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Protochlorophyllide in model systems — An approach to in vivo conditions

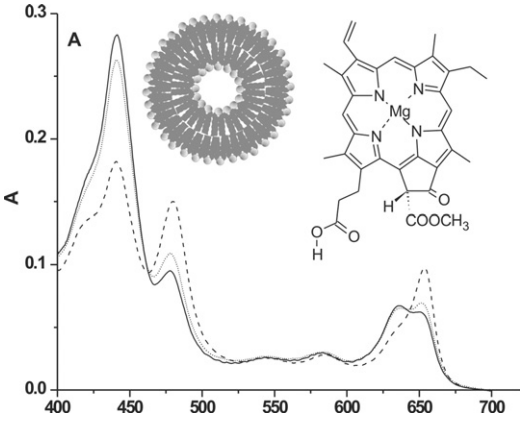
Beata Mysliwa-Kurdziel *, Jerzy Kruk, Kazimierz Strzałka

Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, 30-387 Kraków, ul. Gronostajowa 7, Poland

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.

* Corresponding author at: Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland. Tel.: +48 12 664 6372; fax: +48 12 664 6902.
E-mail address: b.mysliwa-kurdziel@uj.edu.pl (B. Mysliwa-Kurdziel).

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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