Spectroscopic and Thermodynamic Studies of Chlorophyll Containing Monolayers and Vesicles

Part II: Chlorophyll a and Pheophytin a Aggregation on DMPC Vesicles

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Absorption and fluorescence experiments on pheophytin and chlorophyll containing lipid bilayer vesicles are reported. Pheophytin aggregates on the vesicles are established from an additional red shifted band (at 695 nm) in the absorption spectrum. These aggregates contain pheophytin in an arrangement with the molecular planes of the porphyrin rings being parallel and cover about 10% of the vesicle surface. The lipid phase dissolves pheophytin up to a molar ratio of 15% above the lipid phase transition. This solubility limit decreases hardly on solidification of the lipid.

For chlorophyll a containing vesicles the aggregates are not observed in the absorption spectrum. The chlorophyll solubility is about equal to that of pheophytin. This suggests that the phase separation indicated from fluorescence measurements at temperatures below the lipid phase transition does not lead to the formation of strongly bound chlorophyll aggregates.

Introduction

Spectroscopic measurements and calculations have provided evidence that the chlorophyll molecules of the thylakoid membranes are present in fairly large local concentrations of about 1 M [1]. Concentration quenching of the excitation energy is expected to waste the absorbed light energy. There is increasing evidence now that these molecules are distributed nonrandomly [2].

Aggregates of chlorophyll are observed in nonpolar solvents and a similarily between the absorption spectra of highly concentrated chlorophyll solutions and chlorophyll in the thylakoid membranes is found [3]. For that reason specifically organized aggregates of chlorophyll are suggested to be present in the photosynthetic membranes.

The "fluid-mosaic model" of biological membranes established by Singer and Nicolson [4] can also be applied to the chloroplast thylakoids. The photosynthetic membrane is viewed as a lipid bilayer in which the intrinsic proteins are embedded and to which the extrinsic proteins are attached [5]. The lipid bilayer consists of two types of lipids: protein fixed boundary lipid and the fluid mobile lipids which form the matrix of the lipid domains of the bilayer. In the photosynthetic membrane the chlorophylls comprise about 20% of the lipid mass. Since most of the thylakoid lipids contain high proportions of double bonds the photosynthetic membrane is thought to be in the fluid state. The most important lipids are the galacto lipids (about 40% of the total lipid mass) which form the fluid matrix of the membrane [5]. The sulfo lipids and the phospholipids are better suited to form the boundary lipid because they are more saturated. This boundary lipid could also include the chlorophyll molecules. Because of the high fluidity of the lipid matrix, aggregation of chlorophylls within the membrane could only occur if there is a specific chlorophyll-chlorophyll, chlorophyll-protein or chlorophyll-lipid interaction.

Thus further information is required on a specific lipid-chlorophyll interaction. Model systems containing lipids and chlorophylls might reveal phase separation and aggregation phenomena. Therefore we have studied monolayers [6] and bilayer lipid vesicles containing dimyristol-phosphatidyl choline (DMPC) and chlorophylls in fairly large relative concentrations. In part I of this series we established the existence of a phase separation between the lipid and pheophytin on monolayers [6]. Pheophytin has been chosen because of its better chemical stability. Now pheophytin domains are also found in bilayer vesicles from fluorescence quenching experiments [7].

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However, in similar experiments we could not observe aggregated forms of the chlorophylls. This is on the other hand essential for an understanding of the molecular arrangement within those domains containing chlorophyll in large concentrations. Up to now the detection of aggregates has also not been possible by ESR [8], optical [9, 10] and Raman measurements (J. Luisetti, unpublished results) on these model systems.

The direct observation of pheophytin aggregates on vesicles is reported for the first time in this work. These are detected from their characteristic absorption spectrum (maximum at 695 nm) that is red shifted by about 25 nm with respect to the monomeric form. We can thus provide information on the structure of these aggregates and give a more quantitative picture of the phase separation. In addition the results on pheophytin containing vesicles are compared with those on vesicles containing chlorophyll a.

