

A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in *Chlamydomonas reinhardtii*

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Abstract α -Tocopherol concentrations were determined at low and high light intensities and compared with the rate of photosynthesis, photosystem II (PS II) and its reaction center D1 protein. Blocking of tocopherol biosynthesis at the 4-hydroxyphenylpyruvate dioxygenase by the herbicide pyrazolynate led to a quick disappearance of α -tocopherol in high light, as well as of PS II activity and the D1 protein. Homogentisic acid rescued all activities. It is concluded that α -tocopherol has a continuous turnover as a scavenger of the singlet oxygen that arises from the quenching by oxygen of the triplet of the PS II reaction center and triggers the degradation of the D1 protein. Thus tocopherols are essential to keep photosynthesis active. We suggest that this is why plants make and need tocopherols. Chemical quenchers of singlet oxygen, notably diphenylamines, completely protect PS II, prevent D1 protein degradation and keep tocopherol levels even at very high light intensities. This supports the notion that $^1\text{O}_2$ is the intermediate in light triggered D1 protein turnover. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: D1 protein; Diphenylamine; Herbicide; *p*-Hydroxyphenylpyruvate dioxygenase; Photosystem II; Pyrazolynate; Singlet oxygen quencher; Tocopherol

1. Introduction

Tocopherols are localized in plants in the thylakoid membrane of chloroplasts [1]. They are considered general antioxidants for protection of membrane stability [2], including quenching or scavenging singlet oxygen [3]. A detailed function has not been described to date. Here, we assign α -tocopherol a specific and obligatory role in the scavenging of singlet oxygen produced in the quenching of the triplet state of the reaction center of photosystem II (PS II), which would otherwise trigger the degradation of the D1-protein.

The concentration of tocopherols in the chloroplast is light-dependent [2,4] and is increased during senescence [5]. Its biosynthesis (as well as that of plastoquinone) from tyrosine via homogentisic acid [6,7] can be blocked by a mutation in the 4-hydroxyphenylpyruvate (HPP)-dioxygenase [8], by an anti-

sense mutant [9] or deletion [10] in the prenylation steps or by herbicides inhibiting the HPP-dioxygenase [11–13]. This leads to chlorophyll bleaching of the cells, in the case of dioxygenase deficiency attributed to a shortage of plastoquinone in carotene biosynthesis (see [11,13]). In accordance with and extension of the observation by Graßes et al. [9] on the light sensitivity in a phytylation mutant and from their tocopherol deficiency of *Nicotiana* we clarify that the bleaching effect in *Chlamydomonas reinhardtii* in high light and oxygenase inhibition is due to loss of tocopherol and that this is a consequence of an imbalance of tocopherol oxidation and its resynthesis in the continuous turnover of tocopherol as it acts as an $^1\text{O}_2$ scavenger in the degradation mechanism of the D1 protein of PS II.

Furthermore, we show that chemical quenchers of singlet oxygen are effective substitutes for tocopherols in complete protection of D1 protein degradation and maintenance of PS II structure and function even at very high light intensities.

2. Materials and methods

C. reinhardtii was grown autotrophically at 24°C and 70 $\mu\text{E}/\text{m}^2$ (=low light) to a chlorophyll concentration of about 15 μg chlorophyll/ml, which is well below the stationary phase. They were then exposed to the indicated high light intensity. It is important to note that we stop further high light exposure when chlorophyll bleaching sets in. This is because we want to clarify the early events and oxygen species that are the cause and not the consequence of chlorophyll bleaching where uncontrolled and not specified oxygen radicals participate. Inhibitors and quenchers were added at zero time. Optimal concentrations of oxygenase inhibitors (not documented here) block growth of the algae and induce chlorophyll bleaching as in the systems quoted above [8–13]. Therefore, and as discussed below, the oxygenase inhibitors were used at suboptimal concentration to allow for sufficient plastoquinone biosynthesis for photosynthesis and growth at low light. Photosynthesis was measured in vivo by oxygen evolution with CO_2 as substrate. PS I and PS II activities were measured in the membrane fraction after sonication of the cells by oxygen uptake with ascorbate and methyl viologen or by oxygen evolution with ferricyanide as acceptor, respectively. Values are given in μmol oxygen/per mg chlorophyll/h. Tocopherols were separated from an isohexane extraction of freeze-dried cells by high performance liquid chromatography on a Lichrosorb-NH₂-100 column [14]. The D1 protein was immunologically decorated with a rabbit antibody against an overexpressed truncated sequence of the *psbA* gene.

