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Temperature dependence of the lipid packing in thylakoid membranes studied by time- and spectrally resolved fluorescence of Merocyanine 540

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ABSTRACT

The lipid packing of thylakoid membranes is an important factor for photosynthetic performance. However, surprisingly little is known about it and it is generally accepted that the bulk thylakoid lipids adopt the liquid-crystalline phase above −30 °C and that a phase transition occurs only above 45 °C. In order to obtain information on the nature of the lipid microenvironment and its temperature dependence, steady-state and time-resolved fluorescence measurements were performed on the fluorescence probe Merocyanine 540 (MC540) incorporated in isolated spinach thylakoids and in model lipid systems (dipalmitoyl phosphatidylcholine and dioleoyl phosphatidylethanolamine) adopting different phases. It is demonstrated that the degree and way of incorporation differs for most lipid phases – upon selective excitation at 570 nm, the amplitude of the fluorescence component that corresponds to membrane-incorporated MC540 is about 20% in gel-, 60% in rippled gel-, and 90% in liquid-crystalline and inverted hexagonal phase, respectively. For thylakoids, the data reveal hindered incorporation of MC540 (amplitude about 30% at 7 °C) and marked spectral heterogeneity at all temperatures. The incorporation of MC540 in thylakoids strongly depends on temperature. Remarkably, above 25 °C MC540 becomes almost completely extruded from the lipid environment, indicating major rearrangements in the membrane.

1. Introduction

Many physiological processes that take place in the photosynthetic thylakoid membrane as well as a variety of its properties have been demonstrated to depend on temperature. These include oxygen evolution [1], CO_2 assimilation, non-photochemical quenching, aggregation of the major light-harvesting complex LHCII [2], photophosphorylation [2,3], expression of proteins [4], lipid-protein ratio [5] and lipid–protein interactions ([6] and references therein), membrane permeability [7–9], electron transport activity [10–14], proton efflux [15] and the rate of NADP-reduction [16]. The temperature dependences of some of these processes and properties were correlated with (or related to) the mobility of the chloroplast-membrane lipids as measured by EPR spin-label spectroscopy [17–19]. Abrupt changes in the membrane fluidity were also reported at 10–12 °C [20].

It is known that the lipid packing and phase behavior are essential for the operation of violaxanthin de-epoxidase [21–23] and that they are modulated by the xanthophyll cycle pigments solubilized in the bilayer [24]. They might also play a significant role in providing the optimal conditions for diffusion of hydrophobic molecules such as plastoquinol [25,26] or integral protein complexes (e.g. during state transitions [27,28]).

It is generally accepted that the bulk lipid mixture in the thylakoid lamellae exists in the liquid crystalline phase (Lα) above −30 °C [29] and no phase transition is expected to occur up to about 45 °C; above this temperature the thylakoid lipids segregate from the membrane and form non-bilayer structures [30,31]. This, however, does not exclude the possibility of changes in the lipid packing and/or formation of membrane domains with distinct physical properties at lower temperatures. Since the thylakoid membranes contain about 70–80% proteins, and only 20–30% lipids, the lipid packing might also be influenced by changes in the macro-organization of the protein complexes, as indeed indicated by temperature- and light-induced reorganizations in the LHCII-containing chiral macrodomains (see e.g. [32]). The packing of biological membranes in general, as well as the

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nature of lipid–protein interactions, are also largely influenced by the presence of high concentrations of non-bilayer lipids [33,34].

The thylakoid membrane is constituted of four lipid classes – monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol, phosphatidylglycerol and sulfoquinovosyl. It is known that half of the thylakoid membrane lipids (MGDG) are non-bilayer forming lipids (NBL), which are nevertheless believed to be organized predominantly in bilayers assuming the liquid-crystalline phase in the intact membrane [29,35]. Their significance for the macroorganization and function of the membrane is still unknown. It was hypothesized that the presence of NBL is needed for the maintenance of a certain lipid–protein ratio, essential for the optimal functioning of the membrane [36]. The adjustment of the MGDG:DGDG ratio was found to be a way for adaptation to cold stress [37,38]. Phase segregation of thylakoid lipids into the inverted hexagonal phase (H_{II}) was shown to occur in spinach plants grown under low-light conditions [39] and also under various other stress conditions like high temperatures (above 45 °C) [30,31,40], low pH [41] and high concentrations of metal cations [42]. These data show that under certain conditions a marked heterogeneity can be induced in the lipid phase of thylakoid membranes. Our recent ^{31}P-NMR data have shown that heterogeneity is also present in freshly isolated intact thylakoid membranes. It has been shown that the location of the phospholipids is not restricted to the bilayer phase and the lamellar phase co-exists with a non-bilayer isotropic phase, with a strong temperature dependence between 14 and 20 °C [43].

In general, modulations in the lipid packing and co-existence of different lipid phases and domains are rather difficult to detect in complex biological membranes such as thylakoids due to limitations of the available techniques. Here we apply a new approach, the combination of steady-state and time-resolved fluorescence spectroscopy of the lipophylic fluorescent dye Merocyanine 540 (MC540). The spectroscopic properties of this probe have been shown to be sensitive to the phase behavior and lipid packing/spacing in model membranes [44–51], which has been used for identifying domains with different packing in different biological membranes [52–56]. MC540 is incorporated into lipid membranes as monomers [57,58] or forms dimers and higher aggregates on the surface [46,49,59,60].

In this work, we have determined the steady-state and time-resolved fluorescence properties of MC540 in model lipid systems that can adopt different phases, depending on the temperature of the suspension, and have compared them to those in thylakoid membranes. The combination of steady-state and time-resolved fluorescence spectroscopy led to the unequivocal identification of three forms of MC540, with characteristic spectral properties and fluorescence lifetimes, in model lipid systems as well as in thylakoid membranes, which can be assigned to dyes in different microenvironments. Our results reveal that incorporation of MC540 in thylakoids (whose lipids are believed to adopt liquid-crystalline phase, see above) is far less pronounced than in model liquid-crystalline phase and strongly depends on the temperature. Above 25 °C the dye molecules are progressively extruded from their hydrophobic lipid environment, indicating the onset of structural rearrangements in the thylakoid membrane.

2. Materials and methods

2.1. Sample preparation

Liposomes of dipalmitoyl phosphatidylcholine (DPPC) and dioleoyl phosphatidylethanolamine (DOPE) were prepared in 20 mM Tricine (pH 7.8) using the procedure described by Csiszar et al. [61]. MC540 (Fig. 1) was purchased from Sigma-Aldridge Co. The lipids were purchased from Avanti Polar Lipids, USA.