Materials and Methods

Dimyristoyl phosphatidyl choline from Fluka was checked for purity by thin layer chromatography. It was used without further purification. Chlorophyll a and pheophytin a were prepared from spinach and were purified by column chromatography on powdered sugar columns by the method of Smith and Benitez [11]. Lipids plus chlorophyll a or pheophytin a dissolved in chloroform were mixed in a flask and evaporated to dryness under a nitrogen stream. Buffer solution (pH 8.0; 0.1 M) was added and the mixture was dispersed for 10 min by ultra sonication with a Branson sonifier type B12 at P = 30 W.

Fluorescence spectra were recorded with a Schoeffel RPS 1000 spectrometer equipped with a cooled red sensitive photomultiplier. The chlorophyll a and pheophytin a concentration was kept constant at 10^{-5} M. The chlorophyll to lipid ratio was changed by the lipid concentration. The temperature was controlled by a thermocouple.

Absorption spectra were recorded on a dual-beam spectrometer Cary 219. For measurements on different samples the lipid concentration was maintained constant at 3×10^{-4} mol/l. The experiments at room temperature were performed using a reference solution containing the pure lipid vesicles in the same concentration as in the probe cuvette that contained additional pheophytin in varying amount. The baseline drift due to stray light problems could thus be eliminated. For temperature dependent measurements reference and sample cuvette contained the same solution. The reference cuvette was maintained at a constant temperature of 48 °C, *i. e.* well above the transition temperature of 23 °C of the lipid; the temperature of the sample was varied. Thus very small differences in the absorption spectra with respect to $T = 48$ °C could be detected. The accuracy of the measurement of the optical density was better than 3×10^{-4}, the wavelength accuracy was ± 1 nm. Cuvettes with an optical path length of 4 mm were used.

Results and Discussion

1. Pheophytin containing vesicles

1.1. Absorption measurements at room temperature

Fig. 1 gives a comparison of the pheophytin absorption spectrum in a hexane solution with the spectrum of a vesicle containing pheophytin and lipid in a relative molar amount of $c = 1/5$. Whereas in the Soret band region (≈ 400 nm) the spectra are nearly identical, drastic differences are observed near 700 nm. In the vesicle spectrum there appears an additional band at 695 nm that we will later ascribe to pheophytin aggregates. It is this new band that we are going to study in detail.

Fig. 2 gives the absorption spectra in the red region between 600 nm and 850 nm for different pheophytin concentrations. At low pheophytin concentrations ($c < 10\%$) one observes only the main peak at 670 nm which is also observed in the hexane solution spectrum of Fig. 1 and is due to monomeric pheophytin. With increasing c, however, the red

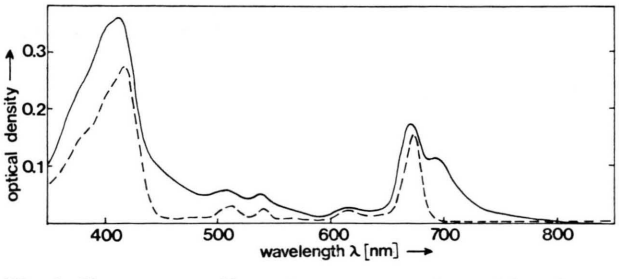

Fig. 1. Upper curve: Absorption spectrum of a vesicle solution containing 3×10^{-4} M DMPC and 1×10^{-4} M pheophytin a ($c = 0.33$). The dashed curve shows the pheophytin absorption spectrum in a hexane solution (10^{-6} M).

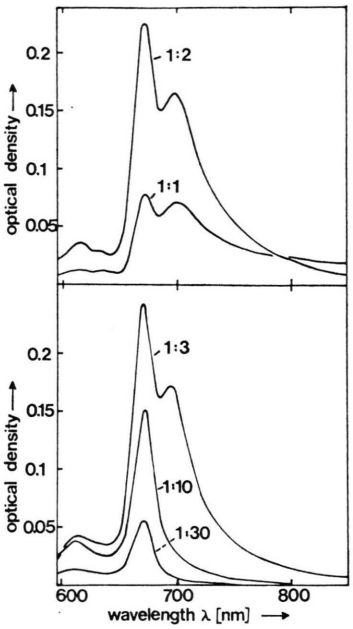

Fig. 2. Absorption spectra of pheophytin containing vesicles for different molar ratios c of pheophytin. The DMPC concentration was kept constant at 3×10^{-4} M.

