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ANTENNA CHLOROPHYLL IN PHOTOSYNTHETIC MEMBRANES
A STUDY BY RESONANCE RAMAN SPECTROSCOPY

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SUMMARY

Raman spectra of antenna chlorophyll *a* and chlorophyll *b* were selectively obtained from chloroplasts of green plants and from monocellular algae, using resonance enhancement in the respective Soret bands of these molecules, at 35 K. It is shown that:

Antenna chlorophyll *a* molecules occur in at least five discrete categories, distinguished by different extramolecular bonding of their 9-keto carbonyl groups.

These vibrational categories are probably identical in nature and number among the different organisms studied, but differ in their relative populations.

Chlorophyll *b* molecules occur in at least two different categories differing by the strength of the interactions of their 3-formyl C = O groups. These vibrational categories also appear as universal.

Most chlorophyll *a* and *b* molecules have their magnesium atoms bound to a single foreign ligand, whose nature may depend on the population considered.

Resonance Raman spectra of antenna structures, including those of organisms devoid of chlorophyll *b*, were compared to resonance Raman spectra of chlorophyll *a* and *b* in monomeric, oligomeric and hydrated polymeric states, at room temperature and at 35 K. No sizable amount of antenna chlorophyll *a* or *b* occurs as dry or hydrated oligomers, or polymers. The antenna molecules are thus necessarily bound to foreign molecules, probably proteins, through H-bonding on their formyl and/or keto carbonyl groups and through bonding of their magnesium atoms.

INTRODUCTION

The organization of chlorophyll molecules in photosynthetic membranes determines the nature of the energy transfer processes from sites of light absorption to reaction centers. Much work has thus been devoted to understanding the arrangement of chlorophyll in higher plants and algae. Particularly, electronic absorption and emission spectroscopies have contributed much of the present knowledge about chlorophyll states in vivo [1]. Data derived from these methods have generally been

taken as indicative of a heterogeneity of antenna chlorophyll *a* molecules arising from differences in environmental interactions [1–5]. Selective fractionation and selective alteration of the photosynthetic apparatus have led to similar interpretations [5].

However, the mere existence of such different categories still remains controversial [6], much less their number and the origins of their differentiation. Current hypotheses that have been forwarded to explain heterogeneity of spectroscopic and structural properties of antenna chlorophyll *a* include differences in binding sites to the membrane [1, 7], simultaneous presence of monomers and/or of oligomers of different sizes [8, 9], simultaneous presence of different types of oligomers [4], or combinations of these hypotheses [10].

Vibrational spectroscopy has yielded considerable knowledge about the interactions assumed by chlorophyll in vitro [11]. Application of these techniques to chlorophylls imbedded in the very complex medium of a photosynthetic membrane necessitates high selectivity in producing vibrational spectra of chlorophyll. Owing to the fact that a very few molecular species of chloroplasts have electronic transitions in the visible region, we recently showed that the resonance effect in the Soret bands of chlorophyll could be used to selectively enhance Raman scattering of vibrational modes of either chlorophyll *a* or of chlorophyll *b* in the antenna of chloroplasts of spinach [12–14]. Amplification of absolute intensities of Raman bands of chlorophyll by factors up to $5 \cdot 10^4$ excludes any contribution in these spectra from non-resonating molecules, including water and proteins [15]. Experimental conditions and the low proportion of reaction center chlorophylls also precludes their significantly contributing to spectra of unfractionated chloroplasts.

The information content of Raman spectra of chlorophyll in preresonance and resonance conditions with respect to the Soret transitions was studied in vitro [12, 15]. It was shown, in particular, that the resonating modes included stretching motions of the 9-keto carbonyl groups of chlorophyll *a* and of chlorophyll *b*, and of the 3-formyl carbonyl of chlorophyll *b*. Resonance enhancement of bands involving vibrational modes of the $Mg\text{-}N_4$ grouping was also evidenced by ^{26}Mg isotope substitution in chlorophyll *a* and chlorophyll *b* [15, 16]. These functional groups are known to play a predominant role in intermolecular interactions of chlorophyll in vitro [11]. No activity was observed, on the contrary, from ester carbonyl groups in position 10, due to their lack of conjugation with the main π electron system of the phorbin ring, which is responsible for the Soret electronic transitions [15]. These groups are known also to participate in building intermolecular associations of chlorophyll in vitro [8, 9, 17, 18]. Finally the extent of participation of C···C and C···N bonds, in resonance Raman active modes of the phorbin skeleton of chlorophyll *a* and *b* were estimated from ^{15}N isotope substitution [16].