Dark-adapted leaves of market spinach were homogenized in a medium containing 20 mM Tricine (pH 7.5), 400 mM sorbitol, 5 mM $MgCl_2$ and 5 mM KCl; the suspension was filtered through 4 layers of cheese cloth and centrifuged for 4 min at 4000 × *g*. The chloroplasts were osmotically shocked in a hypotonic medium containing 20 mM Tricine (pH 7.5), 5 mM $MgCl_2$ and 5 mM KCl, and centrifuged for 5 min at 6000 × *g*. The pellet was finally resuspended in the same medium supplemented with 400 mM sorbitol (resuspension buffer); the chlorophyll (Chl) content was adjusted to 20 μg Chl/ml and quantified according to Arnon [62].

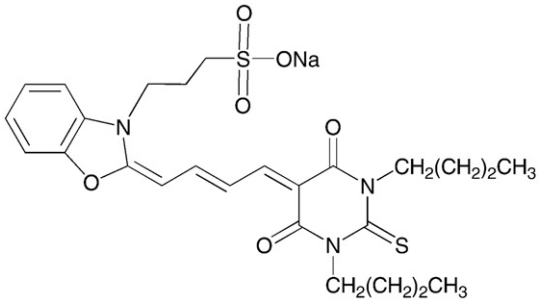

Fig. 1. Structural formula of Merocyanie 540.

Before performing the measurements the liposomes and the isolated thylakoid membranes were kept on ice and MC540 was added from 1 mM ethanol stock solution; the samples were gently stirred in the dark for 30 min. The final concentration of MC540 was 0.6 μM for the lipid suspensions and 0.2 μM for the thylakoid membrane preparations, respectively. During the measurements the samples were thermostated for 15 min at each temperature. For each experiment five independent repetitions were performed.

2.2. Fluorescence spectroscopy

Steady-state fluorescence spectra were recorded with a Jobin Yvon Fluorolog FL3-22 spectrofluorimeter and corrected for the detection sensitivity. In order to correct for the contribution of Chl fluorescence, excitation and emission spectra of thylakoid membranes were recorded in the absence of MC540 and subtracted from the corresponding spectra obtained after the addition of the dye. The emission wavelength for the excitation spectra was set at 580 nm, unless stated otherwise. For the model lipid systems the bandwidth for both excitation and emission was 0.75 nm, and for thylakoid membranes it was 3 nm; the step size was 0.2 nm. The emission spectra were detected using 2 nm bandwidth both for the excitation and the emission monochromators for the lipid systems and 3 nm for thylakoid membranes, the step size was 0.5 nm.

2.3. Fluorescence lifetime measurements

Time-correlated single photon counting technique was used to perform time-resolved fluorescence measurements. A CW diode-pumped, frequency-doubled Nd:YVO_4 laser (Coherent Inc., Santa Clara, CA, model Verdi V10) was used to pump a titanium:sapphire laser (Coherent Inc., Santa Clara, CA, model Mira 900-D in fs mode) that was passively mode-locked and tuned to either 540 or 570 nm. The channel spacing was 5 ps. The excitation intensity was reduced with neutral density filters to obtain a count-rate of 30,000 s^{-1}. The emission filters were two 3 mm thick Schott RG 610 nm cut-off filters for the lipid systems and a 3 mm thick Schott RG 610 nm cut-off filter and an additional Balzers broadband interference filter model K60 for the thylakoid suspensions, thus collecting the fluorescence between 610 and 630 nm in chlorophyll containing (thylakoid membranes) samples. For the fitting procedure, the dynamic instrumental response of the experimental setup was recorded using the fast and single-exponential fluorescence decay (6 ps) of the reference compound

pinacyanol in methanol [63]. Data analysis was performed using the computer program described earlier [64,65]. During the deconvolution procedure the lifetimes for different preparations of the same kind (measured upon excitation at 540 and 570 nm) and certain temperature were linked and their relative amplitudes were free. For example the lifetimes were linked for five samples of MC540 added to DPPC vesicles at 7 °C measured at 540 nm excitation and five samples but measured at 570 nm excitation. In this way the variation of the relative amplitudes corresponding to certain decay component could be followed in the different samples and at different temperatures. The fit quality was evaluated from χ^2, and from plots of the weighted residuals and the autocorrelation thereof (see e.g. [66]). Typical values of χ^2 were 1.1–1.2.

3. Results

Before studying the lipid packing in a system as complex as the thylakoid membrane, we first address the fluorescence properties of MC540 in model systems: DPPC and DOPE, which adopt different phases. It has been shown that the fluorescence yield and spectra of MC540 depend on the lipid phase and packing [46–50]. Here we further extend this approach by combining the steady-state properties of MC540 with its fluorescence lifetime characteristics and apply this information for thylakoid membranes.

Time-resolved fluorescence measurements of MC540 incorporated in lipid systems adopting different lipid phases, to our knowledge, have not been reported previously. The combination of the steady-state and time-resolved fluorescence spectroscopy techniques is particularly informative in cases where different microenvironments co-exist and when the fluorescence yields vary in a broad interval. With steady-state spectroscopy, heterogeneity can be revealed by varying either the excitation or the detection wavelength. However, molecules that have a short excited-state lifetime contribute considerably less to the excitation/emission spectra than the molecules with a longer excited-state lifetime, which makes their identification and determination of their contribution difficult. These can readily be performed with the aid of time-resolved fluorescence measurements, where the amplitude of a decay component is linearly proportional to the number of MC540 molecules in the corresponding environment.

In the following sections, steady-state and time-resolved fluorescence properties of different model lipid systems and of isolated thylakoid membranes will be presented at different temperatures. The temperature dependences will be confined for a range between 5 and 45 °C, the physiological range for thylakoid membranes. Above this temperature, as pointed out in the Introduction, thylakoid lipids segregate in large quantities from the membrane and form extended non-bilayer structures.

3.1. Steady-state fluorescence

3.1.1. MC540 in aqueous environment

In the aqueous phase (resuspension buffer, see Materials and methods) the excitation spectrum of MC540 is characterized by bands at around 500 and 540 nm (Fig. 2, see also [67]). The spectrum is not changing significantly upon varying the detection wavelength. The same holds true also for the emission spectrum – it peaks at 575 nm and the shape is only weakly dependent on the excitation wavelength.

