β-Carotene to zeaxanthin conversion in the rapid turnover of the D1 protein of photosystem II

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Received 9 February 1998

Abstract The carotenoid composition was investigated during enhanced D1 protein turnover in *Chlamydomonas reinhardtii* exposed to high light. After 2 h of high light there was no loss of the D1 protein yet. However, the β-carotene content was significantly reduced. In parallel, an increase of the zeaxanthin content was found, which was higher than can be accounted for by the light-induced de-epoxidation of violaxanthin in the xanthophyll cycle reactions. We therefore assume that β-carotene of photosystem II (PS II) is hydroxylated to zeaxanthin under high light stress. Inhibitors of carotene biosynthesis led to the loss of both PS II activity and D1 protein, indicating the requirement of β-carotene synthesis for the reassembly of PS II in high light. Diuron blocked D1 protein as well as β-carotene turnover. In the presence of chloramphenicol – which allows just one turnover of the D1 protein – 15% of the total β-carotene was lost, calculated to be two β-carotene.

Key words: Carotenoid; D1 protein; Herbicide; Photoinhibition; Rapid turnover; Zeaxanthin

1. Introduction

In addition to their established accessory and protective functions [1] for the photosynthetic apparatus, carotenoids have a structural role in the assembly of both antenna [2] and reaction center [3–5] chlorophyll binding proteins (see [6] for an overview). We have recently observed that the reassembly of photosystem II (PS II) in the rapid turnover of the D1 protein requires carotene biosynthesis [5]. It appeared in these experiments that the two β-carotenes in the reaction center are not available for the reassembly of the D1 protein because they had been modified. Here we report on the possible fate of these β-carotenes in *Chlamydomonas reinhardtii* in high light exposure.

2. Materials and methods

C. reinhardtii strain 2137+ was grown heterotrophically in the presence of 17.5 mM acetate in the medium. 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. Inhibitors were added either directly after inoculation or just before high light illumination at 2000 W/m^2, as indicated in the legends.

For pigment analyses and immunoblotting, cells were collected by centrifugation, resuspended in buffered solution and broken by three sonication pulses of 15 s. Thylakoid membranes were spun down and then either extracted with acetone for HPLC analysis [7] or used for SDS-PAGE and immunoblotting [5]. Photosynthetic activities of such algal preparations have already been described in [5].

3. Results

D1 protein turnover is a well studied phenomenon in both low and high light [8]. It is related to photoinhibition where PS II is inactivated in high light exposure (for review see [9]). The *Chlamydomonas* system used here has been intensively studied by Ohad and colleagues [10]. In the present study we used algae that were still in the logarithmic phase of growth under low light. Under high light exposure for 2–3 h, the resynthesis rate of D1 protein fully compensates for the reassembly of active PS II as seen by immunoblotting and photosynthetic activity (see [5]). Table 1 shows the changes in the carotenoid content under such conditions in a typical experiment. After 2 h high light the amount of β-carotene per chlorophyll decreased by about 32 mmol (= 27%). The decrease of the violaxanthin content by about 46 mmol (= 66%) indicated a high activity of the xanthophyll cycle de-epoxidase. However, the increases of antheraxanthin and particularly zeaxanthin were much higher than can be expected simply from the conversion of violaxanthin. The sum of violaxanthin+antheraxanthin+zeaxanthin (VAZ), i.e. the VAZ pool size, was indeed increased in high light by about 48 mmol. Additionally, the amount of lutein was increased, while the changes in neoxanthin were small, percentagewise. Thus, our data show significant xanthophyll formation in high light. The stoichiometries of the decrease in the β-carotene content and the increase of the VAZ pool size suggest the formation of zeaxanthin from β-carotene under these conditions.

