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Assembly of light harvesting complexes II (LHC-II) in the absence of lutein

A study on the α-carotenoid-free mutant C-2A′-34 of the green alga *Scenedesmus obliquus* [1]

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Abstract

Stable assembly of the major LHC-II proteins (22, 26 kDa) is inhibited in the α-carotenoid-free mutant C-2A′-34 of the green alga *Scenedesmus obliquus*, whereas other LHC-II proteins (22, 23, 29 kDa) seem to be not affected. We conclude that lutein is essential for assembly of the major LHC-II proteins of *S. obliquus*, but not for assembly of the minor LHC-II proteins. It is proposed that in the minor LHC-II proteins lutein is replaced by violaxanthin, zeaxanthin and antheraxanthin.

Keywords: Carotenoid; Chlorophyll; Photosystem II; Photosynthesis

1. Introduction

The light-harvesting complex of Photosystem II (LHC-II) in higher plants and green algae consists of various pigment-protein complexes with polypeptide weights of 20–30 kDa. The LHC-II polypeptides are encoded by a nuclear family of genes now named *Lhcb* (formerly *cab*) [1]; in higher plants at least five distinct LHC-II gene products have been identified [1–3]. The in vivo assembly of functional pigment-protein complexes occurs near to or inside the thylakoid membrane [2]. Recently, the role of Chl *a*, Chl *b* and various carotenoids in the assembly process of the pigment-protein complex has become a matter of intensive research [4–7].

The predominant LHC-II proteins, which presumably form trimeric aggregates within the thylakoid membrane, are often denoted as major LHC-II proteins. Possibly, the major LHC-II proteins are located at the periphery of the LHC-II and connected to the PS II core complex (the CP43/CP47 core antenna plus reaction center complex) via the so-called 'minor' LHC-II proteins [1]. In higher plants there are at least four distinct types of minor LHC-II proteins (CP29, CP26, CP24 and CP22) [3].

Lutein, an α-carotenoid, is the predominant carotenoid of the major LHC-II proteins. It is thought to have an important structural function. The crystal-

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[1] Dedicated to Prof. Dr. Horst Senger on occasion of his 65th birthday.

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lographically determined structure of LHC-II proteins of the major type is suggestive that two luteins form an internal cross-brace in the center of the protein [8]. Reconstitution experiments revealed that assembly of the major LHC-II proteins requires lutein [4–7]. However, recently lutein-deficient mutants of *Arabidopsis* were presented whose LHC-II complexes seem to be not affected [9–11].

A significant fraction of the carotenoids found to be present in the minor LHC-II proteins are violaxanthin, antheraxanthin and zeaxanthin which are involved in the so-called xanthophyll cycle [12,13]. Zeaxanthin, which is formed via deepoxidation of violaxanthin, is assumed to play a role in protective energy dissipation under high-light conditions [12,13].

In this study we present results obtained with the α-carotenoid-free mutant C-2A′-34 of the unicellular green alga *Scenedesmus obliquus*, which is a submutant of the greening mutant C-2A′ [14]. Although this mutant lacks the whole α-carotenoid-pool, i.e., lutein, α-carotene and loroxanthin, it is able to live autotrophically in the light [14]. Furthermore, the photosynthetic capacity and quantum yield are not affected by the complete lack of α-carotenoids [15]. However, in contrast to the lutein-free mutants of *Arabidopsis* [9,11] the Chl *b* content of intact mutant cells is strongly decreased in comparison to the wild type indicating that the LHC-II content is reduced [14,15].

For the green alga *S. obliquus*, Hermsmeier et al. resolved three LHC-II polypeptides [16]. The precise number of distinct LHC-II gene products of *S. obliquus* and their amino acid sequence are still unknown. We found indications that at least five LHC-II proteins are present (see Section 3 and Section 4). In the following the predominant type of the LHC-II proteins is called ‘major’ LHC-II and the less dominant types are denoted as ‘minor’ LHC-II proteins. This nomenclature is not intended to indicate that the amino acid sequences are necessarily similar to the sequences of the respective LHC-II polypetides of higher plants.

