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Protochlorophyllide and protochlorophyll in model membranes — An influence of hydrophobic side chain moiety

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ABSTRACT

In the present work, a comparative study of protochlorophyllide- and protochlorophyll-lipid interaction was performed on liposomes prepared from phospholipids and galactolipids, which had a pigment content varying from 0.1 to 4 mol%. The incorporation of pigment molecules into the lipid bilayer and pigment–pigment interactions were investigated. Protochlorophyllide entered the lipid bilayer spontaneously and showed fluorescence spectra characteristic of its monomers. Similar spectra were observed for protochlorophyll where its concentration was low. However, the fluorescence maxima of protochlorophyll monomers were blue-shifted compared to those of protochlorophyllide by about 5 nm. Protochlorophyll at high concentrations formed transient aggregates that showed an additional fluorescence band with a maximum at around 685 nm, especially in liposomes prepared from phospholipids. For both compounds, the Stern–Volmer constant for KI quenching was much lower in liposomes than in solution, which confirmed the incorporation of these compounds into the lipid bilayer. Two populations of protochlorophyll that differed in their accessibility to quenching by KI were determined, and the proportions between them for different lipids are discussed. Protochlorophyllide showed such heterogeneity only in DPPC membranes. Quenching with 5- and 16-SASL revealed a localization of the porphyrin ring of both Pchl and Pchlide in the polar headgroup area of the lipid bilayer. The side chain of protochlorophyll forced these molecules to localize deeper in the bilayer in the case of DPPC in gel phase.

1. Introduction

Protochlorophyllide (Pchlide) and protochlorophyll (Pchl) are naturally occurring porphyrins in plants. Pchl is more hydrophobic than Pchlide because of a long chain of phytol or its precursors attached to the tetrapyrrole ring (Fig. 1).

The physiological role of Pchlide as an intermediate in chlorophyll biosynthesis has been known for a long time and intensively investigated (for a review see [1–4]). In angiosperms, the reduction of Pchlide to chlorophyllide is catalysed by a photoenzyme, protochlorophyllide oxidoreductase (LPOR, EC.1.3.1.33). The dynamics of deexcitation of the S_1 state of the Pchlide molecule is important for catalysis (reviewed by [5–7]). Pchlide phototransformation is inhibited in angiosperms in darkness, which has a great impact on plant development (for a review see [8–10]). Dark-grown angiosperm seedlings follow the developmental program known as scotomorphogenesis, which involves differences in morphology, plastid structure as well as physiology than those observed during photomorphogenesis [11]. The most important feature of dark-grown seedlings is a lack of photosynthetic structures, which are essential for plant autotrophy, accumulation of Pchlide and lack of chlorophyll, as well as the development of characteristic inner plastid membranes: prolamellar bodies (PLB) and prothylakoids (PT) (reviewed by [12,13]). Under light conditions, Pchlide is continuously synthesized and upon binding to LPOR, immediately reduced to chlorophyllide. Since excited tetrapyrroles are highly toxic, chlorophyll synthesis is strictly regulated by plants at the level of Pchlide synthesis and photoreduction, among others [1,2,9].

Much less is known about the role of Pchl, which seems to be a side product of chlorophyll biosynthesis. LPOR has been found not to accept Pchl as a substrate (for a review see [8,10]). Pchl has been found in large amounts in the inner seed coat of members of the *Cucurbitaceae* family, namely *Cucurbita pepo* [14,15], *Luffa cylindrica* [16] and *Cyclanthera explodens* [17,18], but its physiological role is as yet unknown.

Both Pchlide and Pchl have been found in the inner membranes of etioplasts. Pchl has been detected in PT [19], whereas Pchlide has been found in PT as well as in PLB [20], where it is bound to LPOR, and forms ternary Pchlide:LPOR:NADPH complexes. These complexes are organised in aggregates of different sizes, which is the reason for the observed multiple spectral forms of Pchlide *in vivo* (reviewed by [9,21]). The role of Pchlide aggregation *in vivo* and the organization

Abbreviations: DGDG, digalactosyldiacylglycerol; DPPC, dipalmitoylphosphatidylcholine; LPOR, light-dependent protochlorophyllide oxidoreductase; EYL, egg yolk lecithin; MGDG, monogalactosyldiacylglycerol; n-SASL, spin-labeled stearic acid with a nitroxyl group at the n^{th} carbon atom of the acyl chain; Pchl, protochlorophyll; Pchlide, protochlorophyllide; PLB, prolamellar body; PT, prothylakoids; SUV, small unilamellar liposomes

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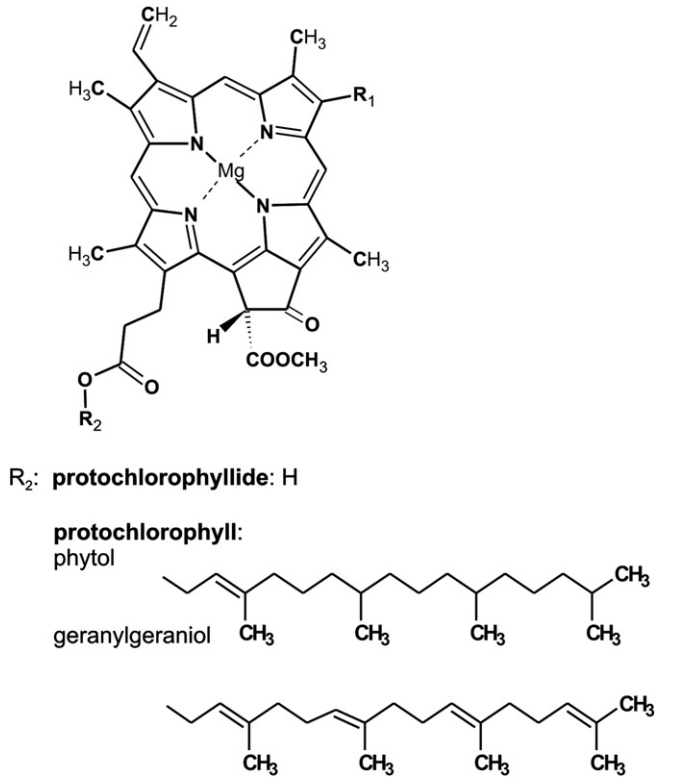

