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Aggregation of the 636 nm emitting monomeric protochlorophyllide form into flash-photoactive, oligomeric 644 and 655 nm emitting forms in vitro

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Abstract

Artificial formation of flash-photoactive oligomeric protochlorophyllide complexes was found in etiolated pea (*Pisum sativum* L. cv. Zsuzsi) epicotyl homogenates containing glycerol (40% v/v) and sucrose (40% m/v). The 77 K fluorescence emission spectra indicated that the ratio of the 644 and 655 nm emitting forms to the 636 nm form increased during 3 to 5-day incubation in the dark at −14 °C. Electron micrographs showed the presence of well-organized prolamellar bodies in the homogenates. The same phenomena were found when the homogenates were frozen into liquid nitrogen and thawed to room temperature in several cycles. Similar treatments of intact epicotyl pieces caused significant membrane destructions. In homogenates, the in vitro produced 644 and 655 nm emitting protochlorophyllide forms were flash-photoactive; the extent of phototransformation increased compared to that in native epicotyls. The newly appeared 692 nm chlorophyllide band showed a blue shift (similar to the Shibata shift in leaves), however this process took place only partially due to the effect of the isolation medium. These results prove that the in vitro accumulated 644 and 655 nm protochlorophyllide forms were produced from the flash-photoactive 636 nm emitting monomeric NADPH:protochlorophyllide oxidoreductase units via aggregation, in connection with structure stabilization properties of glycerol and sucrose.

Keywords: Aggregation; Glycerol; NADPH:protochlorophyllide oxidoreductase (POR); Protochlorophyllide; Sucrose

1. Introduction

In etioplasts of dark-grown angiosperms, protochlorophyllide (Pchlide) is assembled into various complexes with different molecular structures and spectral properties [1,2]. In the 77 K fluorescence emission spectra of leaves of etiolated pea seedlings two main peaks can be observed around 631 and 654 nm at 440 nm excitation. Four Pchlide bands with emission maxima at 630, 640, 654 and 667 nm were described with the help of Gaussian deconvolution of these spectra [3]. The fluorescence spectra of epicotyls of dark-grown pea seedlings significantly differ from those of leaves [4]. The main emission peak at 631 nm is composed of two bands with maxima at 629 and 636 nm; the bands at 644, 655 and 670 nm are only minor components in the spectra [5]. The spectral multiplicity is due to the various molecular interactions of the chromophores, i.e., pigment aggregation [6], different localization in the etioplast inner membranes [6] and their connection to various proteins. These molecular species are named as spectral forms; they are abbreviated in this work as Pxxx, where xxx indicates the fluorescence emission maximum of the given complex. The majority of Pchlide is bound in ternary complexes with the light-dependent NADPH:Pchlide oxidoreductase (POR, EC 1.3.1.33) enzyme units and NADPH [7]. Dimers (P644) [8,9] and oligomers (P655) [6,10] of such ternary units have been described. P655 is an integral component of the prolamellar bodies' (PLB) core membranes, whereas P644 is localized mainly on the surface regions of PLBs [6]. In epicotyls, P636 is abundant, in which Pchlide is in monomeric state [5]. P636 is considered to be a monomeric ternary complex [11] but its localization is not known. A similar, short wavelength emitting protochlorophyll (Pchl, i.e., Pchlide-ester) or Pchlide form with emission maximum at 633 nm is general in etiolated materials [12]. It is localized mainly in the prothylakoid membranes [13]. On the other hand, there is a variable amount of Pchl or Pchlide, which is not bound to POR and is often referred to as "free" pigment [14]. In epicotyls, P629 was suggested to be monomer, non-POR-bound Pchl or Pchlide species. Its equivalent complex with 628 nm emission band was detected in etiolated wheat leaves

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with fluorescence spectroscopy at 10 K [15]. The above-mentioned 667–670 nm emission band is present as a shoulder in the spectra of etiolated leaves [16] and originates mostly from the broad vibrational band of P655 and contains also a (0,0) band with excitation maximum at 674 nm [15]. Consequently, the 667–670 nm emission band is the overlap of the intense and broad vibronic band of P655 and of a low intensity emission band above 674 nm. The identification of this band is very difficult in spectra of epicotyls due to the low intensities and strong overlaps in this spectral region.

The above-described Pchlide complexes have different roles in chlorophyll (Chl) biosynthesis. Both P644 and P655 are flash-photoactive, they transform through short-lived intermediates [17] into 683–684 and 696 nm emitting chlorophyllide (Chlide) forms, respectively [18]. In the initial period of phototransformation (which can be studied at low light intensities) P644 has a higher transformation rate than P655 [19]. Recent studies on pea epicotyl showed that the phototransformation of P636 is even faster than that of P644 (and P655) [11]. Pchlide in P629, P633 and in the majority of P636 can be transformed to Chlide and Chl at continuous illumination of low intensity light during several hours [20]. The phototransformation rates of these forms are influenced by their dynamic interconversions [21], which are allowed by dynamic changes of the etioplast inner membranes in vivo at room temperature [19].

The exact molecular structures and the aggregational state of the Pchlide and Chlide forms are not fully understood. The majority of data indicates the aggregated nature of P644 and P655. CD spectroscopy [6] of isolated etioplast inner membranes and experiments with chemical cross-linkers [10] proved that the P655 is a multimolecular complex of the ternary POR complexes. The oligomerization of maltose-binding protein (MBP)-bound POR and its spectral properties also showed that the long-wavelength forms must be aggregates; the POR dimers are abundant [8]. However, the aggregation number of POR units or that of Pchlide molecules in the native long-wavelength complexes is a subject of discussion. On the basis of energy migration, maximum18 Pchlide molecules were calculated as native or functional units [22].

The connections among the spectral properties of the pigment molecules, their aggregational states and/or interactions with their environment have been studied in chemically purified, protein-free model systems like non-polar solvents [23–25], solid films [26] and micellar solutions of Triton X-100 [27]. These studies indicate that the red shift of the absorption and fluorescence emission maxima of Pchl or Pchlide is connected to the aggregation of the pigments. The experiments with Triton X-100 showed that at least 10 Pchl(ide) molecules are needed for the formation of the 650 nm absorbing form [27]. These works showed that complexes with similar spectral properties could have different molecular arrangements [26]. In vitro aggregation of the POR apoenzyme, NADPH and the zinc analog of Pchlide has been described; plastid lipids and glycerol stimulate this process [28]. The lipid composition and structure have basic role in building up the PLB membranes, which effects also the formation of the P655 [29].