3.1.2. MC540 in DPPC

The main phase transition for DPPC (L_{β}' to L_{α} phase) occurs at about 41.5 °C with a pre-transition (rippled gel phase, P_{β}') at about 33 °C [68]. When MC540 is added to vesicles in L_{β}' phase (temperature below 33 °C) the most striking difference between its excitation spectrum and that of MC540 in an aqueous environment (cf. Fig. 2) is the reduction of the 500 nm band and the presence of an additional band

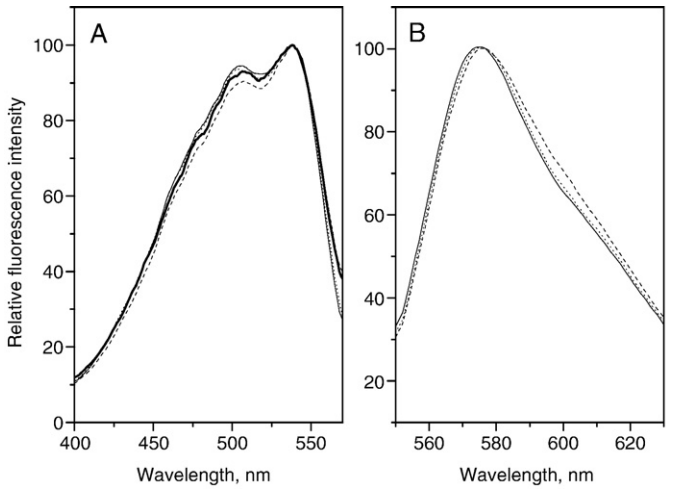

Fig. 2. (A) Excitation spectra of MC540 in resuspension buffer at 7 °C, recorded with the fluorescence emission collected at 572 nm (thick line), 580 nm (thin line), 595 nm (dashed line) and 620 nm (dotted line). (B) Emission spectra of MC540 in resuspension buffer at 7 °C, excited at 480 nm (solid line), 505 nm (dashed line), and 540 nm (dotted line). The spectra are normalized to their maxima.

at 566 nm (Fig. 3A). This latter band has been shown to originate from MC540 monomers incorporated in the lipid bilayer [69].

As reported previously the fluorescence intensity is increasing dramatically upon the gel to liquid crystalline phase transition (Fig. 3B, C see also [44,47,58]) and the fluorescence ratio between the 566 nm and 536 nm bands in the excitation spectra recorded at 580 nm, F_{566}/F_{536} increases from 1.20±0.11 (at 7 °C) – 1.95±0.05 (at 25 °C) in the L_{β}' phase to 2.83±0.08 in the P_{β}' and 2.94±0.04 in the L_{α} phase. Subtraction of the normalized excitation spectrum of MC540 in the L_{α} phase (recorded at 45 °C) from the spectrum in the L_{β}' phase (recorded at 7 °C) results in a difference spectrum very similar to the one of MC540 in buffer (Fig. 2A). The shape of the emission spectrum strongly depends on the excitation wavelength for the L_{β}' phase (Fig. 3C), but remains unchanged for the P_{β}' and L_{α} phases (Fig. 3C). These latter two phases cannot be distinguished from each other by comparing the steady-state properties of MC540 – the excitation (Fig. 3B) and emission (Fig. 3C) spectra have identical shapes.

3.1.3. MC540 in DOPE

The shape of the excitation and emission spectra of MC540 in the H_{II} phase of DOPE (DOPE adopts the H_{II} phase above 3 °C [70] at 7 °C (Fig. 4A) is almost identical to the ones of DPPC in the L_{α} phase at 45 °C ($F_{566/536}$ is 2.81±0.01, as compared to 2.94±0.04 for DPPC at 45 °C, see also Fig. 3B). The fluorescence intensity is linearly decreasing with the increase, as was observed for MC540 in ethanol [47], and the spectral shape remains unchanged. As observed for the L_{α} phase of DPPC, the shape of the emission spectrum does not depend on the excitation wavelength (Fig. 4B).

3.1.4. MC540 in thylakoid membranes

The excitation spectrum of MC540 in the presence of thylakoid membranes at 7 °C (Fig. 5A) is dominated by a band at 566 nm with a shoulder at 536 nm, whereas no pronounced 500 nm band is observed. The ratio F_{566}/F_{536} depends on the concentration of MC540. Over the whole concentration range studied – between 0.04 and 1.2 μM MC540 (at a fixed chlorophyll concentration of 20 μg Chl/ml) – the shoulder at 536 nm is more pronounced than for MC540 in the $H_{II}/P_{\beta}'/L_{\alpha}$ lipid systems. The highest value for F_{566}/F_{536} (2.21±0.09) is obtained at concentrations below 0.2 μM but it is significantly lower than the one observed for the L_{α} phase of DPPC ($F_{566/536}$=2.94±0.04). Above this concentration, the shoulder at 536 nm becomes even more prominent. In the rest of our experiments

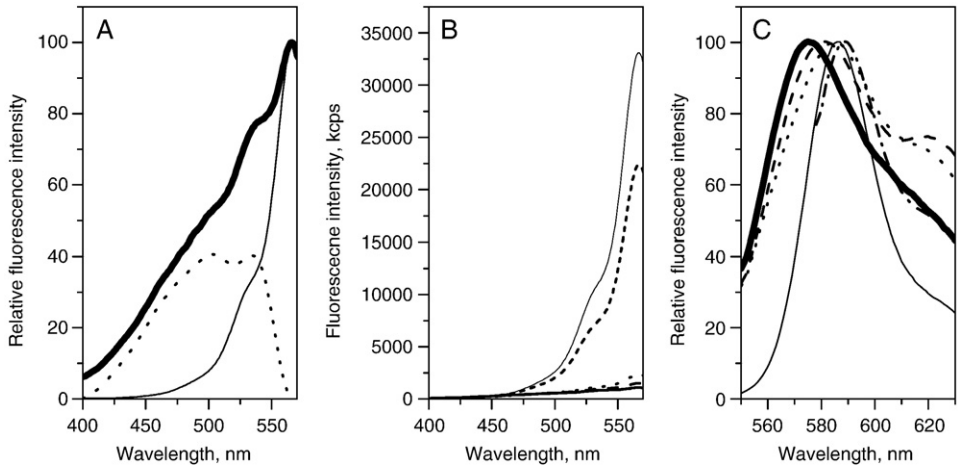

Fig. 3. (A) Excitation spectra of MC540 added to dipalmitoyl phosphatidylcholine (DPPC) vesicles adopting different phases [68]: the gel ($L_{\beta'}$) phase at 7 °C (thick line), and the liquid crystalline (L_α) phase at 45 °C (thin line). The spectra are normalized to their emission maxima. Their difference spectrum is represented by the dotted line. The emission was collected at 580 nm. (B) Temperature dependence of the excitation spectra of MC540, recorded at 580 nm: 7 °C (thick line), 14 °C (dashed line), 25 °C (dotted line), 35 °C (short dashed line) and 45 °C (thin line). (C) Emission spectra of MC540 added to DPPC vesicles in the $L_{\beta'}$ phase at 7 °C, using different excitation wavelengths: 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line) and 570 nm (dash-dotted line). The emission spectrum of DPPC in L_α phase at 45 °C upon excitation 540 nm is presented with thin line. The spectra are normalized to their emission maxima.

we used 0.2 μM MC540, i.e. the highest concentration of MC540 at which $F_{566/536}$ is still 2.21. When the excitation spectrum of MC540 in the L_α phase (DPPC at 45 °C) is subtracted from the excitation spectrum of MC540 in the presence of thylakoid membranes, the resulting difference spectrum (Fig. 5A) resembles but is not identical to the one of MC540 in buffer (Fig. 2A). In addition, the shape of the emission spectrum depends on the wavelength of excitation (Fig. 5B).

