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Organization of protochlorophyllide oxidoreductase in prolamellar bodies isolated from etiolated carotenoid-deficient wheat leaves as revealed by fluorescence probes

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Abstract

Carotenoid importance for membrane organization of NADPH protochlorophyllide oxidoreductase (POR) was studied by comparing interaction of two membrane fluorescent probes with proteins in prolamellar bodies isolated from norflurazon-treated wheat plants (cdPLBs) to those isolated form plants with normal carotenoid amount (oPLBs). The tryptophan fluorescence quenching by 1-anilino-8-naphthalene sulfonate (attached to the surface of membrane lipid phase) and pyrene (situated deep into the fatty acid region of membrane lipids) was used to locate the position of POR molecules toward lipid phase, to analyze their supramolecular organization and the light-induced structural transitions. Our results showed that the pigment–protein complexes of cdPLBs were larger than those of oPLBs. Upon flash irradiation the aggregates of both types of PLB dissociated into smaller units but in cdPLBs this process was accompanied by reorientation of the POR molecules closer to the lipid surface and/or dissociation from the lipids. These results revealed that carotenoid deficiency led to a looser attachment of POR to the lipid phase and its early (in comparison with oPLBs) dissociation from the membranes during the light-induced transformation of cdPLBs. This might be one of the reasons for the inability of carotenoid-deficient plants to form functional plastids.

Keywords: Carotenoid; Fluorescence probe; NADPH-protochlorophyllide oxidoreductase; Norflurazon; Prolamellar body

1. Introduction

In the absence of light angiosperms accumulate, instead of chlorophyll (Chl), mainly its immediate precursor-protochlorophyllide (Pchlide). In many plants, the predominant part of Pchlide molecules forms a ternary complex with the enzyme NADPH-protochlorophyllide oxidoreductase (POR, EC 1.3.1.33) [1–3] catalyzing Pchlide photoreduction and NADPH as a cofactor. POR–Pchlide–NADPH complexes are highly organized in a three dimensional netlike membrane structure—the prolamellar body (PLB) [4–6]. POR can constitute as much as 90% of the PLB protein content. Within PLB the ternary complexes are organized in large aggregates [7–10] which, besides mediating Pchlide reduction, also play a role in PLB assembly and protection against photo-oxidative damages [11–13]. Pchlide within the large aggregates has a fluorescence emission peak at 656 nm and can be photoreduced by a short (ms) light flash. As a result of photoreduction, Pchlide transforms to chlorophyllide (Chlide) which subsequently is esterified to Chl [4–6]. Simultaneously, the large POR aggregates dissociate into smaller units [5,14].

Some recently obtained results indicate that the other big group of plastid pigments—carotenoids, also plays role in PLBs formation and disassembly; particularly, zeaxanthin and violaxanthin molecules might be associated to photoactive POR–Pchlide–NADPH complexes [15].

The identification of carotenoid and chloroplast regulation (*ccr*) mutants in *Arabidopsis* has made it possible to show the

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specific function of certain carotenoids in the formation of the PLBs [16]. The accumulation of prolycopene in dark-grown *ccr* mutants caused an inhibition of PLB formation. A similar effect was also found after treatment of barley leaves with amitrole [17,18]. It was suggested that lutein could be inserted in the membranes and oriented both in vertical and in parallel to them [19], while a similar orientation of prolycopene was regarded as not favouring the curvature of the PLB membrane to the same extent [16]. The membrane lipid composition and curvature [20], as well as the lutein presence, can all be factors important for the proper attachment of POR in the PLBs. In addition, the surface charge densities, which were known to be altered by Norflurazon [21], might influence POR attachment too.

Earlier, we reported that after flash irradiation dark-grown plants with norflurazon-induced carotenoid deficiency accumulated more Chlide than plants with normal carotenoid content [22]. When etiolated plants were grown at room temperature the norflurazon-induced carotenoid deficiency did not cause aberrations in ultrastructure of PLB as seen by electron microscopy [23,18]. However, under increased temperature (30 °C), such plants demonstrated less ordered PLB [18], supporting the idea of Havaux that polar carotenoids are required for plastid membrane stability [24]. Still, the carotenoid importance for POR organization and functioning as well as for the light-induced transitions in PLB remains unclear.

Here, we present results of experiments aimed to elucidate whether the POR-pigment complexes of carotenoid-deficient plants differ from those of plants with normal carotenoid content in their organization and behavior before and after flash irradiation. To achieve our goal, we used a non-destructive method based on energy transfer between the tryptophan (Trp) residues of PLB membrane proteins and two fluorescent probes with different membrane localization-1-anilino-8-naphthalene sulfonate (ANS) and pyrene [25–27]. In wheat, the main PLB protein-POR contains four highly conserved Trp residues: Trp 113, Trp 363, Trp 365 and Trp 390 [6]. The proper membrane association of POR strongly depends on the intactness of C-terminus [28], where three of the Trp (Trp 363, Trp 365 and Trp 390) are located. They are exposed on the protein surface [29] which enables them to interact with the membrane. Birve and co-workers [30] have also suggested that either a β-sheet or an α-helical region containing Trp residues is involved in POR anchoring to membrane. Of the three tryptophans—Trp363, Trp365 and Trp390, located on the C-terminus of POR, Trp363 is a part of a β-sheet and Trp390 is a part of an α-helix. It is also possible for Trp365 to be involved in membrane anchoring since it is located close to a β-sheet. The fluorescent probe ANS localizes at the level of polar heads of membrane lipids and interacts with membrane proteins [31]; the non-polar hydrophobic probe pyrene accumulates in the fatty acid region of the membranes [32]. The energy transfer between POR tryptophans and probes can only occur on distances close to the average size of a lipid monolayer [25–27]. That enabled us to study POR organization and its localization toward the lipid phase of PLB membranes.

