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Isolation and characterization of a xanthophyll-rich fraction from the thylakoid membrane of *Dunaliella salina* (green algae)†

Kittisak Yokthongwattana,‡ Tatyana Savchenko, Juergen E. W. Polle§ and Anastasios Melis*
Department of Plant and Microbial Biology, 111 Koshland Hall, University of California, Berkeley, CA, 94720-3102, USA. E-mail: melis@nature.berkeley.edu; Fax: +1-510-642-4995; Tel: +1-510-642-8166

Received 6th April 2005, Accepted 2nd September 2005
First published as an Advance Article on the web 5th October 2005

Long-term acclimation to irradiance stress (HL) of the green alga *Dunaliella salina* Teod. (UTEX 1644) entails substantial accumulation of zeaxanthin along with a lowering in the relative amount of other pigments, including chlorophylls and several carotenoids. This phenomenon was investigated with wild type and the *zea1* mutant of *D. salina*, grown under conditions of low irradiance (LL), or upon acclimation to irradiance stress (HL). In the wild type, the zeaxanthin to chlorophyll (Zea/Chl) (mol : mol) ratio was as low as 0.009 : 1 under LL and as high as 0.8 : 1 under HL conditions. In the *zea1* mutant, which constitutively accumulates zeaxanthin and lacks antheraxanthin, violaxanthin and neoxanthin, the Zea/Chl ratio was 0.15 : 1 in LL and 0.57 : 1 in HL. The divergent Zea/Chl ratios were reflected in the coloration of the cells, which were green under LL and yellow under HL. In LL-grown cells, all carotenoids occurred in structural association with the Chl-protein complexes. This was clearly not the case in the HL-acclimated cells. A β-carotene-rich fraction occurred as loosely bound to the thylakoid membrane and was readily isolated by flotation following mechanical disruption of *D. salina*. A zeaxanthin-rich fraction was specifically isolated, upon mild surfactant treatment and differential centrifugation, from the thylakoid membrane of either HL wild type or HL-*zea1* mutant. Such differential extraction of β-carotene and Zea, and their separation from the Chl-proteins, could not be obtained from the LL-grown wild type, although small amounts of Zea could still be differentially extracted from the LL-grown *zea1* strain. It is concluded that, in LL-grown *D. salina*, xanthophylls (including most of Zea in the *zea1* strain) are structurally associated with and stabilized by the Chl-proteins in the thylakoid membrane. Under HL-growth conditions, however, zeaxanthin appears to be embedded in the lipid bilayer, or in a domain of the chloroplast thylakoids that can easily be separated from the Chl-proteins upon mild surfactant treatment. In conclusion, this work provides biochemical evidence for the domain localization of accumulated zeaxanthin under irradiance-stress conditions in green algae, and establishes protocols for the differential extraction of this high-value pigment from the green alga *D. salina*.

Introduction

Carotenoids function in a variety of ways in plants, algae and photosynthetic bacteria. They are essential because they maintain the function of photosynthesis through photoprotection of the photosystems (carotenoid-less mutants are always lethal). Carotenes (*e.g.* β-carotene) are associated with the photosystem core and reaction center proteins, whereas a variety of xanthophylls are found in association with the peripheral light-harvesting complex of the photosystems. The most common function among all carotenoids is that of an accessory pigment, serving in the process of light absorption by the highly organized chlorophyll–carotenoid antenna proteins. However, carotenoids also act as efficient quenchers of excitation energy from triplet chlorophyll, as well as neutralize superoxide radicals and singlet oxygen.[1] Beyond a function in photosynthesis, many carotenoids serve as coloring agents in flowers and fruit to attract insects for pollination and animals for seed dispersion. In addition, carotenoids play a significant role in human and livestock nutrition and, thus, in the pharmaceutical, nutraceutical and cosmetic industries. Many carotenoids are precursors to vitamin A[2] while some have anticancer properties.[3]

Plants, and especially microalgae, have been exploited for the commercial production of carotenoids. For example, under conditions of irradiance stress, the green alga *Dunaliella bardawil*[4] and some strains of *D. salina*[5] are noted for their ability to accumulate β-carotene by a process referred to as "carotenogenesis". The green alga *Haematococcus pluvialis* has also been commercially exploited for the production of the xanthophyll astaxanthin.[6] Nevertheless, exploitation of green algae for the commercial production of other carotenoids, such as the high-value zeaxanthin (Zea), was not practical due to the fact that only trace amounts of Zea are normally found in photosynthetic organisms.