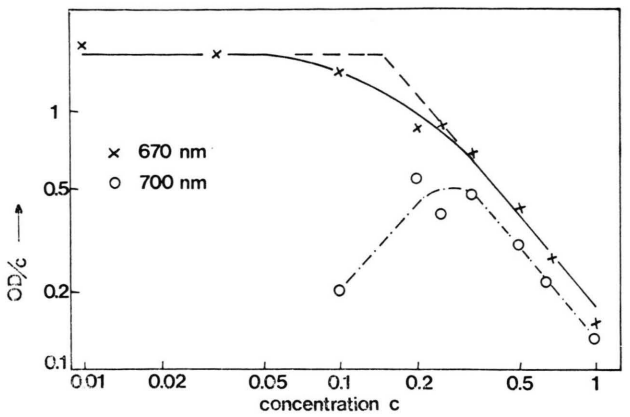

Fig. 3. "Normalized" optical density OD/c as a function of pheophytin concentration c for two different wavelengths. $c=1$ corresponds to a pheophytin concentration of 3×10^{-4} M.

peak at 695 nm appears and its intensity increases to nearly that at 670 nm. On further increasing the pheophytin concentration this red absorption intensity is not raised anymore. Instead the absorption spectrum exhibits additional red shifted components not distinguishable in detail. They are indicated from an increase in the optical density of the solution between 700 nm and 850 nm (*cf.* spectrum 1:1 in Fig. 2).

The optical densities at 670 nm and at 700 nm (OD(670), OD(700)) as a function of chlorophyll : lipid ratio c are given in Fig. 3. Since in the experiments the lipid concentrations was kept constant ordinate in Fig. 3 is given as OD/c. Thus a constant value of the ordinate indicates a proportionality between nominal pheophytin concentration and pheophytin molecules absorbing at the given wavelength. This condition is fulfilled very well for pheophytin concentrations below $c=5\%$ for the absorption peak at 670 nm. This peak is obviously due to pheophytin dissolved in the lipid phase of the vesicle. Hence it follows that at concentrations below $c=5\%$ pheophytin is soluble in the lipid phase. For $c>20\%$ on the other hand OD/c decreases nearly inversely proportional to c. This shows that the pheophytin concentration in the lipid phase has assumed a constant value, its solubility limit X_0. Excess pheophytin is precipitated within the pheophytin domains on the vesicle or into the waterphase where it does not contribute to the absorption at 670 nm.

This solubility limit X_0 is immediately determined from an extrapolation of the straight lines obtained for $c<5\%$ and $c>20\%$ in Fig. 3. This is justified, since ideally for $c\leqq X_0$ the measurements should obey the horizontal line obtained for concentrations where all pheophytin is dissolved in the lipid phase, and for $c\geqq X_0$ the optical density of the pheophytin containing DML domains (OD 670) should remain constant. Hence the straight line with the slope (-1) should be observed on a logarithmic scale. From the intercept one obtains $X_0=0.15\pm0.03$.

The additional peak near 700 nm appears for those concentrations where the normalized monomeric intensity OD/c (670) decreases with increasing concentration (*cf.* Fig. 3). We will show that this is due to pheophytin aggregates on the vesicle. These aggregates are present up to a well defined limiting concentration X_1 that we will determine.

The peak appearing at 700 nm obviously increases with pheophytin concentration. It may be due to a dimerization or due to the formation of larger pheophytin aggregates. The following arguments support the latter point of view:

1) The spectra of Fig. 3, if normalized for identical pheophytin concentration, do not show an isosbestic point. This should be observed for an equilibrium in the monomer-dimer reaction [12].

2) The wavelength of the absorption maximum agrees exactly with that reported by Ballschmiter and Katz [13] for the absorption of pheophytin films, where definitely aggregates are formed. From this one may derive that the red absorption peak of the pheophytin dimer is red shifted by considerably less than 25 nm with respect to the monomer [14].

3) The chlorophyll dimerization in the ground state needs the presence of the central magnesium atom which is lacking in pheophytin. Therefore a much smaller tendency for the dimerization of the latter molecule is observed arising mainly from π-π interactions [15].

4) The additional red shifted band appears at pheophytin concentrations for which the vesicle is supposed to be saturated with pheophytin.