2. Material and methods

Seeds of wheat (*Triticum aestivum* L., cv. Kosack) were soaked for 24 h in water (control plants) or in 10 μmol l^{-1} norflurazon (Sandoz Ltd., Switzerland). Plants were grown in darkness for 7 days in a mixture of peat and sand at 25 °C [33]. PLBs were isolated according to Ryberg and Sundqvist [4]. The isolated PLB were pelleted by centrifugation and resuspended in sucrose-free TES–HEPES buffer (1 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 20 mmol l^{-1} TES, 10 mmol l^{-1} HEPES, adjusted to pH 7.2 with KOH).

Total membrane protein was determined by a color reaction with Coomassie Brilliant Blue G250 [34]. SDS-PAGE of proteins extracted from PLBs was performed according to Santel and Apel [35]. The gel was stained with Coomassie Brilliant Blue R-250 [4]. Molecular masses of PLBs proteins were determined using molecular weight kit 14–70 kDa (SIGMA MW-SDS-70L).

Pchlide, Chlide and carotenoids were extracted in 85% acetone. Pchlide amount was calculated using the molar extinction coefficients of Kahn [36] while carotenoid amount was determined according to MacKinney [37].

Low temperature (77 K) fluorescence spectra were recorded from PLB suspensions with and without probes using a SLM 800 °C fluorescence spectrophotometer upgraded with a SLM 8100 motherboard (SLM Aminco, Urbana, IL, USA) and the accompanying software. Fluorescence emission was measured between 600 and 780 nm with an excitation wavelength of 440 nm. Excitation and emission bandwidths were 4 nm, and the integration time was 0.1 s. All spectra were smoothed 10 times (using a fixed bandwidth, sharp cut-off, three point and low pass digital filter) and corrected for variations in the sensitivity of the photomultiplier.

To cause phototransformation of Pchlide to Chlide, the PLBs were irradiated with 3 flashes of "white light" from a photographic flash (Braun F 800) at a distance of 10 cm (impulse energy 120 J).

To determine the energy transfer from Trp to ANS and pyrene, the PLB samples containing 100 mg l^{-1} protein were mixed with different amounts of probes. Mg-ANS (Serva) was taken from a 1 mmol l^{-1} stock solution (in nano-pure water) and added to the membranes to final concentrations of 5, 7.5 or 10 μmol l^{-1}. Pyrene (Serva) was added from a 1 mmol l^{-1} stock solution (in 95% ethanol) in small portions (1 μmol per min) as described by Dobretsov [25] to final concentrations of 2.5, 5 or 7.5 μmol l^{-1}. In order to achieve a good integration in the membranes, all samples were incubated with probes on ice for 1 h.

The energy transfer between the Trp of PLB proteins and probes was calculated by Trp fluorescence quenching at 330 nm after excitation at 290 nm. Fluorescence emission spectra were recorded between 300 and 500 nm at room temperature using the fluorescence spectrophotometer described above.

Energy transfer occurs if the distance between Trp and probe is less than 1.2 times the Förster radius (R_0), which was equal to 2.6 nm for ANS and to 2.8 nm for pyrene. R_0 values ware calculated by Förster's equation:

$$R_0 = 979\left(k^2 n_r^{-4} Q_d J\right)^{1/6} \quad (1)$$

where Q_d is donors quantum yield, n_r is refractive index of the medium, k^2 is the orientation factor, J is the overlap of the emission spectrum of donor with absorption spectrum of the acceptor. k^2 was determined according to Dale et al. [38,39]. According to Eftink and Ghiron [40], the Trp residues of PLB proteins could be divided into two parts depending on the distance to probes—accessible for quenching (β) and non-accessible for quenching (1-β). A method for calculating β has been described by Eftink and Ghiron [40] and further adapted by Dobretsov [25,41,42]:

$$\beta = a/[F_0/(F_0 - F)]\text{min}. \quad (2)$$

where: a is a constant equal either to 0.75 for pyrene or to 0.65 for ANS [25,38,39]; F_0 is Trp fluorescence in the absence of probes; F is Trp fluorescence in the presence of probes. The values $[F_0/(F_0-F)]$ min were found by using the modified Stern–Folmer plot [43] and represent the maximum possible quenching of Trp fluorescence by an infinite large concentration of the acceptor (the probe).

