Interactions between Photosynthetic Pigments Bound to Lipid and Protein Particles. Spectroscopic Properties

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The spectroscopic (visible) properties of pigment-bearing lipid and protein particles extracted from milk show that: 1) chlorophylls *a* and *b* bound to separate particles can form aggregates provided their relative concentration is high enough. Neither pheophytin *a* nor β-carotene, in the same conditions, form observable aggregates. 2) Chlorophylls *a* and *b* can co-aggregate when they are bound to the same particles. Pheophytin *a* as well as β-carotene seem to prevent the aggregation of chlorophyll *a*. β-carotene has no effect on the aggregation of chlorophyll *b*.

Photosynthetic lamellae, *in vivo*, have an absorbance maximum at 679 ± 1 nm due essentially to the presence of chlorophyll *a* and its structural bounds with the membrane constituants. Chlorophyll *b*, carotenoids and probably pheophytins too [1] contribute also to the spectroscopic properties of the photosynthetic structure. In the lamellae, the excitation is transferred from chlorophyll *b* to chlorophyll *a*, and from carotenoids to chlorophylls [2]. Moreover, chlorophyll aggregates exist in some places of the lamellae: P-700 is probably an auto-aggregate of chlorophyll *a* absorbing at about 700 nm, and P-680 could be a hetero-aggregate of chlorophylls *a* and *b* absorbing around 680 nm. The former is considered to be the energy converter (trap) of photosystem I and the latter the converter of photosystem II. Finally, if the chlorophylls were homogeneously dispersed (solvated) in the lamellae, their average concentration would be 5×10^{-2} M [3].

Dilute solutions of chlorophylls (10^{-5} M) in polar solvents have absorbance about 663 nm (chlorophyll *a*) and 645 nm (chlorophyll *b*), and no interaction between pigments can be found, by absorbance or by fluorescence studies. But when the pigment concentration exceeds 1×10^{-2} M, the absorbance maxima are red-shifted 10 to 15 nm, the bandwidths increase, new bands eventually appear at 710 nm and even at longer wavelengths [4, 5]; there is, in addition, a concentration quenching of fluorescence and excitation transfers between pigment molecules: the excitation of chlorophyll *b* results in a chlorophyll *a*-fluorescence [6]. Therefore, concentrated chlorophyll solutions could be considered as good models for the chloroplast lamellae, at least spectroscopicallywise, except that *in vivo* the pigments are located in structures, at protein-lipid interfaces probably. The aim of the present paper is to evaluate the general role of a macromolecular support in that respect.

The system used here has been described previously [7, 8]: it is made of particles precipitated from fresh cow milk with acetone and resuspended in aqueous buffer solutions.

They are about 2 μm in diameter; they contain about 30% (w/w) lipids containing mostly the $C_{18:1}$ fatty acid. The protein moiety consists of aspartic and glutamic acids as well as of leucine essentially [7].

If the chlorophylls (*a* and *b*) are considered to be dissolved in the particles, aggregations and excitation transfers happen when their concentration exceeds 2×10^{-3} M. If, however, their global concentration in the aqueous suspension is considered, those phenomena occur when the chlorophyll concentration is greater than 5×10^{-6} M. There are, therefore, reasons to believe that pigments can interact within one particle but not from particle to particle.

As a working hypothesis, in this paper, it will thus be stated that the pigments are dissolved in each particle. If the spectroscopic properties studied here show interactions or complex-formation between

Abbreviations: Chl, car, phine are respectively chlorophyll, β-carotene and pheophytin *a*. lp designates the lipid and protein particles and lp_x the lp with bound pigment x.

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pigments, then it will be necessary to evaluate the importance of structuration by the particles. In the opposite case, the working hypothesis will be confirmed, and the pigments will have to be treated as solutes in the particles.

Materials and Methods

Chlorophylls *a* and *b* are prepared according to Strain and Svec [9]. Pheophytin *a* is obtained by acidifying a chlorophyll *a* solution as described by Perkins and Roberts [10]. Synthetic β-carotene is purchased from Sigma.

