert and P. Böger, unpublished). Thus, strong peroxidation by excited Chl was reported after blocking the elctron flow with DCMU (24), whereas O_2^- is vigorously generated by transfer of electrons from PS I via added MV to molecular oxygen (12).

This study was undertaken to obtain a better understanding of the phytotoxic action of Cu. The experimental data allow for a hypothesis on Cu-mediated lipid peroxidation driven by the photosynthetic electron transport and pigment system.

[1] This study was supported by the Deutsche Forschungsgemeinschaft through its Sonderforschungsbereich 138.

[2] Abbreviations: MV (methylviologen), 1,1'-dimethyl-4,4'-dipyridylium dichloride; DAD (diaminodurene), 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea; SOD, superoxide dismutase DABCO, 1,4-diazobicyclo-2,2,2-octane.

MATERIALS AND METHODS

Chloroplasts from fresh spinach leaves (*Spinacia oleracea*, strain Atlanta) grown in the open during fall were prepared according to reference 4 and suspended in 50 mM phosphate buffer (pH 7.5), including 5 mM $MgCl_2$. To obtain chloroplasts with high endogenous O_2 uptake (as in Table V), the harvested leaves were stored at room temperature for 8 h prior to chloroplast preparation.

Chloroplast incubation for the determination of either the light-induced formation of C_2H_6, and C_2H_4 or O_2 was carried out in a Warburg apparatus (Braun, Melsungen) using 150 w/m^2 light, provided by simple tungsten light bulbs. The reaction mixture to measure O_2^- generation included, in a 2-ml reaction volume: 50 mM phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM NH_4Cl, 0.5 mM hydroxylamine, and chloroplast material equivalent to 100 μg Chl. The time of incubation was 10 min. Then, the nitrite formation from the added hydroxylamine due to the presence of superoxide was measured as described (9).

The medium for production of C_2H_6 and C_2H_4 contained 10 mM phosphate buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM NH_4Cl, and chloroplasts equivalent to 200 μg Chl in a 2-ml final reaction volume. Incubation was over a period of 1 h in 10-ml vessels sealed with gas-tight rubber caps. The separation of both gases was performed with a 2-m column of activated alumina using a temperature program from 60 to 140 C and a gas chromatograph (model F22, Perkin-Elmer) equipped with an automatic headspace sampler (20).

Photosynthetic $NADP^+$ and DCIP reduction was performed and measured continuously in 50 mM phosphate buffer (pH 7.5), including 5 mM $MgCl_2$ and 5 mM NH_4Cl, using a split-beam spectrophotometer (Hitachi, model 124) equipped with a 40-w tungsten lamp for actinic cross-illumination. In addition, the $H_2O \rightarrow NADP^+$ reaction mixture contained 0.4 mM $NADP^+$ and 2 μM *Bumilleriopsis* ferredoxin; with DAD as electron donor, this assay included additionally 1 mM sodium ascorbate, 100 μM DAD, and 100 μM DCMU. The DCIP concentration was 50 μM when using the $H_2O \rightarrow$ DCIP system.

O_2 uptake of isolated chloroplasts was measured with a Clark-type O_2 electrode with red light (140 w/m^2) using an RG 610 Schott cut-off filter and a KG 1 heat filter (3).

Each experiment was repeated three times. The data given are from a representative assay. The control values varied by up to 20% according to chloroplast preparation. The variation in the experimental data relative to the control measured after the addition of the compounds indicated in the tables was in the range of 5 to 10% only.

Chemicals, pro analysi grade, were purchased from Merck, Darmstadt. MV and DAD were from Serva, Heidelberg. DCMU was purchased from Riedel de Haen, Hannover. SOD from bovine blood was from Sigma, München; its units are expressed according to reference 16.

RESULTS

Peroxidative hydrocarbon formation in illuminated chloroplast lamellae as a quantitative marker of lipid peroxidation (10) was increased in the presence of Cu(I) or Cu(II) salts (Table I).. In addition, a shift from C_2H_6 to C_2H_4 production resulting in higher C_2H_4/C_2H_6 ratio was observed at higher Cu(II) concentrations in comparison with Cu(I). Formation of C_2H_4 and C_2H_6 was somewhat higher in the presence of MV (Table II). Cu(II) stimulated hydrocarbon output, again favoring C_2H_4 formation. This stimulation was increased by the presence of superoxide dismutase or DCMU.