Pyrazolynate ((2,4-dichlorophenyl)-[1,3-dimethyl-5-[(4-methylphenyl)sulfonyl]oxy]-1H-pyrazol-4-yl]methanone) is produced by Sankyo, Japan. Isoxaflutole (5-cyclopropyl-isoxazol-4-yl-2-mesyl-4-trifluoromethylphenyl ketone) is available from Pestanal Service, Riedel-de Haën, Germany.

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Abbreviations: DPA, diphenylamine; HPP, 4-hydroxyphenylpyruvate; PS II, photosystem II

Table 1
Tocopherol content in *C. reinhardtii*

(a) Dependence on growth time and chlorophyll content in low light (70 $\mu\text{E}/\text{m}^2/\text{s}$)			
Growth time (h)	Chlorophyll ($\mu\text{g}/\text{ml}$)	α -Tocopherol (ng/ml)	γ -Tocopherol (ng/ml)
10		2400	47
14		5180	86
18		7370	
18	17	4350	20
22	20	6670	27
26	21	6800	
42	32 (stationary phase)	11 370	315
(b) Dependence on high light intensity			
Light intensity ($\mu\text{E}/\text{m}^2/\text{s}$)	Time of exposure (h)	α -Tocopherol (ng/ml)	
70	18 h LL	1685	
70+250	18+3 h HL	4653	
70+750	18+3 h HL	4185	
70+1500	18+3 h HL	7136	

3. Results

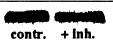
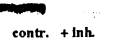
It is well established that the concentration of tocopherols in plants and algae increases in high light [2,4]. Table 1 shows this effect in *C. reinhardtii*. Both α - and γ -tocopherol were detected, the amount of β -tocopherol was not yet quantified. In separate experiments, it is shown that the concentration increases with growth time and is highest in the stationary phase (Table 1a). After growth at 70 $\mu\text{E}/\text{m}^2/\text{s}$ for 18 h and subsequent exposure to higher light the tocopherol concentration rises dependent on light intensity (Table 1b). The values at 26 h and at 750 $\mu\text{E}/\text{m}^2/\text{s}$ are not linear with the others reflecting the relatively poor accuracy of the procedure for tocopherol estimation.

Herbicides which inhibit the HPP-dioxygenase in plastoquinone and tocopherol biosynthesis in higher plants are well established [10–12]. They lead to a bleaching of the plant cells (see [11]). The ‘classical’ inhibitor of HPP-oxygenase, the herbicide Sulcotrione was shown to be a poor inhibitor of *C. reinhardtii* [15]. We found that both pyrazolynate and the active derivative of Isoxaflutole inhibit *C. reinhardtii* growth and eventually bleach the algae very well with identical effects to the experiments described here (only pyrazolynate data are shown). We use, however, a concentration of inhibitors just below maximal inhibition and a time exposure in high light below the onset of bleaching. This allows sufficient plastoquinone biosynthesis for an optimal photosynthesis potential as well as almost undiminished growth in low light in order to study the influence of the inhibitors before chlorophyll bleach-

ing. Plastoquinone concentrations therefore remaining constant in short time high light exposure, the effects described here, are due to a diminished rate of tocopherol biosynthesis.