Resonance Raman spectra of spinach chloroplasts at room temperature indicate that the magnesium atoms of many chlorophyll *a* and chlorophyll *b* molecules are in coordination states similar to those encountered in chlorophyll oligomers or hydrated aggregates. The carbonyl stretching regions (1600–1750 cm^{-1}) appear as complex superpositions of bands, most of them downshifted from the free C = O stretching frequencies. This latter observation suggested the simultaneous existence in vivo of different types of intermolecular binding of the ketone and aldehyde carbonyls of chlorophyll *a* and chlorophyll *b*, respectively [13].

In the present study, we attempted to resolve the 1600–1750 cm^{-1} cluster of bands by working at low temperatures. Our previous observations on spinach were extended to other green plants and to algae, including species containing only a few or no chlorophyll *b* molecules, whose formyl ν (C = O) modes may interfere with the chlorophyll *a* contributions in resonance Raman spectra obtained at 441.6 nm [13]. These spectra were compared to those of chlorophyll *a* and of chlorophyll *b* in monomeric, oligomeric and hydrated polymeric "microcrystalline" states, at room temperature [15] and at low temperature (this work). Direct information was thus obtained on the interactions assumed by chlorophyll in intact photosynthetic structures, which also allowed a test of the validity of some of current hypotheses about the states of antenna chlorophyll in the chloroplast. A preliminary account of the low temperature work on some biological samples has been published [14].

METHODS AND SAMPLES

Spectroscopic methods. All samples were studied as optically dense deposits on microscope coverslips immersed in a flow of gaseous helium at about 35 K. Heat produced at the illuminated site was thus efficiently removed. Moreover, grazing incidence and luminous powers not higher than 5 mW were used. Different lines from an Argon laser and the 441.6 nm emission of He-Cd laser (Spectra Physics) were alternatively employed to achieve optimal selectivity in producing the resonance Raman spectrum of a given molecular species in a given environment. Light scattered at about 90° from the illumination beam was analyzed through a double grating Raman spectrometer (Coderg PHO), with effective resolution from 5 to 8 cm^{-1}. Time constants of 1.5–5 s were used in the detection system. In studying the 1650–1750 cm^{-1} and 200–400 cm^{-1} regions, summation of spectra was achieved in a multichannel analyser.

Samples. Chlorophyll *a* and chlorophyll *b* were prepared and characterized as previously described [15]. Suitable dehydration of samples was achieved by repeated evaporation of solutions in dry carbon tetrachloride followed by heating at 50 °C in a 10^{-4} Torr vacuum for up to 50 h [17]. Solvents were dried either with 3 Å molecular sieve or with calcium hydride. Oligomers in the solid state were obtained by spreading solutions in dry cyclohexane on a coverslip and evaporating them under a stream of dry nitrogen. Illumination of these samples at 457.9 nm yielded resonance Raman spectra arising from $(\text{chlorophyll } a)_n$ oligomers only, despite systematic presence of a second fraction, which spectra were selectively excited at 441.6 nm. A monomeric state is suggested for this fraction, in view of its Soret band being blue shifted with respect to that of oligomers [19], and in view of its Raman spectrum being nearly identical to those of monomeric chlorophyll *a* in solution. Monomeric samples of chlorophylls were obtained by freezing a droplet of solution in acetone before introducing it into the cryostat. Hydrated, "microcrystalline" polymers of chlorophyll were prepared by sonication of dehydrated samples dissolved in cyclohexane with water, at 35 °C. Such preparations of chlorophyll *a* and of chlorophyll *b* presented characteristic electronic bands at 740 and at 681 nm, respectively, at room temperature [17]. Droplets of aggregates suspensions were transferred on a coverslip and frozen before introducing them in the cryostat. No subsequent dehydration of the samples was observed under these conditions.