Glycerol is well known to stabilize or protect protein structures. Various mechanisms have been suggested about the mode of this protection. According to a well-accepted idea, glycerol molecules surround proteins but do not connect directly to the amino acid side chains. Glycerol molecules are preferentially depleted from protein surface layers, which remain enriched in water, so proteins are preferentially hydrated [30,31]. This phenomenon changes the water surface tension and solution viscosity around the protein [32,33], which results in increase in the free energy of the protein. To reduce this thermodynamically unfavourable state, the system decreases the contact surface between protein and solvent [30]. According to a different idea, the small glycerol molecules can enter the interior (hydrophilic cavities) of the protein [32], induce water release from these cavities and cause their collapse. As a result, the protein volume decreases and the number of intramolecular bonds increases [34]. Other explanation is that glycerol as osmolyte induces osmotic potential difference between the bulk solvent and the water in the cleft or core of the protein [33]. Glycerol is able to repel hydrophobic groups of the protein surface; accordingly these groups move to the protein interior, which process also decreases the final volume of the protein [35]. All these structural changes stimulate protein aggregation, too [30].

Besides glycerol, different carbohydrates are known as protein stabilizers [36]. In aqueous solution, sucrose or trehalose provoke preferential hydration [37,38] and similarly to glycerol, water release from protein cavities [33]. On the other hand, sucrose molecules are large and thus have an effect only through the periphery of globular proteins [32].

In this work, homogenates were prepared from dark-grown pea epicotyls in a phosphate buffer containing 40% (v/v) glycerol and 40% (m/v) sucrose. The homogenates were incubated at −14 °C for 2–10 days in the dark or frozen into liquid nitrogen and thawed to room temperature in several cycles. In a series of experiments these treatments were combined. Fluorescence emission spectra of the dark and illuminated samples were measured and the ratio of the Pchlide forms were compared to study the structure-stabilizing role of glycerol and sucrose. To have ultrastructural information, transmission electron microscopy was used for some of the samples.

2. Materials and methods

2.1. Plant material

Epicotyls of 5 to 12-day-old (8–25 cm long) etiolated pea (*Pisum sativum* L. cv. Zsuzsi) seedlings were used in the experiments. Plants were grown in hydroponic culture at room temperature in the dark. The middle 2–3 cm long segments of the epicotyls were collected at dim green light, which was previously tested and did not cause phototransformation.

2.2. Preparation of the homogenates

In each experiment, 3 g of middle segments of the epicotyls were homogenized in 2–3 ml of Na_2HPO_4–KH_2PO_4 buffer (pH 7.2) containing 50% (v/v) glycerol and 50% (m/v) sucrose. The homogenization was done in mortar at 0 °C at dim green light. The homogenate was filtered through 4 gauze layers and the final volume was completed with the buffer to 10 ml.

The water contents of epicotyls were determined with Unicryo-MC 4L condensation trap freeze dry system equipped with Univapo 100 ECH vacuum

concentrator centrifuge (both "Uniequip, Martinsried, Germany) as follows: the fresh weights of 6 epicotyl pieces were measured then the freeze drying procedure was done until the constant mass of the epicotyls were reached. The water contents varied between 79.4% and 94.9% of the fresh mass. In other series of experiments, 3 g of epicotyl pieces were homogenized in the phosphate buffer above containing 50% (m/v) sucrose but no glycerol. This way the extent of the dilution could be identified with refractometer (Modell 1 Carl Zeiss Jena, Germany); the original sucrose concentration of the homogenizing buffer changed to 35.8–38.1%. These values fit to the results of the lyophilization. Considering the water contents of the epicotyls, the final concentrations of glycerol and sucrose were 36.4% (STDEVP 0.8) (v/v) and 36.4% (STDEVP 0.8) (m/v), respectively. (STDEVP stand for standard deviation P - Microsoft-Excel). The initial pH of the phosphate buffer changed to 6.36 in the homogenate. The pH was measured with Orion Model 420A pH meter equipped with micro-electrode 9810 (USA). To prevent protease activity, 100 μl protease inhibitor cocktail for plant tissues (Sigma-Aldrich P9599) was added to the homogenization buffer. In several experiments, glycerol or sucrose or both were left out from the medium. To preserve the integrity of the etioplast inner membranes (what is much less organized in etioplasts of epicotyls than in those of leaves [4]), the homogenization was done directly in the glycerol and sucrose containing buffer and the homogenization was done very quickly, i.e., within 1 min and the temperature of the buffer was 0 °C. The homogenates were studied immediately after the preparation or incubated in the dark for 2–10 days at −14 °C.

2.3. Freeze–thawing experiments

To examine the stability of the native and the in vitro formed Pchlide complexes, epicotyl pieces, fresh or incubated homogenates were frozen into liquid nitrogen and subsequently thawed in the dark to room temperature (during 10 min) and frozen into liquid nitrogen again. The freeze–thawing cycles were repeated 2–5 times in the case of homogenates and the fluorescence emission spectra were measured in every cycle when the sample was in liquid nitrogen. The epicotyl pieces were frozen and thawed only once.

2.4. Illumination

The homogenates were illuminated with Chinon-8000 (Yokohama, Japan) photoflash apparatus; the energy output was 160 J/ 2 ms. In some experiments, the samples were illuminated with tungsten lamp for 10 s at room temperature; the photon flux density was set to 100 μmol m^{-2} s^{-1}.

2.5. Measurement of the Shibata shift

To study the process of the Shibata shift, four-day-incubated homogenates were pipetted into a 1 cm cuvette, thawed to room temperature and illuminated with a tungsten lamp for 10 s. The fluorescence spectra were recorded after 0; 1.5; 3; 6; 10; 15; 20; 25 and 30 min. During this period, the samples were kept in the sample compartment of the spectrofluorometer at room temperature in the dark but were illuminated with the 440 nm excitation light while recording the spectra, which lasted for 1.5 min.