Exposure of plants and algae to suboptimal temperature or irradiance-stress conditions may induce cellular acclimation responses, including changes in the pigment composition of the chloroplast thylakoids and changes in the Chl antenna size of the photosystems.[7–10] In green algae, such acclimation responses are substantially more pronounced than in vascular plants, making them an excellent model system for related investigations. Several publications have reported on irradiance-dependent lowering in the cellular Chl content and attenuation in the size of the light-harvesting Chl antenna size,[10–14] changes in photosystem stoichiometry,[10,11,15,16] and a reversible de-epoxidation of violaxanthin (Vio) to Zea in green algae.[17–25] Accordingly, several algal species are known to accumulate Zea under stress conditions.[17,18,22,25–29] Biochemically, irradiance stress induces a reversible conversion of Vio *via* antheraxanthin (Ant) to Zea, catalyzed by the enzyme violaxanthin de-epoxidase (Vio→Zea).[30] Zea accumulation persists as a property of the HL-acclimated cells, but Zea

† Dedicated to Professor James Barber on the occasion of his 65th birthday.

‡ Present address: Department of Biochemistry, Faculty of Science, Mahidol University, Rama 6 Rd, Bangkok 10400, Thailand.

§ Present address: Department of Biology, Brooklyn College of the City University of New York, 2900 Bedford Ave 200 NE, Brooklyn, NY 11210, USA.

DOI: 10.1039/b504814a

reverts to Vio (a reaction catalyzed by the enzyme zeaxanthin epoxidase), upon removal of the stress conditions that elicited it.

We noted that, under steady-state irradiance-stress conditions, Zea accumulation in *D. salina* was in excess of what could be accommodated within the existing pigment-proteins of the photosystems.[22,31] This observation raised questions regarding the localization of the extra Zea in the HL-acclimated algal cells. In this work, results are presented on the quantitation of chlorophylls and carotenoids in LL- and HL-acclimated *D. salina* wild type and the *zea1* mutant, which constitutively accumulates zeaxanthin and lacks antheraxanthin, violaxanthin and neoxanthin. Further, fractionation studies and domain localization of the carotenoids was implemented in control and irradiance-stressed cells. It is shown that, in HL-grown *D. salina*, there is a substantial pool of carotenoids that are not in close association with the Chl-protein complexes, unlike the case of LL-grown cells, where the carotenoids are integral to these complexes. Specifically, a pool of β-carotene was found to form globules that are extraneous to the thylakoid membrane. Further, a substantial pool of xanthophylls, particularly Zea and lutein (Lut), although bound within the thylakoid membrane, are not associated with the proteins of the peripheral Chl *a*-*b* light-harvesting complex of the photosystems but occupy a distinctly different domain, possibly the thylakoid membrane lipid bilayer. A fractionation protocol is presented for the differential extraction of a xanthophyll-rich fraction, which is substantially free of chlorophyll contaminants.

Materials and methods

Algal culture and growth conditions

The unicellular green alga *D. salina* Teod. (UTEX 1644) was grown photoautotrophically in 1 L Roux-type bottles (path length of 4 cm) at a light intensity range of 50–100 µmol photons m^{-2} s^{-1} (LL) or 2000 µmol photons m^{-2} s^{-1} (HL). The growth medium was an artificial hypersaline described by Pick *et al.*[32] and Kim *et al.*[33] The cultures were gently stirred by magnetic stir bar to prevent cell settling and to ensure uniform illumination.

Measurement of photosynthetic pigments

Chlorophyll (*a* and *b*) and carotenoid content of the samples were determined according to Arnon,[34] with equations corrected as in Melis *et al.*[35] and according to Lichtenthaler,[36] respectively. Detailed pigment composition, including chlorophylls, carotenoids and xanthophylls, was determined by HPLC analysis.[22,37]

Thylakoid membrane isolation and analysis

Thylakoid membranes of *D. salina* were prepared according to Kim *et al.*[33] followed by subsequent sucrose-gradient purification.[38] The functional Chl antenna size of the photosystems was determined by the kinetic-spectrophotometric method developed in this lab.[39] SDS-PAGE and western blot analyses were performed as previously described.[37]