Standard methods were employed in preparing intact chloroplasts of spinach (*Spinacia oleracea*), of barley (*Hordeum vulgare*) and of a barley mutant free of chlorophyll *b* (*Hordeum vulgare* cv. *Donaria*, strain 3613, Gatersleben) [20], as well as of maize mesophyll (*Zea mays*) [21]. The latter material was grown under intermittent light [22], insuring chlorophyll *a*/chlorophyll *b* ratios higher than 6. The monocellular algae *Chlorella vulgare* and *Botrydiopsis alpina*, the latter devoid of chlorophyll *b*, were centrifuged from their culture media and studied as intact whole cells.

RESULTS

(*1*) *Resonance Raman spectra of chlorophylls in vitro, at 35 K*

Cooling of chlorophyll preparations in vitro down to 35 K brought considerable improvement in signal to noise ratios and decrease in bandwidth of resonance Raman bands (Figs. 1 and 4). Resonance Raman spectra of chlorophyll *a* and of chlorophyll *b* in monomeric, oligomeric and microcrystalline states at 35 K were thus used together with room temperature spectra [15] in interpreting those of chlorophyll in vivo.

Comparisons between these spectra often involve comparisons of relative intensities of resonance Raman bands. We have shown previously that variations of intensities with respect to illumination wavelength were nearly parallel for all measurable Raman bands of chlorophyll *a* and of chlorophyll *b* for a single type of association, in Soret resonance conditions [15]. Relative intensities of homologous bands may thus be compared in resonance Raman spectra for different samples excited at different wavelengths without correction for variations of absolute intensities with excitation wavelength.

(1.1) *Chlorophyll a.* (1.1.1) Carbonyl stretching region (1550–1750 cm^{-1}): The 1550–1750 cm^{-1} region yields, in addition to bands arising from stretching motion of the 9-C = O ketonic groups of chlorophyll *a*, three bands close to 1560, 1585 and 1617 cm^{-1} arising from C=C stretching modes of the phorbin ring, without noticeable participation of nitrogen motions [16]. The 1617 cm^{-1} band likely involves stretching motions of the methine bridges [15, 23]. The 1585 cm^{-1} band, once suspected to be due to pheophytin contamination [15], does in fact belong to chlorophyll *a*.

The stretching frequency of non-bonded 9-C = O groups occurs at 1695–1700 cm^{-1} in Raman spectra of chlorophyll *a* in polar solvents at room temperature [15]. At 35 K, this frequency is 1690 cm^{-1} in dry, solid samples and 1682 cm^{-1} for 10^{-2} M solutions in acetone (Figs. 1B and 2A). Downshift in the latter sample may be due to the increase in permittivity of the solvent on cooling. Dry $(\text{chlorophyll } a)_n$ oligomers at 35 K yield a broad band at 1650 cm^{-1}, characteristic of Mg-bound 9-C = 0 carbonyls (Fig. 2B). Its halfwidth is more than 25 cm^{-1}, much larger than the 15 cm^{-1} value observed for room temperature solutions in apolar solvents. This fact is probably connected to the simultaneous presence of aggregates of very different sizes. Microcrystalline aggregates yield a sharp 9-C = O stretching band at 1641 cm^{-1}, which is characteristic of these structures in both their infrared and resonance Raman spectra at room temperature [15, 24]. In spite of its halfwidth of less than 15 cm^{-1}, this band presents no observable structure at 6 cm^{-1} resolution, nor do any of the carbonyl bands of the other samples here described (Fig. 2).

