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Protochlorophyllide in model systems — An approach to in vivo conditions

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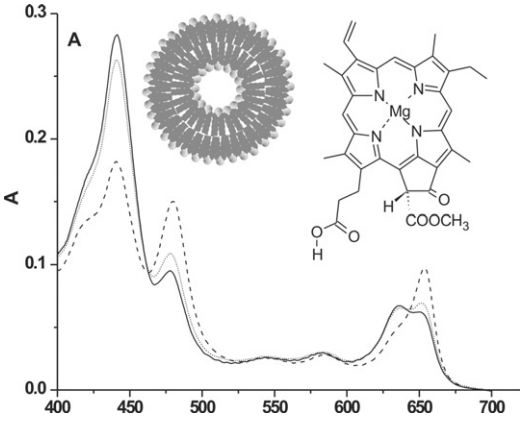
HIGHLIGHTS

- Absorption and fluorescence of protochlorophyllide was studied in model systems.
- Galactolipids facilitate protochlorophyllide aggregation.
- Fluorescence lifetimes of Pchlide aggregates were 0.1 and 1.5–2 ns.
- Fluorescence lifetimes of Pchlide monomers in liposomes were of 4.1–4.6 ns.
- Molecular dynamics of water is important for Pchlide aggregation.

GRAPHICAL ABSTRACT



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ABSTRACT

Absorption and fluorescence properties of protochlorophyllide (Pchlide) monomers and aggregates in various model systems are presented in this study. The absorption and fluorescence maxima, and fluorescence lifetimes of Pchlide monomers were not dependent on liposome composition. Fluorescence quenching experiments using KI and SASLs as fluorescence quenchers, revealed that Pchlide molecules entered a lipid bilayer and were localized close to the polar lipid headgroup area. The process of Pchlide aggregation was evident for high (i.e. at least 9 mol%) Pchlide content in liposomes prepared from galactolipids. To our knowledge, this is the first study of Pchlide aggregation in membrane-mimicking model systems. The aggregates showed absorption maxima at 480 and 650 nm. Fluorescence of the aggregates measured for excitation at 480 nm had a maximum at 656 nm and was characterized with two fluorescence lifetime components, i.e. 0.1 and 1–2 ns. Pchlide aggregates observed in the buffer had similar position of absorption and fluorescence bands to those observed in liposomes, although the overall fluorescence intensity was considerably lower. Some differences in the relative intensity of Soret absorption bands were observed. These results showed that the presence of liposomes decreased the efficiency of the process of Pchlide aggregation. Water bound at the interface region of AOT/isooctane/water reversed micelles induced disaggregation of the Pchlide aggregates indicating that Pchlide aggregates are buried into hydrophilic core of micelles. The results are discussed with respect to the role of lipids in Pchlide aggregation found in plant etioplasts and their significance for light-induced Pchlide photoreduction.

Abbreviations: ALA, δ-aminolevulinic acid; AOT, dioctyl sulfosuccinate sodium salt; Chl, chlorophyll; Chlide, chlorophyllide; DGDG, digalactosyldiacylglycerol; LPOR, light-dependent protochlorophyllide oxidoreductase; EYL, egg yolk lecithin; MGDG, monogalactosyldiacylglycerol; Pchlide, protochlorophyllide; PG, phosphatidylglycerol; PLB, prolamellar body; PT, prothylakoid; SQ, sulfoquinovosyldiacylglycerol; SUV, small unilamellar liposome.

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1. Introduction

Protochlorophyllide (Pchlide) is a porphyrin dye and one of the key intermediates in the biosynthetic pathway of chlorophyll (Chl), the main photosynthetic pigment. Pchlide to Chl conversion involves two reactions. The first is reduction of one double-bond in the porphyrin ring, leading to chlorophyllide (Chlide) formation, and the second is esterification of Chlide by phytol or its unsaturated precursors (for a review see refs. [1–5]). While being excited by light, tetrapyrroles can act as photosensitizers, and thus their synthesis and distribution must be strictly regulated and controlled by plants (reviewed by [6,7]). On the other hand, photosensitizing properties of tetrapyrroles make these compounds a promising object of application in photodynamic therapy (for a review see e.g. ref. [8]).

In angiosperms, Pchlide reduction to Chlide is totally light-dependent and catalyzed by a protochlorophyllide oxidoreductase (LPOR, EC 1.3.1.33), a photoenzyme. Angiosperms accumulate Pchlide in the dark but do not synthesize Chl (for a review see refs. [4,7,9,10]). Nevertheless, Chl synthesis can be continued under subsequent light conditions. Light-triggered reduction of Pchlide plays a key regulatory role in Chl biosynthesis, as well as in angiosperm development, being the first event in de-etiolation, i.e. the process leading to the formation of a photosynthetically active plant. In the absence of light, Pchlide accumulate in etioplasts, which develop instead of chloroplasts and contain a regular paracrystalline lipid structure known as a prolamellar body (PLB; for a review see refs. [11,12]). PLB is surrounded by flat and unstacked membranes called prothylakoids (PT). Among different proteins detected in PLB [13], the most abundant is LPOR, which is found mainly in the form of ternary Pchlide: LPOR:NADPH complexes [14].

Pchlide in vivo shows great spectral heterogeneity, which has been intensively investigated using absorption and fluorescence spectroscopy (for a review see refs. [7,9,15,16]). In general, long-wavelength forms, found in PLBs, were ascribed to aggregates of Pchlide:LPOR:NADPH complexes of different sizes that are stabilized by interaction of π electrons of the neighboring pigment molecules [17]. Short-wavelength forms were assigned to Pchlide which was unbound to the LPOR enzyme and mainly found in PTs [14,18]. The redox state of NADPH in Pchlide: LPOR:NADPH complexes also influences Pchlide spectral properties and contributes to the heterogeneity [19]. Fluorescence lifetime study confirmed this heterogeneity and revealed three lifetime components of 0.25, 1.8–2.0 and 5.5–6.0 ns for Pchlide in etioplast membranes at room temperature, and double-exponential decay at 77 K [20,21]. Due to the equilibrium existing between non-aggregated and aggregated Pchlide forms and the fact that it is difficult to control the conversion of one Pchlide form into another during isolation procedures, the separation of different Pchlide spectral forms by standard biochemical methods appeared impossible. At the same time, the strong overlapping of the fluorescence excitation and emission spectra of individual Pchlide forms is a fundamental difficulty in separating these forms, using different excitation and emission wavelengths. Due to these experimental problems, the nature of the interactions between the components of Pchlide:LPOR:NADPH complexes, the mechanism of their aggregation, their localization in the PLB structure and interactions with the lipid moiety, as well as the role of Pchlide aggregation in the catalytic mechanism of Pchlide photoreduction are still a matter of debate. Moreover, the localization of Pchlide molecules that are unbound to LPOR, but exist somewhere within the PT and PLB membranes, as well as their interaction with the lipid lattice also remains unknown. The functioning of Pchlide and its interaction with the local microenvironment can be studied in vivo but this native system displays all of the properties in a very complex form. Alternatively, these studies can be performed in model systems that simulate in vivo conditions. The presented experiments were aimed at characterizing of Pchlide in various model systems such as liposomes, reversed micelles and organic solvents by absorption and fluorescence study. We focused our attention on the formation of Pchlide aggregates and the effect of lipids on Pchlide–Pchlide interaction.

2. Materials and methods

2.1. Pigments, lipids and solvents

Pchlide was extracted from 6 day-old dark-grown wheat (*Triticum aestivum*) leaves treated with δ-aminolevulinic acid (ALA) and isolated using HPLC, as described previously [22]. Treatment with ALA stimulated Pchlide accumulation in etiolated seedlings. The organic solvents used for liposome preparation were of spectroscopic grade and those for Pchlide purification were of analytical or HPLC grade. Lipids were purchased from Sigma and Lipid Products (South Nutfield, Redhill, Surrey, UK).

