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ORIGINAL PAPER

Solvent effects on fluorescence properties of protochlorophyll and its derivatives with various porphyrin side chains

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Abstract Fluorescence spectra and fluorescence lifetimes of protochlorophyll (Pchl) were measured in organic solvents having different physical and chemical properties and were analyzed taking into account the nonspecific (dependent on bulk solvent parameters), and specific (e.g. H bonds, Mg coordination) solvent–solute interactions. The energy of the fluorescence emission band decreased, while the Stokes shift increased for increasing solvent orientation polarizability, which is a function of both the dielectric constant (ε) and the refractive index (n). The extent of the dependence of the Stokes shift on solvent orientation polarizability was higher in protic (i.e. those able to form hydrogen-binding) than in aprotic solvents. High value of the Stokes shift was also observed in pyridine and methanol, i.e. in solvents hexacoordinating the central Mg atom. The fluorescence decay of Pchl was monoexponential in all of the investigated solvents. The fluorescence lifetime decreased for increasing solvent orientation polarizability from 5.5 ± 0.1 ns in 1,4-dioxane to 3.3 ± 0.1 ns in methanol. Longer lifetime values were observed in the case of aprotic solvents than in protic solvents. The hexacoordination of Mg had no effect on the fluorescence lifetime. The present data are discussed with respect to results found for protochlorophyllide (Pchlide) (Myśliwa-Kurdziel et al. in Photochem Photobiol 79:62–67, 2004), and they indicate that the presence of phytol chain in the porphyrin ring influences the spectral properties of the whole chromophore. This is the first complex analysis comparing the fluorescence emission and fluorescence lifetimes of purified Pchl and Pchlide.

Keywords Fluorescence emission spectrum · Fluorescence lifetime · Protochlorophyll · Protochlorophyllide · Solvent effect · Stokes shift

Abbreviations

Bchl	Bacteriochlorophyll
Bchlide	Bacteriochlorophyllide
Chl	Chlorophyll
Chlide	Chlorophyllide
cP	Centipoises
DMSO	Dimethyl sulfoxide
DV-	Divinyl
EPIM	Etioplasts inner membranes
GG	Geranylgeraniol
MV-	Monovinyl
Pchlide	Protochlorophyllide
Pchl	Protochlorophyll
Pchl(ide)	Mixture of Pchl and Pchlide of unknown proportions
THF	Tetrahydrofuran

Introduction

Protochlorophyll (Pchl) and protochlorophyllide (Pchlide) are natural porphyrin-type compounds. Pchlide is one of

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the intermediates of the Chl biosynthesis process (for a review, see Willows 2003; Schoefs and Franck 2003; Bollivar 2006). Pchl is also formed in dark-grown plants; however, it is only a side product of Chl biosynthesis, and is not involved in Chl formation. In dark-grown angiosperm seedlings, an accumulation of Pchlide takes place within the etioplast inner membranes (EPIMs) (for a review, see Sundqvist and Dahlin 1997). Besides Pchlide, Pchl also has been found in etioplasts; however, it amounts to only a small percentage of the total accumulated porphyrins (Böddi et al. 1989). Considerable amounts of Pchl accumulate in the inner seed coat of some plants of the *Cucurbitaceae*, e.g., *Cucurbita pepo* (Singh 1953; Houssier and Sauer 1969; Böddi et al. 1979), *Luffa cylindrica* (Sundqvist and Ryberg 1983) and *Cyclanthera explodens* (Ryberg et al. 1980). Recently, the presence of Pchl and Pchlide (Pchl(ide)) has also been detected in cabbage heads (Solymosi et al. 2004; Kruk 2005) and in the innermost leaves of buds (Solymosi and Böddi 2006; Solymosi et al. 2006) developing under natural conditions.

Pchl(ide) is present in different molecular complexes in vivo, which have different spectral properties and are designated as Pchlide (or Pchl) spectral forms (for a review, see Böddi 1994; Sundqvist and Dahlin 1997; Schoefs 2001, 2005). This way, the in vivo fluorescence and absorption spectra of Pchl(ide) pigments are complex, and consist of several Gaussian components. The number of these forms, the position and/or amplitudes of their absorption and fluorescence maxima depend on plant species, the stage of leaf development and on treatment of a sample before recording the spectra. However, Schoefs et al. (2000) found similar position and different amplitudes of Gaussian components in the spectra of etiolated bean leaves at different age. The diversity of spectral forms is due to interaction of the π electron system of Pchl molecules with surrounding molecules.

Fluorescence spectroscopy has been commonly applied in studies dealing with the greening process, taking advantage of highly efficient fluorescence of porphyrin and chlorin macrocycles as natural fluorescence probes (for a review, see Schoefs and Franck 2003). Analysis of Pchlide and Chl fluorescence in vivo provides information on the interactions of these pigments with their molecular surroundings. Fluorescence lifetime studies of etiolated seedlings and isolated EPIMs gave several fluorescence lifetime components, ranging from 0.25 to 5.8 ns (Myśliwa-Kurdziel et al. 1999, 2003), which confirmed the spectral heterogeneity of Pchl(ide) in vivo. However, because of the complexity of these systems, the determination of the fluorescence lifetimes of individual Pchlide and Pchl spectral forms in natural systems has been unsuccessful up to now.

