Biochimica et Biophysica Acta, 318 (1973) 4–9

BBA 76398

CHLOROPHYLL IN PHOSPHOLIPID VESICLES

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(Received February 14th, 1973)

SUMMARY

The fluorescence of chlorophyll *a* is a sensitive probe of the liquid–crystalline phase transition of dipalmitoylphosphatidylcholine. Transitions at about 34 and 41 °C are observable in vesicles prepared by ultrasonic dispersion. Similar behaviour is observed if these materials are dissolved in ethanol and water is added to the solution.

INTRODUCTION

The red absorption band and fluorescence parameters for chlorophyll *a* in lipid bilayers were measured in order to reevaluate R_0 in Förster's theory[1,2] of energy transfer between chlorophyll molecules, and to study the spectral changes due to chlorophyll aggregation. In the course of this work it was found that chlorophyll *a* is a sensitive probe of the liquid–crystalline phase transition of L-α-dipalmitoylphosphatidylcholine. It was further discovered that lipid vesicles, with or without chlorophyll could be produced by precipitation with water from ethanol solution. This new method gives vesicles with similar absorption characteristics to those obtained with the established method of ultrasonic dispersion of lipids in water. The present paper deals only with these aspects; aggregation and energy transfer will be discussed in a following paper.

Vesicles are often used as model systems for biological membranes, as reviewed by Bangham[3]; French[4], Goedheer[5], Ke[6] and others have written recent reviews on chlorophyll spectra in the visible region. The absorption and fluorescence of chlorophyll have been studied in solution, in the solid state, in monolayers, in colloidal suspensions, and recently also in lipid bilayers[7–9]. The aim of this work is to learn through observed spectral changes how chlorophyll interacts *in vivo* with lipids, proteins and other pigment molecules. Lipid bilayers (black films) represent a well defined and most useful model system for biological membranes; however, it is difficult to incorporate enough chlorophyll to obtain good spectra, or to study aggregation effects as they are likely to occur at the high concentrations present *in vivo*.

These problems do not exist with lipid vesicles, where it appears possible to incorporate a much larger concentration of chlorophyll and where in any case one

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may use a thicker sample. However, here the difficulty is to produce well defined vesicles surrounded by a single bilayer rather than onion-like structures[10,11], and then to show that the chlorophyll is in these bilayers. We shall show the latter by investigating the liquid–crystalline phase transition of dipalmitoylphosphatidylcholine vesicles by light scattering, chlorophyll absorption and fluorescence. The phase transition occurs in excess water at about 41 °C, as shown by calorimetric measurements[12], X-ray diffraction[13], NMR[14], as well as change in light scattering and 1-anilinonaphthalene 8-sulfonate fluorescence[11]. Above the phase transition the hydrocarbon chains are more mobile than below the phase transition[13], and the directions of the dipole moments due to the choline groups may change as well.

CHLOROPHYLL SPECTRA

To monitor the initial purity of the chlorophyll *a* and its subsequent fate during vesicle preparation, solution spectra were taken in ether and ethanol. The spectrum of chlorophyll varies from solvent to solvent[15] and worker to worker (Table I). The variations between different groups are outside the error of physical measurement and must be partly due to chemical differences of the chlorophyll,

TABLE I

SPECTRAL POSITIONS (nm) OF THE RED ABSORPTION BAND AND THE FLUORESCENCE OF CHLOROPHYLL *a*

From the older literature only the papers are quoted which give both fluorescence and absorption, further references may be found in the book by Rabinowitch[16].

Reference	*Ethanol*		*Ether*	
	Absorption	*Fluorescence*	*Absorption*	*Fluorescence*
Baas-Becking, Koning (1934)[16]	667	675	666	675
Biermacher (1939)[16]	663.5	666	663.0	663.5
Harris, Zscheile (1943)[16]	—	—	660.0	664.5
French (1960)[4]	—	—	662	668
Stensby, Rosenberg (1960)[17]	—	676	—	—
Seely, Jensen (1965)[15]	664.7	—	660.6	—
Goedheer (1966)[5]	—	—	662	669
Brody, Broyde (1966, 1968)[18,19]	664.0	682	—	—
This paper	664.2	672.4	660.1	667.2

such as allomerization or pheophytinization. The latter results in a 5-nm red shift of the fluorescence and the red absorption band while its vibrational satellite shifts to the blue[4]. Allomerization results in a blue shift of the red absorption band and the disappearance of the shoulder on the blue band[20]. In both cases the blue satellite (410 nm) is increased relative to the blue band (430 nm) and the red absorption band. Spectral analysis of our starting material (puriss., sealed tubes from Fluka AG, Switzerland) indicated the presence of 9% pheophytinized and allomerized chlorophyll. This increased to about 20% after vesicle preparation under oxygen exclusion, or prolonged storage in ethanol.