2.2. Liposome preparation

Small unilamellar vesicles were prepared by injection as described by Jemioła-Rzemińska et al. [23] in a Hepes–NaOH buffer (pH 7.5). In short, 25 μl of ethanol solution containing all of the required compounds (i.e. lipids and Pchlide) was slowly injected into 2 ml of a buffer under stirring. The liposomes were prepared from egg yolk lecithin (EYL), or mixtures of plastid lipids: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQ) and phosphatidylglycerol (PG). The plastid lipids were mixed at different proportions for liposome preparation, as indicated in the Results. In particular, MGDG, DGDG, PQ and SQ were mixed at the proportion that was found for PLB by Ryberg et al. [24]. Liposomes were prepared at room temperature.

The pigment content in liposomes varied between 0.1 mol% and 17 mol% (i.e. the following pigment:lipid molar ratios were examined, 1:1000, 1:500; 1:100; 1:50; 1:25; 1:15; 1:10 and 1:5). The final lipid concentration was 0.031 mM in the case of 9–20 mol% of Pchlide or 0.5 mM for the other samples.

2.3. Micelle preparation

Micelles were prepared according to the procedure described in [25]. AOT (dioctyl sulfosuccinate sodium salt) was dissolved in isooctane to the final concentration of 50 mM. A methanol Pchlide solution was evaporated in a glass tube and dissolved in reversed AOT micelles to reach 5 μM Pchlide concentrations. In another experiment, Pchlide was dissolved in isoamyl alcohol and diluted with isooctane to obtain isoamyl alcohol:isooctane mixture of 0.14:1 (v/v). The sample was incubated at room temperature overnight until Pchlide aggregates were formed. Then it was centrifuged at 14000 ×*g* for 30 min. A delicate pellet was suspended in isooctane and supplied with AOT/isooctane reversed micelles to a 20 mM final AOT concentration. The sample was incubated at room temperature for 8–12 h with stirring. Aliquots of water were added to give different molar H_2O/AOT ratios with vortexing repeated after a single water addition.

2.4. Absorption measurements

Absorption spectra were recorded with an SLM AMINCO DW-2000 (Aminco Instruments, USA) spectrophotometer between 380 and 750 nm.

2.5. Fluorescence measurements

Steady-state fluorescence emission spectra were measured in the range of 595 to 750 nm using a Perkin-Elmer spectrofluorometer (LS50B, UK). For the instrument settings see Ref. [26]. The excitation wavelength for a given experiment is indicated in the Results. The spectra were corrected for the baseline and wavelength-dependent

sensitivity of the photomultiplier. The fluorescence maxima agreed to 0.5–1 nm with the independent sample preparation.

The fluorescence lifetime was measured with a multifrequency cross-correlation phase and modulation K2 fluorometer equipped with a 300 W Xenon lamp as a light source and a Pockels-cell modulator (ISS Instruments, USA). Some details of the instrument setting have already been described [26]. Average fluorescence lifetime values with standard deviation were calculated for several (five to seven) independent repetitions of a given sample preparation.

In fluorescence quenching experiments small aliquots (μl) of a quencher stock solution were added to a sample and a fluorescence spectrum was measured. The sample was continuously stirred during the whole experiment. In experiments performed for Pchlide in liposomes, the addition of the quencher was followed by 20-min incubation at room temperature before measurement. The following fluorescence quenchers were used: KI and spin labels, namely 5-doxylstearic acid (5-SASL) and 16 doxyl-stearic acid (16-SASL). 5- and 16-SASL have free radical fragment (nitroxyl group) attached to C-5 or C-16 carbon of stearic acid, respectively. SASLs were added from an ethanolic stock solution (1 M) and the final ethanol concentration in the liposomal samples never exceeded 1% (v/v). The aqueous stock solution of KI (4 M) contained 0.1 mM sodium thiosulfate to prevent I_3^- formation. The presence of these quenchers did not change the Pchlide absorption spectrum.

All the fluorescence measurements were performed at room temperature (22 ± 2 °C). In the case of samples having an optical density (OD) higher than 0.15, the measurements were performed in small cuvettes with an optical path of 0.5 cm.

2.6. Resolution of absorption spectra

The resolution of Q region of absorption spectra (14000–20000 cm^{-1}) of Pchlide in liposomes were carried out with PeakFit software (v 4.0; Jandel, USA). Prior to resolution, the wavelength–frequency conversion was performed. The spectra were smoothed using a five point linear smoothing, and an exponential background was subtracted if needed. The resolution was performed using a uniform type of Gaussian–Lorentzian function for all the components. A minimal number of components that have the r^2 value higher than 0.999 without any systematic errors was accepted. Four or five components usually fulfilled the criteria.

3. Results

3.1. Low concentration of Pchlide in model systems mimicking membrane

In the first part of the experiments, Pchlide concentration was kept between 1 and 5 μM, which corresponded to 0.2 and 1 mol% pigments with respect to lipids. Such a low Pchlide concentration prevented its aggregation. In the case of fluorescence studies, a Pchlide concentration of 0.5 μM (i.e. 0.1 mol%) was examined to ensure that no self-quenching effects were detected. Small unilamellar liposomes (SUV) were prepared from mixtures of chloroplast lipids: MGDG, DGDG, PQ and SQ. Different relative proportions of the lipids were examined, including the one corresponding to the lipid composition of PLB [24]. Liposomes from natural egg yolk lecithin (EYL) were used in this study as they are routinely applied as model membranes. In addition, Pchlide was also examined in MGDG, which tends to form reversed hexagonal phases [27].

The absorption spectra of Pchlide had a shape typical of a porphyrin-like compound in all the membrane-mimicking models that were investigated (Fig. 1). They consisted of two complex bands: a Soret band having a maximum around 440–441 nm and a Qy band with a maximum at 633–634 nm (Fig. 1) and showed almost no differences in the position of the absorption maxima in liposomes of different composition (Table 1). The intensity ratio of the Soret band to the Qy band varied between 4.3 and 5.8. The steady-state fluorescence emission spectra of Pchlide had a main band between 640 and 642 nm, accompanied by a vibrational side-band in the range of 680–690 nm (Fig. 1; Table 1). The Stokes shift calculated for the data from Table 1 was between 152 and 196 cm^{-1}. The fluorescence decay of Pchlide (0.5 μM, i.e. 0.1 mol%) was single-exponential in all the models investigated, and showed a fluorescence lifetime between 4.1 and 4.4 ns (Table 1). In the case of liposomes prepared from plant lipids, some shortening of a Pchlide fluorescence lifetime was noticed in the case of liposomes containing MGDG compared to liposomes prepared from DGDG only.

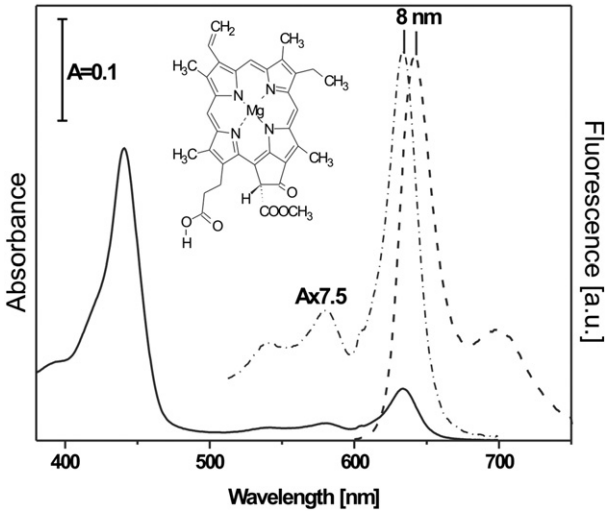

Fig. 1. Absorption and fluorescence emission spectra of Pchlide in liposomes prepared from the mixture of chloroplast lipids MGDG:DGDG:SQDG:PG (51:29:7.7:12.3 mol/mol). Pchlide concentration was 1.3 μM. The chemical structure of Pchlide is inserted in the figure.