In a system where photosynthetic electron transport is blocked by DCMU, lipid peroxidation was stronger than in the control (Table III). Simultaneous addition of Cu(II) again resulted in a stimulation of hydrocarbon formation that could be partially inhibited with 1,4-diazobicyclo-2,2,2-octane, a quencher of singlet oxygen ($^1O_2$) (17). When DCMU was present, MV had no additional effect on Cu-treated or Cu-free chloroplasts.

Table I. *Formation of C_2H_6 and C_2H_4 by Spinach Chloroplasts in the Presence of Cu(I) and Cu(II)*

Cu(I) was added as CuCl; Cu(II) was added as $CuSO_4$.

Additions	C_2H_6	C_2H_4	C_2H_4/C_2H_6
	nmol/mg Chl·h		*ratio*
Control	11.6	0	0
+ Cu(I), 10 μM	7.9	4.5	0.6
+ Cu(I), 50 μM	11.8	29.1	2.5
+ Cu(II), 10 μM	7.4	11.2	1.5
+ Cu(II), 50 μM	5.5	24.6	4.5

Table II. *Influence of Cu(II) on C_2H_6 and C_2H_4 Formation with Isolated Chloroplasts in the Presence of 250 μM MV*

Additions	C_2H_6	C_2H_4
	nmol/mg Chl·h	
Control	14.4	0
+ Cu(II), 50 μM	12.3	28.3
+ Cu(II), 50 μM + SOD, 100 U/ml	14.2	37.7
+ Cu(II), 50 μM + DCMU, 1 μM	29.7	27.1

Table III. *Influence of Cu(II) on C_2H_6 and C_2H_4 Formation with Isolated Chloroplasts in the Presence of 1 μM DCMU*

Additions	C_2H_6	C_2H_4
	nmol/mg Chl·h	
Control	26.0	0
+ Cu(II), 50 μM	33.6	20.1
+ Cu(II), 50 μM + DABCO, 30 mM	24.7	7.0
+ Cu(II), 50 μM + MV, 250 μM	34.4	18.9

Table IV. *Nitrite Formation from Hydroxylamine by Superoxide Generated by Illuminated Spinach Chloroplasts*

Additions	NO_2^- formed
	nmol/mg Chl·h
1. Control	11.2
2. + Cu(II), 50 μM	4.7
3. + MV, 250 μM	37.9
4. + DCMU, 1 μM	0
5. + Cu(II), 50 μM + DCMU, 1 μM	2.9
6. + Cu(II), 50 μM + MV, 250 μM	6.5
7. + Cu(II), 50 μM + MV, 250 μM + SOD, 100 U/ml	4.3
8. + Cu(II), 50 μM + MV, 250 μM + DCMU, 1 μM	3.4

Table V. *Light-induced O_2 Uptake by Chloroplast Material from Aged Spinach Leaves*

Additions	Rate	Light Control
	μmol O_2 uptake/mg Chl·h	*%*
1. Control, light	52	100
2. Control, dark	0	0
3. + DCMU, 1 μM	52	100
4. + Cu(II), 50 μM	11	21
5. + Cu(II), 50 μM + SOD, 100 U/ml	14	27
6. + Cu(II), 50 μM + SOD, 100 U/ml + Azide	21	40
7. + SOD, 100 U/ml	71	137
8. + SOD, 100 U/ml + Azide	87	167

Table VI. *Inhibition of Photosynthetic Electron Transport by Cu Ions with Isolated Spinach Chloroplasts*

Assay plus additions	Rate of $NADP^+$ or DCIP Reduced	Control
	μmol/mg Chl·h	*%*
$H_2O \rightarrow NADP^+$	93	100
+ 10 μM Cu(II)	79	85
+ 30 μM Cu(II)	76	78
+ 50 μM Cu(II)	66	71
DAD/ascorbate → $NADP^+$	78	100
+ 50 μM Cu(II)	66	85
$H_2O \rightarrow$ DCIP	122	100
+ 50 μM Cu(II)	116	95