In this study we compare the pattern of assembled LHC-II proteins of the *S. obliquus* wild type with the mutant C-2A′-34. The results indicate that the hypothesis of an essential lutein requirement for the LHC-II assembly has to be qualified. Lutein is not required for assembly of the minor LHC-II proteins of *S. obliquus*. We propose that in the minor LHC-II proteins lutein is substituted by other xanthophylls.

2. Materials and methods

The mutant C-2A′-34 of the unicellular green alga *Scenedesmus obliquus* was derived by random mutagenesis [14]. Revertants were not observed.

Cultures of the mutant and the wild type (strain D_3) [17] of *Scenedesmus obliquus* were grown heterotrophically for three days as described elsewhere [18]. Then, the cells were exposed to light (100 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 15 h to allow greening of the mutant C-2A′-34, which lacks light-independent protochlorophyllide reductase activity. After 15 h in the light, the mutant shows all pigments of the wild type with exception of the α-carotenoids (α-carotene, lutein and loroxanthin).

Thylakoids were prepared using a cell mill [19] and stored at 77 K. Pigment-protein complexes were separated by two different systems of mildly denaturating green gel electrophoresis: A system modified after Anderson et al. [20] and the system number IV described by Machold et al. [21] and Machold [22]. The former retained most pigments protein-associated but did not separate different types of LHC-II monomers, where the latter yielded an increased fraction of free pigments but resolved several LHC-II monomer bands. Hence, we carried out pigment analysis with pigment-protein complexes obtained by the Anderson gel, and characterized the LHC-II monomers using the Machold system.

The gels were scanned immediately after separation with a densitometer (CD 60, Desaga, Heidelberg, Germany) at 680 nm. For further investigations the bands were cut out of the gel. Room temperature absorption spectra of green gel bands were performed with a dual-beam spectrophotometer with a head-on photomultiplier (UV 3000, Shimadzu, Kyoto, Japan).

For pigment analysis the pigment-protein complexes of green gel bands were electroeluted and extracted with diethylether. After evaporation of the solvent the pigments were transferred into acetone and analyzed by HPLC [23].

Fully denaturating gels (SDS-PAGE) were carried out according to Fling and Gregerson [24]. The proteins were eluted from green gel bands with a buffer

containing 2% SDS, precipitated with 80% aceton and boiled for 5 min in a buffer containing 50 mM DTT, 1% SDS, 0.01% bromophenol blue, 10% glycerol and 50 mM Tris/HCl (pH 6.8). The resolving gel contained 15% acrylamide and was superimposed by a 5% stacking gel. Each lane of the gel was loaded with a volume containing 100 μg of protein. Protein bands were stained with Coomassie brilliant blue.

3. Results

Pigment-protein complexes of the wild type and the mutant C-2A′-34 were separated by mildly denaturating green gel electrophoresis [21]. For wild type and mutant, Fig. 1 shows gel scans (680 nm absorption). The scans were normalized with respect to band 5, which contains CP 43, an essential component of the PS II core. The following bands are resolved (Fig. 1): (1 and 2) PS I with and without LHC-I antenna complexes, respectively, (3) trimers of the so-called 'major' LHC-II proteins probably mixed with some PS I components, (4) the core antenna component CP 47, (5) the core antenna component CP 43, (6 and 7) less prominent 'minor' LHC-II monomers, (8) 'major' LHC-II monomers and (9) free pigment. The identity of the different bands was confirmed by co-electrophoresis of pigment-protein complexes of barley, which are well characterized [21], and by analysis of 77 K fluorescence spectra (data not shown).