3.2. Localization of Pchlide in liposomes

In order to reveal the localization of Pchlide molecules within the lipid bilayer, fluorescence quenching experiments were performed using a polar quencher (KI), as well as spin labeled stearic acids (i.e. 5- and 16-SASL) which are membrane-localized quenchers. The SASL quenchers act at different depths within a lipid bilayer, the first can quench the fluorescence of a fluorophore localized close to the lipid headgroup area whereas the second does so in the membrane interior. A set of fluorescence emission spectra was measured for increasing quencher concentrations for given liposomes. A maximal fluorescence intensity was read from each spectrum. The Stern–Volmer equation (Eq. (1)) was used for the analysis.

$$F_0/F = 1 + K[Q] \quad (1)$$

where: F_0 and F are the maximal fluorescence intensities in the absence and presence of quencher, respectively; K is the Stern–Volmer constant; [Q] is the quencher concentration.

The Stern–Volmer plots and respective Stern–Volmer constants calculated as the slope of the linear fits obtained for Pchlide in liposomes prepared from MGDG:DGDG:SQDG:PG (51:29:7.7:12.3) are shown in Fig. 2, to provide an example. The most effective quenching was observed for 5-SASL, which was about 20 times higher than for 16-SASL. The quenching constant for KI, which is a polar quencher, was four times higher in ethanol than that in liposomes. Similar results of quenching by KI and SASLs were observed for liposomes prepared from other lipids, i.e. DGDG, a mixture DGDG:MGDG (70:30; mol/mol) and EYL. The quenching constants of KI were considerably lower (i.e. 2–3 orders of magnitude) than those of SASLs. SASLs accumulate in the lipid bilayer

Table 1
Absorption and fluorescence maxima, and fluorescence lifetime of protochlorophyllide (Pchlide) in membrane-mimicking model systems. Pchlide concentrations of 5 μM and 0.5 μM were used in absorption and fluorescence measurements, respectively. These concentrations corresponded to 1 mol% and 0.1 mol% pigment content, respectively. Fluorescence measurements were performed for excitation at 440 nm. The temperature was 22 ± 2 °C.

System	Absorption maxima [nm]		A(Soret) / A(Q_y) [±0.2]	Fluorescence maximum [nm]	Fluorescence lifetime [ns]	Stokes shift [cm^{-1}]
	Soret	Qy				
Liposomes						
EYL	440	633.8	5.6	640	4.4 ± 0.1	152 ± 25
DGDG	440.8	633.1	4.5	639	4.4 ± 0.1	170 ± 25
DGDG:MGDG (70:30)	440.2	634.0	5.8	641	4.1 ± 0.2	172 ± 25
DGDG:MGDG:SQ:PG (51:29:7.7:12.3)	440.8	634.0	5.0	642	4.2 ± 0.1	196 ± 25
Hexagonal phases						
MGDG	440	633.5	4.8	640	–	160 ± 25
Reversed micelles						
AOT	440	632	3.3	636	3.5 ± 0.3	100 ± 25

of liposomes and their apparent concentration here is much higher than that calculated for the whole volume of a sample.

3.3. Protochlorophyllide aggregation in liposomes

An increase in the Pchlide content in liposomes up to 6 mol% resulted in a gradual red-shift of the fluorescence emission maximum, maximally of about 4 nm, and in a gradual decrease of the fluorescence lifetime, although, the shape of the absorption spectrum did not change (Fig. S1). These effects are due to concentration-dependent self-quenching, which is the result of a small Stokes shift (Table 1), and it is well known for numerous fluorophores [28].

The formation of aggregates was evident in the case of high Pchlide content in liposomes prepared from galactolipids. As an example, Pchlide aggregation in liposomes prepared from MGDG:DGDG:SQDG:PG (51:29:7.7:12.3) and containing 9 mol% of Pchlide (corresponding to 1:10 Pchlide:lipid molar ratio) is shown in Fig. 3. Aggregate formation was revealed by the appearance of additional absorption bands having maxima at 475–480 and 650 nm (Fig. 3A). The intensity of these bands increased with time after the liposome preparation. The corresponding changes in fluorescence spectra were relatively slow (Fig. 3B, C). Just after liposome preparation, only an asymmetrical broadening of the emission band was observed both for excitation at 440 and 480 nm. On a time-scale of minutes, an additional band with a maximum at 656 nm became evident at 480 excitation, and its intensity increased with time (Fig. 3C). In the case of excitation at 440 nm, a slight blue-shift of the maximum accompanied by a shoulder on the red part of the main band of the spectrum was finally observed (Fig. 3B). For 17 mol% Pchlide in liposomes, the observed changes went faster than for 9 mol% Pchlide, and a fluorescence band at 657 nm was also evident at 440 nm excitation (Fig. S2). Fluorescence lifetime analysis showed a complex fluorescence decay that was easily distinguishable into three components (Table 2). The fractional intensity of the very short component of about 100 ps increased by two during Pchlide aggregation. In that time, the fractional intensity of the second component of 1–2 ns decreased significantly. The third component of several μs was detected in all measurements, however, its value could not be exactly determined with the fluorometer used and it is not discussed here.

The process of Pchlide aggregation proceeded in a similar way in liposomes prepared from DGDG, as well as a mixture of DGDG:MGDG (70:30). In the case of Pchlide in MGDG, aggregate formation took place for higher Pchlide content than in the case of the above mentioned galactolipid liposomes. Tiny effects were found with up to 10 mol% of Pchlide and were observed as a shoulder in the absorption spectrum with a weak fluorescence band detected for excitation at 480 nm. Absorption and fluorescence bands of Pchlide aggregates became evident in the absorption and emission spectra in the case of 17 and 20 mol% of Pchlide in MGDG (not shown). By contrast, the aggregation effects were almost negligible in the case of liposomes from EYL for the

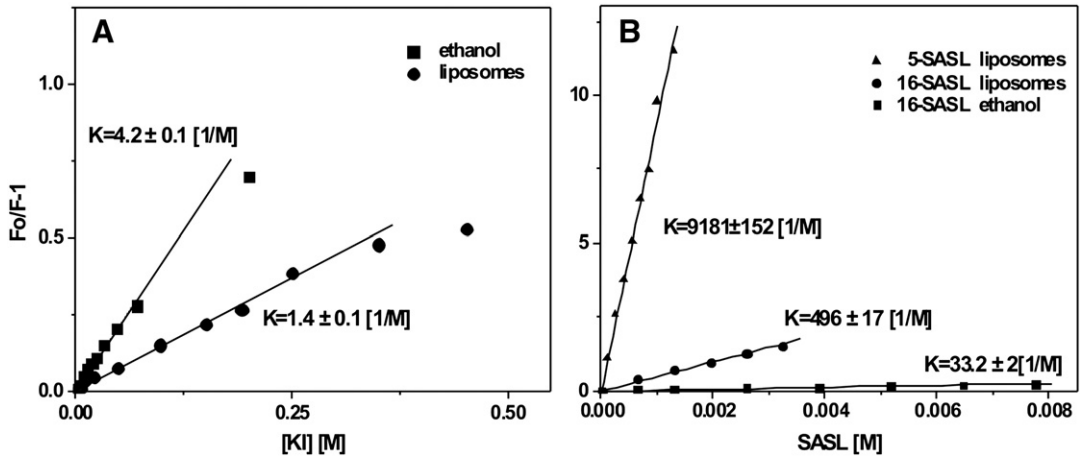

Fig. 2. Stern–Volmer plots of Pchlide fluorescence quenching with KI (A) and SASL probes (B). Pchlide was in liposomes prepared from the mixture of chloroplast lipids MGDG:DGDG:SQDG:PG (51:29:7.7:12.3 mol/mol). Quenching constants are shown in the figures.

