Chlorophyll-Lipid-Interactions in Monomolecular Layers

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Z. Naturforsch. **38 c,** 1003 – 1010 (1983); received June 8, 1983

Chlorophyll, Phase Separations, Fluorescence Spectroscopy, Monolayers, Chlorophyll-Lipid Interaction

Mixed monolayers containing chlorophyll *a* and dimyristoyllecithin (DML) have been investigated by simultaneous thermodynamic, fluorescence and absorption spectroscopic measurements. It has been shown that the solubility limit of chlorophyll *a* in the fluid, as well as in the solid lipid matrix, is above 20 mol%. This contrasts to previous findings for pheophytin *a* containing monolayers and indicates the existance of an interaction between the phospholipid head group and the central Mg atom.

A fluorescence decrease on solidifying the monolayer was observed and it is suggested that it is due to an enhanced self quenching as a consequence of the reorientation of the porphyrin rings, accompanying the phase transition.

I. Introduction

Lipid monolayers are well-suited for use as models of biological membranes, since they allow the investigation of molecular interactions in a defined and related organization and in a unique and controlled environment [1, 2]. One biological membrane where nature and magnitude of the intermolecular interactions are still unknown, but where these interactions are known to be of great importance for the overall organization, is the thylakoid membrane of the photosynthetic unit [3 – 5]. This organization depends a great deal on the interaction of the light harvestig chlorophyll molecules with other chlorophylls, lipids and proteins.

In an effort to study these interactions we prepared and studied model systems composed of chlorophylls and lipids in varying relative amounts [6 – 9]. These studies shall primarily yield information on the chlorophyll-lipid ineraction, but they may also be used to derive data on the interaction of chlorophyll with lipoproteins [10]. The techniques applied are absorption and fluorescence spectroscopy of monolayers spread at the air (argon)-water interface accompanied by simultaneously measuring the surface pressure versus area diagrams. Whereas the latter measurements essentially probe the phase and the molecular density of the monolayer, optical measurements are sensitive with respect to the interaction of the porphyrin rings with their environment.

By absorption spectroscopy on pure chlorophyll monolayers we could prove that at surface pressures above 5 mN/m and at temperatures below 4 °C a crystalline chlorophyll state is formed exhibiting a dimeric chlorophyll arrangement [11]. Fluorescence and thermodynamic measurements of mixed monolayers revealed that pheophytin *a* is soluble in a monolayer of dimyristoyllecithin (DML) up to a limit of 15 mol%. Thus at higher pheophytin content a phase separation within the monolayer exists [6].

The present study presents an extension of the previous one [6] to mixed monolayers of DML and chlorophyll *a*. If focuses on the question of a phase separation in solid as well as in liquid monolayers and gains its special value from a comparison of results obtained with pheophytin *a* and chlorophyll *a* containing systems. As the above molecules differ in the respective absence and presence of the central Mg atom, the influence of this atom can be discussed.

II. Experimental set up and materials

The special value of the experiments described here results from the fact that optical and thermodynamic experiments are performed simultaneously. For this purpose a special film balance was con-

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0341-0382/83/1100-1003 $ 01.30/0

structed [12]. It exists of a rectangular teflon trough (dimension $55 \times 300\ mm^2$) that was incorporated into the sample chamber of the Cary 219 absorption spectrometer. By a mirror system the probing light beam was reflected to pass several times through the surface before entering the detector compartment of the spectrometer. The mirror system can be adjusted to vary the number of passes through the monolayer between 2 and 12 and to vary the angle of incidence of the light beam with respect to the surface normal between 20° and 45°. This variability is of importance especially when performing polarized experiments but for the experiments reported herein it proved optimum to keep the mirrors fixed to allow 6 passes and an angle of incidence of 33°.

Changes in the length of the light pass resulting in a reduced light level at the detector and the wavelength dependence of the baseline can be sufficiently compensated by the electronics of the instrument. The accuracy in measuring the optical density is 5×10^{-4}/monolayer. This means that mixed monolayers with a chlorophyll content above 10 mol% can be investigated.