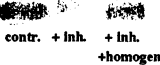
Table 2 shows the effect of the HPP-oxygenase inhibitor pyrazolynate on *C. reinhardtii* grown in low light and added at the beginning of growth. There is no difference in photosynthesis potential and in the amount of D1 protein present. The α -tocopherol content is, however, appreciably lower, though well above a turnover rate. The latter is calculated from the difference minus/plus inhibitor divided by the time of illumination. Exposure of the algae cells to a 20-fold increase in high light for 2 h has a marked effect. After high light in the presence of the inhibitor the photosynthesis potential in vivo is zero, PS II activity in the thylakoid is much decreased, whereas the effect on PS I is much lower. The amount of D1 protein, detected by immunoblotting, is considerably reduced. The α -tocopherol content is low. The turnover rate of tocopherol per hour in high light is more than 10 times compared with that in low light and much higher than the remaining tocopherol concentration (i.e. the pool is almost completely exhausted). Some chlorophyll bleaching has already set in. An exact calculation of the tocopherol turnover rate is somewhat complicated, as there is no linear relation to time. PS II activity provides the singlet oxygen for tocopherol oxidation. However, when the tocopherol level is low, PS II activity declines as well as less $^1\text{O}_2$ being formed. Also, the enzymes in tocopherol biosynthesis are turned on in high light possibly triggered by $^1\text{O}_2$ (see below). Furthermore, as explained above, we use a suboptimal concentration of the di-

Table 2
Influence of an inhibitor (pyrazolynate) of HPP-dioxygenase on photosynthesis, α -tocopherol and D1 protein in low and high light

light intensity	+/- inhibitor 5 μM	chlorophyll change $\mu\text{g}/\text{ml}$	PS rate in vivo $\mu\text{m O}_2/\text{mg chl/h}$	PS II in vitro $\mu\text{m O}_2/\text{mg chl/h}$	PS I in vitro $\mu\text{m O}_2/\text{mg chl/h}$	α - tocopherol content in ng/ml	α -tocopherol turnover rate in ng/h	D1 protein immunoblot
in low light = 70 $\mu\text{E}/\text{m}^2/\text{sec}$								
18 h LL	none	6 to 18	99			4650		
18 h LL	plus	6 to 15	99			1822	157	contr. + inh.
in high light = 1500 $\mu\text{E}/\text{m}^2/\text{sec}$								
18 + 2 h HL	none	18 to 20	90	480	540	5085		
18 + 2 h HL	plus	15 to 11.5	0	30	240	890	2095	contr. + inh.

PS I and PS II were not measured when the photosynthesis rate was high. Pyrazolynate ((2,4-dichlorophenyl)-[1,3-dimethyl-5-[(4-methylphenyl)-sulfonyl]oxy]-1H-pyrazol-4-yl)methanone) was added at zero time.

Table 3
Reversal of dioxygenase inhibition of photosynthesis, α -tocopherol biosynthesis and D1 protein degradation by homogentisic acid

light intensity	+/- pyrazolynate 5 μ M	+/- homogentisic acid 50 μ M	chlorophyll change μ g/ml	PS rate in vivo μ m O ₂ /mg chlorophyll/h	α - tocopherol ng/ml	D1 protein immunoblot
18 h LL	none	none	4 to 18		5270	
18 h LL + 1h HL	none	none			7224	
18 + 1h	none	plus			7625	
18 + 1h	plus	none			815	
18 + 1h	plus	plus			7395	
18 h LL + 2h HL	none	plus	18 to 18	90	7925	 contr. + inh. + inh. + homogent.
18 + 2h HL	plus	none	15 to 11.5	0	465	
18 + 2h HL	plus	plus	18 to 20	81	6217	

LL: 70 μ E/m²/s; HL: 1500 μ E/m²/s. Additions at zero time.

oxygenase inhibitor. With incomplete inhibition of the oxygenase some tocopherol is also formed. These interdependencies are at this time difficult to evaluate for a more precise turnover rate.