Fig. 1 shows the influence of CPTA, an inhibitor of lycopene cyclases, on the carotenoid content. The algae had been preincubated with CPTA for 6 h in low light. The level of carotenoids is then generally lower. In high light, the amount of β-carotene fell, that of zeaxanthin and of the VAZ pool rose, as before. The marked difference in this experiment is that in the presence of the inhibitor there is no longer PS II activity and no D1 protein is observed in the immunoblot (Fig. 2A). Also the increase of lutein was negligible under these conditions. The effect of chloramphenicol (CAP), an inhibitor of chloroplast protein synthesis, was very similar (Table 2, Fig. 2B). There is still a loss of β-carotene, but the D1 protein was no longer present. These two experiments show that the loss of β-carotene and the increase of the

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Abbreviations: CAP, chloramphenicol; CPTA, 2-(4-chlorophenylthio)triethylamine; Diuron = DCMU, dichlorophenyldimethylurea; PS I, photosystem I; PS II, photosystem II; VAZ, sum of (violaxanthin+antheraxanthin+zeaxanthin)

PII S0014-5793(98)00188-4

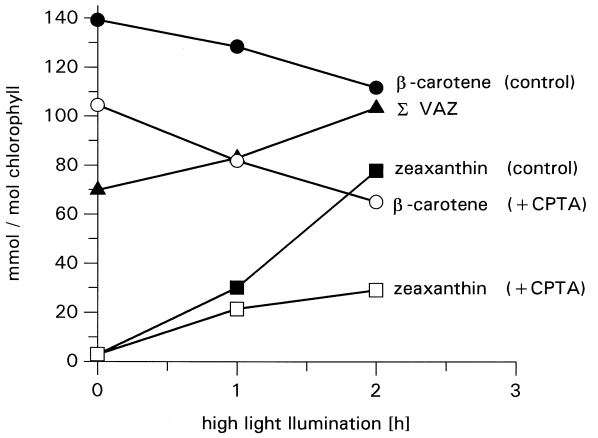

Fig. 1. The effect of CPTA on the changes in the β-carotene and zeaxanthin content of *Chlamydomonas reinhardtii* thylakoids during 2 h high light illumination (2000 W/m^2). 2 μM CPTA was added 5 h before starting the high light treatment. A typical single experiment is shown.

VAZ pool occur independently of carotenoid synthesis and in a step permitted by CAP. The β-carotene value in the sample with CAP should give roughly the amount of β-carotene converted in just one D1 protein turnover. With 15% less than found in the control this is somewhat above what one would expect on the assumption that β-carotene is evenly distributed among PS I and PS II, and that there are eight carotenes present in PS II [16], of which two (= 12.5%) are connected with the reaction center.

In the presence of CPTA, no newly synthesized β-carotene is available for reassembling the D1 protein. By comparison with the CAP experiment (15% of the already present β-carotene is converted in one turnover), the conversion of 30% of the β-carotene in the CPTA experiment indicates that about two more β-carotenes are available for reassembly and a second turnover. However, the origin of these β-carotenes is unclear at present. But definitely not all β-carotene in the membrane can be converted.

Clearly different is the effect of DCMU, which blocks electron flow through the Q_B site and is known to prevent the turnover of the D1 protein [11,12]. In the presence of DCMU there is no β-carotene disappearance (Table 3) and only a very small increase of the VAZ pool size. The increase in zeaxanthin and antheraxanthin is mainly attributable to violaxanthin de-epoxidation, although the de-epoxidase activity is markedly slowed down likely due to a lower pH gradient in the presence of DCMU.