To understand complicated natural systems, a detailed examination of photophysical properties of the pigments in different model systems is required. In these systems, the pigments are purified and their molecular environments can be varied.

The Pchl molecule consists of a porphyrin ring esterified with a long chain alcohol, phytol or its unsaturated biosynthetic precursors, e.g. geranylgeraniol (GG), dihydroGG, tetrahydroGG (Schoefs and Bertrand 2000; Rassadina et al. 2004; Böddi et al. 2005) (Fig. 1). Pchl is soluble in organic solvents but not in water. Protochlorophyllide (Pchlide), the nonesterified Pchl, can be dissolved both in water and in polar organic solvents.

The electrophilic central Mg atom of the tetrapyrrole macrocycle, as well as nucleophilic binding sites, like C=O groups, determine specific interactions of the Pchl molecule with its molecular vicinity, such as hydrogen binding or coordination of ligands. The strongly hydrophobic phytol chain may interact with nonpolar molecules in the pigment environment.

Fluorescence spectra and fluorescence lifetimes for monovinyl- (MV-) and divinyl- (DV-) Pchlide, purified using HPLC, have already been measured in a range of organic solvents of different properties (Kotzabazis et al. 1990; Myśliwa-Kurdziel et al. 2004; Kruk and Myśliwa-Kurdziel 2004). Similar fluorescence study has been performed for Pchl aggregates in Triton X-100 micelles (Böddi et al. 1983; Myśliwa-Kurdziel et al. 2007). Recent studies, performed on Chl (Fiedor et al. 2003) and Bchl (Fiedor et al. unpublished results), showed that phytol can influence the photophysical properties of the porphyrin

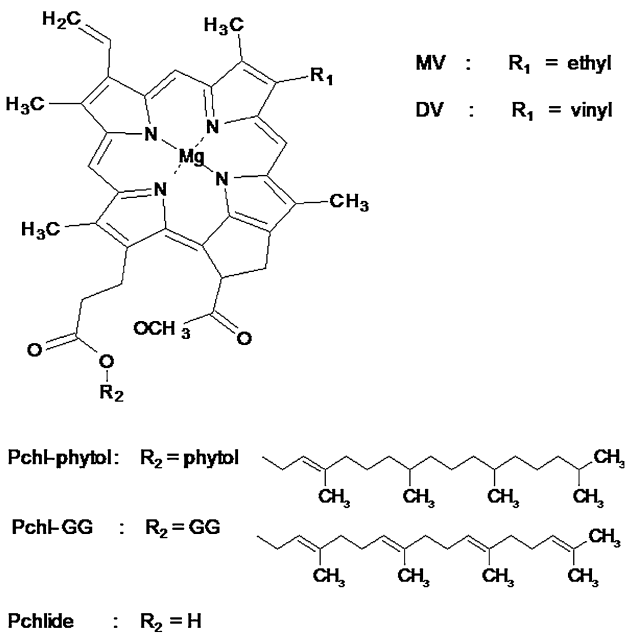

Fig. 1 Structure of protochlorophyll (Pchl) and protochlorophyllide (Pchlide)

macrocycle, and therefore, the results obtained for non-phytylated pigments cannot be extrapolated in all aspects for phytylated ones.

In this work, we have determined the fluorescence characteristics of Pchl in different organic solvents and compared them to those obtained for Pchlide (Myśliwa-Kurdziel et al. 2004) to verify if the phytol may modify the photophysical properties of Pchlide. Furthermore, we examined the effect of different side groups of the porphyrin ring on the photophysical properties of Pchl in naturally occurring protochlorophyllous pigments, MV- and DV-Pchl esterified with phytol or with GG.

Materials and methods

Pigments

Pchl was extracted from inner seed coats of pumpkin (*Cucurbita pepo*, var. Stajer). About 20 seeds were soaked in distilled water for 5 min. The water was discarded prior the addition of 5–10 ml of acetone. This solvent was discarded after 10 s, and pigments were then extracted with two to three portions of acetone (5–10 ml). Total Pchl fraction (mixture of isomers) was separated from carotenoids by column chromatography, as described by Myśliwa-Kurdziel et al. (2007), and used for the experiments as Pchl unless otherwise indicated. In some experiments, the purified individual Pchl isomers were used (Table 2), which were separated using C_{30} RP column (250 × 4.6 mm^2, 3 μm, YMC, Europe GmbH, Schermbeck, Germany) in methanol/ethyl acetate (34/16, vol/vol) at the flow rate of 1.5 ml/min. Pchlide was extracted from ALA-treated etiolated wheat leaves and purified as described in Kruk and Myśliwa-Kurdziel (2004).

Solvents

All the organic solvents used for spectroscopic measurements were of spectroscopic grade. Solvents for Pchl purification were of analytical or HPLC grade.