It is obvious that the mutant lacks LHC-II trimers (Fig. 1A, band 3) and most of the LHC-II monomers (bands 6–8). Closer inspection (Fig. 1B) shows that band 8, which represents the dominating 'major' LHC-II proteins of the wild type, is completely absent in the mutant. The 'minor' LHC-II monomers in

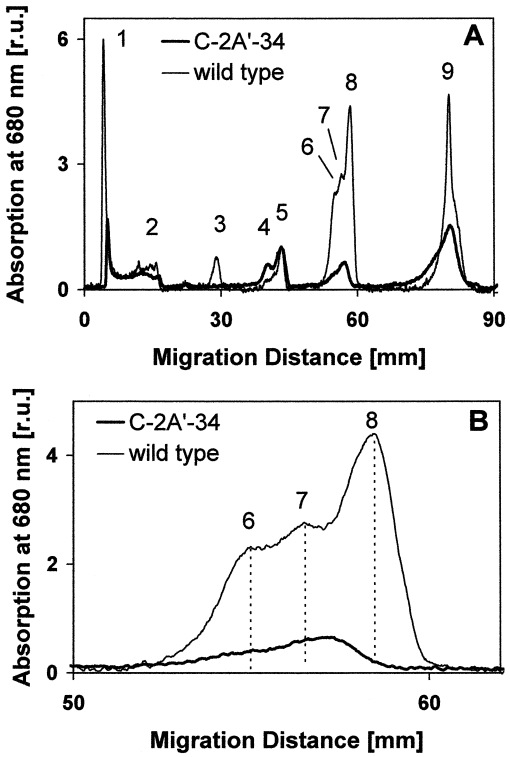

Fig. 1. Absorption scan of a mildly denaturating green gel. Thin line: thylakoids of the wild type; thick line: thylakoids of the mutant C-2A′-34. In A, the complete gel scans are shown; in B, an enlargement of the LHC-II monomer region is displayed. Green gel electrophoresis was carried out according to Machold et al. [21]. The absorption at 680 nm was normalized to the height of peak 5. An assignment of the numbered peaks is proposed in the text.

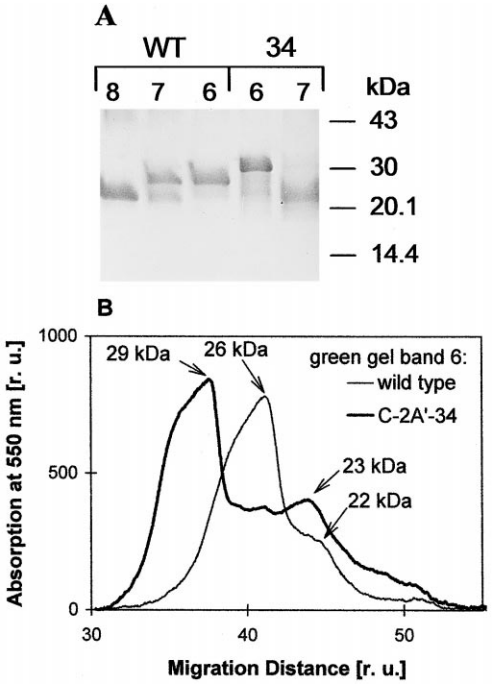

Fig. 2. A: fully denaturating SDS-PAGE of the LHC-II monomer bands (band 6–8 in Fig. 1) cut out of a green gel. Each lane was loaded with 100 μg protein. B: gel scan of the lanes with the proteins of the green gel band 6 of the wild type and the mutant C-2A′-34, respectively. WT, wild type; 34, mutant C-2A′-34, numbers refer to the green gel bands in Fig. 1.