Results

Pigment composition of LL- and HL-grown *D. salina*

D. salina Teod. (UTEX 1644) wild type and *zea1* mutant cultures grow with similar rates under LL (100 µmol photons m^{-2} s^{-1}) and HL (2000 µmol photons m^{-2} s^{-1}) conditions, corresponding to a cell duplication time of about 2–3 per day.[40,41] However, these LL- and HL-acclimated cells have remarkably different phenotypes. When grown under LL, wild type and *zea1* cultures are limited by the low-level of irradiance, they possess a large photosystem Chl antenna size, with 290–300 Chl molecules in PSII and 240–250 Chl molecules in PSI (Table 1), resulting in cultures with a deep green coloration. HL-grown cultures, on the other hand, are acclimated to a state of chronic photoinhibition, in which growth is limited by the high rate of photodamage.[33,40] The HL-grown wild type and *zea1* both have a small Chl antenna size, with 60–65 Chl molecules in PSII and 95–105 Chl molecules in PSI (Table 1), and have a yellowish coloration (Fig. 1, see also Jin *et al.*[22]). Such distinctive phenotypic differences between LL- and HL-grown *D. salina* cultures underline substantial irradiance-induced changes in cellular pigmentation. Table 1 shows the pigment composition of *D. salina* grown under LL- and HL-conditions. LL-grown WT cells contained relatively high amounts of Chl (~42 × 10^{-16} mol $cell^{-1}$) in relation to carotenoids (~21 × 10^{-16} mol $cell^{-1}$). Thus, the Car/Chl ratio in the WT-LL cultures was about 0.5 : 1 and was reflected in the green coloration of the algae. WT-LL-grown cells also had relatively low xanthophyll to chlorophyll ratio (Xan/Chl = 0.4 : 1) (Table 1). There were higher amounts of Neo (~2.3 × 10^{-16} mol $cell^{-1}$) and Vio (~3.7 × 10^{-16} mol $cell^{-1}$) relative to Zea (0.44 × 10^{-16} mol $cell^{-1}$) in these samples (Table 1).

Table 1 Cellular pigment composition in *D. salina* wild type and *zea1* mutant[a]

Pigment content	WT-LL	WT-HL	*zea1*-LL	*zea1*-HL
Chl-PSII	300	65	290	60
Chl-PSI	250	105	240	95
Total Chl	41.51	5.02	51.2	8.20
Total Car	20.63	9.94	18.50	9.92
Car/Chl	0.50	1.98	0.36	1.21
Xan/Chl	0.4	1.65	0.3	1.03
Neo	2.28	0.38	n.d.	n.d.
Vio	3.72	0.18	n.d.	n.d.
Ant	1.07	0.88	n.d.	n.d.
Zea	0.44	3.53	8.33	4.66
Lut	8.95	3.29	6.84	3.77
β-Car	3.54	1.68	3.33	1.49

[a] Cells were grown under low light (LL, 100 µmol photons m^{-2} s^{-1}) or high light (HL, 2,000 µmol photons m^{-2} s^{-1}) conditions. Pigment content is given on a per cell basis (× 10^{-16} mol $cell^{-1}$), determined by HPLC analysis. Both LL- and HL-cell cultures were gently stirred during growth to prevent settling. Results are given as the average of 5 independent measurements, SE in all measurement are less than ±12.4%, n.d.: not detected.

Fig. 1 Cultures of LL- and HL-grown *D. salina*. Under LL-growth conditions, *D. salina* is green due to the dominance of chlorophyll in the photosynthetic apparatus. Acclimation to HL induces changes in pigment content within the algae. The cells have a lower Chl content and assemble a truncated Chl antenna size. Irradiance stress also induces accumulation of zeaxanthin. These changes are reflected in the yellow coloration of the HL-grown cells.

When WT *D. salina* was grown under HL-conditions, cells contained lower amounts of Chl (~5 × 10^{-16} mol $cell^{-1}$) relative to total carotenoid (~10 × 10^{-16} mol $cell^{-1}$). Thus, the Car/Chl ratio in the WT-HL culture was about 2 : 1, and this was reflected in the yellowish coloration of the culture (Fig. 1, see also Jin *et al.*[22]). Under HL-growth conditions, the cellular content of

Neo (0.38×10^{-16} mol cell^{-1}) and Vio (0.18×10^{-16} mol cell^{-1}) was substantially lower than that of Zea (3.53×10^{-16} mol cell^{-1}) (Table 1). These quantitative analyses showed that the WT-HL Zea content (3.5×10^{-16} mol cell^{-1}) was greater by about 800% relative to that in the WT-LL cells (Zea = 0.44×10^{-16} mol cell^{-1}). In consequence of these acclimation adjustments, the Xan/Chl ratio was elevated from 0.4 : 1 in the WT-LL to 1.65 : 1 in the WT-HL.