Absorption spectroscopy

Absorption spectra of Pchl in all the investigated solvents were recorded with a SLM AMINCO DW-2000 (Aminco Instruments, Urbana, IL, USA) spectrophotometer between 400 and 750 nm as in Myśliwa-Kurdziel et al. (2004).

Fluorescence measurements

Fluorescence emission spectra were measured with a LS50B spectrofluorometer (Perkin-Elmer, Beaconsfield, Buckinghamshire, UK). The spectra were recorded in the range between 580 and 780 nm. The data collection frequency was 0.5 nm. The excitation and emission slits were 10 and 5 nm, respectively. In case of each solvent, a fluorescence spectrum was measured with the excitation wavelength corresponding to the respective absorption maximum of the Soret band. The spectra were corrected for the baseline and for the wavelength-dependent sensitivity of the photomultiplier. The fluorescence maxima agreed within 0.5–1 nm in the independent parallel measurements.

Fluorescence lifetime was measured with a multi-frequency cross-correlation phase and modulation K2 fluorometer equipped with a Pockels-cell modulator (ISS Instruments, Urbana, IL, USA). The samples were excited using a 300 W Xenon lamp as light source. Excitation wavelength corresponded to the respective absorption maximum of the Soret band. The fluorescence signal was measured through a 600-nm cut-off filter, with respect to a scattering solution of glycogen (Merck, Darmstadt, Germany) in water having the fluorescence lifetime of 0 ns. The phase and modulation data were recorded for 12 frequencies of modulation of light intensity, ranging from 2 to 225 MHz. The maximal errors in the measured phase shift and modulation were ±0.3 and ±0.006, respectively. Phase shift and modulation data were analyzed using a monoexponential model of fluorescence decay by the nonlinear least-square program (ISS Instruments, USA). An average fluorescence lifetime value and standard deviation were calculated for several (at least three) independent measurements of fluorescence lifetime in a given solvent.

Pchl concentration was at the level of (0.5–1) × 10^{-6} M in all fluorescence measurements, which corresponded to the optical density lower than 0.2 at the respective maximum of the Soret band. Spectroscopic measurements were performed at room temperature (20°C), unless otherwise stated.

Thermostatting of the measuring cell

In the experiment aimed at measurements of the fluorescence lifetime of the pigments at different temperatures, stabilization of temperature was performed using a thermostat (Julabo F32, Germany) connected to the K2 ISS spectrofluorimeter. Solution of Pchl or Pchlide was prepared at room temperature and placed in the sample holder of the K2 spectrofluorimeter cooled down to 5°C. The fluorescence lifetime measurement was performed after equilibration of the temperature. Then, the sample was gradually heated to given temperatures at which lifetime measurements were planned. During the measurement, the temperature was stabilized at the programmed constant level.

Statistics

The experiments were repeated at least three times for each pigment and in each solvent. Each time a freshly prepared pigment solution was used.

Results and discussion

Steady state fluorescence properties of Pchl in organic solvents with respect to solvent orientation polarizability

The fluorescence properties of Pchl were investigated in 15 organic solvents of different physical and chemical properties. In case of each solvent, fluorescence emission spectra and fluorescence lifetimes were measured at room temperature for the same Pchl solution. The Pchl concentration was at the level of (0.5–1) $\times$ 10^{-6} M for all the measurements to avoid pigment aggregation, self-absorption and self-quenching effects.

The fluorescence spectra recorded for various solvents had slightly different shapes, i.e., the half-width of the main band and the relative amplitude of the vibrational sideband to the main band varied. The fluorescence emission maxima were observed between 625 and 643 nm in the different solvents (Table 1). The representative spectra measured for *n*-hexane, acetone, ethanol, pyridine and methanol are shown in Fig. 2.

In the case of a solute molecule having different dipole moments in the electronically excited state and the ground state, absorption of light induces changes of the local electric field and gives rise to a reorganization of solvent molecules within a sphere of action surrounding the excited chromophore molecule. This process, called solvent relaxation, lowers the energy level of the excited fluorophore, which is manifested by a solvatochromic shift of the emission maximum. The extent of the shift depends on the solvent polarity. Different theoretical and empirical polarity scales have been applied to analyze the solvent effects for a set of fluorophores (for a review, see Reichardt 1990). Solvent orientation polarizability (Δf) (Lakowicz 1999), given by Eq. 1, which is a function of the dielectric constant (ε) and the refractive index (n) of a solvent, has been frequently applied as an appropriate polarity scale for fluorescence studies.

$$\Delta f = \frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n^2 - 1}{2n^2 + 1} \quad (1)$$

The wavenumber of the fluorescence maximum linearly decreases with the increase of solvent orientation polarizability if there are no specific solvent–solute interactions (for a review, see Reichardt 1990). In the present study, the fluorescence maximum of Pchl shifted

Table 1 Orientation polarizability, spectral parameters and fluorescence lifetime of Pchl in organic solvents