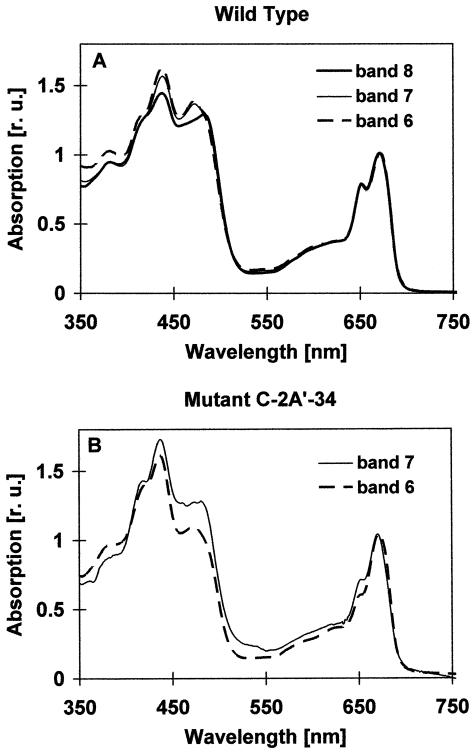

Fig. 3. Absorption spectra of LHC-II monomer bands cut out of a green gel (bands refer to Fig. 1): wild type (A); C-2A′-34 (B). The spectra were normalized to the absorption at 671 nm.

the mutant C-2A′-34 (band 6 and 7) are decreased by about 80% in comparison to the wild type.

Fig. 2A shows a fully denaturating gel (SDS-PAGE) of the different LHC-II monomers (bands 6–8) cut out of the green gel shown before (Fig. 1). For the wild type two major bands are resolved: one with an apparent molecular weight of 22 kDa in band 8 and 7 of the green gel and one with a molecular weight of 26 kDa in band 7 and 6 of the green gel. In the gel scan of the third lane (proteins of green gel band 6) a shoulder with a molecular weight of 22 kDa becomes visible (Fig. 2B).

In the mutant, the dominant LHC polypeptide of the green gel band 6 is characterized by an apparent molecular weight of 29 kDa. The gel scan in Fig. 2B reveals that the band 6 contains also 23 and 26 kDa polypeptides. Band 7 of the green gel contains almost solely a 22 kDa protein. The molecular weight of this polypeptide resembles those of the protein in band 8 of the wild type which is thought to represent the dominating, trimer forming major LHC-II. However, presumably the two proteins are not identical because the migration behavior in the green gel is different. In conclusion, the wild type and the lutein-free mutant differ clearly in their LHC-II pattern (see Section 4).

Room temperature absorption spectra of the green gel bands containing LHC-II monomers from the wild type and the mutant are shown in Fig. 3A and in Fig. 3B, respectively. The spectra were normalized to the Chl *a* peak at 671 nm. In the wild type (Fig. 3A) the ratio between the Chl *b* peak-magnitude (651 nm) and the Chl *a* peak-magnitude (671 nm) remains constant for all LHC-II monomers. The differences in the spectra between 400 nm and 500 nm reflect differences in the xanthophyll pattern.

In the mutant C-2A′-34, both minor LHC-II bands of the green gel show significantly higher 671 to 651 nm absorbance ratios than those of the wild type. This is most obvious for band 6 which contains an unusual 29 kDa LHC-II polypeptide (Fig. 2).

For analysis of the pigment composition of LHC-II monomers, a ‘milder’ green gel system was used in order to reduce pigment release. This system does not

Table 1
Pigment content of the LHC-II monomers of the wild type and the mutant C-2A′-34, respectively

Pigment	Relative pigment content of LHC-II monomers	
	Wild type	C-2A′-34
Lutein	6.5	0
Violaxanthin	0.4	4.5
Antheraxanthin	0.1	2.5
Zeaxanthin	0.2	1.4
Neoxanthin	1.7	3.1
Loroxanthin	0.9	0
Chlorophyll *a*	56	59
Chlorophyll *b*	32	25
α-Carotene	0.8	0
β-Carotene	1.5	3.9
Total pigment content	100	100
Chl *a*/Chl *b*	1.8	2.4
Chlorophyll/carotenoids	7.3	5.3
Xanthophyll cycle pigments	0.7	8.4

The LHC-II monomers were isolated by green gel electrophoresis according to Anderson [20] and the pigments analyzed by HPLC. The LHC-II monomers in this green gel system correspond to the sum of band 6–8 in the green gel shown in Fig. 1. The pigment contents are given in percent of the total pigment content. The standard deviation (four independent measurements) was less than 11% of each value.