In Table 3 the effect of homogentisic acid is shown. It fully reverses the inhibitor effect of pyrazolynate both in low and high light. Photosynthesis potential remains high, the D1 protein is protected, α -tocopherol content remains high and chlorophyll bleaching is prevented. It shows that the inhibitor specifically effects the homogentisic pathway.

The tocopherol turnover demonstrated above is proposed (see Section 4) to be due to the scavenging of singlet oxygen produced in the quenching of the triplet state of P680, which is much increased in high light. Therefore, we tested putative quenchers of singlet oxygen under the conditions in the *C. reinhardtii* system of breakdown of the D1 protein and of PS II in high light and the presence of dioxygenase inhibitors. Table 4 compares a few compounds. Clearly diphenylamine (DPA) and its trifluoro-derivative are very effective in preventing the destructive action of the herbicide on the three parameters measured. Photosynthesis remains high as is the α -tocopherol concentration and the D1 protein is not degraded in spite of very high light. Those compounds with little or no efficiency, the piperidine and histidines, show no clear

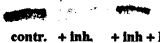
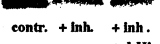
response in the parameters measured. There could be limited access to the site of ¹O₂ formation inside the membrane. The quantitative aspects of chemical quenchers on PS II protection will be further explored.

4. Discussion

The rapid turnover of one of the reaction center polypeptides of PS II, the D1 protein, is long known (see [16–20]). It occurs at any light intensity [18], but is much enhanced by increasing light intensity [19]. It is a consequence of triplet formation of P680, the reaction center of PS II, when the redox state of the plastoquinone pool and of the plastoquinone acceptors Q_A and Q_B of PS II is overreduced. The light triggered loss of D1 protein is compensated in steady state photosynthesis by continuous resynthesis. If the degradation rate is not compensated by the resynthesis rate, PS II proteins disappear (see [17,19]) the pigments come off and chlorophyll photodynamic bleaching sets in leading to further decomposition.

Singlet oxygen appears to be an intermediate in the triggering of the degradation of the D1 protein [21,22]. This singlet oxygen arises from the quenching of P680 triplet [21–25]. This is because surprisingly the two β -carotenes in the reaction

Table 4
Influence of singlet oxygen quenchers on photosynthesis, tocopherol and the D1 protein turnover

Additions in 3 h high light (1500 μ E/m ² /sec)	PS in vivo μ m O ₂ /mg chl/h	PS II μ m O ₂ /mg chl/h	PS I μ m O ₂ /mg chl/h	α -tocopherol ng/ml	D1 protein immunoblot
none	90	480	540	8760	 contr. + inh. + inh. + DPA
+ 5 μ M pyrazolynate	0	30	240	733	
+ " + 5 μ M DPA	101			6830	
+ " + 5 μ M DPA-6	105				 contr. + inh. + inh. + DPA 6
+ " + 10 μ M "piperidine"	30			1830	
+ " + 25 μ M d-histidine	0	24	450	400	
+ " + 25 μ M l-histidine	10	108	450	2199	 contr. + inh. + inh. + d-His + l-His

PS I and PS II were not measured when the total PS rate was high. Additions at zero time. Pyrazolynate: (2,4-dichlorophenyl)-[1,3-dimethyl-5-[[[(4-methylphenyl)sulfonyl]oxy]-1H-pyrazol-4-yl]]methanone; DPA: diphenylamine; DPA-6: 3-trifluoro-diphenylamine; 'piperidine': tetramethylpiperidine.