Solvent	Orientation polarizability	Fluorescence maximum		F_{main}/F_{vibr}	E_{Q_y} (cm^{-1})	Stokes shift (cm^{-1})	Fluorescence lifetime (ns)
		(nm)	(cm^{-1})				
n-Hexane	−0.0014	625.5	15,987	5.4 ± 0.1	16,026	38.4	4.8 ± 0.09
Benzene	0.0020	630.0	15,873	5.2 ± 0.1	15,974	101.4	5.2 ± 0.1
CCl_4	0.0097	629.5	15,886	5.0 ± 0.1	15,974	88.8	3.7 ± 0.1
Toluene	0.0132	630.0	15,873	5.0 ± 0.1	15,974	101.4	5.06 ± 0.08
1,4-Dioxane	0.0213	629.0	15,898	3.8 ± 0.1	16,000	101.7	5.5 ± 0.1
Chloroform	0.1469	635.0	15,748	4.6 ± 0.1	15,898	150.2	3.96 ± 0.08
Diethyl ether	0.1634	626.5	15,962	5.1 ± 0.1	16,064	102.6	5.22 ± 0.07
THF	0.2086	632.5	15,810	3.4 ± 0.1	15,898	88.0	4.92 ± 0.08
Pyridine	0.2138	639.5	15,637	2.7 ± 0.1	15,873	235.8	4.5 ± 0.1
n-Octanol	0.2259	636.0	15,723	4.0 ± 0.1	15,873	149.7	4.6 ± 0.1
Isoamyl alcohol	0.2546	636.5	15,711	3.4 ± 0.1	15,873	162.1	3.98 ± 0.09
DMSO	0.2628	631.0	15,848	3.8 ± 0.1	15,974	126.6	4.7 ± 0.1
Acetone	0.2836	629.0	15,898	4.6 ± 0.1	16,015	117.1	4.94 ± 0.08
Ethanol	0.2893	636.0	15,723	3.2 ± 0.1	15,898	175.0	4.08 ± 0.07
Methanol	0.3079	643.5	15,540	2.2 ± 0.1	15,886	345.6	3.3 ± 0.1

Excitation wavelength corresponded to the respective absorption maximum of the Soret band

Solvents in the table are ordered according to their orientation polarizability, calculated according to Eq. 1 (see "Results and discussion")

F_{main}/F_{vibr} ratio of the amplitude of the main fluorescence band (0–0 transition; F_{main}) to the amplitude of the vibrational sideband (F_{vibr}), E_{Q_y} the wavenumber of Q_y absorption band

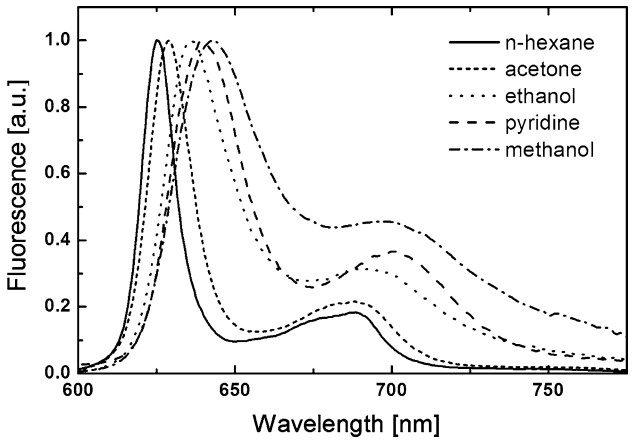

Fig. 2 Representative fluorescence emission spectra of Pchl in *n*-hexane, acetone, ethanol, pyridine and methanol. In case of each solvent, the fluorescence spectrum was measured for the excitation wavelength corresponding to the respective absorption maximum of the Soret band. Pchl concentration was at the level of 0.5–1 $\times$ 10^{-6} M. Spectra were normalized at their maxima

towards long wavelengths (i.e. the wavenumber of the fluorescence maximum decreased) for increasing solvent's orientation polarizability (Table 1). However, this correlation was rather weak and nonlinear (not shown), indicating the presence of specific Pchl–solvent interactions enhancing solvatochromic shift. In case of the Pchl molecule, the C=O group in the fifth ring, located in close vicinity of the π electron system of the porphyrin macrocycle, can serve as an electron donor for H-binding. For the investigated primary alcohols, the fluorescence maximum of Pchl was evidently red-shifted with respect to the maximum observed in aprotic solvents of similar orientation polarizability, for example, 7-nm difference was found between the emission maximum of Pchl in ethanol (protic solvent) and acetone (aprotic solvent) (Table 1). Similar observation was reported for Pchlide (Myśliwa-Kurdziel et al. 2004) and for Chlide and Chl (Fiedor et al. 2003). For *n*-octanol, isoamyl alcohol and ethanol, the energies of the fluorescence maxima were similar, corresponding to the wavenumber of about 15,700 cm^{-1}, whereas the energy for methanol was significantly lower. Considering the investigated alcohols, the relative intensity of the vibrational sideband increased for increasing solvent orientational polarizability (Table 1, Fig. 2).