The decay time of Trp fluorescence was measured by a PRA-System 3000 multichannel equipment (Photochemical Research Association Inc., Canada) by pulse-decay counting method. Hydrogen/nitrogen impulse light source was

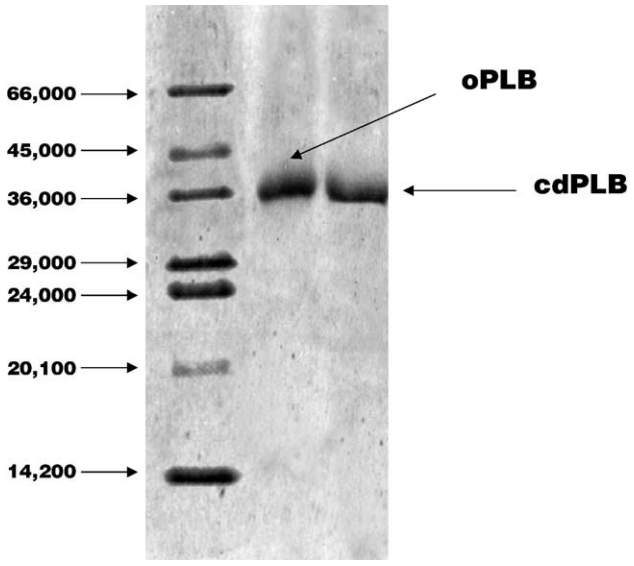

Fig. 1. SDS-PAGE protein profile of PLB isolated from 7 days old etiolated plants with normal carotenoid content (oPLB) and with carotenoid deficiency (cdPLB). The location of POR is indicated.

used producing 1.8 ns long single impulses of excitation light with frequency 15 kHz. Time-dependent photon counting was performed by multichannel analyzer Northårn Tracår TN-1750 upgraded with computer hardware and software by Photochemical Research Association Inc., Canada.

The means of 5 measurements were given for all experiments. All the results were statistically processed and the values of standard deviation and P were calculated. Only results with P less than 0.01 are discussed below.

3. Results

The PLB (carotenoid deficient PLB, cdPLB) isolated from norflurazon-treated wheat had much lower caroteniod content: 2.46 mg g^{-1} protein than that of the control PLB isolated from non-treated leaves: 14.4 mg g^{-1} protein.(original PLB, oPLB). The Pchlide amount was 1.53 and 1.49 mg g^{-1} protein in cdPLB and oPLBs, respectively, SDS-PAGE showed that in cdPLB the main protein was POR (Fig. 1) as it was shown earlier for norflurazon-treated barley plants by Moro et al. [18].

To estimate the potential influence of fluorescence probes on the activity of ternary POR–Pchlide–NADPH complex, the low temperature fluorescence spectra (77 K) of non-irradiated oPLBs and cdPLBs, either in the absence or in the presence of probes, were recorded. In the probe absence, the spectra showed two fluorescence maxima—one at 657 nm corresponding to phototransformable Pchlide and another one at 633 nm originating from non-phototransformable Pchlide (Fig. 2A, B). The presence of 10 μmol l^{-1} ANS did not cause any changes in the pigment spectral forms. In the presence of 7.5 μmol l^{-1} pyrene, a small decrease in the amplitude ratio of Pchlide 657 to Pchlide 633 was observed (Fig. 2A, B). This effect was likely due to the presence of 0.75% ethanol (pyrene was added from 1 mmol l^{-1} ethanol stock solution) because there was no substantial difference between samples with pyrene and those with the same amount of ethanol (Fig. 2A). However, the ethanol concentration in pyrene-containing samples was quite low and did not affect POR enzymatic activity, i.e., Pchlide–Chlide photoreduction (Table 1).

Trp fluorescence spectra of both cdPLB and oPLB containing samples had a maximum at about 330 nm which indicated relatively high polarity of the Trp surrounding [40]. The position of maxima was not affected by probes presence (Figs. 3, 4). After flash irradiation, the fluorescence intensity increased rapidly reaching plateau after the first 15–20 min. The increase in fluorescence intensity of cdPLBs without probes was twice as big as that in the oPLBs (Figs. 3A, 4A). The samples with probes had much lower intensity of Trp fluorescence than those without probes (Figs. 3 and 4). The average decay times (τ) of Trp fluorescence in the presence of both, 5 μmol l^{-1} ANS and 5 μmol l^{-1} pyrene, were 1.6 ns and 1.7 ns for oPLBs and cdPLBs, respectively, while in the samples without probes τ was much higher 2.7 ns. These results were considered as a good proof that the Trp fluorescence quenching was due to the effective energy transfer from Trp to the probes.

The proportion of pyrene bound to the membranes was estimated by testing the quenching of its fluorescence after addition of iodine ions. An equimolar amount of KI was added

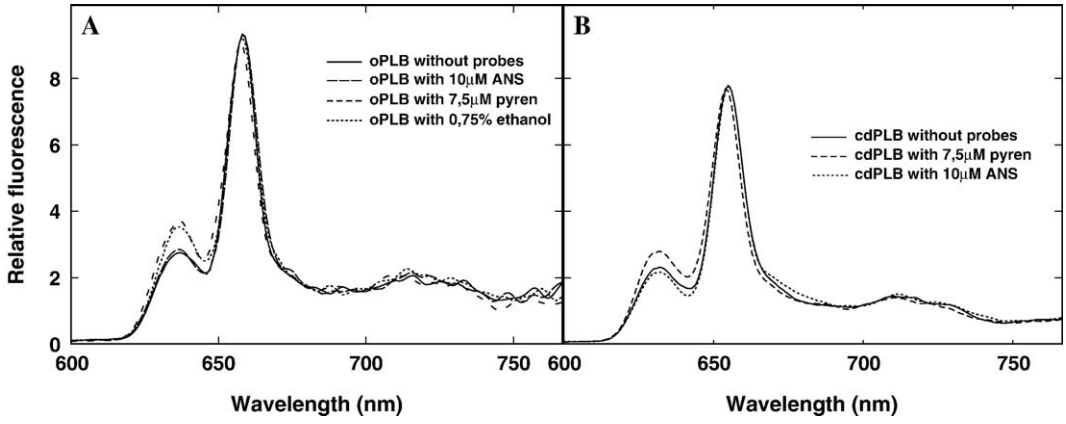

Fig. 2. Fluorescence emission spectra at 77 °K of isolated oPLB (A) and cdPLB (B). The samples were incubated in darkness, on ice for 1 h with or without fluorescence probes. The excitation wavelength was 440 nm. The spectra were corrected for the sensitivity of the photomultiplier and normalized at 657 nm.