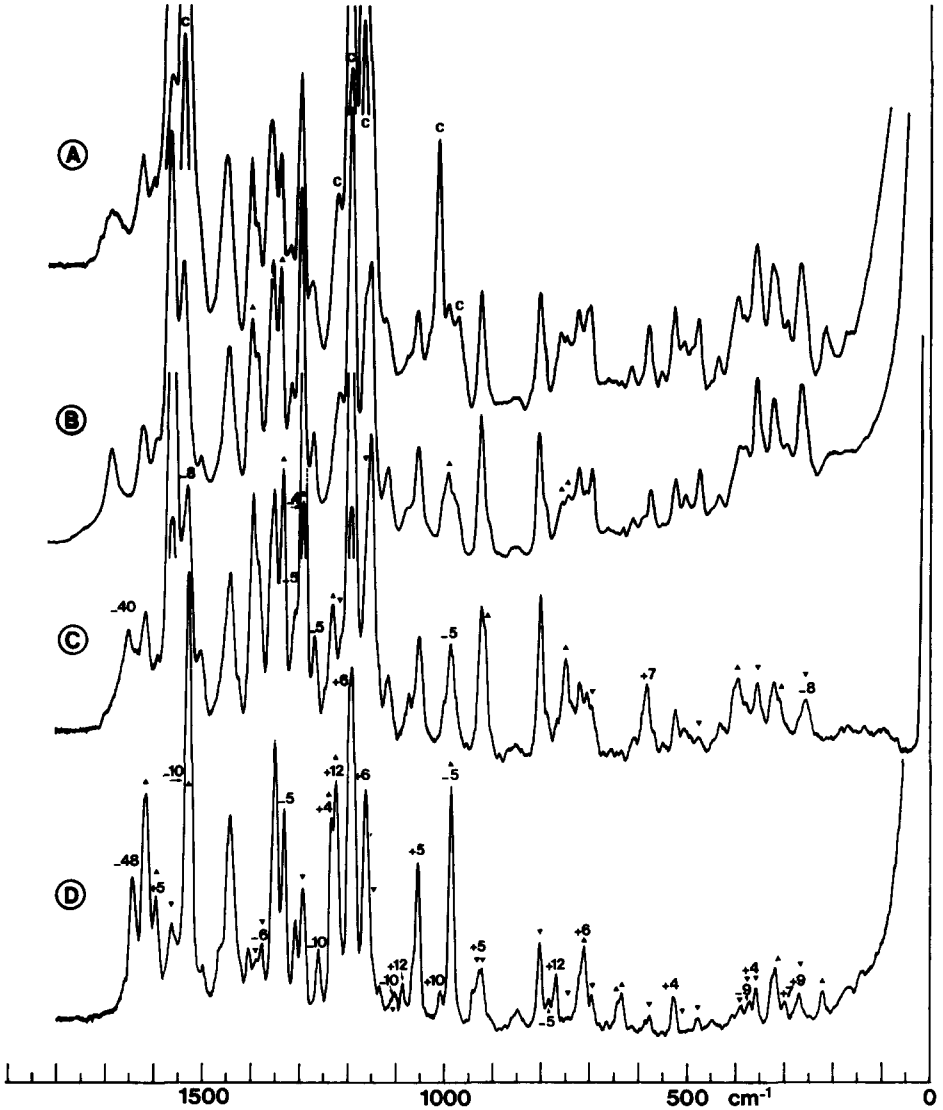


Fig. 1. Resonance raman spectra of chlorophyll *a* at 35 K. A, antenna chlorophyll *a* of whole chloroplasts of mutant barley lacking chlorophyll *b*. C labels bands from carotenoids. B, monomeric chlorophyll *a*, 10^{-2} M in acetone. Arrows indicate bands enhanced in relative intensity with respect to spectra of monomer at room temperature. C, $(\text{chlorophyll } a)_n$ oligomers, desiccated, solid deposit. Numbers and arrows: frequency shifts (cm^{-1}) and variations in relative intensity with respect to monomers at 35 K. D, $(\text{chlorophyll } a, n H_2O)_m$ polymers in cyclohexane. Numbers and arrows as in C. In case of ambiguous correlations with monomer spectra, the lowest possible frequency shifts have been indicated. Excitation wavelengths: A, B, 441.6 nm; C, D, 457.9 nm. Resolution at 1000 cm^{-1} is 8 cm^{-1}.