With the increase of temperature, significant changes are observed both in the intensity and the shape of the excitation spectrum (Fig. 6A). The relative intensity of the band at 566 nm decreases significantly and at 45 °C $F_{566/536}$ equals 1.27±0.12. The intensity of the emission spectrum of MC540 is decreasing with the increase of temperature from 7 °C to 45 °C, but no major spectral change is observed (Fig. 6B, C).

The thylakoid membrane is enriched in proteins and hence it cannot be ruled out that MC540 is not only incorporated in the bulk lipid phase but also in the solvation shell of the proteins. In order to study this in more detail, we have checked whether there is excitation energy transfer between MC540 and the Chl molecules in the pigment–protein complexes of the thylakoids. Emission spectra were recorded both in the absence and presence of MC540 (selective excitation at 570 nm). It was established (data not shown) that there is absolutely no change/increase in the intensity of Chl *a* fluorescence (684 nm), upon excitation in the MC540 absorption region, demonstrating that no detectable energy transfer is taking place and the fluorescence lifetimes of MC540 are not governed by excitation energy transfer but by their direct environment, which is either the water or the lipid phase. However, it should be noted that the possibility of association of part of the MC540 molecules to proteins which do not contain pigments cannot be ruled out.

3.2. Time-resolved fluorescence

3.2.1. MC540 in aqueous environment

Time-resolved fluorescence measurements revealed a mono-exponential decay upon exciting at 540 nm with a lifetime

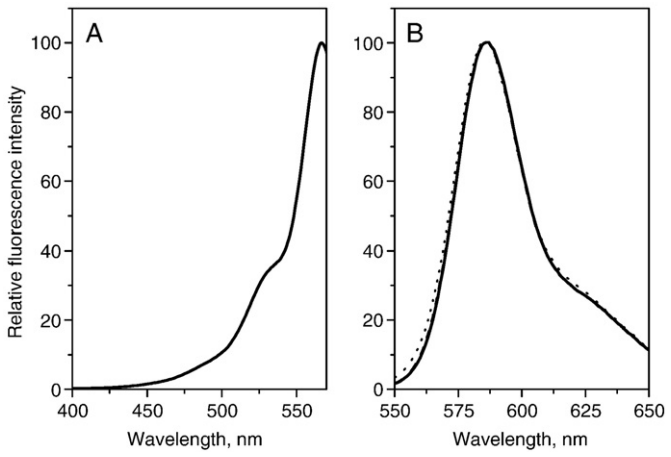

Fig. 4. (A) Excitation spectra of MC540 added to dioleoyl phosphatidylethanolamine (DOPE) adopting the inverted hexagonal phase (H_{II}, [70]), at 7 °C. The emission was collected at 580 nm. (B) Emission spectra of MC540 added to DOPE adopting the H_{II} phase at 7 °C. The excitation wavelengths are: 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line), 570 nm (thin line). The spectra are normalized to their emission maxima.

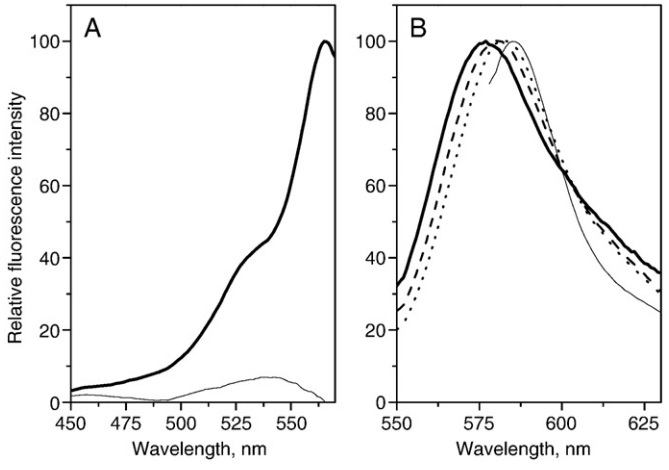

Fig. 5. (A) Excitation spectra of MC540 in thylakoid membranes. The spectra were recorded at 7 °C and the emission was collected at 580 nm. The difference between the spectra of MC540 added to thylakoids and to dipalmitoyl phosphatidylcholine (DPPC) in the liquid crystalline phase (L_α) at 45 °C, collected at 580 nm, is represented by the thin line. (B) Emission spectra of MC540 added to thylakoid membranes at 7 °C. The excitation wavelengths are – 480 nm (thick line), 517 nm (dashed line), 540 nm (dotted line) and 570 nm (thin line). The spectra are normalized to their emission maxima.

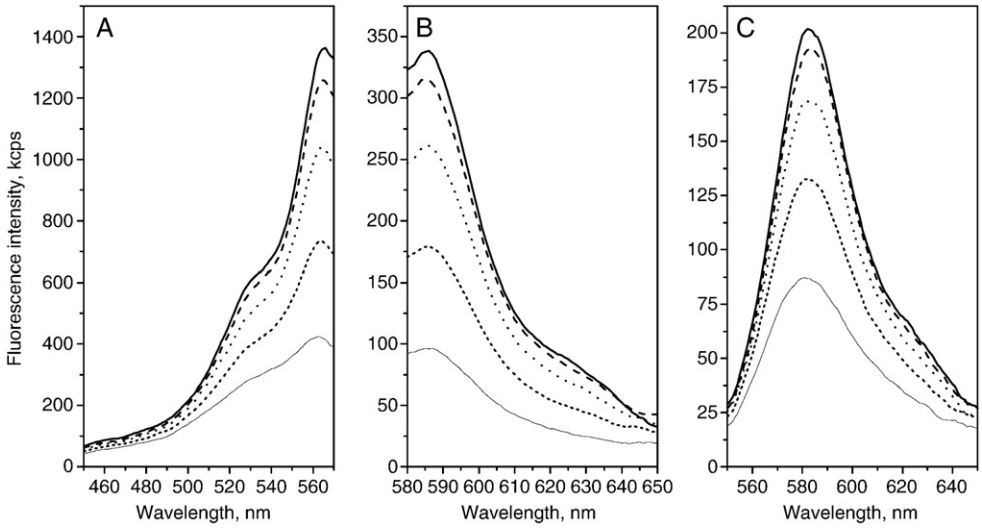

Fig. 6. Temperature dependence of the excitation (A) and emission (B, C) spectra of MC540 added to thylakoid membranes: 7 °C (thick line), 14 °C (dashed line), 25 °C (dotted line), 35 °C (short dashed line) and 45 °C (thin line). The emission was collected at 580 nm. The emission spectra were recorded upon 570 nm (B) and 540 nm (C) excitation.

(somewhat) dependent on the temperature (Fig. 7). At 25 °C it is 110 ps, equal to the one obtained by Mandal et al. [71]. Identical lifetimes were found upon excitation at 570 nm, although a small fraction of an additional longer-lived fluorescence (~1 ns) was also present (data not shown), which is probably due to a small amount of aggregated dye molecules.