The longest wavelength emission maximum was found in the case of methanol (643.5 nm; 15,540 cm^{-1}), which corresponds to low energy of the S_1 state (Table 1, Fig. 2). Similarly, low-energy S_1 state was found in the case of pyridine (639.5 nm; 15,637 cm^{-1}). These solvents have been reported to have the highest fraction of hexacoordinated form of Bchl and Bchlide (Cotton and Van Duyn 1981; Limantanara et al. 1997; Kania and Fiedor 2006). Coordination of a sixth ligand to the central Mg atom of porphyrin macrocycle may be a reason for the decrease of the S_1 energy, which has been shown for fluorescence of Chl and Chlide (Fiedor et al. 2003) and for Pchlide (Myśliwa-Kurdziel et al. 2004). THF was also described as hexacoordinating solvent for Bchl (Limantanara et al. 1997; Kania and Fiedor 2006). However, no specific effects were observed for the position of fluorescence maximum in this solvent either for Pchlide (Myśliwa-Kurdziel et al. 2004) or for Pchl (Table 1). The nature of the external ligand is different for pyridine and THF, which may explain their different effect on the S_1 energy. Stronger red-shift of Q_y absorption energy in the case of nitrogen-containing ligand (e.g. pyridine) than in oxygen-containing ligand (e.g. THF) has also been shown for Chl (Renge and Avarmaa 1985; Krawczyk 1989).

The energy of the emitted light corresponding to the fluorescence maximum of Pchl in chloroform was similar to that in ethanol and *n*-octanol (Table 1), and it was lower when comparing with other solvents having similar orientation polarizability, which indicates some specific interaction of this solvent with the pigment. The highest wavelength of fluorescence maximum was observed for Pchl in hexane and then for diethyl ether (Table 1).

The fluorescence maxima observed for Pchl in the present study were the same as for Pchlide (Myśliwa-Kurdziel et al. 2004) in the case of toluene, dioxane, diethyl ether, THF, isoamyl alcohol and ethanol. In the case of chloroform, pyridine, dimethyl sulfoxide (DMSO) and acetone, the S_1 energy was higher for Pchl than for Pchlide. The difference of the fluorescence maximum was about 50 cm^{-1}, which corresponds to 1–3 nm blue-shift of the Pchl emission maximum compared to that of Pchlide. For methanol, the maximum fluorescence for Pchl was red-shifted (2 nm) compared to Pchlide (Myśliwa-Kurdziel et al. 2004).

The Stokes shift of Pchl in different organic solvents

A difference in wavenumbers between the absorption and fluorescence maxima, known as Stokes shift, provides information on the change in the magnitude of a fluorophore's dipole moment caused by excitation and is an indicator of the sensitivity of the fluorophore to the solvent's reaction field (Reichardt 1990). Moreover, this parameter reveals the solvation effect both in the ground and the excited states of the chromophore. In the absence of any specific solvent–solute interaction, the Stokes shift is proportional to the orientation polarizability of a solvent and this dependence is described as Lippert relation.

In the present study, relatively small values of the Stokes shift were found (Table 1), which reveals small difference of the Pchl dipole moment in the ground and

excited states. Values of the Stokes shift increased for the increasing solvent orientation polarizability (Fig. 3); however, the nonlinear character of this dependence indicates a strong influence of specific Pchl–solvent interaction.

The smallest value of the Stokes shift (about 40 cm^{-1}) was found for Pchl in hexane, which is an aprotic and apolar solvent, having dipole moment of zero. Thus, the shift between absorption and emission energies reflects only the energy loss in the excited state due to intramolecular vibrational relaxation. For other aprotic solvents benzene, toluene, carbon tetrachloride, dioxane, diethyl ether, THF, acetone and DMSO, a small increase of the Stokes shift was observed for increasing solvent orientation polarizability. A linear regression calculation showed a dependence with a slope of 61 ± 35 cm^{-1} for this group of solvents (Fig. 3); this confirms low sensitivity of Pchl to nonspecific solvation. In the case of alcohols, the Stokes shift was higher than 150 cm^{-1}, and the slope of the linear regression function was more than six times higher than in the case of aprotic solvents. These results point out that, in the presence of H-binding solvents, the Pchl molecule becomes more sensitive to solvent polarizability. The highest value of the Stokes shift (345 cm^{-1}) was observed for Pchl in methanol, which is both protic and hexacoordinating solvent. In pyridine, which is another hexacoordinating solvent, the Stokes shift was also high (236 cm^{-1}). Coordination of the central Mg atom therefore also increases the difference between the absorption and fluorescence energies.

An average Stokes shift determined for the investigated solvents was of 136 cm^{-1}, which was similar to the average value calculated for the results measured for Pchlide (Myśliwa-Kurdziel et al. 2004). However, the solvents used in these two studies are partly different because of different solubility of Pchl and Pchlide. Similar average values of the Stokes shift for these two pigments lead to the conclusion that, in general, phytol does not influence the energy gap between the ground and excited state of Pchl(ide) molecule.