The absorption spectra were taken on a Beckmann Acta III dual beam spectrometer, the fluorescence spectra on a Perkin-Elmer Model MPF-3 fluorescence spectrophotometer. The fluorescence excitation light at 410 nm had a band width of 20 nm; and the spectral resolution of both instruments (4–8 nm) was well below the observed line widths (20–30 nm). The factory-supplied calibrations were checked with both deuterium and mercury lamps.

The red peak at 660.1 nm in ether and 664.2 nm in ethanol is in close agreement with accepted values (see Table I), and shows the difference of 4.1 nm between the two solvents identical to that observed by Seely and Jensen[15]. The rather large spread in the fluorescence peak positions may be partly due to selfabsorption[16] and variation of detector response with wavelength. The former would produce a red shift and the latter a blue shift. Another reason could be the presence of different chlorophyll isomers[4].

PHOSPHATIDYLCHOLINE VESICLES

Egg lecithin vesicles were prepared by dissolving egg lecithin and chlorophyll *a* (ratio 20:1) in benzene, followed by freeze drying; 0.1% lyophilized product was added to a buffer (pH 7.2) of 0.1 M NaCl in 0.01 M Tris–HCl. This buffer has no effect on the spectrum, yet slows chlorophyll deterioration as compared to distilled water. The suspension was sonicated with a 20-KC Branson Sonifier under a nitrogen atmosphere for 1 h under maximum power with the vessel immersed in an ice bath. Further sonication did not reduce the scattered light any further[21]. The solution was centrifuged at $100000 \times g$ for 1 h, which removed about 10% of the chlorophyll *a* and egg lecithin.

In the case of dipalmitoylphosphatidylcholine the powder (Fluka AG) was directly added to the buffer. The chlorophyll microcrystallites were crushed with a spatula against the container walls before sonication. Most of the chlorophyll was removed by centrifugation if the sonication was carried out with ice–water cooling. Allowing the suspension to warm to about 50 °C during sonication overcame this difficulty. According to Huang's work[10] on egg lecithin a large fraction of the vesicles should have a molecular weight of about $2 \cdot 10^6$ and consist of spheres of about 250 Å diameter, surrounded by a single bilayer. If the same procedure was followed with chlorophyll but without lipid, negligible fluorescence occurred.

DATA AND DISCUSSION

The absorption spectrum of chlorophyll *a* in dipalmitoylphosphatidylcholine vesicles is red shifted compared to the ethanol solution spectrum. This brings the peak positions closer to the obtained spectrum *in vivo*[4]. In addition there is an increased scatter, particularly at short wavelengths, which increases further for aged vesicle suspensions, presumably due to larger vesicle diameters resulting from vesicle fusion. The fluorescence intensity increases at the phase transition of the lipid, while the scattering background decreases (Figs 1 and 2). A secondary phase transition at about 34 °C may also be seen (Fig. 2); this was also observed recently by NMR and dilatometry[14]. The nature of these phase transitions is not clearly understood. The simplest suggestion is that the fluorescence decreases below the phase transition,

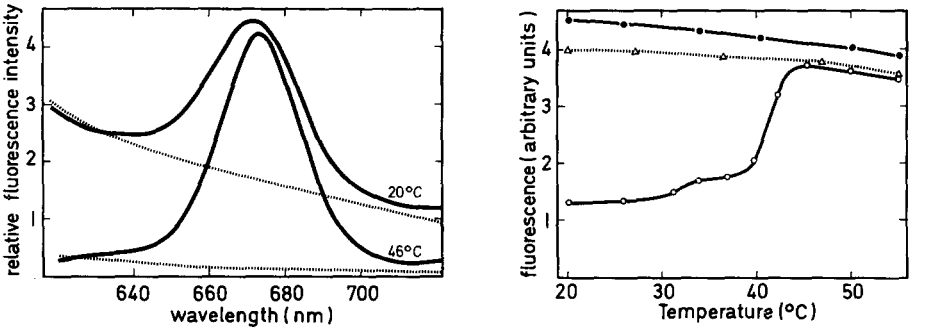

Fig. 1. Fluorescence spectrum of chlorophyll *a* ($\approx 10^{-6}$ M) in dipalmitoylphosphatidylcholine ($\approx 10^{-5}$ M) vesicles in water (———) and scatter due to vesicles without chlorophyll (· · · · · ·) at temperatures below and above the lipid phase transition (41 °C).