Cells of the *D. salina zea1* mutant, which constitutively accumulate zeaxanthin and lack antheraxanthin, violaxanthin and neoxanthin, showed the same basic acclimation properties to LL- and HL-growth conditions as the WT (Table 1 and Fig. 1). The only systematic difference between WT and *zea1* was the lack of Neo, Vio and Ant, and the constitutive expression and accumulation of Zea, which was always the dominant xanthophyll in the *zea1* strain (see also Fig. 2). It is of interest to note in this respect that, similar to the wild type, a HL-acclimation of the *zea1* mutant entailed a lowering in the cellular Chl content, resulting in a 3.4-fold increase in the Car/Chl ratio and a corresponding increase in the Xan/Chl ratio (Table 1). In consequence of this acclimation, the Xan/Chl ratio was elevated from 0.3 : 1 in the *zea1*-LL to 1.03 : 1 in the *zea1*-HL.

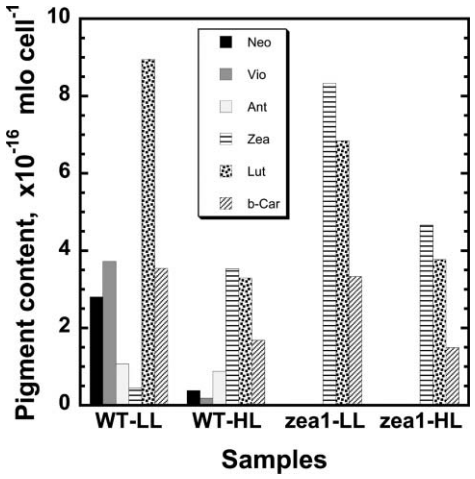

Fig. 2 Bar diagram of the absolute concentration of carotenoids in wild type and *zea1* strains of *D. salina*, acclimated to LL- or HL-growth conditions. Note the relative dominance of Neo and Vio in the WT-LL and the dominance of Zea in the *zea1*-LL or *zea1*-HL acclimated samples.

The above results, showing dominance of zeaxanthin and lutein relative to chlorophyll in the HL-acclimated samples, in combination with the substantially diminished Chl antenna size of the photosystems, raised a question on the localization of these xanthophylls, both in the WT-HL and *zea1*-HL. Under LL-growth, all xanthophylls are thought to be functionally associated with the Chl *a-b* light-harvesting complexes in the thylakoid membrane[42–44] and, therefore, structurally stabilized within these complexes. Under irradiance stress (2000 μmol photons m^{-2} s^{-1}), thylakoid membranes possess diminished amounts of Lhcb and Lhca proteins[45] and the Chl antenna size of the photosystems is substantially smaller than that of the LL-grown cells without a concomitant proportional reduction in the amount of xanthophylls (Table 1). Thus, HL-thylakoids do not possess sufficient amounts of Chl-protein to accommodate the existing xanthophylls, and especially the relatively abundant amounts of Zea. The question of Zea localization in the HL-samples was investigated in detail, see below.

Cellular fractionation and thylakoid membrane isolation from LL- and HL-grown *D. salina*

Models on the association of xanthophylls with the Chl *a-b* light-harvesting complex in chloroplast thylakoids postulate the presence of 3–4 xanthophylls per 10–13 Chl ($a + b$) molecules.[42,43] A xanthophyll (Neo + Vio + Anth + Zea + Lut) to total Chl (Xan/Chl) ratio between 0.3 : 1 and 0.4 : 1 in WT-LL and *zea1*-LL (Table 1) is thus consistent with the notion that most, if not all, xanthophylls are in association with the Chl *a-b* light-harvesting complex. However, in the HL-grown cells, the Xan/Chl ratio ranged between 1.03 : 1 and 1.65 : 1, suggesting an over-abundance of xanthophylls, far greater than the number required to saturate Chl *a-b* light-harvesting proteins that are present in the HL-thylakoids.[45] Three basic questions were raised by the above observations and subsequently investigated in this work: (i) Are all xanthophylls bound to the thylakoid membrane in HL-grown cells? (ii) If so, are these xanthophylls associated with the Chl-proteins of the photosystems in the HL-thylakoid membrane? (iii) Are there hitherto unknown thylakoid membrane domains capable of containing the excess Zea?