Comparing systematically the results for Pchl and for Pchlide from the literature (Myśliwa-Kurdziel et al. 2004), the Stokes shift was higher for Pchl than for Pchlide in diethyl ether, chloroform, pyridine and methanol. In the case of pyridine and methanol, this difference resulted from both different Q_y absorption maximum and fluorescence maximum of these two pigments. The presence of the phytol chain influences the energy levels of the porphyrin macrocycle in these two solvents; it decreases the S_0 and increases the S_1 levels. For chloroform and diethyl ether, the difference in the Stokes shift between Pchl and Pchlide was mainly caused by difference in absorption maximum.

Fluorescence lifetime values of Pchl in organic solvents

The fluorescence decay of Pchl measured in a series of organic solvents was analyzed with a monoexponential model, which was adequate in all cases. The fluorescence lifetimes varied within 3.3–5.5 ns (Table 1). Similar range of fluorescence lifetimes was observed for Pchlide in organic solvents (Myśliwa-Kurdziel et al. 2004).

No simple correlation between the fluorescence lifetimes and solvent orientation polarizability was observed

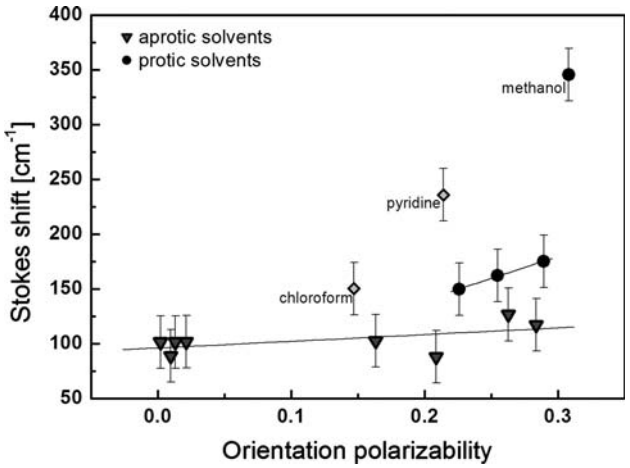

Fig. 3 Stokes shift for Pchl in organic solvents, calculated from absorption and fluorescence maxima shown in Table 1. Orientation polarizability (Δf), defined according to the Eq. 1 (for details, see the "Results and discussion"), is listed in Table 1. The *lines* were drawn by linear regression calculation for aprotic solvents [*fitted line*: $(61 \pm 35) \times \Delta f + (96 \pm 6)$; $R^2 = 0.34$] and for isoamyl alcohol, octanol and ethanol [*fitted line*: $(398 \pm 17) \times \Delta f + (60 \pm 4)$; $R^2 = 0.99$]

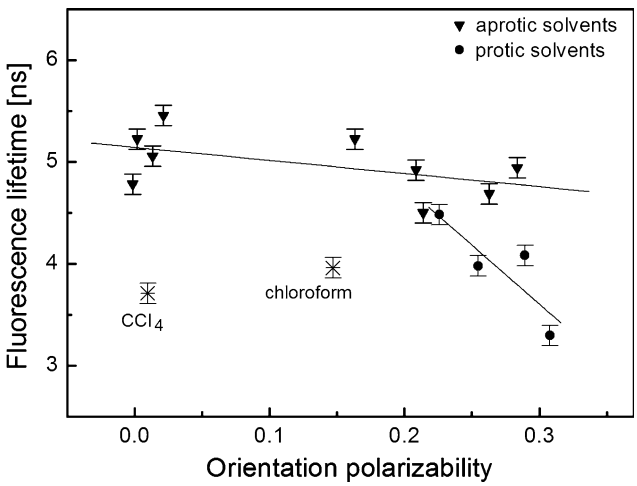

Fig. 4 Fluorescence lifetime for Pchl in organic solvents. Orientation polarizability (Δf) is defined according to the Eq. 1 (for details, see the "Results and discussion"). *Fitted lines*: $(-1.29 \pm 0.81) \times \Delta f + (5.14 \pm 0.13)$, $R^2 = 0.27$ for aprotic solvents; $(-11.56 \pm 4.94) \times \Delta f + (7.07 \pm 1.34)$, $R^2 = 0.74$ for alcohols

(Fig. 4). Pchl fluorescence lifetime was higher in aprotic than in protic solvents (alcohols). The highest value was observed for 1,4-dioxane (5.5 ± 0.1 ns) and for diethyl ether (5.22 ± 0.07 ns). In aprotic solvents, the lowest fluorescence lifetime was found for pyridine (4.5 ± 0.1 ns). In alcohols, the fluorescence lifetime decreased to 3.3 ± 0.1 ns (for methanol).