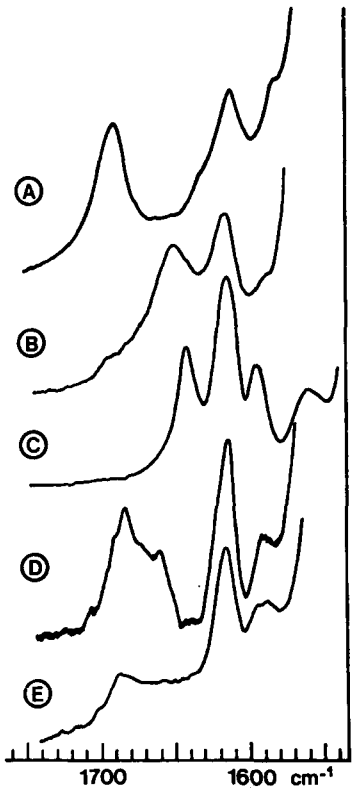


Fig. 2. Resonance Raman spectra of chlorophyll *a*, region of carbonyl stretching modes, averaged by summation. A, monomer in acetone, room temperature, excitation 441.6 nm. B, (chlorophyll $a)_n$ oligomers, dessicated solid deposit, 35 K, excitation 457.9 nm. C, (chlorophyll *a*, $n H_2O)_m$ polymers, 35 K, excitation 457.9 nm. D, antenna chlorophyll *a* in intact *B. alpina*, 35 K, excitation 441.6 nm. E, same sample as in D, 35 K, excitation 454.5 nm. Resolution: A–E, 7 cm^{-1}.

(1.1.2) 700–1550 cm^{-1} region: Resonance Raman bands of chlorophyll observed in this region are most likely to arise from in plane stretching (1000–1600 cm^{-1}) and angular modes of the conjugated C⋯C and C⋯N bonds of the phorbin macroring [15]. About four-fifths of these bands were sensitive to ^{15}N isotope substitution, indicating activity of complex modes involving simultaneous motions of several atoms. Rather pure C⋯C stretching modes were observed above 1520 cm^{-1} only, while the highest shifts affected bands in the 1100–1200 cm^{-1} range, which should then mainly involve stretching motions of C⋯N bonds [16]. Many of these bands are sensitive to change in the association state of chlorophyll, not only because of structural changes resulting from bonding of functional groups, but also as a consequence of π electronic intermolecular interactions, which take place when aggregation brings phorbin rings closer together.

This second effect probably accounts for the differences observed between resonance Raman spectra of monomeric chlorophyll *a* in frozen concentrated solutions in acetone, with respect to those of chlorophyll *a* in polar solvents at room

temperature. These differences include relative enhancement of bands at 1391, 1331, 757 and 745 cm^{-1} (Fig. 1B).

Desiccated, self-associated chlorophyll *a* at 35 K yields spectra presenting further differences with respect to those of monomeric chlorophyll *a* at low temperature (Fig. 1C). These (chlorophyll *a*)$_n$ oligomers are thus characterized by downshifted bands at 1527 cm^{-1} (−8 cm^{-1}), 1289 (−4) and 1266 cm^{-1} (−5 cm^{-1}). Bands at 1289, 1228, 986 and 750 cm^{-1} are significantly enhanced with respect to other skeletal bands, while bands at 1215, 1160 and 695 cm^{-1} are decreased in relative intensity. All of these bands arise from modes with significant participation of nitrogen atoms. Very similar spectral changes follow the formation of (chlorophyll *a*)$_n$ oligomers in non-polar solvents at room temperature (ref. 15 and Lutz, M., unpublished).