The above facts indicated that pheophytin present in excess of its solubility limit is precipitated within the vesicle. But these domains of aggregated pheophytin are present only to some limiting extent, *i. e.* there exist no vesicles containing pheophytin in large excess. This can be derived from the curve OD/c (700) that assumes a maximum near $c = 0.30$ and decreases for $c > 0.30$ with increasing pheophytin concentration. From Fig. 3 one extrapolates that the total pheophytin concentration $X_0 + X_1$ on the vesicle amounts to 30%. Thus X_1 is about 15%. Assuming approximately equal area per molecule of DMPC and pheophytin one then calculates that $X_1/(1 + X_0 + X_1) = 12\%$ of the vesicle surface is covered with domains containing almost exclusively pheophytin.

Since we have shown that the DMPC parts of the vesicles contain a limiting fraction X_0 of monomeric pheophytin molecules and another comparable fraction X_1 of aggregated pheophytin molecules one may ask what happens if further pheophytin is added. It is segregated out into the water phase thereby forming microcrystals. These are likely to cause the increase in the optical density between 700 nm and 850 nm in the spectrum labelled 1:1 in Fig. 2.

Having observed the aggregated state in the absorption spectrum we are also able to comment on its structure. From a comparison of the absorption spectra of films of chlorophyll a and of pheophytin a Ballschmiter and Katz concluded that the larger π-π interaction in pheophytin a is responsible for the larger red shift of its aggregate absorption [13]. This is understood on the basis of the better planarity in the Mg free molecule. The molecular planes may then approach one another more closely. It also shows that both a coplanarity as well as a short distance of the porphyrin rings are necessary for the observation of the long wavelength peak in the absorption spectrum. We conclude that within the pheophytin aggregates on the vesicle the molecules are arranged in closely packed stacks with parallel planes of the porphyrin rings.

The solubility limit X_0 is also nearly identical with the value determined for the fluid lipid phase of DML monolayers at the air water interface [6]. For these systems $X_0 = 18\% \pm 5\%$ was determined from thermodynamic as well as from fluorescence measurements. This indicates that pheophytin is equally distributed over the inner and the outer monolayer of the vesicle. In this context it is remarkable that the pheophytin monolayer under high surface pressure (13.5 dyn/cm) shows the additional band near 700 nm in its absorption spectrum [19]. Obviously also the compressed monolayer contains pheophytin aggregates in an arrangement as described above.

The monolayer experiments presented in part I of this series also show that pure pheophytin domains are stabilized by their environment [16]. It is therefore conceivable that the bilayer lipid vesicle contains pheophytin domains, too. This means that pheophytin not solubilized in the lipid phase is at least not completely segregated into the water phase.

The phase separation described above was previously derived from measurements of the fluorescence intensity, the concentration dependence of which could not be described by the empirical law observed for a homogeneous solution [17]. But these data were less accurate with respect to a quantitative evaluation and furthermore they did not reveal directly the aggregated state.

1.2. Absorption spectra as a function of temperature

Fluorescence measurements suggested a decrease of the pheophytin solubility in the lipid on going from the liquid to the solid phase [7]. If this was true this might also be observed from the temperature dependence of the absorption spectra. Therefore we measured the absorption spectra for different temperatures between 7 °C and 50 °C, *i. e.* above and below the lipid phase transition temperature of $T_t = 23$ °C. However, we could not detect any drastic changes in the absorption spectra. In order

to look for more subtle changes which could reflect details of the phase transition we recorded difference spectra for different temperatures using as reference the same solution, but at 48 °C.

Fig. 4 gives a typical example for these measurements, taken for a solution with a pheophytin:lipid ratio of 1:3. We should stress that the changes observed in the spectra are of the order of 1% of the optical density of the sample. We realize that the aggregate band at 700 nm increases with decreasing temperature. Signals of Fig. 4 with a → at the short wave length side and at the long wavelegth side are due to a bathochromic shift of the band. This shift is obviously observed for the bands at 670 nm and at 410 nm. However, these shifts which are probably due to the pheophytin-lipid interaction do not change significantly near the transition temperature. Therefore their measurement yields neither a good probe to detect the phase transition nor does it give insight into changes in the molecular arrangement near the transition temperature.