In order to investigate the localization of the different carotenoids in HL-grown WT and *zea1* mutant of *D. salina*, cells were subjected to a fractionation protocol, as outlined in Fig. 3. Briefly, cells were disrupted by sonication and debris was removed upon centrifugation at 3000 *g* for 5 min. The pigment-containing supernatant was further centrifuged at 100 000 *g*

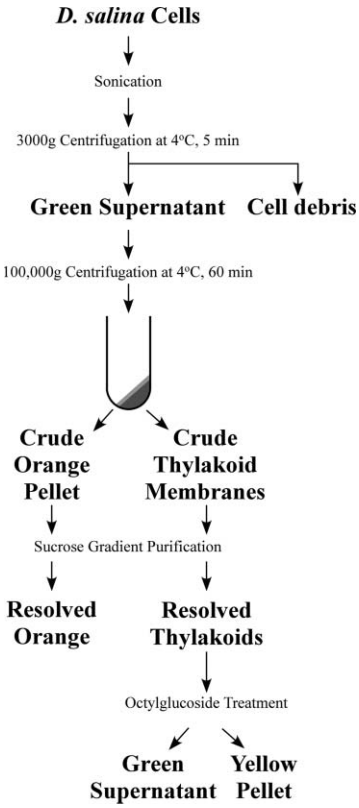

Fig. 3 Flow-chart presentation of a *D. salina* fractionation protocol for the isolation of carotenoid enriched fractions. Cells were suspended in hypotonic buffer containing 50 mM Tricine–NaOH pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl and sonicated for 1 min on ice. The crude sonicated extracts were centrifuged at 3000 *g* for 5 min to remove cell debris. The pigment-containing supernatant was further centrifuged at 100 000 *g* for 1 h. Following this centrifugation, the clear supernatant was discarded. The pellet consisted of a loosely packed 'crude orange' (top layer). Underneath it was the tightly packed 'crude thylakoid' membrane pellet. The 'crude orange' and the 'crude thylakoid' pellets were separated and purified by inverse-step sucrose-gradient centrifugation.[38] At the end of this centrifugation, a 'resolved orange' fraction and a 'resolved thylakoid membrane' fraction were obtained. The resolved thylakoids were suspended in a buffer containing 250 mM Tris, pH 6.8, and 10 mM EDTA at a Chl concentration of ~250 μM, and incubated with 2% (final concentration) octylglucoside (OG) for 15 min on ice (0 °C). Centrifugation at 20 000 *g* yielded two distinct fractions from the OG-treated HL-thylakoids, a soluble 'green supernatant' and a 'yellow pellet'.

for 1 h, yielding two distinct layers of pigmented material in the pellet. On top was a loose 'crude orange' pellet. Underneath it was the tightly packed 'crude thylakoid' membrane pellet. The 'crude orange' and the 'crude thylakoid' pellets were separated and further resolved by inverse-step sucrose-gradient centrifugation.[38] At the end of this centrifugation, a 'resolved orange' fraction and a 'resolved thylakoid membrane' fraction were obtained (Fig. 3). The resolved thylakoids were incubated with 2% octylglucoside (OG) for 15 min on ice (0 °C). Centrifugation at 20 000 *g* was sufficient to separate two distinct fractions from the OG-treated HL-thylakoids, a soluble 'green supernatant' and a 'yellow pellet' (Fig. 3). It is important to note that the 'resolved orange' fraction and the OG 'yellow pellet' fraction could not be obtained upon such fractionation of LL-grown *D. salina*. These were specifically found in chloroplasts from HL-grown WT or the *zea1* mutant. Importantly, other surfactants, such as the non-polar dodecylmaltoside and Triton X-100, or low concentrations of anionic SDS and LDS, could also be employed to fractionate HL-thylakoids and to obtain a soluble 'green supernatant' and a 'yellow pellet' fraction. However, use of high concentration of SDS or LDS (more than 5%) resulted in a complete solubilization of all pigment complexes and loss of the ability to differentially isolate the yellow pellet from the green supernatant fraction.