Chlorophyll molecules involved in (chlorophyll *a* · nH_2O)$_m$ aggregates undergo strong π electronic interactions which lead to very important modifications in their resonance Raman spectra [15] (Fig. 1D). The strongest interactions affect bands with high participation of nitrogen motions. As an example, the 1228 and 1213 cm^{-1} bands of dry oligomers are strongly enhanced and upshifted by 5 and 12 cm^{-1}, respectively. The 1150 cm^{-1} band vanishes; the 1115 cm^{-1} band weakens and may be shifted down by 10 cm^{-1}. By contrast with (chlorophyll *a*)$_n$ oligomers, bands with low nitrogen participation are also affected, as the 1560 cm^{-1} band, weakened by a factor of 8, and the 1530 cm^{-1} band, enhanced and downshifted by 10 cm^{-1}. Other spectral differences with respect to unaggregated chlorophyll *a* may be found in Fig. 1D.

A number of doublets or multiplets may be found in the resonance Raman spectra of microcrystalline chlorophyll *a*, e.g. at 1225–1233, 1191–1197, 923–928 and 635–643 cm^{-1} (Fig. 1D). Most of these correlate with bands that are obviously complex in spectra of the monomer and involve accidentally degenerate modes. They should thus not be ascribed to splittings by excitonic coupling.

Spectra of microcrystalline chlorophyll *a* obtained at 35 K differ in several details from those previously observed at room temperature [15]. Discrepancies in frequencies and relative intensities probably reflect differences in relative orientations and/or distances of the phorbin planes brought about by cooling of the sample.

Although most of the changes observed in resonance Raman active modes of the phorbin skeleton upon formation of (chlorophyll *a*)$_n$ or of (chlorophyll *a* · nH_2O)$_m$ aggregates cannot be interpreted presently, they appear systematically related to the formation of a given type of aggregate. Hence, the simultaneous presence of several of these spectral characteristics may be reliably used as a fingerprint for the occurrence of the corresponding type of aggregate.

(1.1.3) 50–700 cm^{-1} region: Many bands observed in this region of resonance Raman spectra of chlorophyll *a* must arise from in plane deformations of the phorbin skeleton [15]. However, at least two bands near 355 and 320 cm^{-1} arise from modes involving motions of the magnesium atom as well as of nitrogen atoms [16]. The latter band particularly was shown to be sensitive to the aggregation state of chlorophyll *a* at room temperature [15]. Other weak, aggregation-sensitive bands near 295 and 215 cm^{-1}, which involve modes with strong nitrogen participation, may involve participation of magnesium motions as well.

Spectra of monomeric chlorophyll *a* in acetone at 35 K are essentially identical

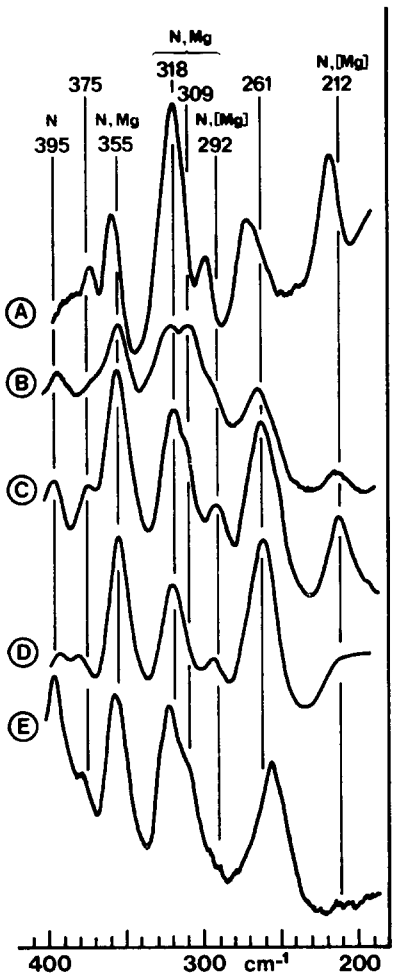


Fig. 3. Resonance Raman spectra of chlorophyll *a*, 200–400 cm^{-1}, averaged by summation. A, (chlorophyll *a*, $n H_2O)_m$ polymers in cyclohexane, 35 K. B, the same as in A, suspension in decane, room temperature. C, in whole chloroplasts of *H. vulgare* 35 K. D, monomer in acetone, 35 K. E, (chlorophyll *a*)$_n$ oligomers, desiccated, solid deposit, 35 K. Excitation wavelengths: A, B, E, 457.9 nm; C, D, 441.6 nm. Resolution: 8 cm^{-1}. Frequencies (cm^{-1}) indicated on top refer to spectrum C. N, Mg: bands shifted on corresponding isotope substitution [16].