The most remarkable result deduced from Fig. 4 is that the concentration of pheophytin aggregates hardly increases on lowering the temperature. This is in marked contrast to what was expect from measurements on monolayers and vesicles that suggested a pheophytin solubility of only a few per cent in the solid phase. Two of the possible reasons for this are given below:

1) On solidifying the lipid matrix excess pheophytin molecules are not segregated out into the aggregated regions. Instead pheophytin enriched regions are formed where the excitonic interactions is too small to account for a red shift of the absorption spectrum. But in these regions self quenching may be effective enough to cause the marked decrease in the fluorescence intensity on solidification of the DMPC phase [7].

2) The decrease in fluorescence intensity on lowering the temperature may be reinterpreted in a way that the lipid transition involves a change in the self-quenching efficiency, not in the local pheophytin concentration. This may be inferred by a change in the intermolecular distance and in the relative molecular arrangement during the phase transition.

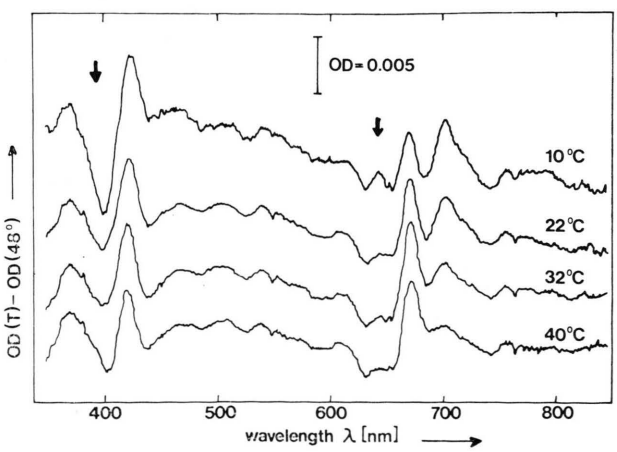

Fig. 4. Difference absorption spectrum measured as described in the experimental section (II). The vesicle solution in reference and sample cuvette contained 3×10^{-4} M DMPC and 6×10^{-5} M pheophytin a ($c=0.2$).

1.3. Fluorescence spectra

In order to investigate whether concentration and temperature dependent changes in the red absorption region are also observed in the fluorescence spectra, we recorded these as a function of the above parameters. A typical result is given in Fig. 5.

Curve (3) in Fig. 5 shows a typical fluorescence spectrum (maximum at 676 nm) of a vesicle containing pheophytin in a small concentration. The shape of this spectrum is independent of temperature. This is not the case for the spectrum taken at large pheophytin concentrations ($c=20\%$, curves (1) and (2) in Fig. 5). In addition to the maximum near 676 nm there also appears a shoulder near 705 nm in the spectrum. On lowering the temperature the shoulder decreases less strongly than the maximum which changes the shape of the spectrum. Since for this concentration the absorption peaks

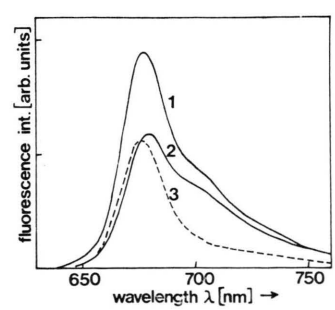

Fig. 5. Fluorescence spectrum of pheophytin containing vesicles.

1: $c=0.33$, $T=32$ °C;
2: $c=0.33$, $T=7$ °C;
3: $c=0.01$, $T=7$ °C.

at 670 nm and at 695 nm are about equally intense this shows that the fluorescence quantum yield of pheophytin in aggregates is smaller than of pheophytin monomers but it is not zero. This is in contrast to measurements on chlorophyll where the fluorescence of aggregates is not observed [18]. It is also conceivable that the lipid solidification affects only the quantum yield of pheophytin in the lipid phase, not the fluorescence of pheophytin aggregates. The strong intensity change near 676 nm therefore supports the assignment of this band to monomeric pheophytin within the lipid, and consistent with this is also the merely slight temperature dependence of the intensity at 705 nm (*cf.* curves (1) and (2) in Fig. 5). Thus the intensity as well as the shape of the fluorescence spectra present probes for the study of the pheophytin environment on a vesicle.