lead to a separation of distinct LHC-II monomer bands. The monomer band, which corresponds to the sum of bands 6–8 of Fig. 1, was cut out of the gel and the pigment content was determined by HPLC-analysis. Table 1 demonstrates that the most striking difference in pigment composition, apart from the loss of α-carotenoids in the mutant, is the more than 10fold increased content of xanthophyll cycle pigments (violaxanthin, antheraxanthin, zeaxanthin) in the LHC-II monomers of the mutant. Apparently, lutein (6.5% of total pigment content in the wild type) is substituted by xanthophyll cycle pigments (8.3% of total pigment content in the mutant).

4. Discussion

The comparison of abundance and characteristics of LHC-II proteins from an α-carotenoid free mutant of *S. obliquus* with the wild type reveals: (1) The lack of α-carotenoids (mainly lutein) leads to a decrease in the number of LHC-II proteins per PS II by more than 80%; seemingly, the lack of lutein affects the ability to form stable LHCs. (2) The whole LHC-II pattern of the mutant is changed with respect to the wild type; in particular, the dominating major LHC-II protein is missing in the mutant and other LHC-proteins, which are hardly detectable in the wild type, determine the LHC pattern of the mutant. (3) LHC-II proteins with a low Chl *b* content can be assembled with a high yield without lutein. These findings explain the low Chl *b* content in the mutant cells in vivo (Chl *a*/Chl *b* = 6.5 in mutant cells in comparison to Chl *a*/Chl *b* = 3.1 in the wild type [15]).

The LHC-II antenna system of plants involves various polypeptides. For most higher plants 5–6 LHC-II gene products have been identified [1–3]. For *S. obliquus*, the number and sequences of the involved gene products are unknown.

To rationalize the results shown in Fig. 1, Fig. 2 and Fig. 3, we tentatively assume the presence of at least five distinct LHC-II monomers in *S. obliquus*, which will be denoted as LHCα to ϵ (Table 2). In the following, the abundance and characteristics of these LHC-II types are discussed.

The dominant LHC-II protein of the wild type (LHCα) is the 22 kDa protein found in the major LHC-II band of green gels (band 8). The second prominent LHC-II protein in the wild type is the 26 kDa protein (LHCδ) of the green gel band 6. Band 7 of the green gel seems to be contaminated with LHCα and LHCδ (see below). The mean Chl *a*/Chl *b* ratio of the wild type LHC-II monomers is 1.8 (Table 1). We do not know the Chl *a*/Chl *b* ratios for the individual bands 6, 7 and 8 of the green gel shown in Fig. 1. However, the absorption ratio A_{671}/A_{651} (A_{671} and A_{651}, absorption at 671 nm and 651 nm, respectively) should be closely related to the Chl *a*/Chl *b* ratio. Because this ratio is identical for the green gel bands 6, 7 and 8 ($A_{671}/A_{651} = 1.3$), we conclude that all three bands contain predominantly LHC-II proteins with a Chl *a*/Chl *b* ratio of about 1.8 (Table 1).

In the mutant, band 7 of the green gel contains a 22 kDa protein (LHCβ). LHCβ differs from LHCα

Table 2
LHC-II proteins proposed for *Scenedesmus*

	Green band	Apparent molecular mass(kDa)	Chl *b* content	Wild type	Mutant C-2A′-34	Role of lutein for LHC-II assembly
LHC*α*	8	22	high	++	−	essential
LHC*β*	7	22	low	+	++	not required
LHC*γ*	6	23	?	(+)	+	not required
LHC*δ*	6	26	high	++	+	enhanced yield?
LHC*ϵ*	6	29	very low	(+)	++	not required