In other experiments, the ratios of the Chl(ide) forms were compared after 30 min dark incubation subsequent to illumination. Middle segments of etiolated epicotyls were illuminated with a tungsten lamp for 10 s and then kept in the dark at room temperature for 30 min and frozen into liquid nitrogen. Four-day-incubated homogenates were illuminated with a photoflash apparatus and treated as above. In these experiments, the fluorescence emission spectra were measured at 77 K.

2.6. Pigment extraction

Five epicotyl pieces were halved longitudinally. One half of each segment was illuminated with white light of 100 μmol m^{-2} s^{-1} photon flux density and the pigments were extracted immediately with 80% acetone in a mortar at 0 °C in the dark. The other halves of the epicotyls were homogenized in 50% (v/v) glycerol and 50% (m/v) sucrose containing buffer. The initial pH of the buffer was 7.2. The homogenates were incubated for 4 days at −14 °C in the dark. Then they were illuminated as above and pure acetone was immediately added. The final concentration of the acetone was 80%.

In other experiments, the pigment contents of the fresh homogenates were compared to those of cold-incubated or freeze–thawed samples. In each case, 0.3 cm^3 homogenate was added to 2.7 cm^3 diethyl ether and was shaken in the dark for 2 h. The fluorescence emission spectra of the diethyl ether phase of the extractions were recorded with 430 nm excitation and the amplitudes of the emission maxima were compared at 627 nm. (These experiments were repeated with 90 and 80% acetone – final concentrations – but the precipitate formation disturbed the quantitative comparisons).

2.7. Fluorescence spectroscopy

The fluorescence emission spectra were recorded using a Jobin Yvon Horiba FluoroMax-3 (Paris, France) spectrofluorometer. The emission and excitation slits were 2 and 5 nm, respectively. The excitation wavelength was 440 or 460 nm. The integration time was 0.1 s, the data frequency was 0.5 nm. The mean of three spectra was automatically calculated in all cases; the exception was the measurement of the Shibata shift at room temperature, when only two spectra were averaged in each case.

2.8. Computer analysis of the spectra

The spectra were exported in ASCII format. Five point linear smoothing, baseline correction and in some cases Gaussian resolution were carried out with the software SPSERV V.11 (copyright Bagyinka, Cs., Biological Research Centre of HAS, Szeged, Hungary). The Gaussian resolution was done in wave number function. The three dimensional and topological projections of the fluorescence spectra were visualized with the software SURFER Version 5.02 (Golden Software, Inc., Colorado, US).

2.9. Electron microscopy

The samples were fixed in 2% glutaraldehyde for 3 h in the dark. In the case of homogenates the samples were then centrifuged for 5 min at 16,000 g. The pellet was resuspended and mixed in drops of cooling agar solution, the agar plates were then cut into small pieces. All samples were postfixed in 1% OsO_4 for 2 h. The fixatives were buffered with 70 mM Na–K phosphate buffer (pH 7.2). After dehydration in alcohol series, the samples were embedded in Durcupan ACM resin (Fluka Chemie AG). Ultrathin sectioning was achieved on a Reichert Jung ULTRACUT E microtome (Vienna, Austria). The sections were stained for 5–5 min with uranyl acetate dissolved in methanol and then with lead citrate according to [39] and were visualized by a Hitachi 7100 TEM (Japan) at 75 kV accelerating voltage.

3. Results

Biological membranes sensitively react to freeze–thawing cycles, for example samples frozen into liquid nitrogen and subsequently thawed to room temperature often loose their integrity. To study this effect on etiolated pea epicotyls, 4 cm long segments were dissected from the central regions of 15–18 cm long seedlings. 0.5 cm long segments were used for electron microscopic investigations and the next 3,5 cm long segments were frozen into liquid nitrogen. After measuring the fluorescence emission spectra of these samples, they were thawed to room temperature. 0.5 cm segments were used for electron microscopic studies and the remaining 3 cm segments were re-frozen into liquid nitrogen and fluorescence spectra were recorded again. The fluorescence spectra showed the decrease of the fluorescence intensity in the whole studied region. The relative amplitude decrease of the 655 nm band was remarkable (Fig. 1). The freeze–thawing cycle caused drastic

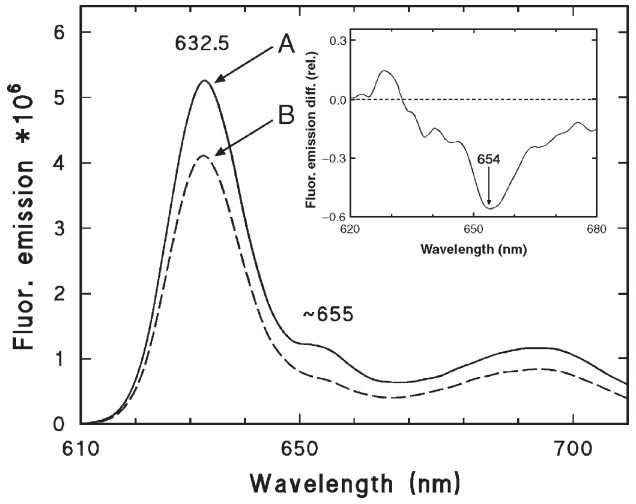

Fig. 1. 77 K fluorescence emission spectra of a segment of an etiolated pea epicotyl: the sample was frozen into liquid nitrogen (A) and subsequently thawed to room temperature in the dark during 10 min and frozen into liquid nitrogen again (B). Inset: "B–A" difference spectrum. To show the relative intensity change of the band around 655 nm, the spectra were normalized at 632.5 nm before calculating the difference spectrum. The excitation wavelength was 440 nm.

damage in all of the membranes in all studied cells of the epicotyls. The control (untreated) samples contained regular cell structures with etioplasts (Fig. 2A), some of them had well-developed PLBs (Fig. 2A inset). The freeze–thawing cycle disintegrated most of the membranes in the cells (Fig. 2B); the membrane fragments around starch grains may have originated from disrupted PLBs (Fig. 2B inset).