When performing fluorescence experiments the excitation light is arranged so that it reaches the surface vertically through a quartz window in the bottom of the trough. The light beam from a 450 W Xe-lamp traverses a suitable filtering system (the water bath and a Scott SKF 7 to excite chlorophyll *a* into the soret band) and is then focused onto the water surface. Detection is achieved from above the surface at an angle of 30° with respect to the surface normal. Fluorescence emission is collected through a combination of lenses in a tube and focused onto the entrance slit of monochromator (Jobin Yvon H20). To reject stray light from the excitation, the monochromator is supported by a cut-off filter (Schott RG610).

Light passing the monochromator is detected by a red-sensitive, cooled photomultiplier (RCA C 31034) operated in the photon counting mode. The sensitivity of the equipment was sufficient to investigate emission spectra of monolayers containing chlorophyll in a molar ratio as low as 0.1 mol%.

During the measurements the pressure could be varied or kept constant and measured to an accuracy of 0.2 mN/m.

Special care was taken to keep chlorophyll stable during the measurements. Therefore the trough was placed in an argon atmosphere. The water was deoxygenated before use by rinsing with nitrogen and the light level in the environment was reduced as much as possible. In addition the intensity of the excitation light had to be reduced during the fluorescence measurements. The adequacy of these measures was proven by optical measurements: (i) The intensity ratio of the blue (440 nm) and the red (678 nm) absorption band of the chlorophyll monolayer in the expanded state amounts to 1.18 demonstrating the high purity of the material [13]. This value as well as the absolute intensities changed by less than 10% during a time of 3 h [12]. (ii) Fluorescence measurements were performed over times of about 30 min. During such a period the fluorescence intensity decreases by less than 5% and the shapes of the spectra do not change at all. Without protective measures the fluorescence intensity would have decreased by more than a factor of two within 20 min [12].

With this experimental set up, measurements can be performed in different ways: (1) The molecular density of the monolayer may be changed continuously while measuring the pressure and the optical signal. Measurements of this type, where the optical signal is the fluorescence intensity at the maximum wavelength of chlorophyll emission (680 nm), are presented in Ch. III.

(2) The optical spectra are measured at constant pressure and molecular density. This type of operation proved to be especially important when measuring absorption spectra at different pressures and temperatures [11].

Chlorophyll *a* and DML were prepared as described previously [14]. The water used was demineralized and additionally filtered (Millipore QTM). The pH was maintained at 7.8 using a Borax buffer. The films were prepared by spreading a *n*-hexane solution containing the two components in the desired molecular ratio on the water surface at the measuring temperature. Measurements were started 10 min after spreading, *i.e.* after evaporation of the solvent.

III. Results

Figs. 1 through 3 show typical results of simultaneous measurements of fluorescence intensity and surface pressure as a function of molecular area. Fig. 1 gives data on a monolayer containing chloro-

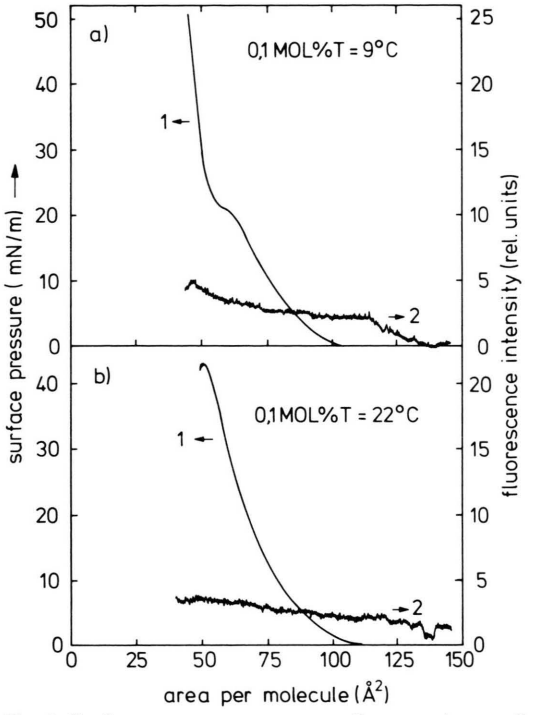

Fig. 1. Surface pressure versus area diagrams (curves 1) and fluorescence intensity versus area diagrams (curves 2) of DML monolayers containing 0.1 mol% chlorophyll *a*. Temperatures: 9 °C (Fig. 1 a) and 22 °C (Fig. 1 b).