2. *Chlorophyll a containing vesicles*

In order to find out whether the above results can be transferred to chlorophyll containing vesicles we also investigated the latter systems. These experiments showed agreements, but also considerable differences in the behaviour of the two different chlorophylls and will be discussed below:

1) With increasing chlorophyll concentration there appears no additional red shifted band neither in the absorption nor in the fluorescence spectra. This shows that chlorophyll a aggregates in a structure like that observed for pheophytin are not present on the vesicle. This is conceivable since the aggregation behaviour of both molecules is entirely different [13, 15].

2) Within an accuracy of 2 nm the absorption maximum (668 nm) does not depend on concentration. This result differs from those obtained on Chl a/phosphatidyl choline liposomes where with increasing chlorophyll concentration a red shift of about 4 nm was observed [9] and shows that the chlorophyll-chlorophyll interactions in the latter model systems differ from those on lipid vesicles.

3) The solubility of chlorophyll a in the lipid phase may be determined from Fig. 6. It shows a plot of the optical density at 668 nm, analogous to the one of Fig. 3. Like in section III 1 a one obtains a solubility limit X_0 (Chl) = 20%. This is about the same value as that obtained for pheophytin.

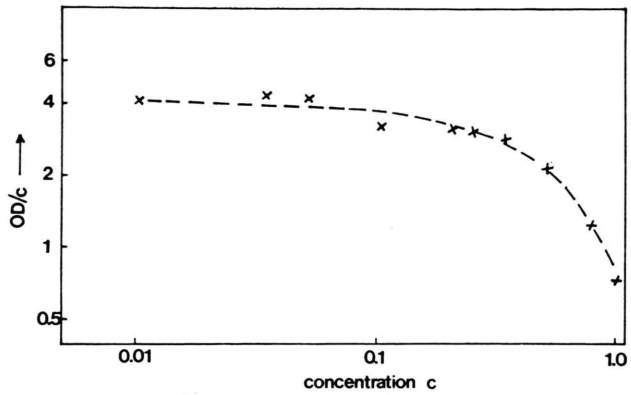

Fig. 6. "Normalized" optical density OD/c as a function of chlorophyll a concentration c at 670 nm. $c=1$ corresponds to a chlorophyll concentration of 3×10^{-4} M.

4) On reducing the temperature below the lipid transition temperature (from 40 °C to 7 °C) the absorption intensity decreases by only about 10% for chlorophyll concentrations $c > 3\%$ and no discernible red shift is observed. This shows that, like the pheophytin, also the chlorophyll solubility on the vesicle is not decreased drastically. This result is surprising since the fluorescence intensity of these vesicles decreases by about a factor of five indicating a phase separation [7]. This may be understood if the chlorophyll absorption spectra within the two phases are identical. It suggests that in the chlorophyll enriched domains the chlorophyll-chlorophyll interaction is weak enough not to influence the absorption spectrum. A similar suggestion was also made in the pheophytin case. There we also offered another explanation that the fluorescence intensity might decrease on solidification of the monolayer due to a change in the intermolecular interaction within the lipid phase. However, we do not believe that such a change suffices to explain the extremely large change observed in the chlorophyll fluorescence.

Concluding Remarks

From thermodynamic and from fluorescence measurements on mixed monolayers containing dimyristoyl phosphatidyl choline and pheophytin in different amounts recently we could establish the existence of phase separation between lipid and pheophytin. The results are comparable with those reported here on bilayer vesicles.

A phase separation is predicted from our earlier study investigating the concentration dependence of the fluorescence intensity below the lipid phase transition temperature [7]. This is valid for both chlorophyll a and pheophytin a. Above the lipid phase transition only pheophytin a seems to form strongly bound aggregates. In good agreement with the monolayer experiments a solubility limit of about 15 mol-% is found for pheophytin in the bilayer. The aggregates which are formed if this concentration is exceeded are now observed directly by a red shift of about 25 nm in the absorption spectrum. This is not the case in chlorophyll a containing vesicles. Again a solubility limit of about 15 mol-% is obtained and a phase separation must be assumed from self quenching experiments below the lipid phase transition temperature.