To study whether glycerol and sucrose could prevent this membrane destruction, middle epicotyl segments were homogenized in phosphate buffer containing 40% (v/v) glycerol and 40% (m/v) sucrose. The 77 K fluorescence emission spectra of the freshly prepared homogenates were similar to those of control epicotyl pieces (Fig. 3A and B curves A); the relative amplitude of the 655 nm band however, had a slight increase (Fig. 3A and B curves B). In this experiment the emission spectra were measured with 440 and 460 nm excitations because the former wavelength allows the observation of the short-wavelength Pchlide forms while the 655 nm form can be better identified at 460 nm excitation [40]. The electron micrographs of the homogenates showed that intact etioplasts were present in the homogenates and the PLB membranes were well preserved (Fig. 4A).

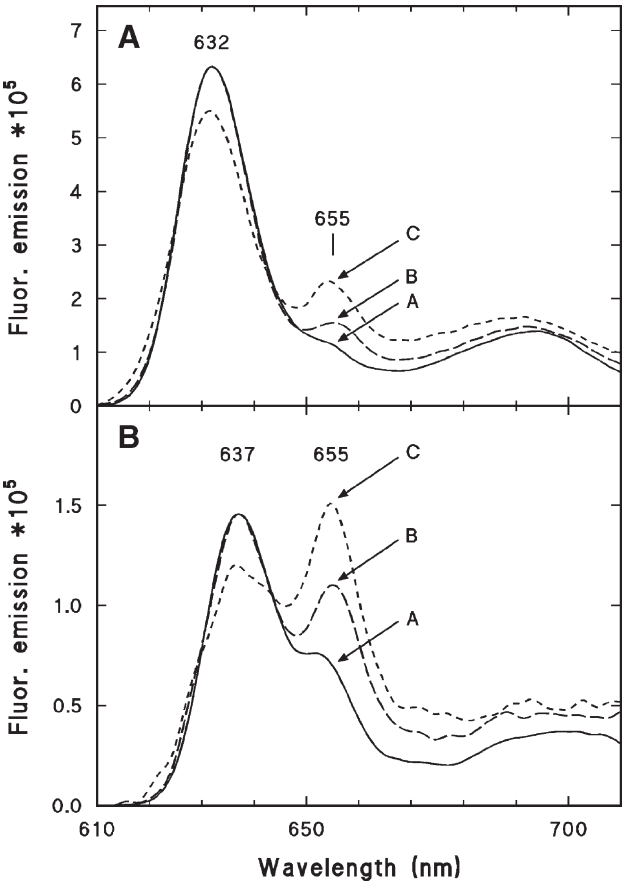

Fig. 3. 77 K fluorescence emission spectra of an etiolated pea epicotyl (A), and of a homogenate prepared from etiolated epicotyls. The spectrum of the homogenate was measured immediately after preparation (B) then it was incubated in the dark at −14 °C for 4 days (C). Spectrum A was normalized at the maximum of the B spectrum, the amplitudes of spectra B and C were not altered. The excitation wavelengths were 440 nm (A) and 460 nm (B).

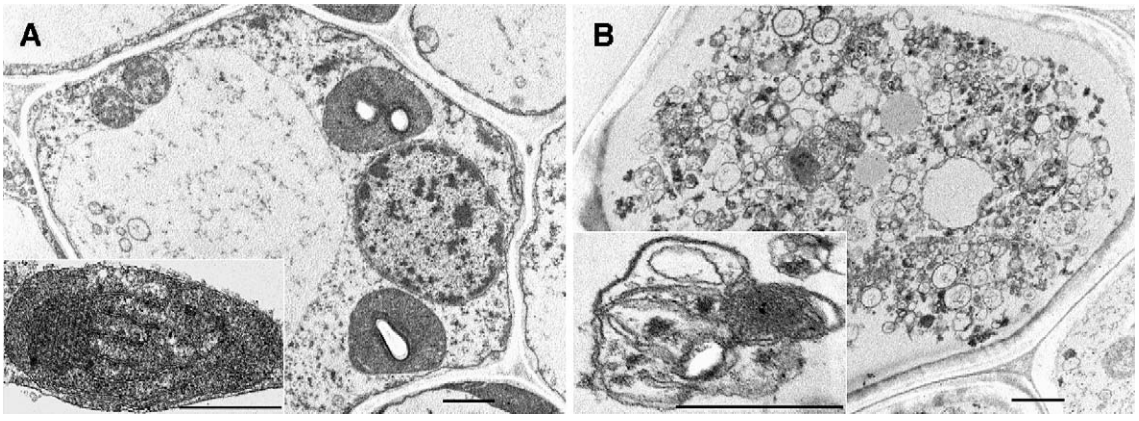

Fig. 2. Electron micrographs of cells from an etiolated pea epicotyl before (A) and after freeze–thawing treatment (B). The insets emphasize the intact (A) and disintegrated (B) prolamellar body membranes. The bars indicate 1 μm.