phyll in a trace amount. The pressure *versus* area diagram at 9 °C shows an abrupt change in the slope at 20 mN/m corresponding to the so-called main transition of the lipid, a transition from a fluid to a solid state. This transition is not expected at a temperature of 22 °C, as is well-known for DML [15]. The fluorescence intensity *versus* area diagrams (curves 2) frequently show discontinuities at very low pressures and at surface areas above 125 Å^2/molecule. These are probably due to inhomogeneities of the monolayer at these low pressures. These inhomogeneities have recently been observed by fluorescence microscopy [16]. The increase in intensity on decreasing the area per molecule from 100 Å^2 to 50 Å^2 is due to an increase in density of fluorescing molecules. The main transition is, however, not observed in the fluorescence data for low chlorophyll content.

The latter observation does not hold if the chlorophyll content is increased, *e.g.* to 2.5 mol%, as is demonstrated in Fig. 2. Whereas the pressure *versus* area diagrams show a broadened main transition at low temperatures (9 °C), it is more pronounced in the fluorescence data: The fluorescence intensity decreases drastically on solidifying the lipid matrix. Such a behaviour has also been observed for pheophytin containing monolayers and for chlorophyll or pheophytin containing vesicles [6, 7, 17]. Then it was suggested that the abrupt intensity change is due to a phase separation within the monolayer or the vesicle. We will, however, suggest in Ch. IV that this does probably not hold for chlorophyll containing monolayers.

On further increasing the chlorophyll content the intensity change on solidification can even exceed a factor of 3. Thus for a chlorophyll content of 20 mol% the main transition of DML can be no longer observed from the pressure data. This is because it is broadened and this broadening may also explain why the changes in fluorescence intensity are not as abrupt as shown in Fig. 2. Comparing the fluorescence intensity *versus* area diagrams for the two temperatures (9 °C and 22 °C) it is remarkable that at higher temperatures a fluorescence decrease is also observed on increasing the pressure, although it is now less pronounced. At 22 °C, however, the main transition is not expected to occur. Thus the intensity change is not linked

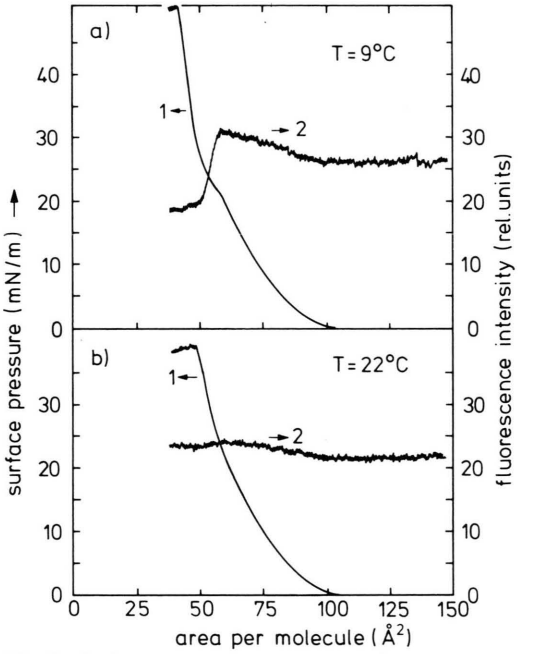

Fig. 2. Surface pressure versus area (curves 1) and fluorescence intensity versus area (curves 2) of DML monolayers containing 2.5 mol% of chlorophyll *a*. Temperatures: 9 °C (Fig. 2 a) and 22 °C (Fig. 2 b).