The rate of superoxide generation with chloroplast material under the influence of various additions is given in Table IV as nitrite formed from hydroxylamine by oxidation with superoxide. MV-treated chloroplasts gave the highest O_2^- concentrations (line 3). The superoxide level, however, was strongly decreased by Cu ions in normal and MV-treated chloroplasts (lines 2 and 6). When SOD was given additionally, this inhibitory effect was intensified (line 7). With DCMU alone present, the concentration of O_2^- was zero (line 4), and only very small amounts could be detected after Cu(II) addition (line 5).

Chloroplasts isolated from aged leaves exhibited a DCMU-insensitive O_2 uptake in the light (Table V). In this case, Cu(II) yielded a strong inhibition of O_2 uptake that could be partially relieved by SOD and even better by SOD plus azide (lines 5 and 6). SOD alone increased the O_2 uptake, so did azide (lines 7 and 8).

Cu salts of the concentrations used in this investigation partially inhibited photosynthetic electron transport (Table VI). With 50 μM Cu(II), inhibition was 30% in the $H_2O \rightarrow NADP^+$ reaction, whereas PS I reaction showed 15% and the $H_2O \rightarrow$ DCIP system a 5% inhibition only.

DISCUSSION

High Cu concentrations that lead to strong lipid peroxidation in intact algal cells (20) also stimulate peroxidation in isolated spinach chloroplasts (Table I). Such a Cu concentration of 50 μM also inhibited photosynthetic electron transport. In contrast to reference 6, only a small decrease in transport in the PS-II region by copper ions could be observed in our chloroplast preparations (Table VI), which agrees with the results of Shioi *et al.* (22). The degree of Cu inhibition of the PS-I region and the overall reaction were in the same range as reported (22).

Using chloroplasts with high electron transfer to O_2 in the light, Cu decreases O_2 uptake (Table V). An analogous reaction with

Fe(III) was mentioned (11). In the presence of SOD and azide for catalase inactivation, however, this Cu effect is partially reversed. This finding suggests that superoxide formed reacts with Cu(II) to give Cu(I) and molecular O_2, thereby lowering the O_2 uptake balance. The O_2 uptake balance is determined by four parameters: photosynthetic O_2 evolution, O_2 uptake by superoxide anion formation from O_2, and liberation of O_2 either by dismutation of superoxide (to H_2O_2 and O_2) or through reduction of Cu(II) by O_2^- (to Cu[I] and O_2). With SOD present, the last reaction cannot occur substantially by lack of O_2^-, thereby lowering liberation of O_2 which leads to increased O_2 uptake.

Further evidence for this conclusion is given in Table IV where it is shown that Cu(II) reduces the O_2^- level in spinach chloroplasts. Apparently, through reduction of Cu(II) by superoxide radical, a certain level of Cu(I) is established in the chloroplast.

Hydroxyl radicals (OH·) are the most likely candidates to start the lipid peroxidation cycle (13). Evidence was given by Beauchamp and Fridovich (2) showing that the C_2H_4 generation from methional is dependent on hydroxyl radicals. They can be formed from O_2^- via H_2O_2 and a Fenton-type reaction with Fe(II) or Cu(I) (14). The Haber-Weiss reaction of O_2^- with H_2O_2 to give OH· is unlikely to occur in biological systems (1). In chloroplasts with good O_2^- formation through MV reduction, Cu salts apparently stimulate OH· production and the rate of peroxidation, especially when SOD is added (Table II). A similar stimulation was observed after addition of ferredoxin and SOD to chloroplasts (25). The starter reaction of lipid peroxidation is an abstraction of H· from α-linolenic acid by the hydroxyl radical. Subsequently, the resulting linolenate radical initiates a chain reaction by binding molecular oxygen to give the peroxy radical that in turn abstracts H· from another linolenic acid molecule and forms linolenate peroxide. This reaction sequence is concluded by data from reference 8; origin and concentration of hydroperoxides should be determined in a separate investigation. The peroxide molecule is reduced to the alkoxy radical lin-O· in a second Fenton-type reaction involving Cu(I) or Fe(II), and splits by β-scission to the corresponding aldehyde and an ethyl radical (ref. 7; *cf.* also ref. 10). The ethyl radical reacts with Cu(II) to produce C_2H_4 and with Cu(I) to produce C_2H_6 (8), thus explaining the higher C_2H_4/C_2H_6 ratios in the presence of Cu(II) as compared to Cu(I) (Table I).