3.2.2. MC540 in DPPC

Fitting of the time-resolved fluorescence decay curves revealed three decay times in most cases, and the relative amplitudes were different for 540 nm and 570 nm excitation. The lifetimes depended strongly on the lipid phase – at temperatures below 33 °C, where the L_{β}' phase is adopted, they were about 2 ns, 0.8 ns and 150 ps (Fig. 8A). Under these conditions the relative contribution of the shortest component (about 150 ps) was particularly pronounced upon 540 nm excitation (Fig. 8B) and it could be ascribed to MC540 in the aqueous phase. The two long components (2 ns and 0.8 ns) are ascribed to MC540 associated with the lipids since their relative contributions are enhanced significantly upon 570 nm excitation (Fig. 8B). The relative amplitudes of these two components were equal in the L_{β}' phase and upon the transition to P_{β}' and L_{α} phases the relative amplitude of the 2 ns became significantly higher. The phase transition from L_{β}' to P_{β}' also resulted in a significant decrease of the relative amplitude of the 150 ps component and its disappearance in the L_{α} phase, demonstrating that no MC540 remains in the water phase. Apparently the dye molecules were easily dissolved in the hydrophobic lipid environment in both the P_{β}' and L_{α} phases. In order to check whether the relative amplitudes of the two slow components are dependent on the excitation wavelength, we compared the normalized relative amplitudes (Fig. 8C). For the L_{β}' phase (7–25 °C) the relative amplitude of the 0.8 ns component is somewhat increased upon 540 nm excitation, as compared to 570 nm excitation, respectively. For the P_{β}' and L_{α} phases no change in the relative amplitudes of the 0.8 and 2 ns components was observed upon variation of the excitation wavelength.

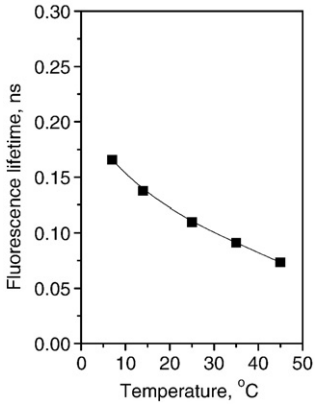

Fig. 7. Fluorescence lifetime (τ) obtained after analysis of the fluorescence decay traces of MC540 in the aqueous phase (resuspension buffer, see Materials and methods) at different temperatures. For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods. The fluorescence decay curves were recorded upon excitation at 540 nm. The samples were thermostated for 10 min at each temperature.

3.2.3. MC540 in DOPE

The fluorescence decay curves of MC540 added to DOPE at different temperatures were fitted with 3 components. The 200 ps component, indicative of MC540 in the water phase, was clearly resolved upon excitation at 540 nm (amplitude, 20%) but was absent upon 570 nm excitation (Fig. 9). Again, the longest component (about 2 ns) had by far the largest amplitude as was observed for the L_{α} phase for DPPC at 45 °C, and normalization of the relative amplitudes of the two long components revealed no dependence on the excitation wavelength (Fig. 9C).

3.2.4. MC540 in thylakoid membranes

Three lifetime components were found in the time-resolved measurements – at 7 °C they are approximately 2.0 ns, 1.0 ns and 170 ps (Fig. 10A), very similar to the values determined in DOPE and DPPC at this temperature. The 170 ps component had a very high relative contribution, about 50% even upon 570 nm excitation and upon 540 nm excitation it reaches a value of about 85% (Fig. 10B). Moreover, the relative amplitude of the 1 ns component was comparable to the one of the 2 ns component (Fig. 10C).

Upon increasing the temperature the relative contribution of the 170 ps component increased substantially and its relative amplitude became larger than 70% even upon 570 nm excitation (Fig. 10B), indicating the extrusion of the dye molecules from the thylakoid membrane. The relative amplitude of the 1 ns component was somewhat enhanced upon 540 nm excitation (Fig. 10C). The amplitudes of the two long-lived components showed a clear temperature dependence – the relative amplitude of the 2 ns

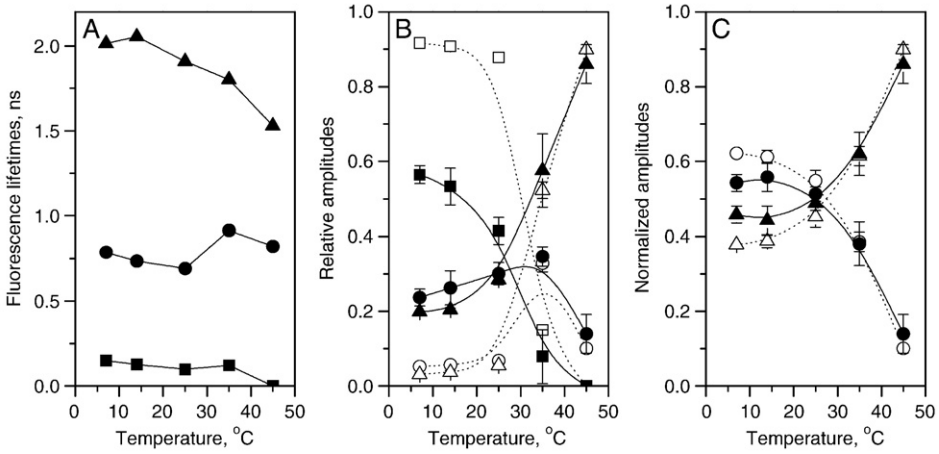

Fig. 8. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to dipalmitoyl phosphatidylcholine (DPPC) adopting different phases at different temperatures (gel phase ($L_{\beta'}$) below 33 °C, "rippled" gel phase ($P_{\beta'}$) at 33 °C and liquid crystalline (L_{α}) above 41.5 °C, [68]): (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, △) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols) excitations). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

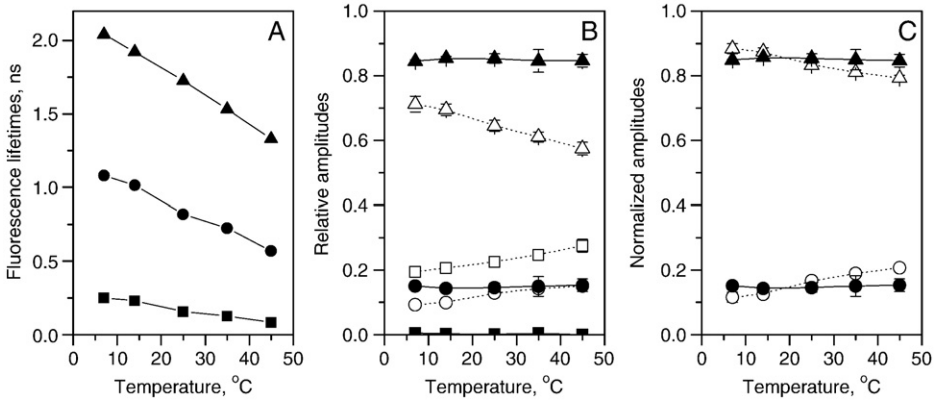

Fig. 9. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to dioleoyl phosphatidylethanolamine (DOPE) adopting inverted hexagonal phase (H_{II}) at different temperatures [70]: (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, △) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols) excitations). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