center PS II seem to be inefficient in quenching P680 triplet [23,26,27], as against the antenna complexes where chlorophyll triplets are efficiently quenched by carotenoids [28]. Likely this is due to a shifted orientation of the (two) carotenes in the D1/D2 protein complex towards the reaction center in PS II, the distance too short for orbital overlap, unlike the situation in the purple bacteria reaction center where the (one) carotenoid does quench [29]. Our results here indicate that tocopherol takes the place of the carotenes in eliminating most of the $^1\text{O}_2$ formed in PS II by the quenching of P680* by $^3\text{O}_2$. In scavenging the oxygen radical the tocopherol is oxidized and the chromane ring opened, a re-reduction is not possible. In $^1\text{O}_2$ scavenging the tocopherol is irreversibly consumed. Continued resynthesis of tocopherol is required to keep the concentration sufficient for PS II protection, i.e. there is continuous turnover of the tocopherol pool.

In this paper, by limiting the resynthesis rate by an inhibitor of HPP dioxygenase and increasing the degradation rate by high light, the turnover cycle was interrupted. The loss of tocopherol was observed and a turnover rate could be estimated. The loss of tocopherol was correlated with the loss of PS II activity and the D1 protein. This connects the physiological role of tocopherol to the turnover of the D1 protein. With this we assign tocopherol not just a general antioxidant effect in the chloroplast but a specific and obligatory role in maintaining PS II active, a function necessary at any light intensity as the P680 triplet is formed even at low light [18,25]. We expect that the enzymes involved in tocopherol biosynthesis are under control of PS II and are overexpressed under high light. Indeed, in *Arabidopsis thaliana* in high light ($250 \mu\text{E}/\text{m}^2$) tyrosine aminotransferase, the first enzyme in the homogentisic pathway to tocopherol, is induced by a factor of three [30,31]. It was also shown that the tocopherol level is much increased under these conditions and declined in the presence of Sulcotrione [32]. The induction of the HPP-dioxygenase is shown to occur under senescence [5], a situation with similarity to stress conditions.

The bleaching of chlorophyll is the phenotype of inhibition by herbicides [12,13] or mutation [8] of the HPP-dioxygenase. It was so far explained by a deficiency in carotene biosynthesis for protection from unspecified oxygen radicals. This was taken as another indication for the still controversial role of plastoquinone participating in the oxidation of phytoene by the phytoene desaturase [33,34]. However, the results here explain the bleaching not by plastoquinone but by tocopherol deficiency. The failure of protection of PS II from singlet oxygen, generated in PS II, triggers the degradation of the D1 protein and the disassembly of PS II subunits. This liberates free chlorophyll for photodynamic bleaching.

We have tested chemical quenchers of singlet oxygen (see [23]) under conditions where limited tocopherol concentrations lead to the rapid disappearance of PS II activity and D1 protein degradation. In diphenylamines we found very effective compounds. Even under high light intensities and limited tocopherol synthesis they completely prevent D1 protein degradation, PS II activity and photosynthesis in vivo is maintained. Also the level of tocopherol remains high. The concentration of DPAs used is below that shown to be necessary for PS II inhibition [35]. Substituted diarylamines are shown to be also ADPR reagents in the accelerated deactivation of the water splitting enzyme system Y [36]. The pre-

sumed singlet oxygen quenchers in the *Chlamydomonas* cells were also tested in an artificial chemical oxygen quenching system ($^1\text{O}_2$ generated by $5 \mu\text{M}$ rose Bengal in water, illuminated with yellow light and measured by the bleaching of crocetin at 422 nm). The results show clearly that the two DPAs as well as others, are highly effective quenchers, whereas the histidines are not (S. Berry and A. Trebst, in preparation).

It has been shown that the induction of astaxanthin globuli in *Haematococcus pluvialis*, is prevented by DPA [37]. This was taken as an indication for the participation of singlet oxygen in the induction process. We speculate that the chloroplast system may require some singlet oxygen to escape scavenging by tocopherol as $^1\text{O}_2$ might be involved in a signal pathway to regulatory systems in communicating the state of PS II structure and function to responsive elements (see [20,38,39]) in the acclimation to high light and possibly other stress situations.

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