Fig.1. Chemical structure of protochlorophyll (Pchl) and protochlorophyllide (Pchlide). R_1 is CH-CH_2 or CH_2—CH_3 in the case of divinyl- and monovinyl-pigment, respectively.

of Pchlide:LPOR:NADPH complexes in PLB is currently under discussion [4]. In chloroplasts, Pchlide has been found in outer envelope membranes [22].

Lipids inside etio- or chloroplasts are a natural environment for Pchlide:LPOR:NADPH complexes, as well as for unbound Pchlide and Pchl. Enzymes catalyzing synthesis of Pchlide and Pchl were found to be associated with membranes [see 1,2 for a review]. Model lipid systems should be helpful in an understanding of the molecular mechanisms of pigment–lipid interaction, as well as in elucidating the role of lipids in the formation of pigment aggregates *in vivo*. The present study was aimed at a comparative investigation of Pchl and Pchlide in liposomes to reveal the effect of the hydrophobic side chain of Pchl molecules on pigment incorporation and its interaction with the lipid bilayer. The liposomes were prepared from galactolipids, i.e. lipids that build PLBs and PTs *in vivo*. Alternatively, liposomes prepared from phospholipids were also used. The localization of Pchl and Pchlide within the lipid bilayer was studied taking advantage of fluorescence quenching experiments using various fluorescence quenchers.

2. Materials and methods

2.1. Pigment isolation

Pchlide, i.e. monovinylprotochlorophyllide, was extracted from 6 day-old dark-grown wheat (*Triticum aestivum*) leaves treated with δ-amino levulinic acid, and purified using HPLC according to the method described by Kruk and Mysliwa-Kurdziel [23]. Pchl was isolated from inner seed coats of pumpkin (*Cucurbita pepo*) and purified as described in [24,25]. Freshly prepared stock solutions of Pchlide in methanol and of Pchl in acetone were used.

2.2. Solvents and lipids

All the organic solvents used for liposome preparation were of spectroscopic grade. Solvents for Pchl and Pchlide purification were of analytical or HPLC grade. Lipids were purchased from Sigma Aldrich and Lipid Products (South Nutfield, Redhill, Surrey, U.K.). SASLs were purchased from Sigma Aldrich.

2.3. Liposome preparation

Small unilamellar liposomes (SUV) were prepared using the injection method according to the protocol given by [26]. Liposomes were made from EYL (egg yolk lecithin), DPPC (dipalmitoylphosphatidylcholine), DGDG and DGDG:MGDG mixtures (85:15 and 70:30) (DGDG and MGDG stand for digalactosyldiacylglycerol and monogalactosyldiacylglycerol, respectively). A proper aliquot of Pchl in acetone (or Pchlide in methanol) was added to lipids in chloroform and evaporated together, then the liposomes were prepared. This protocol was used routinely, and all the results shown in this paper are based on this method. The relative content of Pchl or Pchlide in liposomes varied between 0.1 to 4 mol%. Lipid concentration during liposome preparation was 0.5 mM. Liposomes were used for experiments just after the preparation. Then they were incubated at room temperature with stirring for 4–24 hours and re-examined again. Preparation of liposomes was repeated at least 5 times for a single pigment:lipid ratio, and for each type of lipid.

It should be mentioned here that we also tested other methods for pigment addition to liposomes. First, a thin film of Pchl (or Pchlide) was prepared in a conical glass tube. Liposomes prepared by injection methods were added to the tube and vortexed until the film was dissolved. A rather poor solubility of the Pchl film in liposomes was observed, especially for a high amount of Pchl, and this was the reason for unsatisfactory reproducibility of the results. Next, an aliquot of pigment in ethanolic solution was injected into the liposomes and gently mixed. Then samples were incubated at room temperature for 2 hours. This protocol did not provide reproducible results for samples containing Pchl either.

2.4. Fluorescence measurements

Fluorescence emission spectra were measured using a Perkin-Elmer spectrofluorometer (LS50B, UK). The spectra were recorded in the range of 595 to 750 nm. Samples were excited at 440 nm using 5-nm excitation and emission slits. The data collection frequency was 0.5 nm. The spectra were corrected for the baseline and wavelength-dependent sensitivity of the photomultiplier. The fluorescence maxima observed for independent repetition of liposome preparations agreed within 0.5–1 nm between corresponding samples. Fluorescence lifetime measurements were performed using a multifrequency cross-correlation phase and modulation K2 fluorometer, for excitation at 440 nm. The instrument setting and the analysis of phase and modulation data were as already described [25]. Immediately before fluorescence measurements, samples were diluted with a buffer or alternatively, fluorescence measurements were performed in small cuvettes, having an optical path of 0.5 cm. This was done to assure the optical density of samples of around 0.1 at 440 nm. Any time dependent changes of the spectra caused by dilution were not observed under experimental conditions. All the spectroscopic measurements and sample incubations were performed at room temperature (22 ± 2 °C).

2.5. Fluorescence quenching

Fluorescence quenching was performed with I^- (KI) and 5- and 16-doxyl stearic acids (SASL). 5-SASL and 16-SASL, with free radical fragment (nitroxyl group) attached to C-5 or C-16 carbon of stearic acid, can quench fluorescence at different depths within the lipid bilayer. The first