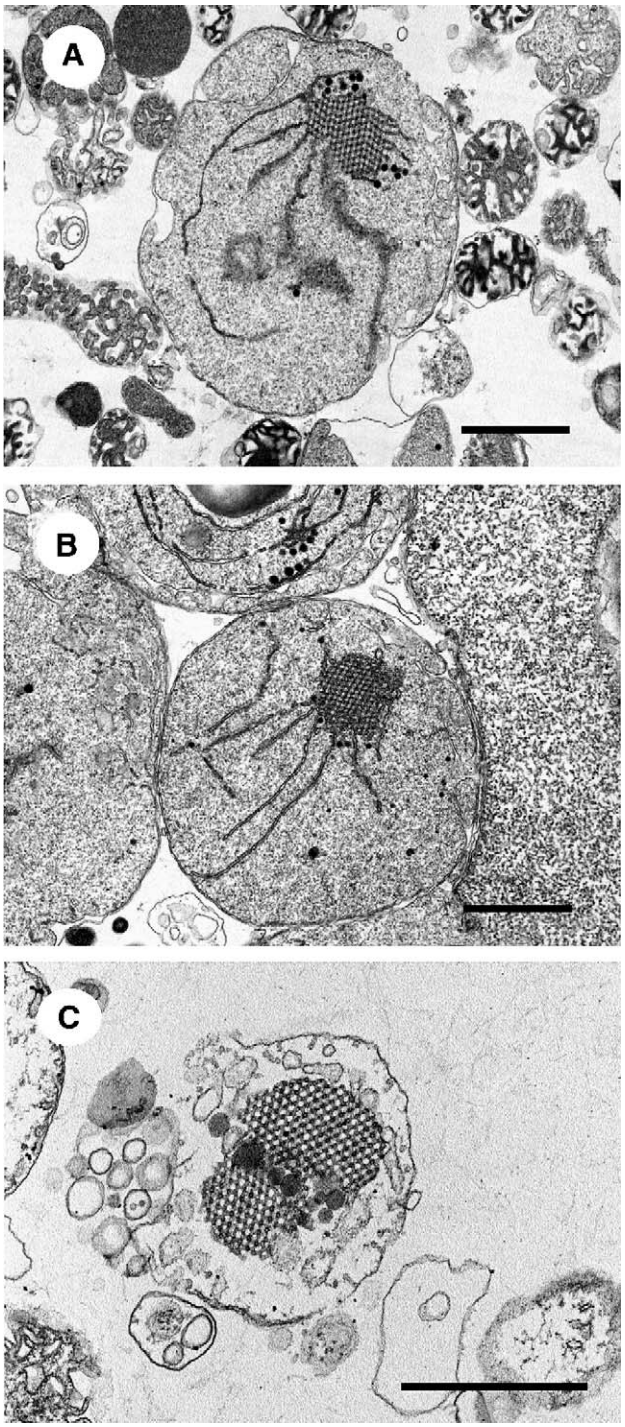

Fig. 4. Electron micrographs of homogenates of etiolated pea epicotyls. Homogenate directly after the preparation (A), the same homogenate after 5-day incubation in the dark at −14 °C (B). Freshly prepared homogenate treated with five freeze–thawing cycles. The medium was phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose. The bars indicate 1 μm.

The stability of the 655 nm emitting Pchlide form was studied. Epicotyl homogenates were stored at −14 °C in the dark, samples were taken regularly and their 77 K fluorescence emission spectra were recorded. The relative amplitude of the 655 nm emission band increased up to 4–6 days; afterwards it showed a slow decrease. The spectra recorded after 4-day incubation

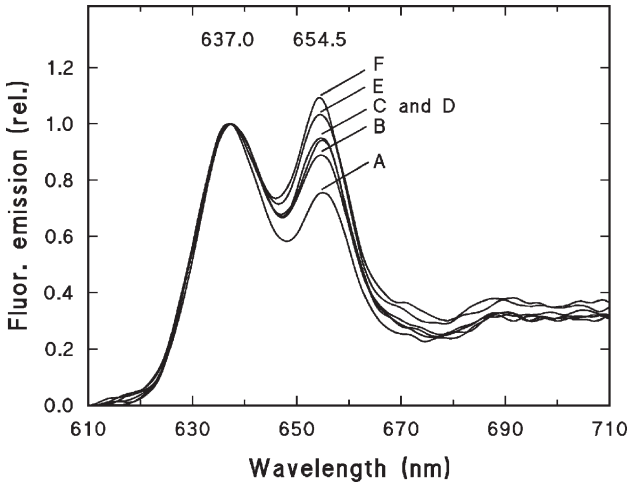

Fig. 5. The effect of freeze–thawing cycles on the 77 K fluorescence emission spectra of a freshly prepared homogenate of pea epicotyls. The homogenate was frozen into liquid nitrogen (A) and subsequently thawed to room temperature in the dark during 10 min and frozen into liquid nitrogen again (B). This treatment was repeated four more times (curves C–F). Phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose was the medium. The spectra were normalized at 637 nm. The excitation wavelength was 460 nm.

are presented in Fig. 3A and B curves C. The electron micrographs showed the presence of intact etioplasts and PLBs with regular membrane structures in the homogenates incubated for 5 days (Fig. 4B).

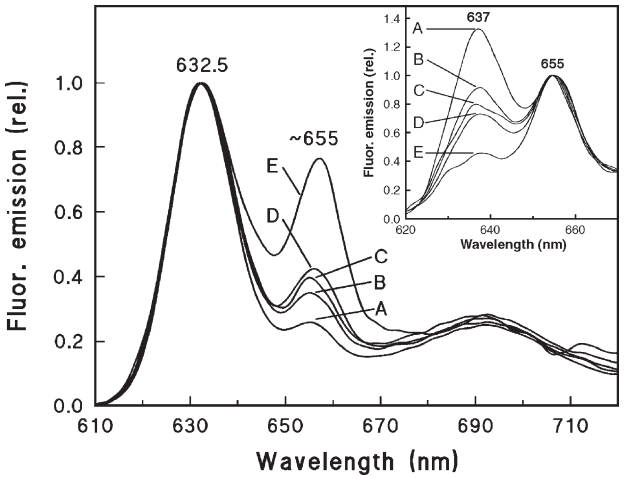

Fig. 6. The effects of 4-day incubation at −14 °C, freeze–thawing cycles and their combinations on the P655 formation in homogenates of etiolated pea epicotyls. The 77 K fluorescence emission spectrum of the freshly prepared homogenate (A). The spectrum of the same homogenate after five freeze–thawing cycles; the samples were frozen into liquid nitrogen and thawed to room temperature during 10 min in the dark in each cycle (B). Spectrum of the sample "B" after 4 days incubation in the dark at −14 °C (C). Another sample of the "A" homogenate was incubated for 4 days after the preparation and then frozen into liquid nitrogen (D). Spectrum of the sample "D" after two freeze–thawing cycles (E). Phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose was the medium. The excitation wavelength was 440 nm. The spectra were normalized at their maxima. Inset: 460 nm excitation spectra of the same samples. The spectra were normalized at their maxima at 655 nm.