Table 1
Flash irradiation-induced reduction of Pchlide in samples containing isolated PLB with and without probes

PLB type	No probes	Fluorescence probes			0.75% ethanol
		ANS (10 μmol l^{-1})	Pyrene (7.5 μmol l^{-1})	Both probes	
oPLB	76±1.2	76±1.4	75±2.1	74±1.8	75±2.0
cdPLB	82±2.6	81±1.8	81±1.6	81±2.3	81±2.9

Aliquots were taken from different samples before and immediately after irradiation and extracted with acetone to determine amounts of Pchlide. The photoreduction was calculated in percentage according to the formula: $100\times(P_D-P_L)/P_D$ where P_D and P_L were the amounts of Pchlide (in mg g^{-1} protein) before and after irradiation respectively.

to a sample containing 5 μmol l^{-1} pyrene. The absence of any significant quenching of pyrene fluorescence (results not shown) indicated that most of the pyrene molecules were embedded in the lipid phase of the PLBs and they could not be reached by the iodine ions [25].

The concentration of ANS bound to PLB was calculated taking into account that the bound probe absorbs at 390 nm and has a fluorescence emission in the region of 460–480 nm [25,31]. We determined that in non-irradiated oPLBs containing 10 μmol l^{-1} ANS (total concentration), 1.84 μmol of probe was bound. The amount increased to 2.39 μmol 10 min after irradiation, and reached 2.57 μmol at the end of the experiment (30 min) (Fig. 5). In the non-irradiated cdPLBs containing 10 μmol l^{-1} ANS (total concentration), 1.46 μmol were bound. Within the first 5 min after irradiation the amount of bound probe raised to 3.46 μmol. For the rest of the experimental period (30 min), the increase in bound ANS reached 4.1 μmol (Fig. 5).

These data were used to calculate the amount of the Trp residues accessible for quenching (β). The results showed that in non-irradiated cdPLBs 10% of the Trp were available for quenching by pyrene, 20%—by ANS (Fig. 6A) and in samples containing both probes β was 27%. After a flash irradiation the Trp quenching accessibility for both probes rapidly increased and 5 min later reached 52% for pyrene, 43% for ANS and 77% for the two probes. After 10 min, a decrease in the Trp accessibility for pyrene was observed and continued until the end of the experiment (30 min). The values of β increased up to 81% in the ANS-containing samples and up to 89% in the samples with the two probes (Fig. 6A).

In non-irradiated oPLB, 58% of the fluorescent Trp residues were accessible for quenching by pyrene, 52% – by ANS and 86% – when the probes were added together (Fig. 6B). After irradiation, the quenching by pyrene increased to 82%, by ANS to about 72% and by both probes up to 92% (Fig. 6B).

4. Discussion

As it was described in the introduction, the tryptophan residues of POR were involved in protein membrane anchoring. We used distance-dependent Trp fluorescence quenching by one fluorescence probe localized on the membrane surface and one solubilized in lipid in order to determine the relative position of Trp residues toward the lipid phase of PLB. Although this method does not allow recognition of particular Trp residues, it can be used to draw conclusions about the relative position of POR molecules in the membrane lipids.