Pigment composition of the isolated fractions

D. salina LL- and HL-thylakoids from WT and *zea1* mutant were subjected to HPLC for quantitative pigment analysis. The Chl and xanthophyll composition of the resolved HL-thylakoids was about the same as that of the HL-cells in both WT and *zea1* mutant (results not shown), suggesting that the majority of the xanthophylls in the HL-samples are thylakoid membrane-bound. Treatment of the LL-thylakoids of WT and *zea1* mutant with a range of octylglucoside (OG) concentrations did not yield a significant differential separation of carotenoids from the Chl-proteins, suggesting that the former are integral components of the Chl-protein complexes. (Minor amounts of Zea could be extracted upon OG treatment from the LL-grown *zea1* strain.) However, treatment of the HL-thylakoids of either WT or *zea1* mutant with octylglucoside (OG), followed by centrifugation, resulted in the separation of a substantial 'yellow pellet' from the 'green supernatant' (Fig. 3). In the WT-HL sample, the 'green supernatant' contained about 96% of the initial Chl and 63% of the initial carotenoid, whereas the 'yellow pellet' accounted for ~3% of the initial Chl and 36% of the initial carotenoid (Table 2). In the *zea1*-HL sample, the 'green supernatant' contained about 86% of the initial Chl and 50% of the initial carotenoid, whereas the 'yellow pellet' accounted for ~12% of the initial Chl and 50% of the initial carotenoid (Table 2). These results suggested that a substantial fraction of xanthophylls in the WT-HL and *zea1*-HL occupy a thylakoid membrane domain that is distinct from that of the Chl-proteins.

The 'resolved orange' fraction, which was initially released into the medium upon sonication of HL-grown *D. salina*, was enriched in β-carotene (~57% of the total carotenoid content) and Zea (~31% of the total carotenoid content). Additional pigments present were Lut (7%), while the remainder was accounted for by other xanthophylls (Table 3). This fraction was obtained as a soft pellet, layered on top of the tightly packed thylakoid membranes following mechanical disruption and centrifugation. The prompt release of the β-carotene-rich fraction upon sonication may suggest occurrence as a carotenoid-containing globule that is localized in the chloroplast stroma. Small oily globules are known to sequester β-carotene in the chloroplast of irradiance-stressed *D. bardawil*,[46] ostensibly serving as a screen against harmful irradiance effects. It is possible that a similar structure may also form in the chloroplast of irradiance-stressed *D. salina*, giving rise to the 'orange fraction' reported here. However, the absolute amount of β-carotene in irradiance-stressed *D. salina* Teod. (UTEX 1644) is much less than that reported in irradiance-stressed *D. bardawil* strains.

Detailed analysis of the WT-HL carotenoid content in the OG-derived 'yellow pellet' revealed that this fraction was enriched in Zea (~55%) and Lut (~34%) and depleted of β-carotene (3.6%, Table 3). Taken together, results in Tables 2 and 3 suggest that the OG treatment differentially extracted a substantial portion (35–50%) of the Zea and Lut pools that are contained within the HL-thylakoids.

Table 2 Balance sheet of pigments in resolved HL-thylakoids, OG 'green supernatant', and OG 'yellow pellet' derived from HL-acclimated *D. salina* wild type and *zea1* mutant[a]

Fraction	Resolved WT-HL-thylakoid	WT-OG green sup	WT-OG yellow pellet	Resolved *zea1*-HL-thylakoid	*zea1*-OG green sup	*zea1*-OG yellow pellet
Chl (%)	100	95.7	3.2	100	85.5	12.0
Total Car (%)	100	62.6	36.0	100	50.2	49.7

[a] Chl and Total Car content in the resolved 'HL-thylakoids' of *D. salina* were normalized to 100. Following OG extraction, pigment content in the 'green supernatant' and the 'yellow pellet' fractions were quantified relative to that of the corresponding 'HL-thylakoids'. Note that the sum of pigments recovered upon OG fractionation does not quite sum up to 100% due to minor pigment loss in the course of fractionation and sample processing. Results are the mean of 4 independent measurements, SE in all measurement are less than ±1.8%.

Table 3 Carotenoid composition of the resolved orange fraction (released upon sonication of the cells), and of the thylakoid membrane OG-extracted 'green supernatant' and 'yellow pellet' fractions[a]

Pigment	Resolved orange	Yellow pellet (OG-treated fraction)	Green supernatant (OG-treated fraction)
Neo	1.21	0.53	3.84
Vio	0.42	1.20	0.79
Ant	3.73	6.00	4.97
Zea	30.71	54.94	37.32
Lut	7.35	33.75	41.02
β-Car	56.58	3.58	12.06

[a] The resolved "orange" fraction showed a Car/Chl ratio of 7.14 : 1 (mol : mol). Pigments in each fraction were normalized to 100 for total carotenoid content. Results are the mean of 5 independent measurements, SE is less than ±11.8%.

