Role of carotene in the rapid turnover and assembly of photosystem II in *Chlamydomonas reinhardtii*

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Abstract Inhibitors of the phytoene desaturase in carotene biosynthesis were tested in the enhanced rapid turnover of the D1 protein of photosystem II in high light exposure of *Chlamydomonas reinhardtii* cells. After 1 h high light on heterotrophically grown cells in the presence of norflurazon or fluridone, photosynthesis activity in vivo and PS II activity in vitro is lost. The D1 protein has disappeared. PS I activity is not affected, nor is the D2 protein. It is concluded that β-carotene is essential for the assembly of the D1 protein into functional photosystem II. It is suspected that bleaching of β-carotene in the reaction center of PS II by high light destabilizes the structure and triggers the degradation of the D1 protein.

Key words: D1 protein; Fluridone; Norflurazon; Photoinhibition; Phytoene desaturase; Rapid turnover

1. Introduction

β-Carotene is a component of the reaction center of photosystem II. Highly purified reaction center preparations of photosystem II with just the D1/D2 polypeptide and cytochrome *b*559 as reported in 1987 [1] contain two molecules of β-carotene per two pheophytines [2]. In the homologous reaction center of purple bacteria a carotene derivative is observed in the X-ray structure [3] close to a monomeric bacteriochlorophyll in the active arm. The role of carotene in a photosystem is seen so far in the photoprotection of the reaction center from triplet states and singlet oxygen [4,5].

The results in this paper show a further obligatory role for carotene in the assembly and stability of the photosystem II protein complex. For studying assembly the rapid turnover of the reaction center polypeptide of PS II, the D1 protein, is followed in *Chlamydomonas reinhardtii*. In this rapid turnover the D1 protein is continuously degraded, but also resynthesized and reassembled into a functional PS II. The basic properties of the rapid turnover of the D1 protein in low light have been established in *Spirodella* by M. Edelman and his collaborators [6,7]. Enhanced turnover of the D1 protein in *Chlamydomonas* in high light has been extensively studied by Ohad and his collaborators [8,9] and is usually related to the less well defined photoinhibition. We tested known inhibitors of phytoene desaturase [10] – sometimes called bleaching herbicides – in the rapid turnover of the D1 protein under high light conditions in *Chlamydomonas reinhardtii*. If carotene synthesis is blocked by such inhibitors of phytoene desaturase, like norflurazon, the repair of photosystem II in the rapid turnover of the D1 protein in high light on *Chlamydomonas reinhardtii* cells is no longer possible. No stable D1 protein or functional PS II is present.

The results clarify events in the rapid turnover and in photoinhibition.

2. Methods

Chlamydomonas reinhardtii strain 2137+ was grown heterotrophically according to [11], i.e. the medium contained 17.5 mM acetate. After inoculation with 4 μg chlorophyll/ml the cells were used after growth at 70 W/m^2 white light when they had grown to about 16–20 μg chlorophyll/ml, well before they had reached the stationary phase. For the photosynthesis rate in vivo, oxygen evolution in whole cells was measured after the addition of bicarbonate in an oxygen electrode. For the PS I and PS II rates, the cells were broken by short sonication pulses and the membrane fraction spun down and washed [12]. PS I was followed by oxygen uptake with the autoxidizable methylviologen as acceptor and diaminodurene/ascorbate as electron donors in an oxygen electrode. PS II was measured spectroscopically of K-ferricyanide reduction in the presence of catalytic amounts of the lipophilic mediator methylenedioxydimethyl-*p*-benzoquinone. All are standard methods in photosynthesis research. The inhibitors were added either at the beginning of 2000 W/m^2 high light or during growth (see figures).

3. Results

Chlamydomonas reinhardtii cannot grow autotrophically in the presence of norflurazon or fluridone (data not shown). But the cells can grow heterotrophically in the light, i.e. when acetate is present. The growth rate is about 60% of a control without inhibitor. Norflurazon or fluridone added to heterotrophically grown cells (before growth has entered the stationary phase) and then exposed to high light intensity (30 times growth light) there is a complete loss of photosynthesis capacity in vivo (i.e. oxygen evolution in whole cells when bicarbonate added) in 2 h high light (Fig. 1). Thylakoids prepared from these cells have lost about 60% of PS II activity but there is no loss of PS I activity (Table 1). The amount of the D1 protein detected in an immunoblot is greatly reduced (see below). The reason for loss of oxygen evolution in vivo in spite of potentially active PS II is thought to lie in the poise of cyclic electron flow, as discussed below.