Independent of OH· formation from O_2^- via electron transport, this radical can also be produced by excited Chl either through singlet oxygen or by a more direct mechanism (15). This fact explains why peroxidation is not retarded with DCMU in the presence of MV when photosynthetic electron transport is totally blocked (Table II). C_2H_6 and C_2H_4 formation is high in Cu-treated chloroplasts (Table III) when superoxide formation is inhibited (Table IV). 1,4-Diazobicyclo-2,2,2-octane, an effective quencher of singlet oxygen ($^1O_2$) (17), can overcome DCMU-stimulated peroxidation, thus indicating that $^1O_2$ in addition to superoxide radical is a likely precursor of OH·.

Cu ions participate in a series of reactions leading to lipid peroxidation. First, they inhibit the photosynthetic electron transport both at the reducing side of PS I and, particularly in algae (21), at the oxidizing side of PS II (5), thus providing for O_2 species that are precursors of the highly reactive hydroxyl radical (Fig. 1, I). The block after PS I enables better electron transfer to O_2 and subsequent O_2^- formation that occurs at small but significant rates in untreated chloroplasts (1). Second, Cu in high concentrations competes effectively with catalase for the consumption of H_2O_2. This Cu-catalyzed Fenton-type reaction yielding OH· seemingly proceeds mainly within the chloroplast and may be the favored reaction since catalase is not present in the organelle (18). Third, as a consequence of the Cu-inhibited electron flow from H_2O to PS II, we assume that the excited Chl gives rise to OH· with singlet oxygen as an intermediate in an unknown manner.

The hydroxyl radicals start a peroxidative chain reaction that produces α-linolenate peroxide in a scheme that is outlined in Fig. 1, II and III. Then, Cu again is involved in a second Fenton reaction with this peroxide. At the end of these reaction steps, both Cu(II) and Cu(I) react with the ethyl radical originating from the β-scission of the ω-3-alkoxy radical to give either C_2H_4 or C_2H_6 (Fig. 1, IV).

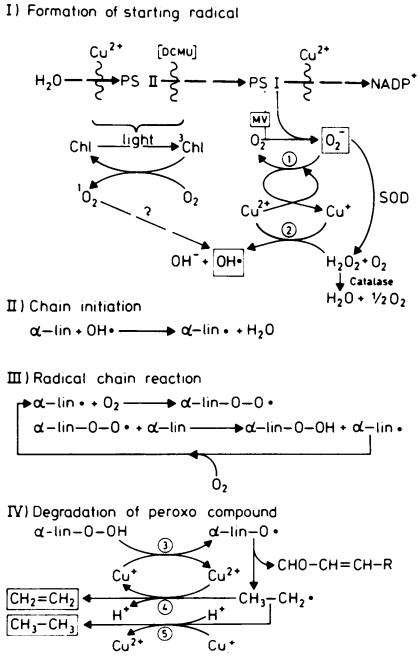

FIG. 1. Copper-mediated lipid peroxidation in the intact cell. In I, the influence of Cu ions on electron transport in the region of PS II and PS I is indicated, as well as the influence on formation of OH· radical from superoxide anion (reactions 1 and 2). Parts II to IV represent the peroxidative reaction steps. Here, Cu ions are necessary for the degradation steps of part IV (reactions 3, 4, and 5). α-lin: α-linolenate; α-lin-O-O·: α-linolenate peroxy radical (ω-3-peroxy radical); α-lin-O·: ω-3-alkoxy radical. For further explanation, see "Discussion."

Acknowledgments—Due thanks are expressed to Dr. K.-J. Kunert for many helpful suggestions and to Mrs. S. Kuhn for expert technical assistance.

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