Both in protic and aprotic solvents, the fluorescence lifetime decreased with the increase of the solvent orientation polarizability. The slope of this decrease was definitely larger in the case of alcohols than in aprotic solvents (Fig. 4), indicating that for solvents able to form H-bonds, the fluorescence yield strongly depends on the solvent orientation polarizability. The shortening of the fluorescence lifetime in protic solvents, as compared to the values found in aprotic solvents, means that the deactivation of the S_1 state via fluorescence becomes less efficient in the presence of H-bonds. The shortening of the Pchl fluorescence lifetime was also observed in $CHCl_3$ (chloroform) and CCl_4, probably due to some collisional quenching.

In most studied solvents, the fluorescence lifetime of Pchl was longer than that measured for Pchlide (Myśliwa-Kurdziel et al. 2004). The highest difference of the fluorescence lifetime of Pchl and Pchlide was found in pyridine. The fluorescence lifetime of Pchl was 4.5 ns and that of Pchlide was 4.2 ns in this solvent (Myśliwa-Kurdziel et al. 2004).

Longer fluorescence lifetime has been observed for Chl than for Chlide (Fiedor et al. 2003) and also for Bchl than for Bchlide (Fiedor et al. unpublished results). However, in the case of methanol and ethanol, the fluorescence lifetime is longer for Chlide and Bchlide than for Chl and Bchl, respectively. In methanol, the fluorescence lifetime was longer for Pchlide (Myśliwa-Kurdziel et al. 2004) than for Pchl in this work (Table 1), but this is not valid for ethanol.

In the case of Bchl and Bchlide, the fluorescence lifetime in dioxane, THF and pyridine is longer than that in other solvents (Connolly et al. 1982a; Fiedor et al. unpublished results). Similar relation has been observed for Chl and Chlide; however, the differences in the fluorescence lifetime values were smaller in this case (Connolly et al. 1982b; Fiedor et al. 2003). For Pchl (Table 1) and Pchlide (Myśliwa-Kurdziel et al. 2004), the highest lifetime values were found in dioxane and diethyl ether. This indicates that in contrast to Bchl and Chl, hexacoordination of the central Mg atom does not cause any increase of the fluorescence lifetime in the case of Pchl and Pchlide. This difference might be explained by the difference in delocalization of the π electron system of the porphyrin ring as compared to chlorin or bacteriochlorin rings.

Influence of viscosity and temperature on the fluorescence lifetime of Pchl and Pchlide

Fluorescence lifetime of Pchl decreased in alcohols in the following order: *n*-octanol > isoamyl alcohol ≅ ethanol > methanol (Table 1), which is also the order of decrease of the viscosity of these solvents. The effect of solvent viscosity on the fluorescence lifetime of Pchl and Pchlide was examined in *n*-octanol, which is the most viscous among the investigated alcohols (10 cP at room temperature). The viscosity of *n*-octanol was changed by increasing the temperature within a range from 5 to 60°C, which corresponded to the decrease of the viscosity of *n*-octanol from 12 to 2 cP. The fluorescence lifetime decreased upon heating in the case of both Pchl and Pchlide pigments (Fig. 5). The effect was stronger in the case of Pchl than Pchlide. For comparison, fluorescence lifetime was measured in the same heating conditions for the pigments in ethanol, which has a viscosity of 1.1 cP at room temperature (i.e. ten times lower than *n*-octanol) and in which the viscosity changes are insignificant upon heating. In such system, the direct influence of temperature on fluorescence lifetime can be analyzed. The results (Fig. 5) show only small changes of Pchl fluorescence lifetime in ethanol, and no changes for Pchlide (not shown) for increasing temperature.

The results of this experiment point out that the phytol side chain bound to the porphyrin macrocycle causes an increase of the fluorescence lifetime in a viscous medium. At low viscosity (ethanol), the fluorescence lifetimes of Pchl and Pchlide are similar, and temperature has only a weak influence on Pchl(ide) lifetime. Long fluorescence lifetime of Pchl has been observed for Pchl in neat Triton X-100 (Myśliwa-Kurdziel et al. 2007), which is also a very viscous medium. This suggests that the long hydrophobic

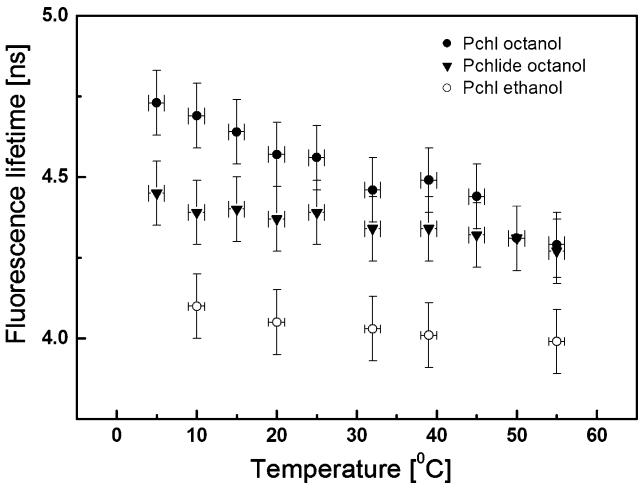

Fig. 5 Fluorescence lifetime of Pchl and Pchlide measured at different temperatures in octanol and ethanol. Excitation: 440 nm