When a culture grown heterotrophically for about 16 h (i.e. not yet in the stationary phase) in the presence of an inhibitor and therefore already β-carotene depleted (see [13] for extensive documentation by HPLC in an etiolated higher plant system) is illuminated in high light, then the inhibitory effect is even stronger. The disappearance of PS II activity and of the D1 protein is complete in 1 h (Fig. 2 using fluridone and Fig. 3 with norflurazon). The amount of D2 protein is not smaller (Fig. 3) but will decrease in longer high light illumina-

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Abbreviations: DAD, diaminodurol; DCMU, dichlorophenyldimethylurea; Fecy, K-ferricyanide; MV, methylviologen; PS II, photosystem II

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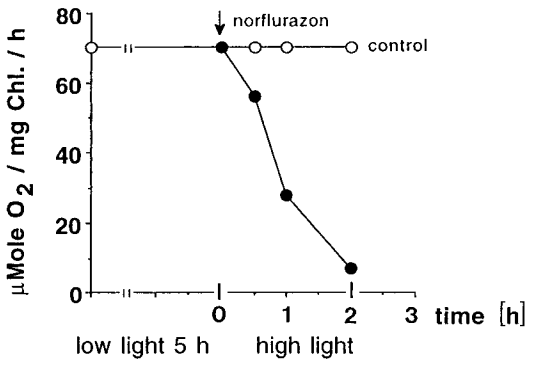

Fig. 1. Effect of norflurazon on oxygen evolution of intact *Chlamydomonas reinhardtii* cells in a high light period. *Chlamydomonas reinhardtii* was grown with acetate in the medium and gased with 5% CO_2/air for 5 h. Then 0.8 µM norflurazon was added and the light intensity increased to 2000 W/m^2.

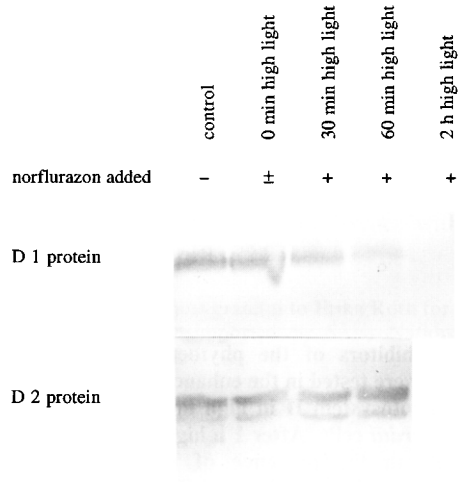

Fig. 3. Effect of high light on the two reaction center protein subunits of PS II of *Chlamydomonas reinhardtii* grown for 16 h heterotrophically in the presence of 0.8 µM norflurazon. Immunoblot with antibodies against a truncated D1 protein (sequence from amino acid 160 to the carboxy end expressed in *E. coli*) and against an oligopeptide of amino acids 235–241 of the D2 protein. Conditions as in Fig. 2.

tion (data not shown). Also even under these conditions PS I is only slightly affected after 2 h high light. After 3 h high light there is massive chlorophyll degradation. In Fig. 4 the influence of the addition of norflurazon or fluridone during or after heterotrophic growth is compared before and after subsequent high light. Cells grown with inhibitor gradually loose photosynthesis activity in vivo to zero after 16 h. Also PS II activity (measured in vitro) is lost, but only to about 30% (for a similar experiment see Fig. 1 and Table 1). In high light the residual PS II activity in these cells is gone. Inhibitor added (arrow) after the growth phase (where there is loss of PS activity neither in vivo nor in vitro) at the onset of high light then photosynthesis in vivo is lost in the high light phase. PS