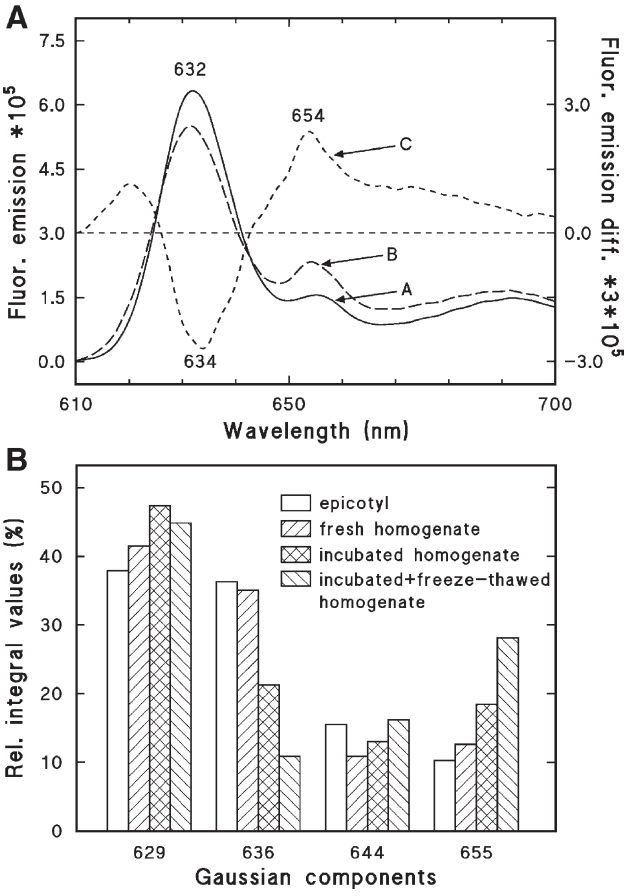

Fig. 7. (A) 77 K fluorescence emission spectra of a homogenate prepared from etiolated pea epicotyls. Spectrum of the freshly prepared homogenate (A) and of the same homogenate after 4 days dark incubation at −14 °C (B). Curve C shows their difference spectrum "B–A". Phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose was the medium. The excitation wavelength was 440 nm. (B) Relative integral values of the Gaussian components in the 77 K fluorescence emission spectra of etiolated pea epicotyls and differently treated homogenates prepared from etiolated epicotyls. Epicotyl: empty bar; freshly prepared homogenate: right-hatched; incubated at −14 °C in the dark for 4 days: crosshatched; freeze–thawed in two cycles after 4 days incubation: left-hatched. The sum of the integral values of the four different Gaussian components (their maxima are indicated along the X axis) was taken to 100%. Phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose was the medium. Spectra recorded with 440 nm excitation wavelength were used for the Gaussian deconvolution.

The freeze–thawing cycles did not cause the decrease of the relative amplitude of the 655 nm emission band in the spectra of freshly prepared homogenates. On the contrary, the ratio of the 655 nm to the 636 nm band (clearly observed at 460 nm excitation) gradually increased after each cycle (Fig. 5). The damage of the etioplast envelope membranes were observed in the electron micrographs of these samples, but the PLB lattice were well preserved (Fig. 4C).

To study if pigment degradation occurred during the 4-day incubation or the freeze–thawing treatments, the total pigments were extracted from the fresh and treated homogenates with diethyl ether. The amplitude changes of the 627 nm emission band had a variation less than 2.5% indicating that no considerable degradation took place (data not shown).

The effects of the 4-day incubation at −14 °C and of freeze–thawing cycles on the production of P655 enhanced each other when the two treatments were applied in sequence. However, the extent of stimulation was the greatest when the samples were first incubated then treated with freeze–thawing cycles (Fig. 6).

To understand the reason of the relative increase of the 655 nm emission band, the spectra of the freshly prepared and 4-day incubated homogenate (aliquots of the same preparation) were compared and their difference spectrum ("4-day incubated minus fresh") was calculated. Since the spectra of the same sample in the same sample tube were recorded, the absolute values and their differences are compared in Fig. 7A. The difference spectrum had a negative band at 634 nm and a positive one at 654 nm. These results agree with the Gaussian analyses of the spectra; the spectrum of a fresh homogenate was deconvoluted into Gaussian components with maxima at 629, 636, 644 and 655 nm. The same calculations were done on the spectrum of the same homogenate after 4 days of dark incubation and on the spectrum of an intact epicotyl piece. The integrals of the components were calculated and compared. The relative decrease of the 636 nm and the parallel increase of the 655 nm band were found (Fig. 7B).

To examine if sucrose or glycerol alone could stabilize the 655 nm emitting Pchlide complex, epicotlys were homogenized in buffer containing either 40% sucrose or 40% glycerol and the homogenates were incubated at −14 °C for 4 days. The 77 K fluorescence spectra of the freshly prepared homogenate with only sucrose showed a remarkable decrease of the 655 nm band. Further decrease was observed in the spectra of the 4-day incubated samples. The glycerol alone did preserve the structure, i.e., the amplitude of the 655 nm band was similar to that in

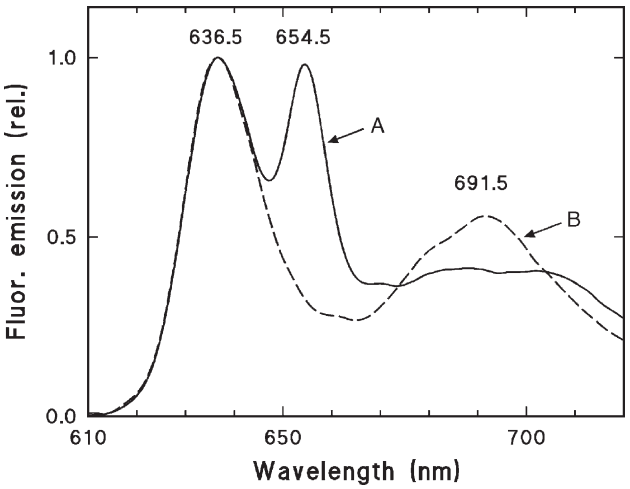

Fig. 8. 77 K fluorescence emission spectra of incubated (for 4 days in the dark at −14 °C) epicotyl homogenates before (A) and after (B) flash illumination. The spectra were normalized at their maxima. The excitation wavelength was 460 nm. Phosphate buffer (pH 6.36) containing 36.4% (STDEVP 0.8) (v/v) glycerol and 36.4% (STDEVP 0.8) (m/v) sucrose was the medium.