The LHC-II proteins were denoted as *α*, *β*, *γ*, *δ* and *ϵ* according to their apparent molecular mass in SDS-PAGE. The second column refers to the green gel bands shown in Fig. 1. The molecular mass as determined by SDS-PAGE is shown in column 3. The Chl *b* content (column 4) was estimated on basis of the absorption spectra shown in Fig. 3. The symbols in the 5th and the 6th column indicate the abundance of the LHC-II type in the wild type and the mutant, respectively: ++, predominant; +, present; (+), possibly present; −, not present.

of the wild type with respect to its Chl *b*-content (high Chl *a*/Chl *b* ratio, $A_{671}/A_{650} = 1.5$) and the different migration behavior in the green gel. In the wild type the green gel band 7 seems to be contaminated by the proteins found in band 8 and 6 (LHCα, LHCδ), but it presumably also contains a particular LHC-II protein, the LHCβ.

Apart from LHCβ the mutant shows a 29 kDa protein (LHCϵ) in band 6 of the green gel ($A_{671}/A_{650} = 1.7$). In this band also a 26 kDa protein (LHCδ or a different LHC-II protein with the same molecular weight) and a 23 kDa protein (LHCγ) are present. Because these two proteins are less prominent in the green gel band 6 than LHCϵ no conclusions with respect to its Chl *b* content can be drawn. Thus, it is not possible to distinguish whether the 26 kDa proteins in the mutant and the wild type are identical. Assuming their identity we have to conclude that the yield of assembly in the mutant is decreased in order to explain the lower LHCδ-content of the mutant.

Presumably, all LHC-II proteins of the mutant are also present in the wild type to some extent. We assume that LHCϵ and LHCγ are also present in the green gel band 6 of the wild type (see gel scan in Fig. 2B).

The results presented above reveal that the loss of α-carotenoids (lutein, α-carotene, loroxanthin) in *S. obliquus* leads to a loss of LHC-II proteins which are the predominant LHC-II types (LHCα, LHCδ) of the wild type organism (Fig. 1, bands 3 and 8). This finding is suggestive that lutein plays an important structural role in the major LHC-II. Kühlbrandt et al. solved the crystal structure of the major LHC-II trimers of higher plants and found two luteins in the center of each monomer [8]. In reconstitution experiments with denaturated LHC-II proteins lutein was found to be essential for stable LHC-II folding [4–6]. Our results confirm that lutein is essential for the assembly of the major LHC-II proteins. It is noteworthy that even in the intact cell with its optimal conditions for protein assembly the major LHC-II proteins require specifically lutein for formation of stable pigment-protein complexes.

Interestingly, however, lutein is not essential for the assembly of all LHC-II gene products. LHCβ, LHCγ and LHCϵ are assembled in the absence of lutein. In these LHC-II proteins of the mutant the content of violaxanthin, antheraxanthin and zeaxanthin is tenfold increased. We propose that the xanthophyll cycle pigments assume the function of lutein in the LHC-II proteins found in the mutant. Accordingly, the xanthophyll cycle pigments could have an important structural function apart from their role in light protection and energy dissipation [12,13]. Presently, however, we cannot decide whether also in the wild type the xanthophyll cycle pigments play an important role with respect to assembly or stability of the minor LHC-II polypeptides.

Pogson et al. [11] also assume that xanthophyll cycle pigments replace lutein in the lutein-deficient mutants of the higher plant *Arabidopsis*. Interestingly, in the *Arabidopsis* mutants the loss of lutein is not accompanied by a decrease of the Chl *b* content indicating that lutein may also be substituted in the major LHC-II. The conclusions of Pogson et al., however, have not been substantiated by analysis of the LHC-II polypeptide composition.

A structural function of violaxanthin was also deduced from experiments on LHC-II reconstitution [4–6] where high yields of LHC-II refolding were solely achieved in the presence of lutein, violaxanthin and neoxanthin. However, LHC-II reconstitution with a reduced yield was also possible in the absence of violaxanthin. Thus, elucidation of the role of xanthophyll cycle pigments for LHC-II assembly and its stability requires further investigations.

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