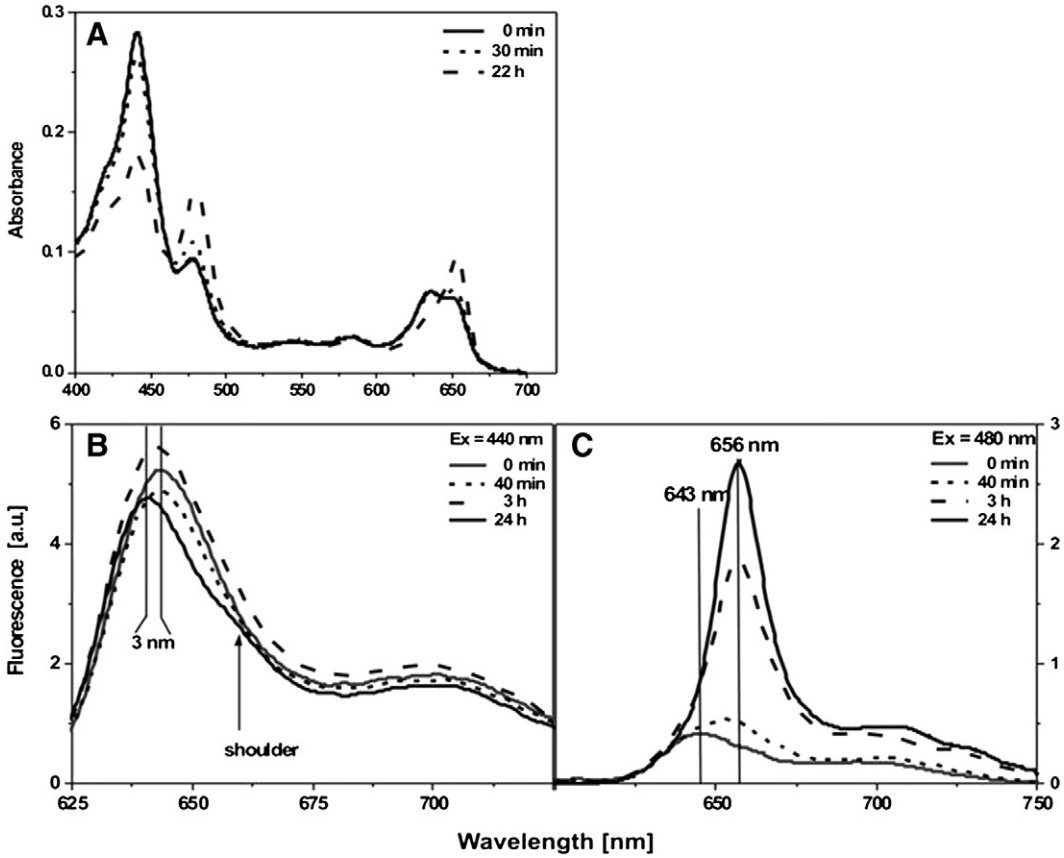

Fig. 3. Pchlide aggregation in liposomes prepared from MGDG:DGDG:SQDG:PG (51:29:7.7:12.3 mol/mol) for 10 mol% of Pchlide, as observed in absorption (A) and fluorescence spectra (B, C). The fluorescence spectra were measured for excitation at 440 and 480 nm, as indicated in figures.

whole investigated range of Pchlide content. In the case of 9 mol% of Pchlide in EYL liposomes, the fluorescence decay was still monoexponential with the lifetime of 2.4 ns.

To investigate more deeply Pchlide aggregation in liposomes, some control experiments were performed for Pchlide in a buffer (Hepes 25 mM, pH 7.5), for the same Pchlide concentrations as those in the liposomes containing 9 mol% of Pchlide and shown in Fig. 3A–C. Pchlide was added to the buffer in the same way as in the case of the liposomes, i.e. by injection of 25 μl of ethanol solution of Pchlide. The Soret band of absorption spectrum of Pchlide in the buffer (Fig. 4) differed significantly from spectra measured in liposomes (Fig. 3A). Pchlide in the buffer had the highest absorption at 475 nm, whereas bands at 420 and 440 nm were only slightly distinguished. The maximum of the Qy band was found at 650 nm, like for Pchlide in liposomes. One fluorescence band with a maximum at 655–660 nm was observed for excitation both at 440 and 480 nm (Fig. 4). The fluorescence intensity of Pchlide in the buffer was more than 10 times lower than that observed for corresponding concentration of Pchlide in liposomes. This weak fluorescence intensity agreed well with the short fluorescence lifetime of 0.1 ns that was dominant (see Fig. 4).

The addition of liposomes (up to 31 μM of lipid concentration) resulted in some significant changes within the Soret region of the absorption spectrum. The intensity of the band with a maximum at 440 nm increased, whereas that with a maximum at 475 nm decreased, and finally the shape of the spectrum resembled the absorption spectrum of Pchlide originally prepared in liposomes (Fig. 3A). The addition of liposomes to Pchlide in the buffer, also caused a significant increase (8–15 times) in fluorescence intensity, as well as the significant decrease in the fractional intensity of the 0.1-ns fluorescence lifetime component together with the increase in the fraction of the other component.

Table 2
Fluorescence lifetimes measured for Pchlide aggregates in liposomes prepared from MGDG:DGDG:SQDG:PG (51:29:7.7:12.3 mol/mol). The best fit of the experimental data was observed for a triple-exponential decay. Two components (t_1 and t_2), and their fractional intensities (f_1 and f_2) are shown in the table. The third component of several μs was beyond the resolution of the instrument and is not shown.

Pchlide content [mol%]	Time after liposome preparation	Exc [nm]	t_1 [ns]	f_1	t_2 [ns]	f_2
10	0	440	0.20 ± 0.08	0.15	1.85 ± 0.02	0.59
		480	0.12 ± 0.05	0.17	1.56 ± 0.05	0.15
	70 min	480	0.17 ± 0.05	0.31	2.2 ± 0.5	0.10
	24 h	480	0.09 ± 0.08	0.35	2.2 ± 0.7	0.08
20	1 h	440	0.2 ± 0.1	0.30	2.0	0.2
		480	0.15 ± 0.05	0.33	1.12 ± 0.3	0.1

3.4. Gaussian components of Q region of Pchlide absorption spectrum

The Q region of absorption spectra of Pchlide in liposomes was resolved into Gaussian–Lorentzian components. Results of the resolution are shown in Supplementary materials (Table S1 and Fig. S3). In the case of 1–6 mol% of Pchlide in galactolipid liposomes, a minimal number of components that provided a good resolution of the spectra was four. The maxima of the fitted bands (i.e. ν_2–ν_5) were found at 634, 612, 581 and 544 nm (each ± 1 nm). The typical Q band of a porphyrin absorption spectrum is composed of two Q_y (i.e. 0–0 and 0–1) and two Q_x (0–0 and 0–1) bands. The component with the maximum at 634 nm can be ascribed do Qy (0–0) absorption band. In the case of Pchlide content of 9 and 17 mol%, five components provided a good resolution. The additional band (i.e. ν_1) was observed at 650–654 nm and originates from Pchlide aggregates. In these samples, the band ν_3 was shifted to