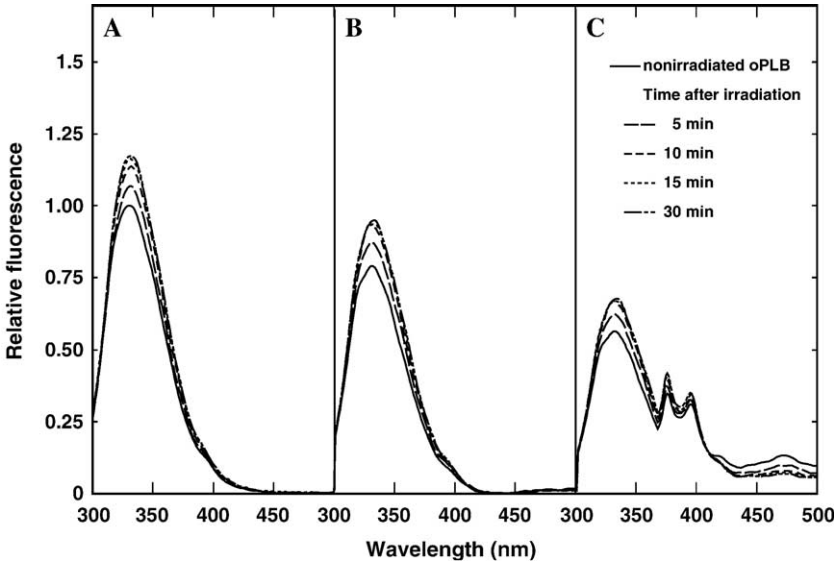

Fig. 3. Changes in tryptophan fluorescence of isolated oPLB and its quenching by the probes. Samples were taken from oPLB incubated in darkness on ice for 1 h. The membranes presented in panels B and C were incubated with 5 μmol l^{-1} ANS or 5 μmol l^{-1} pyrene respectively. After recording the spectra of non-irradiated oPLB, they were irradiated with 3 flashes of white light and kept in darkness on ice. Fluorescence was recorded every 5 min during the following 30 min. Because the fluorescence reached plateau after 15 min, the spectra registered between 20 and 30 min are represented by the spectrum recorded at 30 min. The excitation wavelength was 290 nm.

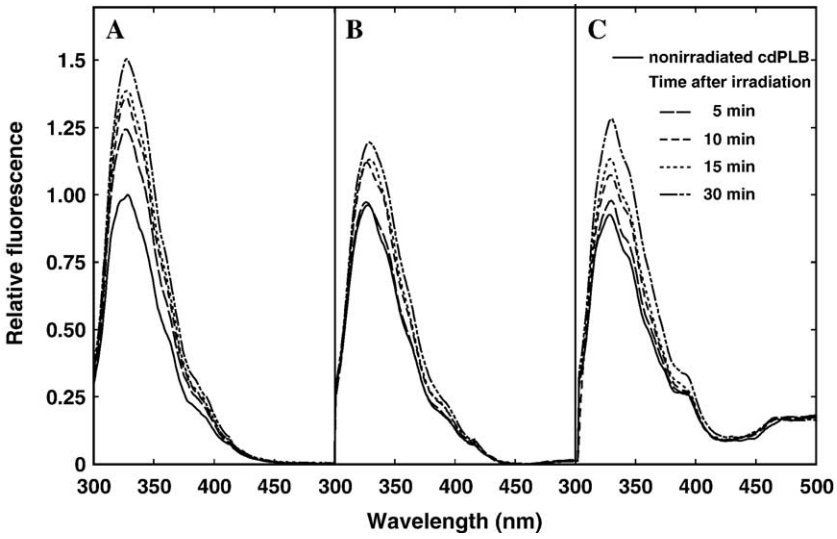

Fig. 4. Changes in tryptophan fluorescence of isolated cdPLB and its quenching by the probes. Samples were taken from cdPLB incubated in darkness on ice for 1 h. The membranes, presented in panels B and C were incubated with 5 μmol l^{-1} ANS or 5 μmol l^{-1} pyrene, respectively. After recording the spectra of non-irradiated cdPLB, the samples were irradiated with 3 flashes of white light and kept in darkness on ice (C). Fluorescence was recorded every 5 min during the following 30 min. Because the fluorescence reached plateau after 20 min, the spectra registered between 20 and 30 min are represented by the spectrum recorded at 30 min. The excitation wavelength was 290 nm.

According to our results before irradiation 58% of Trp residues in oPLBs were accessible to the lipid-buried pyrene. After irradiation, the accessibility increased to 82% of the Trp residues, which suggests that they were localized within the lipid phase. On the other hand, about 52% of Trp residues in non-irradiated oPLB and 72% of them in irradiated once were accessible to the surface-localized ANS, This might be only possible if the predominant part of Trp residues had been situated on the level of polar heads making them accessible to both probes.

After irradiation, the Trp fluorescence intensity in oPLB samples without probes increased with about 20%, which could be explained if more Trp residues emit fluorescence. This fact is especially interesting when we take in account that the light-induced increase in Trp residues accessibility for quenching is in the same magnitude. One can ask where were these Trp residues situated in non-irradiated oPLB? We think that both changes were due to changes in aggregational state of POR protein. It is known that POR, together with Pchlide and NADPH, is organized in large aggregates in PLB membranes [6–10,15]. Within the complexes, the assembled POR molecules were tightly attached to each other and Trp residues in the inner part of the complexes are partially overshadowed from excitation light and do not emit fluorescence. On the other hand, 20% of emitting Trp residues of these complexes were also located beyond the distance which allows energy transfer from Trp to the probes. Upon irradiation POR, aggregates dissociated into smaller units [5,6,14] which significantly decreased overshadowing and made more emitting Trp accessible for quenching. These conclusions are also supported by the dynamic of light-induced increase of concentration of binding sites for ANS (Fig. 5) [27].

Many authors consider the aggregation of POR molecules in membranes containing high percentage of nonbilayer lipids as a prerequisite for PLB formation [8,12,44–47]. Since the ultrastructure [18,23] and Pchlide spectral properties according to Moro et al. [18] and our data (Fig. 2B) of cdPLB are similar to that in oPLB, we can assume that POR in non-irradiated cdPLB, exactly like in oPLB, is organized in large aggregates. Judged by Trp residues accessibility for quenching, the organization of POR aggregates in non-irradiated cdPLB differed significantly from that in oPLB because in cdPLB samples even in the presence of both probes about 70% of the Trp (vs. 20% in oPLB) were not accessible for quenching (Fig. 6A). Such effect is possible if the POR aggregates in cdPLB are larger (build of more tightly attached to each other POR molecules) and hence contain more emitting Trp located beyond the distance, which allows