4. Discussion

There are two β-carotenes in the reaction center of PS II bound to the D1 and the D2 protein [13,14]. It could not be shown that these two carotenes are involved in triplet quenching of P680 [15]. But as shown earlier they are obligatory in the assembly of PS II both in the greening of etiolated tissue [4] and in the reassembly of PS II in the rapid turnover of the D1 protein [5]. The results of this work indicate that during high light enhanced turnover of PS II β-carotene is hydroxylated to zeaxanthin. We find that up to 30% of the total β-carotene is converted in time when an inhibitor prevents new synthesis. This appears to be more than there is β-carotene in the reaction center of PS II, as there is also β-carotene in PS I and in the core antenna proteins [16]. Either there is an excess of β-carotene or possibly some of the core antenna proteins can provide some in the reassembly of the D1 protein. The rapid hydroxylation of the β-carotene explains why inhibition of carotene biosynthesis by norflurazon at the phytoene desaturase level (not shown in this paper, but see [5]) or by CPTA at the lycopene cyclases [17,18] leads to an inactive PS II and the disappearance of the D1 protein. There is no longer β-carotene available for reassembly and the D1 protein, likely synthesized, is not stable in the unassembled state. PS I is not affected at shorter times of exposure to high light and carotene limitation. Only when as a consequence of unassembled subunits of PS II chlorophyll liberation and degradation sets in then also PS I starts to become inactivated [5].

DCMU, an inhibitor of PS II, is known to block D1 protein turnover [11,12]. DCMU also prevents the β-carotene conversion in high light, showing the correlation of D1 protein and β-carotene turnover. CAP as inhibitor of D1 protein synthesis leads to the disappearance of the D1 protein but affects β-carotene conversion only slightly, because CAP does not prevent the degradation of the D1 protein present. Together both experiments indicate that β-carotene conversion occurs during degradation of the D1 protein, independent of the PS II reassembly.

High light is known to induce the violaxanthin cycle in which the de-epoxidation of violaxanthin leads to an accumulation of antheraxanthin and zeaxanthin [19,20]. This is clearly seen also in our experiments here. Most importantly,

Table 1
Changes in the carotenoid composition in *Chlamydomonas reinhardtii* after 2 h high light treatment

Pigment	mmol pigment per mol chlorophyll (*a*+*b*)		
	controls	2 h high light	light-induced difference
Neoxanthin	83.3 ± 0.7	75.0 ± 1.2	−8.3
Violaxanthin	69.5 ± 1.3	23.7 ± 0.3	−45.8
Antheraxanthin	7.3 ± 0.6	31.0 ± 1.1	+23.7
Zeaxanthin	4.1 ± 0.6	75.2 ± 2.1	+71.1
Lutein	229 ± 7	289 ± 6	+60.0
Chlorophyll *b*	289 ± 1	275 ± 2	−14.0
Chlorophyll *a*	711 ± 1	725 ± 2	+14.0
α-Carotene	19.7 ± 2.9	17.5 ± 3.3	−2.2
β-Carotene	120 ± 8.2	87.8 ± 5.3	−32.2
Σ VAZ	80.9 ± 2.0	129 ± 2	+48.1

The data represent the mean values ± S.D. obtained from five different measurements of one sample from a typical experiment. The α-carotene content was estimated with the conversion factor determined for β-carotene.

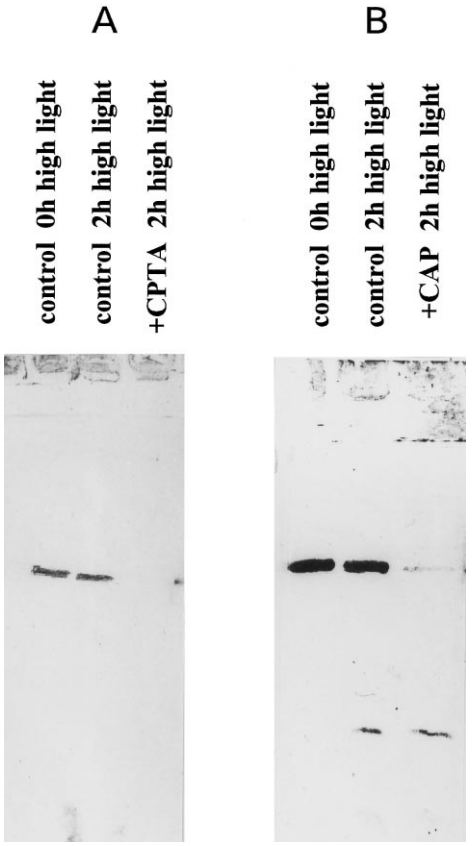

Fig. 2. The effect of 2 μM CPTA (A) and 600 μM CAP (B) on the D1 protein content of *Chlamydomonas reinhardtii* thylakoids after 2 h high light illumination (2000 W/m^2). The relative amount of D1 protein was determined by immunoblot analysis. The same total amount of protein was loaded on each lane. CPTA or CAP was added 5 h or 6 h, respectively, before starting the high light treatment.

however, there is more zeaxanthin formed than can be accounted for from the loss of violaxanthin.