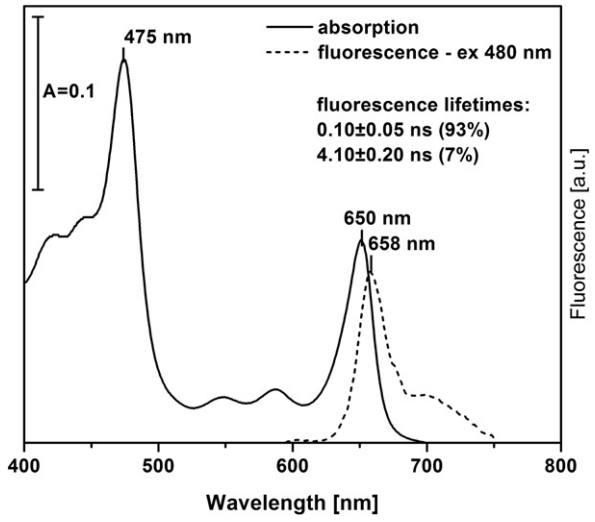

Fig. 4. The absorption and fluorescence spectra of Pchlide in a buffer (Hepes 25 mM, pH 7.5). The sample was prepared by the injection of ethanolic Pchlide solution into the buffer by analogy to liposome preparation (see Materials and methods). Pchlide concentration 4 μM. Ethanol concentration: 1%.

615–616 nm (compared to 612 nm found for 1–6 mol% Pchlide content). Components found in the case of 1–6 mol% of Pchlide in EYL liposomes, showed similar respective parameters, however the ν_3 center was at 621–622 nm. In the case of 9 and 17 mol% of Pchlide in EYL liposomes, five components were found, even though it was not obvious judging from the measured spectrum. However, the additional component was centered at 642 ± 2 nm, which is blue-shifted compared to the respective component found in galactolipid liposomes.

The amplitudes of the respective bands were similar for galactolipid liposomes containing 1–6 mol% of Pchlide whatever was the liposome composition (Fig. 5 and Fig. S4). The dominating Q_y (0–0) band at 634 nm had the relative amplitude at the level of 0.63–0.68 and the other bands at the level of 0.1–0.2. A slightly different relative amplitudes were found for Pchlide in EYL liposomes, but still the band with the maximum at 634 nm was the highest. In the case of samples containing 9 and 17 mol% Pchlide, where Pchlide aggregation was observed, the appearance of the ν_1 band was related to the decrease of the amplitude of the ν_2 band (Fig. 5; Fig. S4). Amplitudes of the other bands (ν_3–ν_5) did not changed significantly. This effect was obvious for Pchlide in galactolipid liposomes, because it was easily seen in the measured absorption spectra (Fig. 3A). However, resolution showed the band of Pchlide aggregates also for EYL liposomes containing 9 and 17 mol% of Pchlide but of lower amplitude than that found for Pchlide aggregates in galactolipid liposomes. What is more, resolution of the absorption spectra showed that the process of Pchlide aggregation starts at a certain Pchlide content in liposomes, as estimated here at about 9 mol% of Pchlide. This process is also time dependent and hence the exact Pchlide concentration that initiate the aggregation process could not be more accurately determined.

Resolution of absorption spectrum of Pchlide in the buffer shown in Fig. 4, provided the bands with the maxima at 653, 643, 637, 588 and 551 nm (each ±1 nm). The position of the maxima of the identified bands (Table S1), as well as their relative amplitudes (Fig. 5) differed significantly from the results found for Pchlide in liposomes.

3.5. Pchlide aggregation in organic solvents

The formation of Pchlide aggregates was observed in a mixture of isoamyl alcohol and isooctane (0.14:1, v/v), both in absorption (Fig. 6A) and fluorescence (Fig. 6B) spectra. The aggregation was initially observed as shoulders of the absorption bands (Fig. 6A) of increasing intensity with time. Finally, the absorption bands resulting

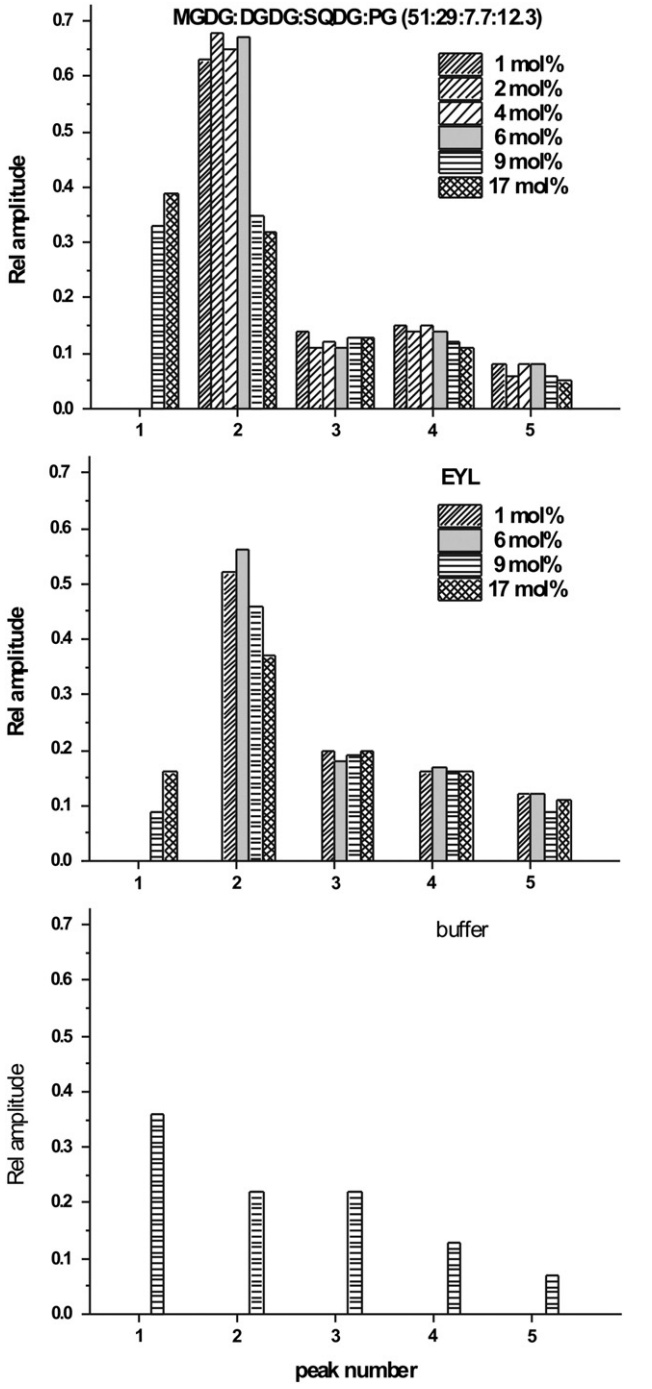

Fig. 5. Relative amplitudes of Gaussian–Lorentzian components found within the Q region of the absorption spectrum of Pchlide in liposomes and in a buffer (Hepes, 25 mM, pH 7.5). Peak parameters are listed in Table 3.

from Pchlide aggregates showed maxima at 481 and 657 nm. For excitation at 440 nm, the fluorescence maximum was found at 638 nm just after sample preparation and it was blue-shifted after 24 h of aggregation (Fig. 6B). The blue-shift was accompanied by an increase in the intensity of vibrational side-band (i.e. between 660 and 680 nm). Excitation at 490 nm selectively excited a weak fluorescence of Pchlide aggregates (Fig. 6B) having a maximum at 680 nm. The fluorescence lifetime was 3.6 ns at the beginning of the aggregation process (Table 3). Then two fluorescence lifetime components were found, although excitation at 440 and 490 nm gave different results. For excitation at 440 nm, the fractional intensity of the long (3–4 ns) component decreased with aggregation time, whereas that of the short one increased, and the