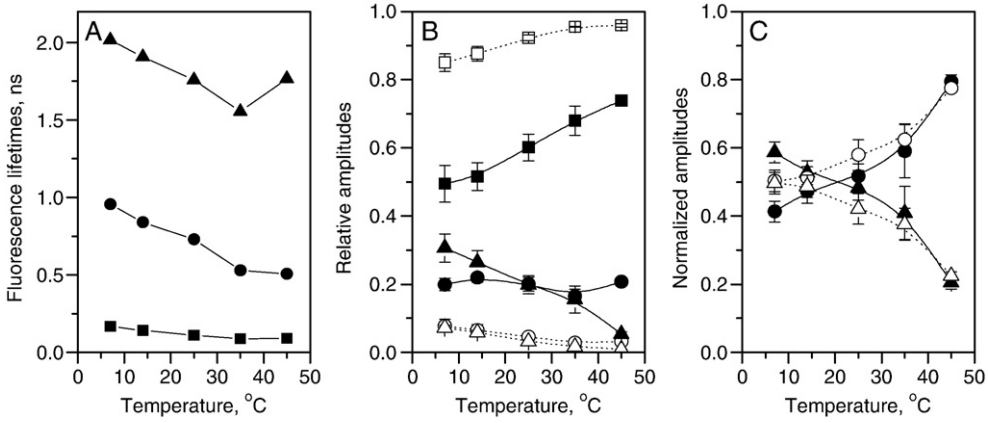

Fig. 10. Parameters obtained from the analysis of the fluorescence decay traces of MC540 added to suspension of thylakoid membranes: (A) fluorescence lifetimes (For the global fitting procedure the lifetimes for the different measurements at certain temperature were linked, thus resulting in only a single value, and the respective amplitudes were free; see also Materials and methods); (B) their relative amplitudes upon 570 nm (full symbols) and 540 nm (open symbols) excitation and (C) normalized amplitudes of the two long components (2 ns (▲, △) and 1 ns (●, ○) resolved upon 540 nm (open symbols) and 570 nm (full symbols)). The error bars represent the standard error. Solid (for 570 nm excitation) and dashed (for 540 nm excitation) lines serve as a guide to the eye to follow the changes in the amplitudes of the fast, intermediate and slow components as a function of temperature. The samples were thermostated for 10 min at each temperature.

component decreased with the increase of temperature, most prominently above 25 °C.

4. Discussion

In this work, in order to obtain information on the lipid packing in thylakoid membranes, we have performed time-resolved and steady-state spectroscopy measurements on thylakoids and model membranes. This combination of fluorescence measurements, which to our knowledge is applied for the first time in this systematic manner, has revealed the existence of different microenvironments in different model systems. These data, in turn, can help us to assess the lipidic microenvironments in the thylakoid membranes. These types of measurements may thus complement the information for the lipid phase of the thylakoid membranes at physiological temperatures, i.e. before the break-down of the membrane impermeability and the segregation of the lipids from the membrane, and the denaturation of proteins. It is believed that the bulk lipid mixture in thylakoid membranes exists in liquid crystalline phase [29] and, although marked temperature dependences were reported (see Introduction), phase transitions were detected only above 45 °C [30,31]. Also labeling of thylakoid membranes with the fluorescent probe DPH [72] and the EPR probe TEMPO [40] showed a monotonous increase in the fluidity of the membrane upon increasing the temperature from 10 °C to 45 °C. In contrast, our previous work pointed towards a more complex behavior and organization/packing of the lipid molecules; ^{31}P-NMR measurements showed a change in the bulk phospholipid behavior between 7 °C and 20 °C and revealed the presence of small non-bilayer structures in thylakoids co-existing with the lipid bilayer at all temperatures [43]. Here we show that steady-state and time-resolved MC540 measurements also reveal different microenvironments for the dye in model lipid systems and in intact thylakoids in the physiological range of temperatures; these will be discussed below.

4.1. MC540 in different lipid systems

The fluorescence spectra of MC540 have already been shown to be sensitive to the lipid packing and phase behavior of different model lipid systems [46,47,49,50]. In short, it was established that the fluorescence yield is substantially increased upon relocation of MC540 from aqueous to lipidic environment and there is a red-shift in the absorption and fluorescence spectra [44,58,73]; moreover the transition from gel to liquid crystalline and from liquid crystalline to inverted hexagonal phases were also correlated with higher fluorescence yields [47,50]. Here we further extend this knowledge by exploring the excitation wavelength dependence of the spectral shapes of MC540 in different environments.

In buffer solution the excitation spectrum is very similar to the absorption spectrum of MC540 in water [49,58,73–75] and does not change substantially upon detection at different wavelengths (Fig. 2A). Correspondingly the emission spectrum also changes only slightly with the variation of the excitation wavelength (Fig. 2B), reflecting a rather homogeneous dye solution. It was reported that the spectral properties of MC540 depend on the presence of salts [76]. Under our experimental conditions no change in the spectral properties of MC540 was observed when solved in the buffer used for the preparation of the model lipid systems (20 mM Tricine, pH 7.5) or the one for the thylakoid membranes (20 mM Tricine, pH 7.5, 400 mM sorbitol, 5 mM $MgCl_2$, 5 mM KCl).

In contrast to MC540 free in the water phase, for MC540 in the L_{β}' phase a strong wavelength-dependence was observed – the emission maximum varies between 575 nm and 589 nm (Fig. 3C), revealing a large heterogeneity in the MC540 population, i.e. the existence of different microenvironments for the dye. Moreover, in accordance with previous studies [46,49] we also observe MC540 dimer formation in the L_{β}' phase of DPPC, reflected by a distinct fluorescent band at 620 nm (Fig. 3C). The transition to the P_{β}' and L_{α} phases leads to a pronounced change in the shape of both the excitation and emission spectra – the 566 nm band becomes the dominating one in the excitation spectra (Fig. 3A, B) and no 620 nm band is observed in the emission spectra (Fig. 3C). The same holds true for the H_{II} phase (Fig. 4). For the P_{β}', L_{α} and H_{II} phases no heterogeneity can be detected upon varying the excitation and detection wavelength, indicating that the spectra are largely dominated by one spectral form of MC540, which is attributed to MC540 monomers incorporated into the hydrophobic lipid environment [57,58]. The excitation spectra for P_{β}', L_{α} and H_{II} phases showed no evidence for the presence of MC540 molecules free in the buffer, as shown by the absence of a well discernible band at 500 nm; however those could be detected by time-resolved measurements (see below).

Furthermore, our work correlates the steady-state characteristics of MC540 with its time-resolved fluorescence properties and assigns the different fluorescence lifetime components to distinct micro-environments of the dye.