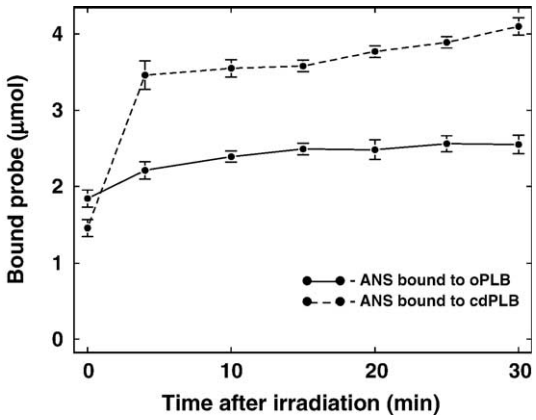

Fig. 5. Binding of ANS to isolated oPLB and cd PLB before and after irradiation. Samples were incubated on ice in darkness for 1 h with 5 μmol l^{-1} ANS. The samples (without the control) were irradiated with 3 flashes of white light and kept in darkness on ice. The fluorescence was measured at 470 nm with the excitation wavelength 390 nm every 5 min during the following 30 min.

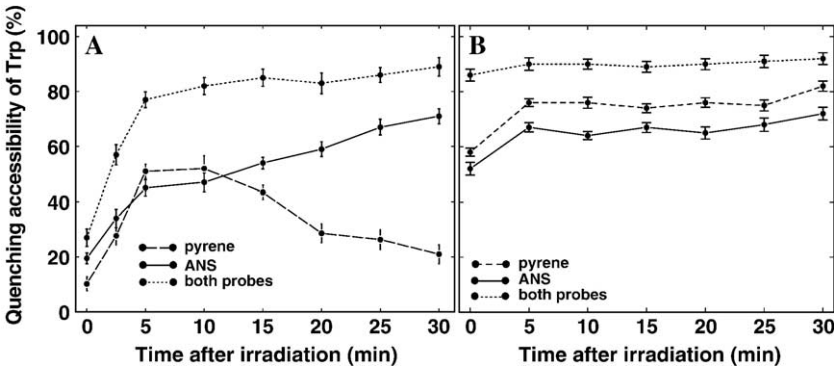

Fig. 6. Light-induced changes in the accessibility of tryptophan residues in the membrane proteins of cdPLB (A) and oPLB (B) for quenching by the fluorescent probes. The quenching of tryptophan fluorescence at 330 nm (excitation 290 nm) in the presence of different concentration of fluorescent probes was used to calculate quenching accessibility of tryptophan residues.

transfer to the probes. The alternative could be that the inaccessible Trp residues belong to the POR molecules situated in inner regions of cdPLB which have not still been reached by probes. But this is quite unlikely since the samples were incubated with probes for 1 h before the start of measurement and pyrene molecules were shown to have high lateral mobility of at least 3×10^{-8} cm s^{-1} [48].

Flash irradiation of cdPLB caused a rapid increase of Trp fluorescence which in samples without probes was with 50% (Fig. 4). As we mentioned above, we think that reason for these changes was partial overshadowing of the Trp within the aggregates. The fact that in cdPLBs the increase of Trp fluorescence was twice as big as that in oPLBs supports the assumption that POR aggregates of cdPLB are larger, i.e., before irradiation more Trp residues were shadowed within the aggregates.

The light-induced processes in cdPLB followed similar pattern to that in oPLB but were much more intensive. We assume that in the absence of carotenoids the separation of POR molecules to smaller units proceed faster. This was seen mainly by rapid increase (within 5 min) of Trp accessibility for quenching with 42% for pyrene and 23% for ANS. In the samples containing both probes, the Trp quenching accessibility reached a level similar to that in irradiated oPLB. The concentration of binding sites for ANS also increased dramatically within 5 min with 57% (Fig. 5).

However, after the first 5 min ongoing changes in accessibility of Trp for quenching started to differ again from those in oPLB. After the 10th min (after the flash irradiation), the Trp accessibility for pyrene started to decrease and at the end of the experiment it was reduced to 21% while the accessibility for ANS increased significantly to 81% (Fig. 6A). Such significant decrease of Trp accessibility for the lipid-localized pyrene, accompanied by increase of accessibility for the water-soluble ANS, can be only explained by a substantial portion of Trp residues moving on the membrane surface and/or even leaving the lipid phase.

The presented results reveal that carotenoid deficiency results in looser attachment of POR to the lipids phase which is in agreement with recent findings of Moro and co-workers [18] and its premature (in comparison with oPLBs) dissociation from the membranes during the light-induced transformation of cdPLBs. That might be one of the reasons for the inability of carotenoid-deficient plants to form functional plastids [17].