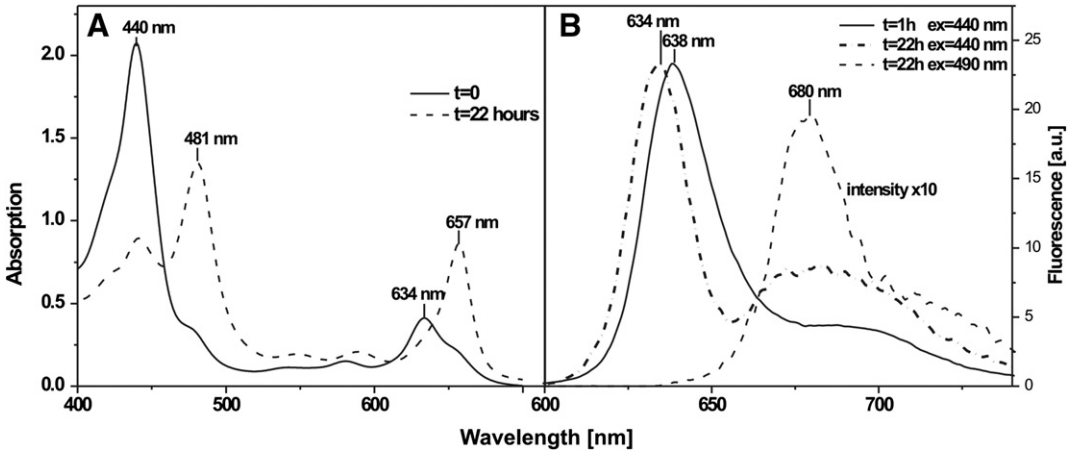

Fig. 6. The absorption (A) and fluorescence emission (B) spectra of Pchlide aggregates in a mixture of isoamyl alcohol:isooctane (0.14:1, v/v). Pchlide concentration was 10 μM.

value of the short component decreased. Selective excitation of aggregates at 490 nm resulted in lifetimes of 2.5 and 0.12 ns, and these did not change with time. The fractional intensity of the 0.12-ns component increased with aggregation time. Pchlide aggregation in this system was reversible upon the addition of a drop of methanol (not shown).

Pchlide aggregation was also observed in chloroform (Fig. S5). The absorption bands of Pchlide aggregates showed maxima at 470–473 nm and 651 nm. These bands were evident immediately after sample preparation and did not change with time. Excitation at 440 nm resulted in a fluorescence spectrum having a maximum at 635 nm, whereas excitation at 475 nm revealed an additional weak fluorescence band of Pchlide aggregates with a maximum at 658 nm (not shown). The fluorescence lifetime of 3.4 ns (Table 3) originate from Pchlide emitting at 635 nm, whereas a short fluorescence lifetime component of 0.1 ns is characteristic for the band having maximum at 658 nm.

3.6. Protochlorophyllide in AOT/isooctane/water reversed micelles

The absorption and fluorescence of Pchlide monomers or aggregates were investigated in reversed AOT micelles at different water content. In these types of micelles, the polar head groups of the surfactant molecules form the core of the micelle and the hydrophobic fragments are exposed to organic solvent [29]. Water molecules accumulate in the polar core and the properties of this droplet are different than in the bulk aqueous phase, among other interactions that between water molecules and the ionic groups of AOT is strong.

Absorption maxima of 5 μM Pchlide in reversed micelles of AOT were found at 440 and 632 nm, and the spectrum was similar to Pchlide monomers in organic solvents and liposomes (Table 1). A fluorescence emission spectrum with a maximum at 636 nm and a fluorescence lifetime of 3.5 ± 0.3 ns were obtained for excitation at 440 nm. A batochromic shift of the fluorescence maximum was observed for increasing molar H_2O/AOT ratio and finally a maximum was found at 642 nm for H_2O/AOT = 16. An increase in water did not cause any significant changes in the absorption spectra (not shown).

In the next experiments, Pchlide aggregates in isooctane were added to AOT/isooctane reversed micelles. The absorption maxima were observed at 481 and 657 nm, indicating that the aggregates were still present in the AOT reversed micelles (Fig. 7A, a curve "0"). Fluorescence maxima were found at 660 nm and 633.5 nm for excitation at 480 nm (Fig. 7B), and 440 nm (Fig. 7B, insert), respectively. The predominant fluorescence lifetime measured for excitation at 480 nm was very short: 0.15 ± 0.1 ns (f = 0.8), whereas the other component was 1.45 ± 0.13 ns (f = 0.2).

The addition of water caused disaggregation of Pchlide aggregates, manifesting in the appearance of bands at 440 nm and 634 nm in the absorption spectrum as well as a band at 640 nm in the fluorescence spectrum for excitation at 480 nm (Fig. 7). The intensity of these bands increased with time, and finally the spectra resembled those of monomeric Pchlide. An increase in fluorescence lifetimes to 0.5 ± 0.2 and 3.7 ± 0.8 ns was associated with the spectral changes. The fractional intensity of the short component significantly decreased upon the subsequent addition of water aliquots from 0.8 to 0.03, and finally most (i.e. 92%) of the fluorescence was associated with the 3.7-ns component.

4. Discussion

4.1. Protochlorophyllide monomers

Absorption and fluorescence spectra measured for low Pchlide concentration in liposomes (Fig. 1) were similar to those in organic solvents [30–32], which indicates the presence of Pchlide monomers. The liposome composition does not significantly influence the photophysical properties of Pchlide because only small differences in the position of absorption and fluorescence maxima were found among liposomes prepared from different lipids. Fluorescence maxima

Table 3
Fluorescence lifetime measured during Pchlide aggregation in organic solvents. Pchlide concentration was 2 and 10 μM in the case of chloroform and the isoamyl alcohol:isooctane mixture, respectively.

Solvent	Exc 440 nm				Exc 475/490 nm**			
	τ_1 [ns]	f_1	τ_2 [ns]	f_2	τ_1 [ns]	f_1	τ_2 [ns]	f_2
Isoamyl alcohol/isooctane	(0.14:1)							
t = 0*	3.6 ± 0.2	1			–	–	–	–
t = 2 h*	4.1 ± 0.1	0.7	1.9 ± 0.1	0.3	2.5 ± 0.4	0.3	0.12 ± 0.09	0.7
t = 24 h*	3.1 ± 0.1	0.6	0.3 ± 0.1	0.4	2.6 ± 0.4	0.12	0.13 ± 0.09	0.88
Chloroform	3.5 ± 0.1	1			3.4 ± 0.1	0.5	0.1 ± 0.09	0.5

* The time between the start of the aggregation and the onset of the measurement. For chloroform, Pchlide aggregation was observed immediately after the sample preparation.
** The excitation was 475 nm for chloroform and 490 nm for the isoamyl alcohol:isooctane mixture.