Additional zeaxanthin formation has been observed before in experiments with higher plants [21]. Also β-carotene destruction in high light has been known for decades. Already in the 1950s Hager [22] and Sironval and Kandler [23] showed that in photoinhibition β-carotene disappears before chlorophyll and these two pigments long before xanthophylls are touched. More recently the reduction of the β-carotene pool has been reported, for example by Demmig-Adams [21]. Our

Table 2
Influence of chloramphenicol (CAP) on the changes in the β-carotene content and the xanthophyll cycle pigments during 2 h high light treatment

Pigment	mmol pigment per mol chlorophyll (*a*+*b*)		
	controls	2 h high light	
		−CAP	+CAP
Violaxanthin	70.8	21.4	31.6
Antheraxanthin	3.4	15.0	30.6
Zeaxanthin	2.2	63.6	43.4
β-Carotene	127.4	102.8	108.0
Σ VAZ	76.4	100.0	105.8

CAP (600 μM) was added 6 h before starting the high light treatment.

Table 3
Influence of DCMU on the changes in the β-carotene content and the xanthophyll cycle pigments during 2 h high light treatment

Pigment	mmol pigment per mol chlorophyll (*a*+*b*)		
	controls	2 h high light	
		−DCMU	+DCMU
Violaxanthin	70.4	24.3	53.0
Antheraxanthin	8.3	31.6	20.2
Zeaxanthin	4.8	65.5	21.5
β-Carotene	94.8	51.3	106.2
Σ VAZ	83.5	121.4	94.7

DCMU (1 μM) was added immediately before starting the high light treatment.

results correlate the loss of β-carotene in high light with zeaxanthin formation in excess to the violaxanthin cycle and show that this is due to the D1 protein turnover.

The rapid hydroxylation of β-carotene in D1 protein degradation implies that in the disassembly of PS II β-carotene is immediately accessible to the hydroxylase. This indicates that the β-carotene synthesized from phytoene via lycopene finds a safe route bypassing the hydroxylase when it is used for the assembly of PS II (also for PS I but there it seems not to be obligatory [4,5]). When bound to a protein β-carotene remains protected and – as shown here – is not readily available for exchange to another carotene binding protein. Therefore continuous carotene biosynthesis is essential even in a steady state because of the turnover of PS II. This makes the system so vulnerable to inhibitors of carotene biosynthesis. In high light carotene biosynthesis is enhanced (complicating the calculation of the relative proportions of zeaxanthin from violaxanthin, from β-carotene and from normal biosynthesis). Likely an increased synthesis rate due to a higher PQ/PQH2 ratio is limiting the PQ-dependent phytoene desaturase as PS II is inactivated by the turnover. A similar argumentation for an active biosynthesis in assembly processes is made for chlorophyll where also only newly synthesized chlorophyll can be used for reassembly of the D1 protein in spite of the large excess of chlorophylls in the antenna [24]. Therefore, chlorophyll also has to be inserted on a route protecting it from chlorophyllase.

The role of the violaxanthin cycle is seen in exciton quenching of chlorophyll by zeaxanthin in the antenna [21,25,26]. It might be speculated that the zeaxanthin formed from β-carotene in the reaction center allows exciton quenching in the core antenna. But we do not know yet where the zeaxanthin formed from the reaction center carotene is located. The induction of early light-inducible proteins [27] and in particular of zeaxanthin binding proteins in *Dunaliella* [28] in high light may absorb excess carotenoids.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB 189, TP B13) and by the European Union (C11.CT94-0085).

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