Protein composition of the individual fractions

Of interest for further analysis were the 'resolved orange' and OG 'yellow pellet' fractions, which predominantly contained β-carotene and Zea–Lut, respectively. Proteins were extracted from these fractions upon solubilization with a buffer containing 250 mM Tris–HCl (pH 6.8), 20% glycerol, 7% SDS, 10% β-mercaptoethanol and 2 M urea and subjected to SDS-PAGE. For comparative purposes, total cell extract and total thylakoid membrane protein from LL- and HL-grown *D. salina* were also loaded on the SDS-PAGE. Fig. 4 shows that the SDS-PAGE protein profile of the 'resolved orange' and the OG 'yellow pellet' fractions were substantially different from those of the intact cells or thylakoid membranes. The 'resolved orange' and the OG 'yellow pellet' fractions were enriched in several unique protein bands of unknown origin (marked by numbers on the right margin of the respective lane in Fig. 4). These unknown proteins did not cross-react with any reaction center or light-harvesting complex specific polyclonal antibodies available in this laboratory, thus excluding origin from the photosystem complexes. This was especially true for the 'resolved orange' fraction, which is enriched in β-carotene, and which did not contain even trace amounts of the major photosynthetic electron-transport proteins. The dominant protein band at around 32 kD in this fraction (band 2, Fig. 4 'resolved orange' lane) could be a component of a β-carotene-containing structure. For example, the carotene-globule-associated protein (Cgp) has a molecular weight of ~38 kD.[46] Also, fibrillin, a ~32 kD protein, is known to be associated with carotenoid globule structures in higher plants.[47] However, antibodies specific to the higher plant fibrillins did not show a positive cross-reaction with this protein band (results not shown).

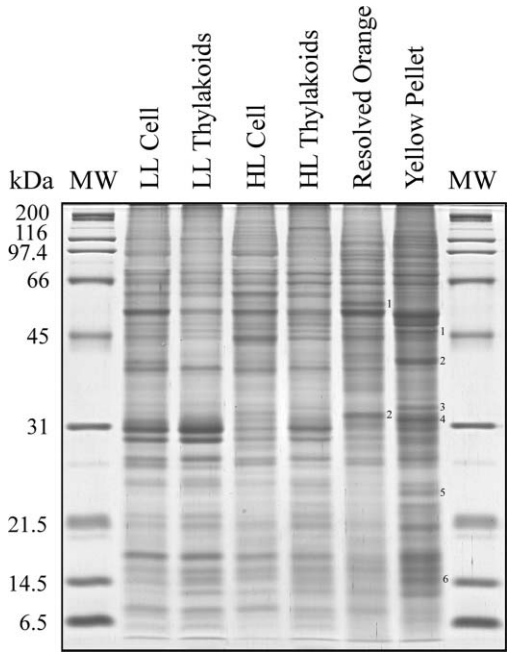

Fig. 4 SDS-PAGE profile of *D. salina* proteins. Samples were solubilized in a buffer containing 250 mM Tris–HCl pH 6.8, 7% SDS, 2 M urea, 20% glycerol, 10% β-mercaptoethanol. The solubilized samples were subsequently resolved in SDS-PAGE. Lanes were loaded with approximately 20 μg of protein. Protein bands were visualized upon Coomassie staining of the gels. Numbers on the right-hand margin of the 'orange pellet' and the 'OG yellow pellet' lanes mark those protein bands that were enriched in these fractions. The identity of these proteins is not currently known.

The OG 'yellow pellet' contained trace amounts of photosynthetic electron-transport proteins based on western blot analyses (not shown). It also contained the Cbr protein, which is proposed to be a Zea-binding protein that functions in a manner analogous to that of ELIPs.[48] Based on the SDS-PAGE profile analysis (Fig. 4), Cbr migrated to about the ~21 kD position.

Discussion

In LL-grown *D. salina* wild type and the *zea1* mutant, xanthophylls are structurally associated with the Chl-proteins and cannot be differentially extracted from the thylakoid membrane. Under HL-growth conditions, there is an overabundance of xanthophylls relative to Chl in the thylakoid membrane. Zea forms upon de-epoxidation of Vio and accumulates under such conditions, whereas the amount of Chl and of the light-harvesting proteins is substantially down-regulated. The fairly easy differential separation of Zea, and to a lesser extent of Lut, from the Chl-proteins in the HL-acclimated thylakoids suggests that these xanthophylls occupy a different domain in the HL than in the LL-grown samples. This domain is easily separated from the Chl-proteins upon mild surfactant treatment of the isolated thylakoids. Further supporting evidence concerning the dual localization of xanthophylls in different thylakoid membrane domains was provided from the analysis of the *zea1* mutant of *D. salina*. This strain constitutively accumulates Zea under all growth conditions and lacks all other β,β-epoxycarotenoids (Neo, Vio and Ant). Thus, the dynamically regulated reversible xanthophyll cycle does not function in this strain. Nevertheless, the *zea1* strain, very much like the WT, was subject to all aspects of the HL-acclimation, including a greater Xan/Chl ratio, a smaller photosystem Chl antenna size, and appearance of the Zea-rich 'yellow pellet' upon OG treatment of the HL-acclimated thylakoids. Analysis in this work suggested that, under LL-growth conditions, most Zea in the *zea1* mutant is found in structural association with the fully developed Chl *a-b* light-harvesting antenna and probably in place of the missing Vio and Neo. As such, Zea could not be differentially extracted from the Chl-proteins of the LL-grown samples upon a surfactant treatment of the mutant thylakoids.