The particles are obtained by precipitating milk with acetone [11]. If the acetone contains pigments, the precipitated particles are coloured.

To know the quantities of bound pigments, they are extracted from the coloured particles with 80% acetone in water (v/v) and the absorbance of the solution is measured. In the cases where pigments are mixed on the particles, three possibilities must be considered: the concentrations of mixed chlorophylls *a* and *b* are given by

$$c_a = 0.01302\ A_{665} - 0.0029\ A_{647}$$
$$c_b = 0.0243\ A_{647} - 0.0065\ A_{665}$$

where A is the absorbance at the wavelength (nm) given in subscript, and c_a, c_b are the concentrations of chlorophylls *a* and *b* respectively (g/l). The coefficients are re-established for each series of experiments. The values given above are averages. Mixtures of chlorophyll *a* and pheophytin *a* are resolved by (in diethyl-ether)

$$c_{ph} = 0.0340\ A_{668} - 0.0245\ A_{662}$$
$$c_a = 0.0205\ A_{662} - 0.0145\ A_{668}$$

where the c_a and c_{ph} (pheophytin *a* concentration) are given in g/l. In both cases, the lightpath through the solutions is 1 cm. When chlorophylls are mixed with β-carotene on the particles, the pigments are first transferred from 80% acetone to petroleum ether, then the solution is chromatographed (Whatman paper n° 1, petroleum ether 40 ° – 60 °C; Benzene; Acetone 42; 2; 5 by volumes). The carotene is eluted in acetone and its concentrations given by

$$c_{car}, \text{ g/l} = 220\ A_{454}$$

The apparent extinction coefficients (ε_{app}) are calculated by dividing the absorbance of a suspension at a given wavelength (that of the red maximum, for chlorophylls and pheophytin) by the molar concentration of the pigment in the suspensions.

The concentration of proteins is measured according to Lowry *et al.* [12]. In the present paper, the relative contents of pigments are given by

$$r_{gx} = \frac{\text{weight of pigment x}}{\text{weight of protein}}$$

in the aqueous particle suspensions. In all our experiments, the protein concentration is usually kept at 500 mg/l.

On the emission spectra, band I is the short wavelength band and II is the long-wavelength band. The two bands are considered symmetrical and the I/II ratio is the ratio of the band-heights.

The milk used is whole cow milk. All the solvents and products are purchased from Merck A.G. (pro analysi = reagent grade). The aqueous suspensions are buffered at pH = 8.

Results

1. Reminder of the absorbance maxima in suspension of single pigment-bearing particles

Chlorophyll *a*: For $r_g < 7 \times 10^{-3}$, the λ_{max} is 670 nm. For $r_g > 3.8 \times 10^{-2}$, there are two bands: one at 672 nm and the other at 740–750 nm. Between those 2 r_g values, there are two bands also: one at 670 nm and the other, visible only by difference spectroscopy, at 705–710 nm [7]. Chlorophyll *b*: The critical value of r_g is 7×10^{-3}. Below, the absorbance maximum is at 653 nm. Above, there are 2 bands, one at 653 nm and the other at 690 nm [7]. Pheophytin *a*: The absorbance maximum is at 672 nm whatever the r_g is, in the range of r_g values given above (Techy, unpublished). β-carotene: The absorbance maxima are at 446 nm and 496 nm for $1 \times 10^{-3} < r_g < 60 \times 10^{-3}$ (Dinant, unpublished).

In the following descriptions and discussions, only the wavelengths higher than 650 nm will be considered.