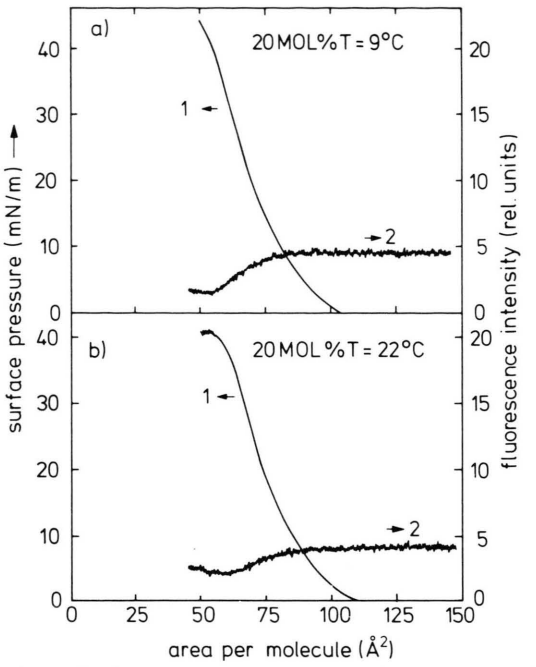

Fig. 3. Surface pressure *versus* area (curves 1) and fluorescence intensity *versus* area (curves 2) of DML monolayers containing 20 mol% of chlorophyll *a*. Temperatures: 9 °C (Fig. 3a) and 22 °C (Fig. 3b).

directly to the phase transition but to processes, supposedly reorientation of molecules, that also accompany the transition but that may also occur by simply compressing the film without changing its state.

The fluorescence intensity as a function of chlorophyll content is given in Fig. 4 for a low pressure (1 mN/m, Fig. 4a) and a high pressure (35 dynes/cm, Fig. 4b) where the monolayer is expected to be in a solid state. The qualitative shape of the curves is well-known and understood: The fluorescence increase with increasing chlorophyll content at low concentrations is simply due to an increasing surface concentration of fluorescing dyes, while the decrease at higher concentrations is due to an enhanced self-quenching of the chlorophylls. The information obtained from this curve will be discussed in Ch. IV.

Due to the strong self-quenching of the excitation the chlorophyll fluorescence could be observed only up to a chlorophyll content of 40 mol%. Within that limit, no changes were observed in the wavelength of maximum fluorescence or in the intensity with concentration (0.1–40 mol%), temperature (9 °C to 22 °C) or pressure (0–35 mN/m).

The surface pressure versus area diagrams measured at 9 °C for different concentrations are given in Fig. 5. For a chlorophyll content below 20 mol% one observes a change in the slope corresponding to the main transition of DML, whereas this is not observed at higher concentrations. The latter diagram shows that the pressure P_k at which the monolayer becomes unstable decreases with decreasing lipid content.

Similar behavior is also observed at higher temperatures and suggests that chlorophyll monolayers are stabilized by the presence of the lipid.

Information on phase separations and compositions of different phases is expected from an inspection of the partial volumes V_p which in this case correspond to the partial area A per molecule. V_p at constant pressure is given by the change dA of molecular area while changing the chlorophyll content by dX according to

$$V_p = dA/dX .$$

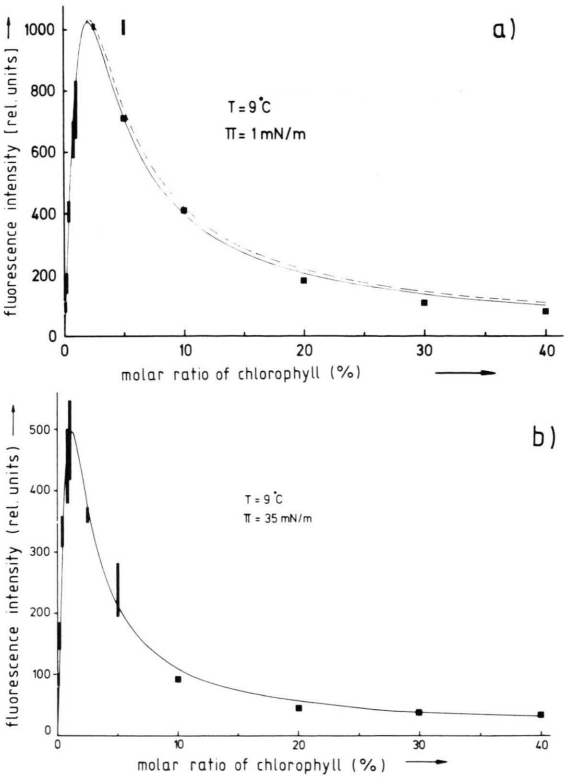

Fig. 4. Fluorescence intensity as a function of chlorophyll concentration X for a fluid monolayer ($T = 9$ °C, pressure 1 mN/m, Fig. 4a) and for a solid monolayer ($T = 9$ °C, pressure 35 mN/m, Fig. 4b). The curves are calculated using Eq. (1) (see Ch. IV) and fitting the quenching constant *a*.