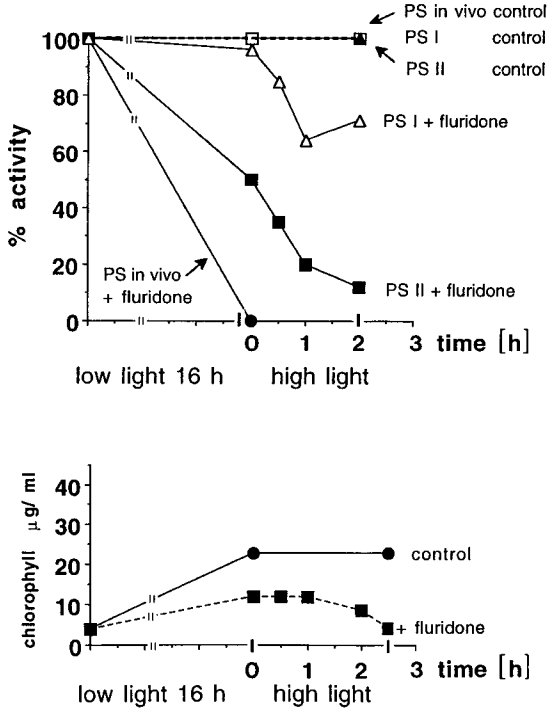

Fig. 2. Effect of fluridone on photosynthetic reactions in *Chlamydomonas reinhardtii* in a high light period. *Chlamydomonas reinhardtii* was grown as in Fig. 1 but with 0.8 µM fluridone already during growth. After 16 h low light, high light was turned on as in Fig. 1. PS I and PS II were measured after the cells had been broken by sonication and membranes centrifuged down.

II activity measured in vitro is lost to about 60%, whereas there is no PS II activity in the cells grown with an inhibitor.

The results show that depletion of carotenes by inhibitors of the phytoene desaturase has a profound effect on the assembly of PS II. In the repair of PS II after photoinhibition and degradation of the D1 protein newly synthesized D1 protein is reassembled again into functional PS II. However, in the inhibitor treated cells, i.e. carotene depleted cells, there is no PS II activity and no D1 protein, i.e. reassembly had not been possible.

4. Discussion

The role of carotenoids in photosynthesis is well established. They are primarily accessory pigments in the antenna proteins and are protecting the chlorophylls (see reviews in [4,5]).

The results presented above show that β-carotene in the reaction center, in addition to its photoprotection, has a further role in the stability of the structure of PS II, because in *Chlamydomonas reinhardtii*, deficient in carotene biosynthesis by inhibiting the phytoene desaturase, PS II is quickly inactivated in high light and the D1 protein disappears. This can be explained by considering the known properties of D1 protein turnover. In the control the loss of the D1 protein in high light is repaired by the incorporation of new D1 protein, which restores the function of PS II. However, when the inhibitors of carotene biosynthesis have depleted the carotene pool the repair of PS II by incorporation of new D1 protein is no longer possible. The loss of the D1 protein and of PS II function indicates that either the translation of new D1 protein (via translational arrest, see Mullet [14,15]) or its assembly into structurally competent PS II is prevented in the absence of carotene. In either case, the correct binding of β-carotene is obligatory for assembly and for stabilization of the folding process. But then it also assures the continued

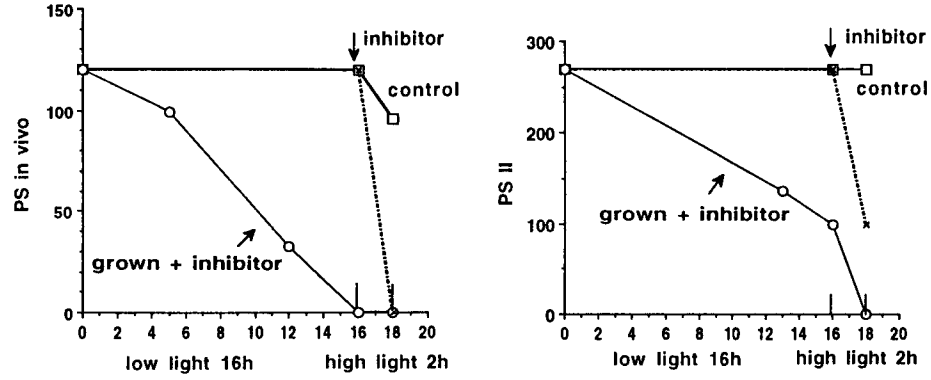

Fig. 4. Photosynthesis activity in vivo and in vitro in *Chlamydomonas reinhardtii* when norflurazon or fluridone was added already during heterotrophic growth or when high light is turned on. 0.8 μM inhibitor was added to cells with 4 μg chlorophyll/ml or after 16 h growth just before high light was turned on for 2 h from 16 to 18 h. PS activity in vivo is oxygen evolution, when CO_2 is added. PS II activity is ferricyanide reduction in a membrane fraction.

stability of PS II; if bleached in high light (and oxygen) the protein structure is destabilized. It is assumed that this role of β-carotene in the stability of PS II is a consequence of the required precise binding and orientation of the two coupled β-carotenes for their protective role in the reaction center.