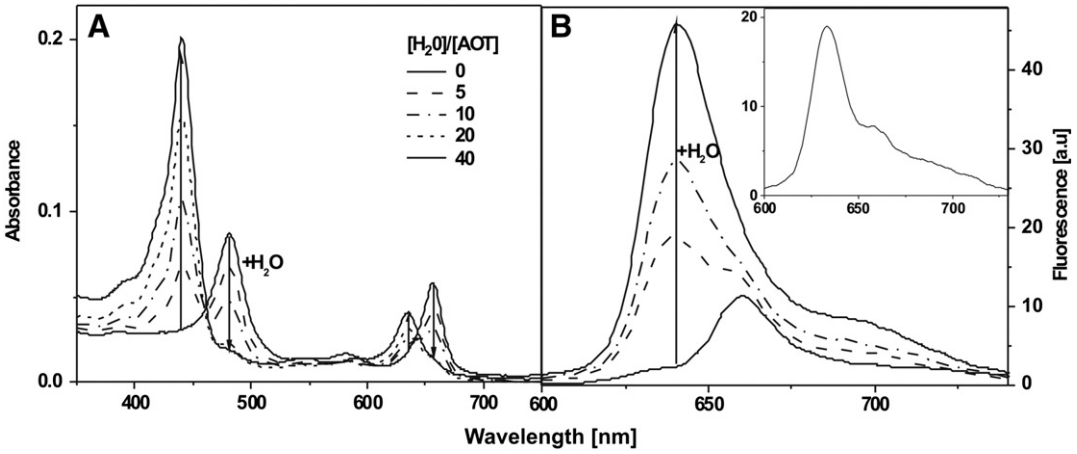

Fig. 7. The absorption (A) and fluorescence emission (B) spectra of Pchlide in AOT/isooctane reversed micelles containing different amounts of water. Pchlide concentration: 5 μM. Excitation wavelength: 480 nm. H_2O/AOT molar ratios are shown in the figure. Insert — the fluorescence spectrum for excitation at 440 nm and H_2O/AOT = 0.

of Pchlide in liposomes (Table 1) were similar to the maximum in methanol and in water, and were red-shifted with respect to other solvents [32]. This red-shift was due to specific solvation effects e.g. resulting from hydrogen bonds and coordination of the central Mg ion. The S_1 state of a Pchlide molecule with an intramolecular charge-transfer character [33] is stabilized by Pchlide solvation, especially via hydrogen bonds interaction [34] and shows different dynamic of the S_1 state de-excitation in protic and aprotic solvents [35–38]. All the above considerations pointed to specific Pchlide solvation in liposomes, manifested by the lowering of the S_1 state energy as well as the relatively large Stokes shift (Table 1), although not as large as that found for methanol [32]. A gradual red-shift of the fluorescence maximum of Pchlide that has been observed in this study for increasing water content in AOT/isooctane/water reversed micelles is another confirmation of Pchlide sensitivity to specific solvation via hydrogen bonding. What is more, the observed effect is an indication that Pchlide molecules occupy a hydrophilic core of reversed micelles, i.e. close to polar head group of AOT molecules. Fluorescence quenching experiments presented here for Pchlide in liposomes showed that Pchlide molecules entered the membrane bilayer, because their fluorescence was quenched with 5-SASL and KI, and in the latter case, the K value was lower in liposomes than in solution (Fig. 2). Therefore, the interface area of liposomes and/or the head-group area of the lipid bilayer is the most probable site of Pchlide localization.

The positions of absorption bands found for Pchlide in liposomes (Table 1) are red shifted as compared to the maxima observed in the majority of investigated organic solvents, except pyridine [26,32], and similar to those found for low concentration of Pchlide in water [32]. Therefore, judging only from the position of the absorption maxima one cannot state about the amount of Pchlide bound or entered to liposomes. On the other hand, quenching experiments (Fig. 2), as well as the observed tendency of Pchlide to aggregate in water [32] and the fact that no Pchlide aggregates were found up to 6 mol% of Pchlide in liposomes (Fig. 5; and Table S1; see also further discussion) allow us to conclude that all Pchlide molecules are associated with liposomes in the presented experiments.

Short-wavelength Pchlide spectral forms in vivo originate from Pchlide molecules that remain unattached to LPOR, and thus are not converted to Chlide upon a short illumination. Maxima of Qy absorption and emission bands of these Pchlide forms, are similar to the results presented here for Pchlide monomers in liposomes (Table 1). This similarity together with the results of fluorescence quenching experiments (Fig. 2) led us to conclude that Pchlide monomers in vivo must be localized within the hydrophillic region of a membrane bilayer or at least attached to the membranes strongly enough not to be lost during the isolation procedure. However, fluorescence lifetimes ascribed to short-wavelength Pchlide forms in etioplast inner membranes, which were between 5.3 and 6 ns [20], were definitely longer than those found in liposomes in the present study (Table 1) and in organic solvents and water [32,34,37]. Longer fluorescence lifetimes reflect a higher fluorescence quantum yield, which means that Pchlide–lipids interaction decreases the probability of nonradiative Pchlide de-excitation, e.g. via thermal de-excitation, although the nature of this interaction cannot be unambiguously stated at present. In etioplast membranes, some interaction of Pchlide with other membrane components such as carotenoids or proteins cannot be excluded.

Pchlide is a porphyrin. Thus, the long fluorescence lifetime of the S_1 Pchlide excited state would favor its phototoxicity. Indeed, plant seedlings that accumulate preferentially short-wavelength Pchlide forms during their growth in darkness are sensitive to excess light (for a review see ref. [7]).

4.2. Formation of protochlorophyllide aggregates

The ability of Pchlide molecules to form aggregates in organic solvents [30,31,39,40] and in solid films [41] has been known for a long time. In general, self-aggregation of porphyrins affects their spectral properties, due to interaction between transition dipole moments of molecules being in close proximity, which results in coupled electronic transitions. Two types of aggregates have been described: *H*- and *J*-aggregates where the monomers show face-to-face and side-by-side arrangement, respectively [42]. In the present work, the formation of Pchlide aggregates in organic solvents, liposomes and in a buffer has been shown. In all these systems, aggregation was manifested as additional absorption bands which were red-shifted as compared to Pchlide monomers (Figs. 3, 4 and 6) and thus indicating the formation of *J*-aggregates. However, additional experiments are required to determine the exact geometry of the aggregates.

Pchlide aggregates in organic solvents were in the past described as nonfluorescent or fluorescing only at 77 K [40]. In the present work, we have shown that fluorescence of Pchlide aggregates can be selectively excited at wavelength corresponding to the maximum of the absorption band that appeared in the course of aggregation, although the fluorescence signal is very weak (Fig. 6; Fig. S5). Short fluorescence lifetime found in these samples (Table 3) confirmed the low fluorescence quantum yield of aggregates. Studies on the aggregation of chlorophyll and bacteriochlorophyll in organic solvents also showed strong fluorescence quenching, reflecting an increased probability of nonradiative ways of de-excitation (for a review see ref. [43]).

The formation of Pchlide aggregates observed for high Pchlide content in galactolipid liposomes was time-dependent (Fig. 3; Fig. S2). Thus, it probably required some reorganization of Pchlide–lipid system. Pchlide molecules, which occupy the interface region of liposomes can easily move within the bilayer and favor a Pchlide–Pchlide interaction. Resolution of absorption spectra revealed that up to 6 mol% of Pchlide in liposomes, the absorption band of Pchlide aggregate was not detected, but observed for Pchlide content higher than 9 mol% (Fig. 5; Fig. S4). The absorption maximum of Pchlide aggregates in EYL liposomes (Fig. 5; Table S1), was blue-shifted compared to that in galactolipid liposomes, which indicates formation of lower-size aggregates in EYL compared to galactolipid liposomes. Judging from the relative bands intensities (Fig. 5), it can be estimated that about 50% of Pchlide in galactolipid liposomes and less than 20% in EYL liposomes is aggregated.

Pchlide aggregates formed in a buffer had the maxima of Q absorption and fluorescence bands at similar positions to those observed in galactolipid liposomes (compare Figs. 3 and 4). Resolution of absorption spectra of Pchlide in a buffer into Gaussian–Lorentzian components showed that the relative intensity of the bands at 650–654 nm and originating from Pchlide aggregates was the highest, which was not the case of liposomes (Fig. 5). Moreover, the resolution revealed also the components centered at 642 nm and detected in EYL liposomes, while not found in galactolipids. This observation indirectly show that Pchlide aggregates formed in liposomes remain associated with the bilayer.