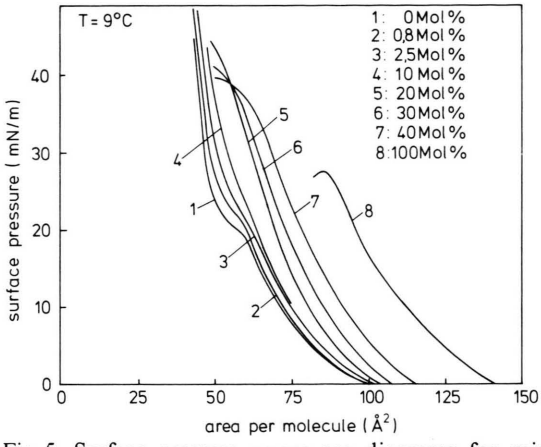

Fig. 5. Surface pressure *versus* are diagrams for mixed monolayers of chlorophyll *a* and DML in varying relative concentrations ($T = 9\,°C$).

One therefore obtaines V_p as the slope in the graph showing the molecular area as a function of chlorophyll content X. Two representative graphs are given in Fig. 6 for a temperature of 9 °C and pressures of 10 mN/m and 35 mN/m. A phase separation would be revealed from a discontinuity in V_p as a function of X. This is apparently not observable at low pressures where V_p is nearly constant. At high pressures, however, V_p changes considerably with concentration. Yet, it is hard to distinguish, whether at a concentration of 20 mol% V_p changes abruptly, as expected for a phase separation, or if a continuous change in the molar volume occurs.

The summary of the pressure data, Table I, shows for 2 temperatures and different pressures, the differences in molecular area of chlorophyll and lipid and the corresponding partial areas V_p. At high temperatures the partial areas and the differences in molecular areas agree and are apparently independent of pressure. For low temperatures, where V_p is not constant, a value is given in the last column that corresponds to V_p for chlorophyll concentrations below 20 mol%. It is observed that for pressures below 10 mN/m V_p and the differences in molecular area agree, as is the case at high temperatures. At high pressures, however, when the lipid is in the solid state, the values in the two last columns no longer agree. Apparently incorporation of chlorophyll effects an expansion of the lipid lattice. This expansion is only possible if chloro-

Table I. Comparison between the differences V in molecular areas of chlorophyll *a* and DML and the partial molecular area $V_P = dA/dX$ for 2 temperatures (9 °C, 22 °C) and different pressures for chlorophyll content of 20 mol%.

Surface pressure [mN/m]	Difference in molecular area [Å²]	Partial molecular area V_P [Å²]	Difference in molecular area [Å²]	Partial molecular area V_P [Å²]
	$T = 22\,°C$		$T = 9\,°C$	
1	33	35	41	43
10	34	34	41	41
30	36	36	38	70

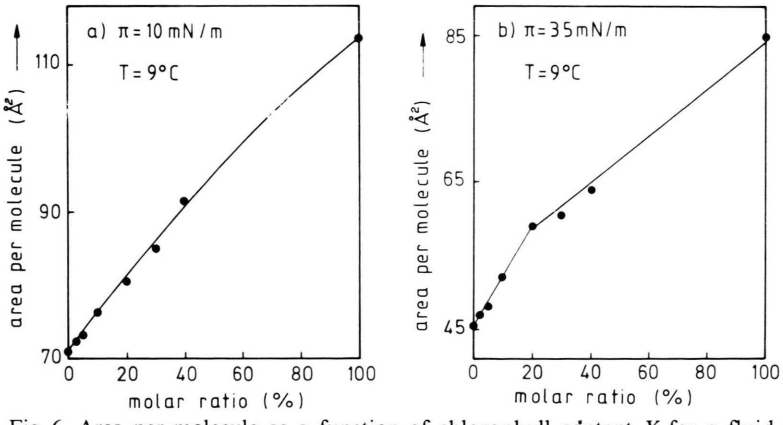

Fig. 6. Area per molecule as a function of chlorophyll content X for a fluid monolayer ($T = 9\,°C$, pressure 10 mN/m, Fig. 6a) and for a solid monolayer ($T = 9\,°C$, pressure 35 mN/m, Fig. 6b). The lines are only guides to the eye. In special, the abrupt change in the slope in Fig. 6b at a chlorophyll content of 20 mol% is not proved at all. As pure chlorophyll monolayers are not stable at a pressure of 35 dynes/cm the data point for $X = 100$ mol% had to be deduced from extrapolation of low pressure data.

phyll dissolves in the lipid matrix. Where it to segregate and form chlorophyll domains, the partial molar volume would correspond to the difference in molecular area.