The same influence of norflurazon on PS II accumulation was reported by Markgraf and Oelmüller [13] in the greening of etiolated mustard seedlings and the same conclusion was already drawn that carotene is required for the assembly of PS II. A similar role of pigment orientation in the assembly of PS II in rapid turnover as reported here for carotene is also likely for chlorophyll synthesis as Feierabend and Dehne [16] observed in the rapid turnover of the D1 protein in mature rye leaves. Probably iron in the quinone binding area of PS II is also required for the proper folding and association of the D1 to the D2 protein to each other.

The need for carotene in PS II is reminiscent of recent data on the role of lutein in the in vitro reconstitution of light harvesting proteins [17].

The experimentation here used the well known phenomenon of rapid turnover of the D1 protein, in which this protein is both rapidly degraded and also resynthesized [7]. The rapid turnover is enhanced in high light. Both degradation and the reassembly rates are increased and therefore they can be studied more easily in a shorter time period. High light leads to photoinhibition of PS II, if the resynthesis rate is no longer competent. The system as used here has been extensively studied in *Chlamydomonas* by Ohad et al. [8,9] as well as in isolated thylakoid systems [18–20]. The trigger for the photodegradation of the D1 protein is not known, but assumed to be damage to the protein [18]. In what way it is damaged is not established nor is there agreement on the primary cleavage site nor is a specific cleavage protease identified. The results here indicate that the destabilization of the PS II structure by loss of one or both (see [21]) of the carotenes in the reaction center is the trigger. Indeed, Barber and Telfer [21,22] have shown that the reaction center carotenes are destroyed by singlet oxygen generated by the reaction center chlorophylls in high light. From the results here on the role of the carotenes in assembly and stability one can now conclude that the bleaching of β-carotenes destabilizes the PS II structure and misorients the D1 protein. The D1 protein is then degraded by one of the proteases that recognize misfolded proteins. These may not be necessarily very specific for a particular misfolded or unassembled protein nor for a primary cleavage site (see Adam for a recent review [23]). If β-carotene is present newly translated (but obviously not the misfolded) D1 protein is reassembled into a functional PS II. This mechanism for enhanced D1 protein degradation holds for the high light conditions used here. But it may also be true for low light conditions.

Heterotrophically grown *Chlamydomonas reinhardtii* was used in the experiments here. These cells do not depend on photosynthetic carbon assimilation as there is acetate present. But the cells do show photosynthesis activity in vivo. This activity in vivo is lost when the algae are grown in the presence of norflurazon or fluridone (Fig. 4). In thylakoids measured after about 12 h growth the activity of PS I is not reduced at all. The activity of PS II is diminished in the inhibitor grown cells when compared with the control. In vitro then activity of both PS II and PS I is measurable but obviously not used in vivo in linear electron flow for complete photosynthesis. We suggest that the ratio of PS I to PS II that remains is the one necessary for the poise of cyclic electron flow through PS I. As known from the poise of cyclic photophosphorylation in vitro [24,25] the rate of electrons from PS II into the plastoquinone pool has to be greatly diminished but not completely blocked to compensate for leakage of electrons from the cyclic system (usually done by adding suboptimal concentrations of DCMU) to allow the reduced ferre-

Table 1
Effect of norflurazon on photosynthetic activities in *Chlamydomonas reinhardtii* after 2 h high light

	μmol O_2 PS in vivo	μmol Fecy PS II in vitro	μmol MV PS I in vitro	D1 protein in % immunoblot
Control	48	221	300	= 100
+0.8 μM norflurazon	0	100	300	40

Conditions as in Fig. 1, i.e. norflurazon present in high light. Activities are per mg chlorophyll and hour and are measured either in vivo or in vitro as explained in Section 2. The D1 protein antibody is explained in Fig. 3.

doxin a chance to find oxidized plastoquinone to complete the cyclic flow. The physiological poise of cyclic photophosphorylation in vivo is unknown. If the assumption is valid then the rapid turnover of the D1 protein has a functional role in reducing PS II activity to a ratio to PS I less than 1:1 in order to allow cyclic electron flow to occur as well. But even cyclic photophosphorylation alone needs some PS II activity – as observed here – or another source of electrons, such as from a NADH-plastoquinone dehydrogenase.

There is a further interesting aspect in that the phytoene desaturase is plastoquinone dependent in higher plants [26,27]. The redox state of plastoquinone could limit carotene biosynthesis, which limits PS II assembly, which poises the redox state of plastoquinone. This way the amount of PSII could be adjusted to the light conditions via the rapid turnover of the D1 protein and β-carotene in its assembly.

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