The shape of the absorption spectrum in the Soret range for Pchlide in a buffer (Fig. 4) also revealed that Pchlide in this system is totally aggregated, while in the presence of lipids there is some equilibrium between monomers and aggregates (Fig. 3A), which is difficult to distinguish by analysis of the Qy band only. Analysis of the Soret absorption band gives more precise information about Pchlide aggregates, although such analysis would be difficult in vivo because the bands of the aggregates have the same position as the carotenoids which are present in PLB (for a review see ref. [12]).

Sytina et al. [44] have recently observed Pchlide aggregates in neat water having similar spectral properties to those presented in this work for Pchlide in the buffer (Fig. 4). Several subpopulations of C═O groups with varying hydrogen bond strength were found, however, their origin have not been elucidated [44]. Hydrogen bonds may be formed between Pchlide molecules in the aggregates or between Pchlide and the surrounding solvents. The latter would be a good explanation of the fact that Pchlide aggregation was observed in the case of galactolipid liposomes and in the buffer. Even though the position of the fluorescence maxima of Pchlide aggregates in the buffer and in liposomes were similar (Figs. 3 and 4), fluorescence intensity was much higher in liposomes than in the buffer and fluorescence decay was characterized with different parameters (Table 2 and Fig. 4). These results show that in liposomes the equilibrium is shifted towards Pchlide monomerization and prove indirectly that Pchlide aggregates are buried in or attached to the bilayer.

Fluorescence lifetimes found for Pchlide aggregates (Tables 2 and 3) fit well with the fluorescence lifetime components found in isolated etioplast membranes, i.e. 0.25 ns and 1.6–2.2 ns [20]. The short component in liposomes (Table 2) was even shorter than in vivo, although the Pchlide photoreduction that occurs in vivo decreases the probability of S_1 state de-excitation via fluorescence. The complex fluorescence decay observed in the case of the selective excitation of Pchlide aggregates with 480 nm, indicates that the complexity is a natural property of fluorescence of Pchlide aggregates. These results confirm the previous observation that Pchlide having a fluorescence maximum at around 655 nm in isolated etioplast membranes may also show complex decay; this had already been suggested [20], but could not be proven under those experimental conditions.

The Qy absorption and fluorescence spectra of Pchlide aggregates in liposomes were similar to those observed for photoactive Pchlide in etioplasts [45], although in the latter the bands were much broader and unstructured, and consisted of several components. The aggregates of the ternary Pchlide:LPOR:NADPH complexes may be different in size, which is an explanation for the multiple spectral forms in vivo [7,45]. Pchlide species having the absorption/fluorescence maxima at 638/642 nm were suggested to originate from dimers of Pchlide:LPOR:NADPH complexes, whereas those with absorption/fluorescence maxima at 650/657 from large aggregates of ternary complexes [17]. In spite of numerous studies on the description of Pchlide spectral forms in vivo, the molecular structure and the degree of aggregation of Pchlide:LPOR:NADPH complexes, as well as the nature of Pchlide–surrounding interactions are still under debate. Even if the spectral properties of Pchlide aggregates in liposomes studied here had covered the spectral range of Pchlide spectral forms in vivo, one has to remember that in vivo Pchlide is bound to the active site of the enzyme and this binding at least partially changes the structure and photophysics of the aggregates.

There exists some equilibrium among various Pchlide spectral forms in vivo [7]. A dynamic equilibrium is also observed during PLB isolation and this is why a single Pchlide spectral form is difficult to obtain. The present results showed that Pchlide easily aggregated in galactolipid liposomes for high Pchlide content, although liposomes prevented Pchlide aggregation as compared to Pchlide in the buffer. The paracrystalline lipid structure of PLB consists of tubules formed by the lipid bilayer, which are joined by the lipids organized in reversed lipid phases. The lipid bilayer divides the structure into two continuous interlocking aqueous compartments [46]. Therefore, the PLB structure seems to be important for maintaining the proper proportion between Pchlide monomers and aggregates. Galactose residues of galactolipids in PLB provide a polar and protic microenvironment, where Pchlide molecules are easily solvated at hydrophyllic region of lipid bilayer and may be bound to LPOR, which is a membrane-attached protein [47–49]. The necessity of a lipid environment for LPOR catalytic activity has been demonstrated [50]. PLB structure may also be important in maintaining the equilibrium between aggregates of ternary Pchlide:LPOR:NADPH complexes of different sizes.

The assembly and localization of photoactive Pchlide:LPOR:NADPH complexes within the PLB structure is still a matter of debate (see ref. [10] for a review). Changes in PLB structure and lipid composition observed in vivo during light-induced PLB dispersion as well as transient PLB formation during the first days of greening [51] might be crucial for the regulation of the proportion between aggregated and non-aggregated Pchlide, which would in turn be vital for the reassembly of Pchlide:LPOR:NADPH complexes and for efficient Pchlide to Chlide transformation. On the other hand, the rapid aggregation that was observed in the case of high relative Pchlide concentrations in liposomes and the fact that the fluorescence of the aggregates is strongly quenched may be important for protection against photooxidation.

Pchlide aggregates in AOT/isooctane/water reversed micelles were easily broken up on the addition of water (Fig. 7). A gradual addition of water to reversed micelles provide opportunity to observe molecules with various states of hydration and simulate well situations present in water restricted environments prevailing at membrane interfaces. Two distinct water domains emerge in the polar core, i.e. “bound” water immobilized in the region of ionized sulfosuccinate head groups and “bulk” water in the inner region of micelles [52]. In the case of a low H_2O/AOT molar ratio, only “bound” water is observed. The observed disaggregation process, which was initiated at low water content, indicates that Pchlide aggregates were localized within the hydrophilic core of micelles, where they were hydrated by water molecules. These results confirm our finding about localization of Pchlide aggregates at the interface region of liposomes. However, even for the highest investigated H_2O/AOT molar ratio, any water-induced Pchlide aggregation was observed, which indicates that Pchlide monomers are not released to the “bulk” water region but remain associated with polar group of AOT.

The process of Pchlide aggregation or disaggregation of Pchlide aggregates depend on the nature of the lipid or surfactant, the relative amounts of Pchlide, buffer and lipids, as well as the dynamic of water present in the system.

5. Conclusions

In the presented studies, it was shown that Pchlide molecules locate close to the hydrophilic headgroup area of the lipid bilayer and in the hydrophilic core of AOT inverted micelles. In the case of high Pchlide content in galactolipid liposomes, Pchlide formed aggregates of similar absorption and fluorescence maxima but of stronger fluorescence than aggregates in a buffer. Galactose residues of galactolipids providing a polar microenvironment for the Pchlide molecule, seem important for the aggregation process. Pchlide aggregation in EYL liposomes was revealed only with resolution of the absorption spectrum. Water bound at hydrophilic core of reversed AOT micelles, induced the process of disaggregation of Pchlide aggregates, which indicates that Pchlide aggregates are located close to polar head groups of the surfactant and that not only a presence of water but also the molecular dynamics of water molecules is important for Pchlide aggregation.

Elucidation of Pchlide properties in various model system is of great importance for understanding of its properties, organization and functioning within plastid membranes in vivo. The present results pointed the role of galactolipids of PLB in vivo in regulating the proportion between Pchlide aggregates and monomers, which is important for the proper assembly of ternary photoactive Pchlide:LPOR:NADPH complexes and for efficient Pchlide to Chlide photoreduction.

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