In the lipid systems studied here, MC540 exhibits three different fluorescence lifetime components, which in most cases decrease with the increase of the temperature, similarly to MC540 in buffer solution (Fig. 7) due to enhanced photoisomerization [67]. However, their relative amplitudes depend exclusively on the local environments of MC540 and on the physical state of the lipid system (Figs. 8B, 9B and 10B). The different lifetime components will briefly be discussed.

4.1.1. The short-lifetime component – MC540 in the aqueous phase

The short-lifetime component (113 ps–214 ps) is found with different relative amplitudes in the various lipid-containing systems (Figs. 8, 9 and 10) but it is by far the dominating component for MC540 in buffer (Fig. 7). In the aqueous buffer phase at 25 °C a value of 110 ps is observed, identical to the one determined by Mandal et al. for MC540 in water [71]. In the studied lipid systems its relative contribution was always higher upon 540 nm than upon 570 nm excitation (Figs. 8B, 9B and 10B). This is in accordance with the expectations (as judged by the absorption spectrum of MC540 in aqueous environment, where the absorption at 570 nm is far less than at 540 nm [49,58,73–75], see also Fig. 2), and the short lifetime component thus represents MC540 molecules which remain free in the aqueous phase. Therefore, it can also be used as an indicator of the accessibility of the lipid membranes for MC540. We have established that the L_{β}' phase is particularly inaccessible for the dye molecules (Fig. 8), presumably due to the tight packing of the lipids. In all other model lipid phases studied, the short component was not present or had small amplitude (Figs. 8 and 9).

4.1.2. The long-lifetime components – MC540 interacting with lipids

The 1 ns and 2 ns components are originating from MC540 in hydrophobic environments (in the lipid phase) and hence provide information about the lipid packing and phase behavior. These lifetimes might be assigned to two discrete populations of the molecules, reflecting two different microenvironments. It is also possible that there is a broad distribution of lifetimes due to incorporation of MC540 in a variety of environments with only small differences in their physical properties (dielectric constant, lipid packing).

4.1.3. Discrete vs. broad lifetime distributions

According to the discrete lifetime distribution model, the two components should be assigned to MC540 residing in different environments. The 1 and 2 ns lifetimes are somewhat similar to the ones obtained by Aramendia et al. [77] for dimyristoyl phosphatidylcholine vesicles in the L_{α} phase at 20 °C – 1.87 ns and 410 ps. It should be pointed out that these authors did not resolve the 200 ps component. Therefore, the 410 ps might be a weighted average of the values of 200 ps and 1 ns, which were resolved in the present study.

Similarly to Aramendia et al. [77] we assign the 2 ns component to MC540 incorporated deep in the bilayer with parallel orientation with respect to the lipid molecules and the 1 ns component to surface-associated MC540 oriented perpendicular to the membrane. This is also substantiated by the fact that red-shifted absorption and fluorescence originate from MC540 located in an environment with a lower dielectric constant [58]. Moreover, a higher relative amplitude is observed for the 1 ns component upon 540 nm excitation in the case of $L_{\beta'}$ and thylakoids. This is not detected for the $P_{\beta'}$, L_α and H_{II} phases, probably due to the pronounced dominance of the 2 ns component. Thus, it can be concluded that the red-most emission upon 570 nm excitation originates mainly from the 2 ns component (MC540 in hydrophobic lipid environment) and the blue shifted emission obtained after excitation at 540 nm from the 1 ns component (more hydrophilic environment), respectively.

As mentioned above the relative amplitudes of the two slow components are strongly dependent on the lipid phase – the 2 ns component is substantially more pronounced in the $P_{\beta'}$, L_α (Fig. 8) and H_{II} (Fig. 9) phases. This reveals that the majority of the MC540 molecules are incorporated completely between the lipid molecules. In the case of the $L_{\beta'}$ phase on the other hand, the 2 ns and 1 ns components have almost equal amplitudes (Fig. 8). In accordance with this, the fluorescence intensity ratio $F_{566/536}$ is significantly higher for the $P_{\beta'}$, L_α and H_{II} phases (2.81–2.94) than for the $L_{\beta'}$ phase (1.20–1.95), thus indicating that a large fraction of the MC540 molecules penetrates less well into the bilayer and remains closer to the more polar environment of the lipid headgroups.

In the case of a broad distribution of lifetimes, the weighted average lifetime of the two long components discussed above should be considered. The average lifetime estimated in this way is not dependent on the excitation wavelength (Fig. 11) and the obtained values are of the order of the ones obtained by Onganer et al. [67] by phase modulation fluorometry. It is longer for the H_{II} and L_α phases than for the $L_{\beta'}$ phase. Mandal et al. [71] have shown that the fluorescence lifetime and quantum yield are significantly lower in aqueous than in polar environment and this was attributed to the increased rate of photoizomerization in polar solvents. This is in line with our observations – for the $L_{\beta'}$ phase the majority of the MC540 molecules remains on the surface of the vesicles and are thus exposed to the aqueous phase or remain free in the buffer phase, allowing fast photoisomerization, causing a shortening of the average fluorescence lifetime (Fig. 11). On the other hand the MC540 molecules that are buried in the hydrophobic lipid environment of H_{II} and L_α phases exhibit longer average fluorescence lifetimes due to the suppressed non-radiative processes (Fig. 11).

4.2. MC540 in thylakoid membranes

4.2.1. Accessibility

The partitioning of MC540 in the thylakoid membrane at different temperatures can in principle be followed by the ratio of the 570 nm and 530 nm absorption peaks ($A_{570/540}$), as suggested by Bakalcheva et al. [78]. However, this approach could not be applied here – due to the low concentration of MC540 and its strong absorption overlap with Chl no good quality difference absorption spectra could be obtained. Instead, the fingerprint (566 nm band in the excitation spectra) for monomeric MC540, and the fluorescence intensity ratio $F_{566/536}$ was used in order to probe the microenvironments of MC540 when incorporated in the thylakoid membranes. A concentration of MC540 was used that results in the highest $F_{566/536}$ ratio (and thus the highest amount of MC540 monomers located in the thylakoids) without the formation of sizeable amounts of MC540 dimers. The incorporation of MC540 appeared to be far less pronounced in thylakoids than in the model L_α phase. For comparison $F_{566/536}$ for thylakoids at 7 °C is 2.21 ± 0.09, whereas for DPPC in the L_α phase it is 2.94 ± 0.04. The combined steady-state and time-resolved fluorescence spectroscopy measurements reveal that a relatively large fraction of the MC540 molecules remains close to or free in the aqueous phase and are not incorporated in the thylakoid membrane. This could not reliably be inferred from the steady-state spectra due to the low fluorescence quantum yield of the short-lived component (Figs. 5 and 10). The lifetime measurements, showed however that even upon 570 nm excitation the relative amplitude of the shortest lifetime component (100–200 ps), and thus the fraction of water-exposed MC540 molecules is very high – it is 50% upon 570 nm and 85% upon 540 nm excitation, respectively (Fig. 10B). The thylakoid membrane thus appear to be less accessible for MC540 than the L_α, $P_{\beta'}$ (Fig. 8) and H_{II} (Fig. 9) phases in model systems, while the amplitude of the short component is close to the one observed for the $L_{\beta'}$ phase (Fig. 8). This might either be due to the tight lipid packing in the thylakoid membrane or to the presence of large amount of proteins whose surface charges might prevent the incorporation of MC540 in their vicinity and thus decrease the lipid area available for MC540.