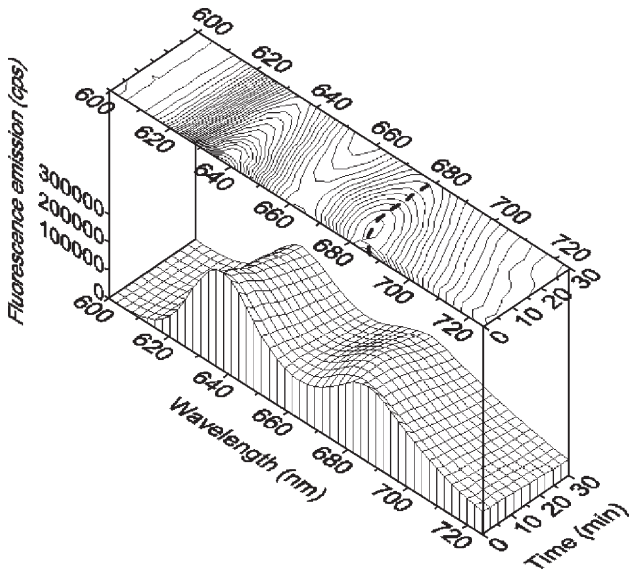

Fig. 9. Blue shift of the 687 nm emission band in the room-temperature fluorescence emission spectra of an epicotyl homogenate incubated at −14 °C for 4 days then illuminated with white light of 100 μmol m^{-2} s^{-1} photon flux density for 10 s and then incubated in the dark and re-illuminated with the excitation light of the spectrofluorometer during recording of the spectra. The three dimensional and topological projections were calculated from spectra measured 0; 1.5; 3; 6; 10; 15; 20; 25; 30 min after the illumination. The excitation wavelength was 440 nm.

spectra of intact epicotyls, however, no amplitude increase of this band was found after 4-day incubation (data not shown).

To study the photoactivity of the 655 nm emitting Pchlide form produced during the dark incubation at −14 °C, the homogenates were illuminated with 2 ms white light flash of 160 J and were frozen into liquid nitrogen within 5 s. The 77 K fluorescence emission spectra showed the disappearance of the 655 nm emission band and the parallel increase of an emission band around 690 nm (Fig. 8). Similar results were found after the illumination of the 655 nm emitting Pchlide form produced via freeze–thawing cycles (data not shown).

The increase in the extent of Pchlide photoreduction was found when the pigments were extracted from illuminated epicotyl segments and from 4-day incubated and subsequently illuminated homogenates. The emission spectra of the pigment extractions were measured at room temperature; the spectra were normalized at 630 nm (i.e., at the emission band of Pchlide and Pchl). Thus the amplitude at 670 nm (the emission band of Chlide and Chl-a) correlated with the Chl(ide) concentrations. This experiment showed that the concentration of Chl(ide) was around twice as high in extractions prepared from homogenates as that in epicotyl extractions (data not shown).

Four-day incubated homogenates were thawed to room temperature and pipetted into 1 cm cuvette. The samples were illuminated with light of 100 μmol m^{-2} s^{-1}. As a result, an emission band corresponding to oligomeric Chl(ide) complexes appeared at 687–688 nm in the room-temperature fluorescence spectrum. A gradual blue shift of this band – similar to the Shibata shift in spectra of illuminated native etiolated leaves – was observed in the function of time after the illumination. In this experiment, the sample was kept at room temperature and the emission spectra were recorded at the same temperature. The Chl(ide) emission band shifted to 679 nm within 10 min (Fig. 9).

Only a partial “Shibata shift” took place in the glycerol- and sucrose-containing buffer. While it was almost complete in intact epicotyls within 30 min, the obvious presence of the 690 nm band (the initial position of the Chlide band immediately after the flash illumination) was observed even after 30 min room-temperature incubation of the illuminated samples when 4-day incubated homogenates were studied and fluorescence spectra were measured at 77 K. However, the clear identification of the amplitudes of the Chlide or Chl emission bands was difficult because of the strong overlaps between the Chlide and Chl bands and vibronic satellite bands of non photo-transformed Pchlide forms. The Shibata shift was even less complete in the case of freshly prepared homogenates, when the samples were cooled to 77 K, thawed and kept at room temperature for 10 min in three cycles (data not shown).

4. Discussion

The spectral multiplicity of Pchlide forms and their interconversions among one another are well documented in leaves [41]. The dynamic equilibrium between the 635 and 650 nm absorbing Pchlide forms was suggested [21]. Recently, a scheme was published in which the interconversions of 629, 636, 644 and 655 nm emitting Pchlide forms are suggested in etiolated pea epicotyls [11]. The question can be raised if these transformation processes could be explained by aggregation and/or disaggregation of Pchlide containing molecular units. On the basis of the high sensitivity of the 655 nm form at heat treatment [42,4,43] or at freeze–thawing treatment [42], disaggregation has been proposed to explain the blue shift of the absorption and/or fluorescence maximum of this form [44]. When etiolated barley leaves were treated with freeze–thawing cycles, the shift of the 650 nm absorption maximum took place to 635 nm, however, this process was prevented in the presence of 30% glycerol [44]. The appearance of P655 has been considered as a prerequisite of the well-organized PLB membrane structure [45] but the direct connections between the membrane structures and spectral properties have not been clarified yet. According to the above-described explanations, freeze–thawing treatments disturb the tubular structures of PLB membranes and make them osmiophilic in the case of etiolated bean leaves [42]. Similarly to these phenomena in leaves, the PLB membranes in intact pea epicotyls were also sensitive to heat [4] or freeze–thawing treatments (Fig. 2), and in parallel the emission band at 655 nm disappeared (Fig. 1).