IV. Discussion and Conclusions

The data presented above provides partial answers to the following questions:

(i) To what degree is chlorophyll a soluble in the phospholipid membrane?
(ii) Why is the lipid phase transition observable from fluorescence measurements?
(iii) Concerning the intermolecular interactions what are the differences between chlorophyll *a* and pheophytin *a*?

Ad (i): Discussing a phase separation between DML and chlorophyll *a* the two cases of a fluid and of a solid lipid monolayer have to be distinguished. The former is observed at high temperatures (*e.g.* 22 °C) at all pressures and at low temperatures and low pressures (*e.g.* 9 °C, 10 mN/m or 1 mN/m), whereas the solid phase exists only at low temperatures and high pressures (*e.g.* 9 °C, 30 mN/m). In a prior study we proved the existence of a phase separation between pheophytin *a* and DML in the solid as well as in the fluid state [6]. The strongest argument for this was the dependence of the fluorescence intensity I on pheophytin concentration.

For an ideally soluble system I can be described by a relation [4, 18, 19]

$$I = \frac{\text{const} * X}{1 + aX^2} \tag{1}$$

with the quenching constant $a = 1/X_{1/2}^2$, $X_{1/2}$ being the concentration where the fluorescence quantum yield drops to half of the value observed at low concentrations.

In case of a phase separation into pheophytin enriched and DML enriched domains the fluorescence results from contributions of both phases. As their relative portions depend linearly on pheophytin concentration this also holds for the total fluorescence intensity. This linear dependence of fluorescence intensity on pheophytin content was indeed observed for concentrations above 20 mol%, whereas at lower concentrations the data were well described by Eq. (1) [6]. From this also the solubility limit for pheophytin in DML enriched domains could be determined. It amounted to 15 ± 5 mol% in the fluid phase and was estimated to drop to about 2 mol% in the solid phase.

Analyzing the chlorophyll data in a similar way, an inspection of Fig. 4 reveals no linear dependence between fluorescence intensity and chlorophyll content at any temperature or pressure for chlorophyll concentrations above 2 mol%. Instead all data can be fitted by Eq. (1), indicating a chlorophyll solubility up to at least about 30 mol% in the solid as well as in the fluid lipid phase.

In accordance with this, the measurement of the partial areas V_p does not support a phase separation between chlorophyll *a* and the fluid DML phase. The result, however, that the partial area V_p does not change with chlorophyll concentration is expected for an ideally miscible as well as a phase separated system. Those data obtained for the solid DML phase are therefore more conclusive (Fig. 6b): Although the change in V_p at 20 mol% chlorophyll content cannot be proved to be discontinuous, and thus establish a phase separation, the molecular areas determined for chlorophyll concentrations $\geqq 20$ mol% reveal two facts:

(1) Incorporation of chlorophyll into the solid DML matrix yields a considerable distortion of the lattice. The widening of the lattice is even larger than necessary to cope with the larger molecular area of chlorophyll compared to DML. In this respect chlorophyll differs from pheophytin, because incorporation of the latter molecule yields no increase in the lattice constant [6]. This indicates that the two molecules are embedded into the lipid matrix in a different way.

(2) At least up to concentrations of 20 mol% chlorophyll is soluble within the solid DML monolayer. We therefore assume an even better solubility for the fluid monolayer as in most cases the solubility increases on fluidizing a monolayer.

In conclusion, in contrast to the findings with pheophytin *a* containing DML monolayers our data give no evidence on a phase separation between chlorophyll *a* and DML. Instead a solubility limit well above 20 mol% in the fluid lipid is suggested and of at least 20 mol% in the solid lipid.