4.2.2. Heterogeneity

Variation of the excitation wavelength in the steady-state spectra of MC540 added to thylakoids reveal heterogeneity in its spectral properties (Fig. 5C) and thus the presence of more than one type of local environment for MC540. Moreover, the difference spectrum presented in Fig. 5A also indicates that a fraction of MC540 is not well incorporated in the membrane. It also shows that these MC540 molecules are located in a rather hydrophilic environment since the difference spectrum is blue-shifted in comparison with the spectrum of monomeric MC540 in a lipid environment. This, in principle, might be due to the presence of MC540 molecules free in the aqueous phase. However, they have a very short lifetime and thus do not contribute substantially to the steady-state spectra. Furthermore, as mentioned above the ratio F_{566}/F_{536} is significantly lower than the one observed for the L_α phase. These observations show that the bulk lipid phase behavior in thylakoids can not be described satisfactorily with the properties of the L_α phase and suggests that either the lipid packing is different from the one in the L_α phase or different lipid domains with distinct properties co-exist in the thylakoid. This is in agreement with our recent ^{31}P-NMR data, obtained on spinach thylakoid membranes, which revealed a substantial degree of heterogeneity in the lipid phases of the thylakoid membranes at temperatures below 45 °C [43].

4.2.3. Lipid packing

When using the discrete lifetime distribution model, it can be seen that up to 25 °C the relative amplitudes of the 1 ns and 2 ns

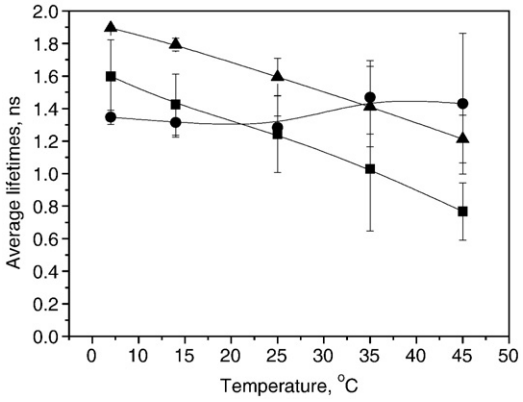

Fig. 11. Average lifetimes obtained from the weighted lifetimes of the two longest decay components (~2 ns and ~1 ns) for MC540 in dipalmitoyl phosphatidylcholine (DPPC, ●), dioleoyl phosphatidylethanolamine (DOPE, ▲) and thylakoids (■) upon 570 nm excitation at different temperatures. The error bars represent the standard error. Excitation at 540 nm resulted in essentially the same average lifetimes.

components are almost equal (Fig. 10C), resembling very much the situation for the L_{β}' phase (Fig. 8C). Above 25 °C, the 1 ns component becomes the dominant one (Fig. 10C). This was not observed for any of the model lipid phases studied, and it indicates the onset of a rearrangement of the bulk lipid matrix of the thylakoids – the MC540 which was buried inside the membrane becomes extruded from this hydrophobic environment and hence localized in an environment with higher water content. This conclusion is corroborated with the changes in the steady-state spectral properties between 25 °C and 45 °C – a strong reduction of the intensity of the 566 nm peak (hydrophobic environment), as compared to the 536 nm band (Fig. 6).

The temperature dependence of the average lifetime of the two long components (Fig. 11) does not correspond to the ones determined for model lipid systems in different phases. The nature of this effect is still unclear; the high protein content might influence the MC540 fluorescence lifetime to some extent. It is also worth mentioning that the temperature dependence of the average lifetime for thylakoid membranes is linear and thus does not reveal any major phase transition. However, it does reveal rather complex behavior of the bulk lipid matrix in the thylakoid membrane, since it differs from the mono-phasic model lipid systems. It has been shown that the decrease in $A_{570/540}$ is proportional to the increase of the dielectric constant [78]. In the present experiments the significant decrease in $F_{566/536}$ for thylakoids between 7 and 45 °C should be regarded as a decrease in the $A_{570/540}$, which thus indicates relocation of MC540 to an environment with higher dielectric constant. Taking into account also the strong reduction of the fluorescence intensity upon an increase in temperature (Fig. 6), it can be concluded that MC540 is extruded from the hydrophobic lipid membrane at high temperatures. The observed changes in the excitation spectra of MC540 in the suspension of thylakoid membranes with the increase of temperature strongly resemble the absorption changes observed by Mateasik et al. induced by increasing the negative surface charge of model lipid membranes [79]. Their data show that electrostatic repulsion prevents the penetration of MC540 into the lipid membrane. The surface charge of thylakoids is also negative and it has been shown to arise mainly from the exposed charged amino acids of the membrane proteins [80,81]. Thus a rearrangement of the protein complexes and/or the lipid molecules upon the increase of temperature might lead to more surface-exposed charges and consequently lead to extrusion of MC540 from the membrane. This hypothesis is further supported by the data of Dobrikova et al. [82] showing that in the temperature range 20–30 °C changes in the permanent dipole moment (transversal charge asymmetry) and the electric polarizability (motion of charges in the diffuse electric double layer) of pea thylakoid membrane occur, which have also been shown to strongly depend on the supramolecular organization of LHCII [83].

5. Conclusion

The steady-state and time-resolved spectroscopic data shown here demonstrate that in a system as complex as the thylakoid membrane the lipophylic fluorescent probe MC540 is exposed to different microenvironments and thus the thylakoid membrane can not be described satisfactorily with the properties of simple, mono-phasic model lipid systems. This conclusion is in line with our earlier results, based on ^{31}P-NMR studies, indicating heterogeneity in the bulk lipid phases of thylakoid membranes [43]. The results presented here also reveal a strong temperature dependence of the spectral properties of MC540 in the thylakoid membranes. At temperatures between 25 and 45 °C rearrangements in the bulk lipid phase occur leading to relocation of MC540 into more hydrophilic environment and its extrusion from the membrane. The formation of inverted hexagonal lipid phase (H_{II}), i.e., the segregation of lipids from the membrane, and the degradation of the pigment–protein complexes occur at higher temperatures, at about 45 °C [30,31,40] and 65–70 °C [84], respectively, thus the changes detected by MC540 can not be correlated with the above mentioned events. Most probably they are due to internal protein rearrangements or changes in the lipid–protein interactions in the otherwise intact and functional membrane. For example they might be associated with the destacking of the membranes at about 30 °C, observed by negative staining electron microscopy (data not shown) and consequent changes in the macroorganization of the thylakoid membranes.

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