Acknowledgments

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References

[1] R.P. Oliver, W.T. Griffiths, Identification of the polypeptides of NADPH-protochlorophyllide oxidoreductase, Biochem. J. 191 (1980) 227–280.
[2] R.P. Oliver, W.T. Griffiths, Pigment–protein complexes in illuminated etiolated leaves, Plant Physiol. 70 (1982) 1019–1025.
[3] K. Apel, H.J. Santel, T.E. Redlinger, H. Falk, The Pchlide holochrome of barley (*Hordeum vulgare* L.). Isolation and characterization of the NADPH:protochloriphyllide oxidoreductase, Eur. J. Biochem. 111 (1980) 251–258.
[4] M. Ryberg, C. Sundqvist, Characterization of prolamellar bodies and prothylakoids fractionated from wheat etioplasts, Physiol. Plant. 56 (1982) 125–132.
[5] M. Ryberg, K. Dehesh, Localization of NADPH-protochlorophyllide oxidoreductase in dark-grown wheat (*Triticum aestivum*) by immuno-electron microscopy before and after transformation of the prolamellar bodies, Physiol. Plant. 66 (1986) 616–624.
[6] C. Sundqvist, C. Dahlin, With chlorophyll pigments from prolamellar bodies to light-harvesting complexes, Physiol. Plant. 100 (1997) 748–759.
[7] B. Böddi, M. Ryberg, C. Sundqvist, The formation of a short-wavelength chlorophyllide form at partial phototransformation of protochlorophyllide in etioplast inner membranes, Photochem. Photobiol. 53 (1991) 667–673.
[8] B. Wiktorsson, M. Ryberg, S. Gough, C. Sundqvist, Isoelectric focusing of pigment–protein complexes solubilized from non-irradiated and irradiated prolamellar bodies, Physiol. Plant. 85 (1992) 659–669.
[9] B. Wiktorsson, S. Engdahl, L.B. Zhong, B. Boddi, M. Ryberg, C. Sundquvist, The effect of cross-linking of the subunits on NADPH-protochlorophyllide oxidoreductase on the aggregational state of protochlorophyllide, Photosynthesis 29 (1993) 205–218.
[10] W.P. Williams, E. Selstam, T. Brain, X-ray diffraction studies of the structural organisation of prolamellar bodies isolated from Zea mays, FEBS Lett. 422 (1998) 252–254.