Absorbance maxima of particles bearing two pigments

When Chl *a* ($r_g = 30 \times 10^{-3}$) is mixed on particles with either Chl *b* or car, a 750 nm-absorbing species appears if the r_g total = 40×10^{-3}. It must be noted

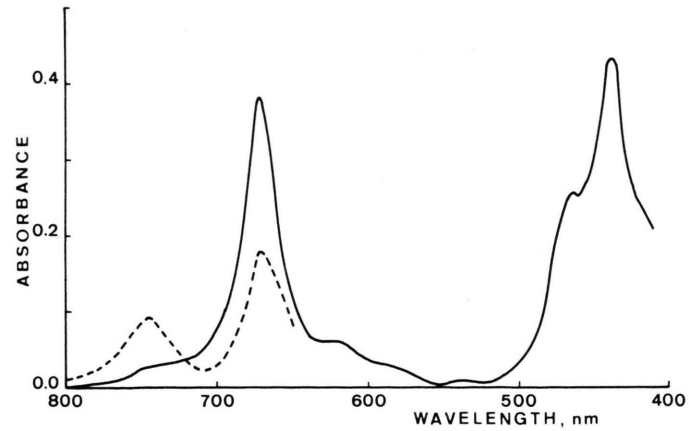

Fig. 1. Absorbance spectra of a suspension of particles bearing Chl *a* and Chl *b* together. $\frac{a}{b} = 2.5$, r_g Chl $a = 3.1 \times 10^{-2}$, r_g Chl $b = 1.2 \times 10^{-2}$. Solid line: spectrum made immediately after the preparation is made. Broken line: after 24 hours at 0 °C in the dark.

that it was not possible to detect a 690 nm- or a 710 nm-band in mixtures of Chl *a* and Chl *b*: such bands could be hidden by the wide (though not intense at so low a r_g) band at 750 nm (Fig. 1).

In the present conditions, no long-wavelength-absorbing Chl *a* has appeared in the presence of phine *a*, but the r_g values used are usually small (maximum = 11.4×10^{-3}), far below those where they could appear.

Finally, *β*-carotene lowers the concentrations of the 690 nm- and of the 710 nm-absorbing species in mixture with Chl *b* and with Chl *a* respectively. Since *β*-carotene facilitates the appearance of 750 nm-absorbing Chl *a* on the particles, this result seems to mean that the forms absorbing at 710 nm and at 750 nm are different by the conditions of their formation and, probably, their nature as well.

2. *Variations of* ε_{app} *in chlorophyll a-chlorophyll b mixtures on the same particles*

The values of ε_{app} are measured at the λ_{max} of the pigments fixed separately on the particles, *i. e.* 670 nm and 653 nm (chlorophylls *a* and *b* respectively).

It appears (Table I) that for chlorophyll *b*: chlorophyll *a* ratios greater than 0.9, ε_{app} for chlorophyll *a* is lowered by the presence of chlorophyll *b*; on the

Table I. Measure of the apparent molar absorption coefficients ε_{app} of Chl *a* and Chl *b* fixed separately and together on lipid and protein particles.

System	$\frac{\text{Chl } b}{\text{Chl } a}$	r_g Chl $a \times 10^3$	r_g Chl $b \times 10^3$	ε_{app} Chl *a*	ε_{app} Chl *b*
				[$\times 10^{-7}$ cm²/mol]	
lp Chl *a*		19.5	–	4	–
lp Chl *b*		–	8.5	–	2.5
lp Chl *a* Chl *b*	0.45	19.5	8.5	3.75	2.5
lp Chl *a*		28	–	3.75	–
lp Chl *b*		–	28	–	<2
lp Chl *a*		17.5	–	3.9	–
lp Chl *b*		–	15.3	–	2.4
lp Chl *a* Chl *b*	0.90	17.5	15.3	3.3	2.4
lp Chl *a*		32.8	–	3.5	–
lp Chl *b*		–	32.8	–	<2
lp Chl *a*		15	–	4.05	–
lp Chl *b*		–	18	–	2.15
lp Chl *a* Chl *b*	1.2	15	18	3.1	2.3
lp Chl *a*		33	–	3.5	–
lp Chl *b*		–	33	–	<2
lp Chl *a*		11	–	4.2	–
lp Chl *b*		–	19.5	–	2.5
lp Chl *a* Chl *b*	1.8	11	19.3	2.8	1.9
lp Chl *a*		30.5	–	3.75	–
lp Chl *b*		–	30.5	–	<2