Nevertheless these aggregates are not observable in the absorption spectra. This must be due to the magnesium atom being present in the chlorophyll a molecule. Coordination of this Mg-atom by the lipid phosphate group would expect to maintain the chlorophyll in a monomeric form. An aggregation with an interaction distance of about 20 Å would lead to a shift in the absorption spectrum of about 5 nm at the most [20] as it is observed by Lee [9] in bilayer vesicles. Such an interaction between the lipid and the chromophore can not be expected in case of pheophytin because of the lack of a Mg-atom. A strongly bound aggregate is formed. An interaction distance of about 5 Å leads to a red shift of at least 20 nm [20]. This is observed in our absorption measurements. Therefore it seems reasonable that it might be not possible to find absorption bands of chlorophyll aggregates in a mixed chlorophyll-lipid system in the absence of any proteins. Moreover it is shown that pheophytin results could only be transferred to chlorophylls to a very limited extent. More work has to be done in artificial systems containing chlorophylls including the chlorophyll-protein interaction.

Proposal of an Alternating Model for the Organization of the Antenna Chlorophyll

The visible absorption spectra of *in vivo* chlorophyll is red-shifted relative to *in vitro* monomeric chlorophyll in organic solvents. This may be due to protein-chlorophyll interaction or/and to chlorophyll self aggregation.

Two main models for the antenna chlorophyll organization were developped in the last years:

1) According to Thornber [21] the chlorophylls are specifically conjugated with proteins. The photosynthetic unit is composed of chlorophyll-protein complexes (*e. g.* the light harvesting chlorophyll a/b protein) whereas the remaining chlorophyll (about 20%) is called an "unsubstantiated" compound. Anderson [5] proposed a model where the chlorophylls are part of the lipid halo surrounding the proteins.

2) Katz *et al.* (for a review see ref. [2, 3]) advanced the hypothesis that chlorophyll oligomers are an appropriate model for the *in vivo* antenna chlorophyll. In their model the chlorophylls are oriented orthogonal to form a highly regular and precisely oriented array. It could be held by a protein matrix through the interaction of nucleophilic amino acid side chains.

There are some objections against these models:

1) There is no doubt about the existence of the chlorophyll-protein complexes. But the excess of chlorophyll molecules is by no means unsubstantial.

2) Chlorophyll molecules being part of a lipid halo would not show a red shift in the absorption spectrum.

3) The oligomer model for antenna chlorophyll does not use chlorophyll-protein interaction at all, and these must be surely important.

4) The oligomer structure is reported to exist only in the absence of nucleophiles. This is not given in the membrane.

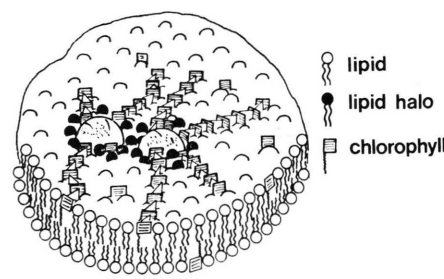

Fig. 7. Model proposal for the organization of antenna chlorophylls. Arrays of oligomeric chlorophylls are attached to a chlorophyll-protein complex. The lipid halo surrounding the proteins includes chlorophyll molecules. These may act as terminals for the extended aggregates and may serve as a connection to the protein bound chlorophylls. The aggregates are stabilized by hydrophobic chain-chain interaction. A second stabilizing effect comes from the nucleophilic binding of the polar lipid headgroups to the Mg-atom of the porphyrin ring.

By the use of absorption and fluorescence spectroscopy we have demonstrated the existence of a solubility limit for Chl a and pheophytin a in lipid bilayers. Aggregates (in case of pheophytin) or enriched domains (in case of chlorophyll a) are formed. This shows that a hydrophobic milieu is not necessary for the formation of oligomers. Our determination of the orientation of chlorophylls in the lipid matrix [11, 22] suggests that oligomers are stabilized through the interaction between porphyrin rings and the polar groups of the phospholipids (or galactolipids and sulfolipids) in the membrane. A second contribution comes from the hydrophobic interaction between the phytol chain and the lipid fatty acid chains. Due to the lipid-chlorophyll interaction studies we propose an alternative model for the antenna chlorophyll: The oligomers (as proposed by Katz *et a.*) constitute the basical units of the antenna chlorophyll. Its terminal chlorophyll could be bound to the nucleophilic groups of the reaction centre proteins. Some chlorophyll molecules together with lipids form a "halo". The excess of chlorophyll molecules precipitates within the lipid matrix to form aggregated arrays. Our model, demonstrated in Fig. 7, could be considered in an intermediate state between the models mentioned above. This somewhat speculative model may be taken as a basis of discussion.

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