Ad (ii): Having demonstrated a rather high solubility limit of chlorophyll *a* in the fluid as well as in the solid lipid we have to explain the decrease of monolayer fluorescence on solidification (*c.f.* Figs. 2 and 3). In case of pheophytin containing monolayers

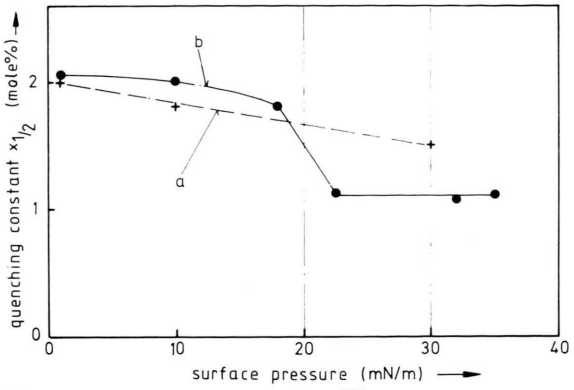

Fig. 7. Concentration $X_{1/2} = 1/\sqrt{a}$, corresponding to halving of the fluorescence quantum yield due to self quenching, as a function of surface pressure at 22 °C (curve a) and at 9 °C (curve b). The measurement points were obtained from a fit of Eq. (1) to the data as shown in Fig. 4.

this decrease was also observed and ascribed to a segregation of the dye into pheophytin domains that do not fluorescence due to enhanced self quenching. In contrast to this we suggest that for chlorophyll containing monolayers self quenching does not increase due to increased local chlorophyll concentration but due to a change in the quenching constant, represented by $X_{1/2}$. To quantify this, Fig. 7 gives $X_{1/2}$ as a function of surface pressure for a temperature of 22 °C, where the phase transition of DML does not exist, and of 9 °C where the phase transition occurs at pressures between 20 mN/m and 30 mN/m. One observes that, whereas at high temperatures $X_{1/2}$ only gradully decreases with increasing pressure the phase transition also causes an abrupt increase in the quenching constant $a = 1/X_{1/2}^2$ of about a factor of 3–4. This increase cannot be explained by the change in molecular diffusion as this decreases on solidification and would even cause a decrease in a. A more probable explanation resides in the fact that the phase transition also involves a reorientation of molecules. This is assumed to bring the planes of the porphyrin rings into closer contact by aligning the planes perpendicular to the surface and thus increasing the probability of forming (quenching) statistical pairs.

Ad (iii): As chlorophyll *a* differs from pheophytin *a* only by the presence of the central Mg atom a comparison of the intermolecular interactions of the two molecules is expected to shed light on the role of the central atom:

- The much better solubility of chlorophyll *a* in the lipid matrix compared to pheophytin can be ascribed to the interaction of the phospholipid head group with the Mg atom. This solubilization may arise from a binding of the phosphate group with the metal atom and may also occur in phosphate containing proteins, not only in lipids [10].
- Self quenching in chlorophyll containing monolayers is much more efficient than in pheophytin containing monolayers ($X_{1/2} = 1 - 2$ mol% for chlorophyll *a* compared to 6 mol% for pheophytin *a* in DML). This may be ascribed to a chlorophyll-chlorophyll-interaction causing the preformation of molecular pairs that finally yield those statistical pairs responsible for quenching. This interaction may involve the Mg atoms, the keto groups and water bridges.
- This latter interaction is also responsible for the formation of dimeric aggregates that were established by us [11] from absorption measurements on chlorophyll *a* films. A different type of aggregate with a red shifted absorption band exists in pheophytin a monolayers only in the collapsed state [20].
- Chlorophyll *a* monolayers are stable up to much larger pressures (25 mN/m) than pheophytin *a* monolayers (15–20 mN/m). This is also assumed to be due to an additional stabilization due to the Mg–H_2O interaction.
- The stronger interaction of the porphyrin rings of chlorophyll *a* compared to those of pheophytin *a* is also reflected in the position of the maximum of the red absorption band: in going from a hydrocarbon solution to the monolayer the pheophytin band shifts from 670 nm to 674 nm, whereas the chlorophyll band shifts from 663 nm to 678 nm.

In summary a considerable contribution of the Mg atom to the interactions of chlorophyll with other chlorophylls, with lipid and with water is observed in many of the features presented above.

Acknowledgements

Thanks are due to H.-J. Galla for the preparation and purification of chlorophyll *a* used in the experiments and to D. A. Cadenhead for critically reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft Mo 283/5, 7.

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