These observations suggested accumulation of xanthophylls in novel, distinct and hitherto unknown domains of the thylakoid membrane in irradiance-stressed green algae. Alternatively, one might postulate movement between different domains for the reversible xanthophyll cycle carotenoids (Vio and Zea) in the course of the dynamic epoxidation/de-epoxidation cycle. More specifically, Vio could be released from the Chl-proteins of the LHC-II during its de-epoxidation to Zea and become associated with another domain of the thylakoid membrane, one that does not require the presence of the Chl *a-b* light-harvesting complex. Such interpretation of the results is consistent with recent observations by Hieber *et al.*,[49] who postulated a movement of xanthophylls in the thylakoid membrane during the dynamically regulated xanthophyll cycle. Results in this work are consistent with localization of Zea directly in the lipid bilayer of the HL-thylakoids. Support for this notion derives from the observation that HL-thylakoids are relatively more difficult to solubilize with surfactants than LL-thylakoids, suggesting a rigidity that is not encountered in the latter. This rigidity may arise from the direct presence of xanthophylls in the thylakoid membrane lipid bilayer. In this structural configuration, Zea may play a role in the stabilization of the lipid bilayer and in the maintenance of membrane integrity under photoinhibition and PSII repair conditions.[50] A functional role of Zea in the lipid bilayer could offer explanation for the relatively high content of xanthophylls in Chl-protein-deficient chloroplasts.[51]

It is of interest to compare the HL stress response of *D. salina*, as described in this work, to that of *D. bardawil*, which is known to accumulate substantial amounts of β-carotene in response to irradiance stress.[4,5,46] Levy *et al.*[52] showed that such extra amounts of β-carotene accumulate in the chloroplast stroma in the form of lipid globule structures, potentially serving as a screen of the excess and presumably harmful irradiance.[46]

This property of β-carotene accumulation is specific to *D. bardawil*. The *D. salina* strain employed in this work, unlike the *D. bardawil*, does not accumulate β-carotene under HL stress conditions. Therefore, Zea accumulation in the thylakoid membrane by *D. salina* and β-carotene accumulation by *D. bardawil* may reflect substantially different strategies, and underlying metabolic processes, in the response to HL stress.

It is possible that small amounts of oily globule structures sequestering β-carotene may also form in *D. salina* under HL stress, albeit to a much lesser extent than that in *D. bardawil*. These could have given rise to the 'orange pellet' isolated in the conduct of our fractionation. Nevertheless, such β-carotene-containing lipid globule structures would account for only a small fraction of the β-carotene pool in *D. salina*, where the majority of β-carotene exists in association with the Chl-proteins of the photosystems in the thylakoid membrane.

Obviously, more work is needed to fully clarify the functional role of the surplus Zea and Lut pigments in the thylakoid membrane of the HL-grown *D. salina*. This article is a first step in this direction. Moreover, the fractionation protocols presented could find application in efforts to extract, in fairly pure form, Zea and Lut pigments from mass-produced *D. salina*. These could find significant commercial application by the nutraceutical and pharmaceutical industries.

Abbreviations

Ant—antheraxanthin; β-Car—β-carotene; Chl—chlorophyll; LHC-II—light-harvesting complex II; Lut—lutein; Neo—neoxanthin; OG—octylglucoside; PS—photosystem; total Car—total carotenoid; Vio—violaxanthin; Zea—zeaxanthin.

Acknowledgements

The work was conducted with the financial support of USDA-NRI FD-2004-35100-14904 grant. TS was supported by a NSF–NATO grant. We wish to thank Dr Kris Niyogi for helpful suggestions and for making available HPLC equipment and Dr Marcel Kuntz for providing fibrillin antibodies. KY was a PhD student supported by a DPST scholarship from the Royal Thai Government.

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