[11] F. Franck, B. Schoefs, X. Barthelemy, K.B. Mysliwa, K. Strzalka, R. Popovic, Protection of native chlorophyll(ide) forms and of photosystem II against photodamage during early stages of chloroplast differentiation, Acta Physiol. Plant 17 (1995) 123–132.
[12] N. Lebedev, M.P. Timko, Protochlorophyllide photoreduction, Photosynth. Res. 58 (1998) 5–23.
[13] G.A. Armstrong, K. Apel, W. Rüdiger, Does a light-harvesting protochlorophyllide a/b-binding protein complex exist?, Trends Plant Sci. 5 (2000) 40–44.
[14] N.N. Artus, M. Ryberg, A. Lindsten, H. Ryberg, C. Sundqvist, The Shibata shift and the transformation of etioplasts to chloroplasts in wheat with clomazone fmc 57020 and amiprophos-methyl tokunol, Plant Physiol. 98 (1992) 253–263.
[15] M.A. Chahdi, B. Schoefs, F. Franck, Isolation and characterization of photoactive complexes of NADPH:protochlorophyllide oxidoreductase from wheat, Planta 206 (1998) 673–680.
[16] H. Park, S.S. Kreunen, A.J. Cuttriss, D. DellaPenna, B.J. Pogson, Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis, Plant Cell 14 (2002) 321–332.
[17] D. Di-Baccio, M.F. Quartacci, I. Moro, F. Dalla-Vecchia, N. La-Rocca, F.N. Rascio, F. Navari-Izzo, Bleaching herbicide effects on plastids of dark-grown plants: lipid composition of etioplasts in amitrole and norflurazon-treated barley leaves, J. Exp. Bot. 53 (2002) 1857–1865.
[18] I. Moro, F. Dalla-Vecchia, N. La-Rocca, F. Navari-Izzo, M.F. Quartacci, D. Di-Baccio, W. Rüdiger, N. Rascio, Impaired carotenogenesis can affect organization and functionality of etioplast membranes, Physiol. Plant. 122 (2004) 123–132.
[19] A. Sujak, J. Gabrielska, W. Grudzinski, R. Borc, P. Mazurek, W.I. Gruszecki, Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: the structural aspects, Arch. Biochem. Biophys. 371 (1999) 301–307.
[20] H. Klement, U. Oster, W. Rüdiger, The influence of glycerol and chloroplast lipids on the spectral shifts of pigments associated with NADPH: protochlorophyllide oxidoreductase from *Avena sativa* L, FEBS Lett. 480 (2000) 306–310.
[21] C. Dahlin, Surface charge densities and membrane fluidities in thylakoids with different degrees of thylakoid appression after Norflurazon treatment, Photosynthesis 41 (2003) 635–639.
[22] G. Yahubyan, I. Minkov, C. Sundqvist, Carotenoid dependence of protochlorophyllide to chlorophyllide phototransformation in dark-grown wheat seedlings, J. Photochem. Photobiol., B 65 (2001) 171–176.
[23] B. Klockare, L. Axelsson, H. Ryberg, A.S. Sandelius, K.O. Widell, The function of carotenoids during chloroplast development: I. Effect of the herbicide SAN 9789 on chlorophyll synthesis and plastid ultrastructure, in: G. Akoyunoglou (Ed.), Photosynthesis V-Chloroplast Development, Balaban Int. Sci. Serv. Philadelphia, 1981, pp. 277–284.
[24] M. Havaux, Carotenoids as membrane stabilizers in chloroplasts, Trends Plant Sci. 3 (1998) 147–151.
[25] G.E. Dobretsov, in: Y. Vladimirov (Ed.), Fluorescence Probes in Investigation of Cells, Membranes and Lipoproteins, Nauka Publ, Moscow, 1989 (in Russ.).
[26] I. Denev, I. Minkov, Membrane protein localization in non-irradiated and flash irradiated prolamellar bodies and prothylakoids, isolated from wheat, measured by fluorescence probes 1-aniline-8-naphthalene sulfonate and pyrene, Bulg. J. Plant Physiol. 22 (1996) 40–52.
[27] I. Denev, I. Minkov, C. Sundqvist, G. Savchenko, E. Kluchareva, Changes in the localization of the proteins in isolated inner membranes from wheat etiochloroplasts under irradiation, measured by 1-aniline-8-naphthalene sulfonate and pyrene, J. Plant Physiol. 150 (1997) 668–673.
[28] H. Aronsson, C. Sundqvist, M.P. Timko, C. Dahlin, The importance of the C-terminal region and Cys residues for the membrane association of the NADPH:protochlorophyllide oxidoreductase (POR) in pea (*Pisum sativum*), FEBS Lett. 502 (2001) 11–15.
[29] H.E. Townley, R.B. Sessions, A.R. Clarke, T.R. Dafforn, W.T. Griffiths, Protochlorophyllide oxidoreductase: a homology model examined by site-directed mutagenesis, Proteins 44 (2001) 329–335.
[30] S.J. Birve, E. Selstam, L.B.A. Johansson, Secondary structure of NADPH:protochlorophyllide oxidoreductase examined by circular dichroism and prediction methods, Biochem. J. 317 (1996) 549–555.
[31] J. Slavik, Anilinonaphtalene sulphonate as a probe of membrane composition and function, Biochim. Biophys. Acta 694 (1982) 1–25.
[32] F. Podo, J.K. Blasie, Nuclear magnetic resonance studies of lecithin bimolecular leaflets with incorporated fluorescent probes, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 1032–1036.
[33] M. Ryberg, C. Sundqvist, The influence of 8-hydroxyquinoline on the accumulation of porphyrins in dark-grown wheat leaves treated with 5-aminolevulinic acid, Physiol. Plant. 36 (1976) 356–361.
[34] J.C. Bearden, Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay, Biochim. Biophys. Acta 533 (1978) 525–529.
[35] H.J. Santel, K. Apel, The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). The effect of light on the NADPH:protochlorophyllide oxidoreductase, Eur. J. Biochem. 120 (1981) 95–103.
[36] A. Kahn, Spectrophotometric quantitation of protochlorophyll(ide): specific absorption and molar extinction coefficients reconsidered, Physiol. Plant. 59 (1983) 99–102.
[37] G. MacKinney, Absorption of light by chlorophyll solutions, J. Biol. Chem. 140 (1941) 315–322.
[38] R. Dale, J. Eisinger, W. Blumberg, The orientational freedom of molecular probes. The orientation factor in intramolecular energy transfer, Biophys. J. 26 (1979) 161–194.
[39] G. Gorbenko, H. Saitob, J. Molotkovsky, M. Tanakad, M. Egashirad, M. Nakanod, T. Handaa, Resonance energy transfer study of peptide–lipid complexes, Biophys. Chem. 92 (2001) 155–168.
[40] M.R. Eftink, C.A. Ghiron, Exposure of tryptophanil residues in proteins. Quantitative determination by fluorescence quenching studies, Biochemistry 15 (1976) 672–680.
[41] G.E. Dobretsov, M.M. Spirin, O.V. Chekrygin, A fluorescent study of a protein localization in relation to lipids in serum low density lipoproteins, Biochim. Biophys. Acta 710 (1982) 172–180.
[42] G.E. Dobretsov, N.K. Kurek, V.N. Machov, T.I. Syrejshchikova, M.N. Yakimenko, Determination of fluorescent probes localization in membranes by nonradiative energy transfer, J. Biochem. Biophys. Methods 19 (1989) 259–274.
[43] M.R. Eftink, C.A. Ghiron, Fluorescence quenching studies with proteins, Anal. Biochem. 114 (1981) 199–210.
[44] E. Selstam, A. Widell-Wigge, Chlroplast lipids and the assembly of membranes, in: C. Sundqvist, M. Ryberg (Eds.), Pigment–Protein Complexes in Plastid: Synthesis and Assembly, Academic Press, 1993, pp. 241–277.
[45] B. Wiktorsson, M. Ryberg, C. Sundqvist, Aggregation of NADPH–protochlorophyllide oxidoreductase–pigment complexes is favoured by protein phosphorylation, Plant Physiol. Biochem. Paris 34 (1) (1996) 23–34.
[46] B.D. Bruce, The role of lipids in plastid protein transport, Plant Mol. 38 (1–2) (1998) 223–246.
[47] C. Dahlin, H. Aronsson, H.M. Wilks, N. Lebedev, C. Sundqvist, M.P. Timko, The role of protein surface charge in catalytic activity and chloroplast membrane association of the pea NADPH: protochlorophyllide oxidoreductase (POR) as revealed by alanine scanning mutagenesis, Plant Mol. Biol. 39 (2) (1999) 309–323.
[48] J.M. Vanderkooi, J.B. Callis, Pyrene: a probe of lateral diffusion in the hydrophobic region of membranes, Biochemistry 13 (1974) 4000–4006.