System	phine *a* / Chl *a*	r_g Chl *a* × 10^3	r_g phine *a* × 10^3	ε_{app} Chl *a* [× 10^{-7} cm²/mol]	ε_{app} phine *a* [× 10^{-7} cm²/mol]
lp Chl *a*		2.5	–	9.5	–
lp phine *a*		–	2	–	2.4
lp Chl *a* phine *a*	0.8	2.5	2	12	2.5
lp Chl *a*		4.5	–	4.8	–
lp phine *a*		–	4.5	–	2.4
lp Chl *a*		2.5	–	9.5	–
lp phine *a*		–	2.6	–	2.4
lp Chl *a* phine *a*	1.05	2.5	2.6	12	3
lp Chl *a*		5.1	–	4.8	–
lp phine *a*		–	5.1	–	2.4
lp Chl *a*		1.6	–	9.5	–
lp phine *a*		–	3.1	–	2.4
lp Chl *a* phine *a*	1.9	1.6	3.1	12	2.5
lp Chl *a*		4.7	–	4.8	–
lp phine *a*		–	4.7	–	2.4

Table II. Measure of the apparent molar absorption coefficients ε_{app} of Chl *a* and of phine *a* fixed separately and together on lipid and protein particles.

contrary, ε_{app} for chlorophyll *b* is the same for a given concentration of pigment, whether chlorophyll *a* is present or not. It must be noted however that ε_{app} for chlorophyll *b* is always higher in the mixture than when the r_{gchlb} is equal to the r_g total.

Dinant and Aghion [7] have argued that the decrease of ε_{app} as r_g increases, is due to the increasing orientation of fixed pigment molecules. Then Table I can be described by saying that chlorophyll *b* induces, in the mixtures, an orientation of chlorophyll *a* more important than the same number of chlorophyll *a* molecules, but that chlorophyll *a* molecules have no noticeable effect on chlorophyll *b* orientation, *i. e.* have less effect than as many molecules of chlorophyll *b*.

3. Variations of ε_{app} in chlorophyll a -pheophytin a mixtures on the same particles

A comparable scheme of interpretation for Table II leads to the idea that pheophytin *a* (λ_{max} = 672 nm) disperses chlorophyll *a* within the particles. It must be emphasized here, however, that the r_g values are relatively very small hence the ε_{app} values are suspect of some error.

4. Variations of ε_{app} in chlorophyll-β carotene mixtures on the same particles

All the cases reported in Table III show that β-carotene has a dispersing effect on chlorophyll *a* in the particles.

Similar experiments done with chlorophyll *b* and β-carotene show absolutely no effect of β-carotene on the ε_{app} of chlorophyll *b*.

5. Fluorescence at room temperature

The increase of r_{gChla} from 7×10^{-3} to 5×10^{-2} is accompanied by two sets of phenomena:

a) long wavelength-absorbing aggregates (710 nm and 750 nm) appear;
b) the I/II ratio (fluorescence at 680 nm and 720 nm) decreases from 8 to 1.6 [7].

As a working hypothesis, a causal relationship is postulated between those two consequences of increasing r_g. In chlorophylls *a* and *b* mixtures, I/II is 20 percent lower than with chlorophyll *a* alone. Pheophytin *a* decreases the I/II ratio in mixtures with chlorophyll *a* by almost 50 percent. β-carotene, on the contrary, causes a slight increase of I/II due to chlorophyll *a*.

System	car / Chl *a*	r_g Chl *a* × 10^3	r_g car × 10^3	ε_{app} Chl *a* × 10^{-7} [cm²/mol]
lp Chl *a*		20	–	4.0
lp Chl *a* car	0.23	20	5	4.4
lp Chl *a*		25	–	3.8
lp Chl *a*		10	–	4.2
lp Chl *a* car	0.46	10	4.6	4.7
lp Chl *a*		14.6	–	4.1

Table III. Measure of the apparent molar absorption coefficients ε_{app} of Chl fixed alone and with β-carotene on the particles.