In this work, the direct transformations of P636 into P644 and P655 were found in epicotyl homogenates. Since the ratio of P636 to P655 is higher in etiolated pea epicotyls than in leaves, the processes above could be clearly seen. The formation rates of P644 and P655 were strongly affected by the mass of epicotyl homogenized in a unit volume (10 ml) of buffer. This can be explained by the ratio changes of the Pchlide forms with the age and along the epicotyl [4] and by the varying water content of the epicotyls. Since the lengths of the seedlings were not the same,

their physiological states were different. Therefore, the sample collection could not be standardized quantitatively, so no mean spectra are shown and no error levels or other statistical calculations were used in this work. The figures show tendencies, which were the same in all experiments.

A very important feature of the P655 produced in homogenates either with incubation or with freeze–thawing cycles or with combinations of these treatments, is that it was fully photoactive upon short time (flash or 10 s) illumination (Fig. 8). Even more, the relative amount of Chlide in homogenates was higher than in intact epicotyls studied in acetone extractions. (The absence of pigment degradation supports the conclusion that flash-photoactive P655 was produced). This indicates that the Pchlide molecules in this artificially produced complex must be localized in the active sites of the POR units and also the NADPH molecules must be in effective positions. The comparison of the ratios of the Pchlide forms (i.e., the integrals of their relevant Gaussian components) showed that P644 and P655 were formed from P636 (Fig. 7B). Recently, the direct photoactivity of P636 was described in intact pea epicotyls [11]. This form was shown to have Pchlide in monomeric state [5] and it has been suggested to be a monomeric POR unit [11]. On the other hand, P644 has been suggested to have dimeric [8,9] and P655 oligomeric structure [6,10]. Thus the P636–P644 and P636–P655 transformations can be taken as pigment and protein aggregation processes. The results of the total pigment extraction, i.e., that no pigment degradation took place during the incubation or freeze–thawing treatments, support that the above described spectral changes can be explained by aggregation and not by different degradation rates of the various Pchlide forms.

On the basis of the structure stabilization and aggregation promoting properties of glycerol described for soluble proteins [30,31] and biological membranes [46], significant effects could be expected on the above-mentioned aggregational states of POR units and thus Pchlide forms, too [44]. Due to their glycerol- and sucrose-enriched environment, POR units must have decreased their volume and they could aggregate in epicotyl homogenates what was shown by the amplitude increase of the 655 nm emission band (Fig. 3A and B). However, aggregation via diffusion is mostly delayed at −14 °C in a viscous environment like the homogenization buffer used in this work. On the other hand, conformational changes of membrane particles holding several P636 units could proceed under these circumstances. Since P636 is presumably localized in lamellar membranes of PTs and/or small and loose PLBs unlike the longer wavelength Pchlide complexes [4] such conformational changes can be of basic importance. The volume reduction caused by glycerol [34] may cause folding or curling up membrane particles which can bring together P636 units to such an extent that the π-electron systems of the Pchlide molecules in their active sites interact and the exciton interaction causes the red shift in their emission maxima to 644 or 655 nm (Fig. 7A and B).

The freeze–thawing treatment could accelerate these conformational changes; whenever the samples were thawed to room temperature, fast molecular movements were allowed. This could force glycerol molecules entering the protein interiors [32] provoking this way volume change and aggregation [34] of the POR-proteins (i.e., the connected Pchlide molecules), causing this way the increase of the 644 and 655 nm bands (Fig. 5). The production of P655 was more effective during the 3–5 days cold incubation than in the freeze–thawing treatments. Interestingly, the combination of the long-term incubation and freeze–thawing cycles was strikingly more effective than these treatments alone (Fig. 6). During the long-term incubation, the glycerol molecules could get close to the positions of POR units or membrane particles what would be important for the conformational changes causing P644 or P655 formation. The freeze–thawing cycles could loose this structure and cause additional conformational changes. Electron micrographs of incubated and freeze–thawed homogenates (Fig. 4b and c) confirm this hypothesis. In the latter case, broken etioplasts were found and thus glycerol molecules could easily reach PLB membranes and membrane-bound proteins. As a consequence, glycerol molecules could enter the inner cavities in the membrane particles and POR units, which might lead to additional P644 or P655 formation. Because of the large size of sucrose, another mechanism can explain its structure stabilizing effect. These molecules surround the membrane particles and/or POR units and cause preferential hydration [37] what delays decomposition or disaggregation. In our experiments, the presence of sucrose enhanced the structure protecting effect of glycerol.

Only a partial “Shibata shift” took place in the sucrose- and glycerol-containing medium. This process has been explained by disaggregation of the PLB membrane particles [47,48] or of the POR units [49] as well as by their conformational changes [10,50]. The structure stabilization effect of the medium was strong enough to prevent the complete progression of the “Shibata shift” in the illuminated samples, which were incubated for 4 days at −14 °C (Fig. 9). Even more, only partial “Shibata shift” took place under freeze–thawing cycles. A total inhibition of this process was described at 87% concentration of glycerol [51]. The fact that the “Shibata shift” partially proceeded in the medium used in this work, indicates that small conformational changes are sufficient for the blue shift [10,50].

Only a part of P636 could be transformed into longer wavelength emitting complexes (Fig. 6, inset) and was flash-photoactive when directly illuminated with 632.8 nm laser light [11]. This indicates the heterogeneity of P636, i.e., the flash-photoactive complexes must be Pchlide molecules bound to the active sites of POR units and a different population of Pchlide is outside of the active site or not-POR bound. Consequently, only the flash-photoactive P636 units did aggregate into flash-photoactive P655. This proves that, at least a part of P636 is monomeric POR unit. In the aggregates, short-distance molecular movements allow the cyclic reaction of the enzyme thus surplus NADPH and Pchlide molecules can “reload” the active sites. The increased extent of phototransformation confirms that the oligomerization gives protection against photooxidation.

The results of this work prove the oligomeric nature of the long-wavelength flash-photoactive Pchlide forms composed of also flash-photoactive monomeric units. The in vitro formation

of P644 and P655 shows the possibility of similar processes in vivo, i.e., the already suggested dynamic interconversions of native Pchlide forms into each other [21]. On the other hand, these results prove the structure stabilization and/or aggregation forcing properties of glycerol and sucrose. Since these compounds are often used during sample preparation in biology, and they modify the native geometries, their presence should be considered and interpreted when the isolated proteins are characterized.

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