Fig. 2. Fluorescence peak intensity corrected for scattered light as a function of temperature in dipalmitoylphosphatidylcholine vesicles in water (○—○), after adding an equal quantity ethanol (●—●), and in egg lecithin vesicles in water (△· · ·△).

since the ordered saturated hydrocarbon chains cause chlorophyll aggregation, which in turn leads to concentration quenching of the fluorescence. This is in agreement with conclusions based on monolayer studies[6]. Two transitions may occur because the choline dipoles rearrange their alignments at a different temperature than the hydrocarbon chain melting (41 °C in excess water). The lipid to chlorophyll ratio was varied from 4 to 100 without any significant spectral changes being noticed. The phase transitions do not occur in egg lecithin vesicles, and they vanish in dipalmitoylphosphatidylcholine vesicles if an equal quantity of ethanol is added to the vesicle in water suspension (Fig. 2). Since the scattered-light phase transition does not vanish (Fig. 3) one must assume that the vesicles are not completely des-

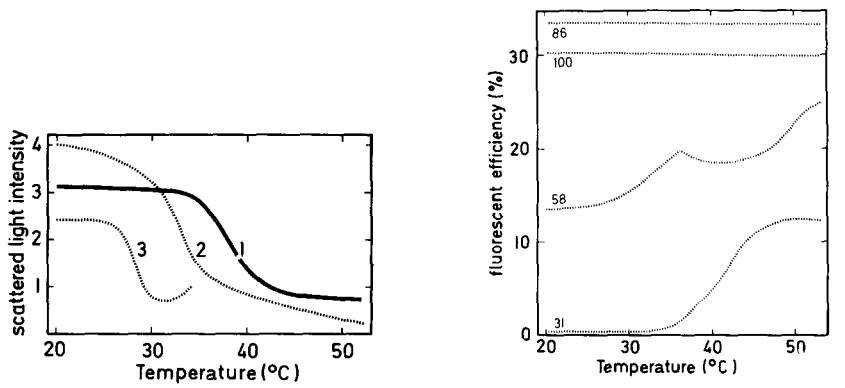

Fig. 3. Scattered light intensity (arbitrary units) at 600 nm as a function of temperature for dipalmitoylphosphatidylcholine vesicles in water, with ethanol added to an ethanol content of 0% (1), 50% (2) and 33% (3). In case 3 flocculation occurred at 34 °C.

Fig. 4. Fluorescence efficiency of chlorophyll *a* corrected for scattered light as a function of temperature in dipalmitoylphosphatidylcholine in various ethanol–water mixtures, with the water added later to obtain mixtures with 100, 86, 58 and 31% ethanol. The efficiency in pure ethanol was assumed to be 30%[22, 23].

troyed by the addition of ethanol; however, rapid fusion seems facilitated. The detailed results are dependent on the speed of ethanol–water mixing.

Two phase transitions are also observed (Fig. 4) if chlorophyll and dipalmitoylphosphatidylcholine are dissolved in ethanol, and the ethanol content is reduced below 60% by the addition of water. The transitions do not occur if egg lecithin is used instead or if no phospholipid is added to the chlorophyll in ethanol. The absorption spectra are also quite different when the lipid is omitted. In continuous scanning of the fluorescence at a rate of warming and cooling of 1 °C per min two phase transitions and hysteresis similar to that observed previously[11] may be seen. The hysteresis disappears if one scans at a lower rate (0.2 °C per min). One finds similar curves for vesicles prepared by sonication, and for lipid *plus* chlorophyll in ethanol with water added. However, there are also differences in detail, showing up in the fluorescence efficiency and extinction coefficient, as well as in the peak position and half-width.

CONCLUSION

Chlorophyll *a* represents a sensitive probe of the liquid–crystalline phase transition of dipalmitoylphosphatidylcholine. Conversely, much can still be learned about chlorophyll–chlorophyll interaction and possibly also chlorophyll–protein interactions which is relevant to photosynthesis, by studying these substances in lipid vesicles at the liquid–crystalline phase transitions. Dissolving the substances in a solvent which is miscible with water and then adding water appears to be a promising alternative to the preparation of vesicles by sonication. However, further work is still required to establish the exact characteristics of vesicles prepared by this means.

ACKNOWLEDGEMENTS

The author would like to thank Professor P. Läuger, Dr G. Pohl and Dr R. Koberstein for their hospitality and for helpful discussions. The award of an Alexander von Humboldt Fellowship is gratefully acknowledged.

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