Discussion

1. Significance of ε_{app} and of its variations

ε_{app} is calculated as if the absorbing molecules were dispersed in the bulk of the aqueous suspension. They are not, actually, but since in all experiments there is the same number of particles per ml, ε_{app} is a multiple of the absorbance coefficient of each particle. Knowing that ε can be viewed as the area offered by the absorbing molecules to the incident flux of photon (ε is expressed in cm^2/mol), ε can statistically represent the orientation of the pigments on the particles. Hence, variations of ε represent variations of the pigment orientation. The relation between the two variations is an inverse one: increasing values of ε mean increasing chances of a meeting of photons with the absorbing molecules and vice-versa. Because the results have shown that a decrease of ε_{app} is concomittant with the appearance of 690 nm and 710 nm-absorbing species, and because those species are chlorophyll aggregates, it is suggested to replace the word orientation by aggregation and to consider the decrease of ε_{app} as a "sieve effect".

2. Chlorophyll aggregation on the particles

With chlorophylls *a* or *b*, as long as $r_g < 7 \times 10^{-3}$, ε_{app} decreases noticeably when r_g increases. When $r_g > 10^{-2}$, ε_{app} remains almost constant (decreases only slightly) with an increasing value of r_g.

It was said that when $r_g > 10 \times 10^{-3}$, Chl *a* is partially aggregated into a 710 nm-absorbing aggregate and Chl *b* forms a 690 nm-absorbing one. Increasing r_g results in a sharply decreasing ε_{app} up to $r_g = 10 \times 10^{-3}$, then ε_{app} varies only slightly. Since (i) ε_{app} is measured at the "monomer" λ_{max} (670 nm and 653 nm for Chl *a* and Chl *b* respectively), (ii) the concentration of long wavelength-absorbing forms increases with r_g at the expense of the "monomer", and (iii) the ε_{app} is measured by dividing the absorbance by the total extracted chlorophyll concentration, the relative constancy of ε_{app} means that the concentration of "monomer" increases, implying probably an equilibrium between "monomers" and auto-aggregates, on lp.

The simultaneous presence of chlorophylls *a* and *b* on the particles decreases the ε_{app} of chlorophyll *a* *i.e.* favors its orientation. This could be interpreted with the knowledge that chlorophylls *a* and *b* at a water-air interface, undergo a one-to-one interaction [13]: the interaction can exist on the particles studied here, too. Of course, this would imply a similar orientation of chlorophyll *b* which is not very conspicuous on Table I but it must be remembered that ε_{app} of chlorophyll *b*-bearing particles varies very little with r_g, anyway.

Pheophytin *a* and β-carotene seem to disperse the molecules of chlorophyll *a* on the particles; incidentally such a result is consistent with the role attributed to the Mg-atom in the aggregation and general interactions of chlorophyll molecules.

β-carotene has no effect on the ε_{app} of Chl *b*. This can be a result of the lack of effect of β-carotene on the fixation of chlorophyll *b*: β-carotene has, on the contrary, a competitive effect on the fixation of chlorophyll *a* [7].

3. Fluorescence at room temperature

The effect of chlorophyll *b* and of β-carotene on the I/II ratio of chlorophyll *a* confirms that on the ε_{app}, as far as aggregation and dispersion of chlorophyll *a* molecules are concerned. However the same is not true with the effects of pheophytin *a*. This could mean that the I/II ratio bears other significances than the aggregation only, but the results are still too scanty to be exploited further.

Conclusion

Absorbance and fluorescence properties show that geometric interactions occur between chlorophyll *a* and *b* molecules, chlorophyll *a* and pheophytin *a*, chlorophyll *a* and β-carotene, chlorophyll *b* and β-carotene when those pigments are fixed and oriented on lipid-and-protein particles in an aqueous suspension. It must be noted that such interactions have never been described with solutions of those pigments, except for excitation transfer of fluorescence. But interactions have actually been described between those pigments in monolayers at interfaces between water and air [13].

It is too early to correlate functional interactions with structural interactions *in vitro* and a fortiori, *in vivo*.

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