Table 2 Fluorescence lifetimes [ns] of Pchls and Pchlides in selected organic solvents

Pigment	Fluorescence lifetime				
	Pyridine	*n*-Octanol	*n*-Propanol	Ethanol	Methanol
MV-Pchl-phytol	4.42 ± 0.1	4.64 ± 0.07	4.16 ± 0.2	4.19 ± 0.06	3.38 ± 0.1
DV-Pchl-phytol	4.51 ± 0.06	4.60 ± 0.1	4.14 ± 0.1	4.14 ± 0.06	3.36 ± 0.02
MV-Pchl-GG	4.4 ± 0.06	4.59 ± 0.1	4.17 ± 0.2	4.12 ± 0.09	3.37 ± 0.12
DV-Pchl-GG	4.6 ± 0.08	4.56 ± 0.1	4.16 ± 0.1	4.09 ± 0.15	3.34 ± 0.1
MV-Pchlide	4.23 ± 0.1	4.5 ± 0.1	4.15 ± 0.15	4.11 ± 0.05	3.54 ± 0.01
DV-Pchlide	4.48 ± 0.1	4.33 ± 0.08	4.11 ± 0.1	4.08 ± 0.06	3.48 ± 0.15

Excitation: 440 nm; emission was recorded through a 600-nm cut-off filter

phytol chain moderates the molecular movements of the excited pigment molecules in a viscous medium and is therefore responsible for the increased lifetime value of Pchl when compared with unesterified Pchlide. The Pchl(ide) form having the fluorescence maximum around 630 nm is regarded as unbound to the active site of Pchlide:NADPH reductase; however, the exact localization of pigment molecules within the EPIMs has not been revealed. The fluorescence lifetime of these short-wavelength Pchl(ide) forms found in isolated EPIMs (5.5–6 ns; Myśliwa-Kurdziel et al. 1999) is longer than the fluorescence lifetime of the pigments in organic solvents. The present data indicate that such a long fluorescence lifetime may result from localization of these Pchl(ide) molecules in viscous environment of EPIMs or from their immobilization.

The effect of different side groups of the porphyrin ring on Pchl fluorescence lifetimes

The question arises as to whether different substituents of the porphyrin ring, i.e. having different degree of unsaturation, influence the fluorescence lifetime. This issue was examined in pyridine and alcohols, because for these solvents, the largest difference of the fluorescence lifetime of Pchl (Table 1) and Pchlide (Myśliwa-Kurdziel et al. 2004) was found. Moreover, the fluorescence lifetimes of Pchl and Pchlide in alcohols strongly depended on the solvent orientation polarizability. Fluorescence lifetimes measured for MV-PChl-phytol, DV-PChl-phytol, MV-Pchl-GG, DV-Pchl-GG, MV-Pchlide and DV-Pchlide are summarized in Table 2.

In the case of pyridine, the fluorescence lifetime of pigments having two vinyl groups on the porphyrin macrocycle (DV-pigments) was longer than the lifetime of MV-pigments. This effect was stronger in the case of Pchlide than Pchl; however, it was observed for both Pchl-GG and Pchl-phytol.

No difference in the fluorescence lifetimes was observed for pigments in alcohols having low viscosity at room temperature (*n*-propanol, ethanol and methanol). In these solvents, the side groups of the porphyrin ring do not influence the fluorescence lifetime. In *n*-octanol, the fluorescence lifetime of MV-Pchlide differs from that of DV-Pchlide. For pigments with alcohol side chain, this difference is eliminated. No difference is observed for MV-Pchl and DV-Pchl both in GG and phytol esters.

Conclusions

Fluorescence emission maximum position, Stokes shift and fluorescence lifetime values were analyzed in relation to nonspecific and specific solvation effect for Pchl dissolved in various organic solvents. In alcohols (H-binding solvents), Pchl molecule is more sensitive to the solvent polarizability than in aprotic solvents, which can be observed both for the Stokes shift and for the fluorescence lifetime (Table 1; Figs. 3, 4). Hexacoordination of the central Mg atom results in an increase of the Stokes shift, and this effect is stronger than that observed in alcohols. On the contrary, hexacoordination of Mg has no effect on the fluorescence lifetime.

Fluorescence lifetime of Pchl is generally longer than that of Pchlide, except in methanol. The wavenumber of the fluorescence maximum of Pchl is higher than that of Pchlide in chloroform, pyridine, DMSO and acetone. In pyridine and methanol, large differences of the fluorescence energy between Pchl and Pchlide were observed, which indicates the effect of phytol on the S_1 energy level of the porphyrin. Different fluorescence lifetimes of Pchl and Pchlide in these solvents also indicate the effect of phytol on the fluorescence lifetime.

The present data clearly show different fluorescence properties of Pchl and Pchlide, especially the fluorescence lifetime in organic solvents, i.e. in the simplest model system. The degree of unsaturation of the alcohol chain and the presence of MV- or DV- side group has a little effect on the fluorescence lifetime.

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