to those previously obtained at room temperature, with bands at 354, 321, 295, 262 and approx. 210 cm^{-1} (Fig. 3D). The 321 cm^{-1} band is sharp and symmetrical, as is its room temperature homologue. This excludes that a change in coordination number of Mg could occur on cooling for any significant proportion of the molecules.

Spectra of self-associated chlorophyll *a* at 35 K differ from the preceding by the presence of additional medium intensity bands at 392 and 582 cm^{-1}. The latter band may alternatively be correlated with the 575 cm^{-1} band of the monomer, although it is more intense (Fig. 1C). As in room temperature spectra, a shoulder occurs at 308 cm^{-1} on the side of a band at 321 cm^{-1} (Fig. 3E). Weak monomer bands at 295 and 210 cm^{-1} almost vanish.

As mentioned in the preceding section, (chlorophyll $a \cdot nH_2O)_m$ polymers at 35 K yield resonance Raman spectra differing in some details from those obtained at room temperature. The main discrepancy in the present region consists in a shift of approx. $+5$ cm^{-1} of the band observed at 307 cm^{-1} at room temperature, which therefore is no longer resolved from the main 318 cm^{-1} band (Figs. 3A and 3B).

Differences in band frequencies and relative intensities occurring in this region between microcrystalline and monomeric chlorophyll *a* at 35 K are to be found in Fig. 1D. Additional bands are also observed at 635, 372 and 218 cm^{-1}.

Hence, both room and low temperature spectra of chlorophyll *a* (and of chlorophyll *b*, cf. section 1. 2. 3), when self associated with and without interposition of water, present an additional component near 310 cm^{-1}. An infrared band at the same frequency also appears to be characteristic of self aggregated chlorophylls and is attributed to stretching of the Mg ... O = C (9) bonds bridging two chlorophyll molecules [25]. Such an assignment probably cannot be accepted for the corresponding resonance Raman bands. Actually, no band of the resonance Raman spectra of monomeric chlorophyll in pure acetone can be ascribed to stretching of Mg ... O bonds, although under these conditions the magnesium atoms of most molecules are bound each to two carbonyl groups of acetone molecules [15, 26]. Such Mg-ligand bonds indeed assume orientations nearly perpendicular to the phorbin plane [8, 9], and are not conjugated to the main π electron system of the phorbin skeleton. Hence they are unlikely to be directly active in the present Raman spectra, which were obtained by resonance with in-plane polarized, π-π^* electronic transitions. Therefore, the additional resonance Raman component near 310 cm^{-1} most probably arises from a motion of the Mg-N_4 grouping, probably parallel to the phorbin plane [15]. This spectral variation, as well as those affecting other nitrogen and magnesium-sensitive bands upon aggregation, should then arise as a consequence of, and should be taken as indicative of, a distortion of the conformation of the Mg-N_4 group when the coordination number of magnesium is lowered from six to five [14].

(1.2) *Chlorophyll b.* Resonance Raman spectra of chlorophyll *b* in monomeric, oligomeric and hydrated polymeric states at 35 °K are reproduced in Fig. 4. These spectra will not be described in detail, but only the characteristics which may be of help in interpreting spectra of chlorophyll *b* in vivo. In fact, many bands of the latter, particularly in the region of stretching modes of phorbin, contain contributions from carotenoids at low temperature and are not easily compared to their in vitro homologues (see lower).

(1.2.1) Carbonyl stretching region (1620–1750 cm^{-1}): Stretching motions of the ketone and formyl carbonyl groups of monomeric chlorophyll *b* at 35 K give rise to resonance-enhanced Raman bands at 1700 and 1664 cm^{-1}, respectively, in desiccated samples, and at 1692 and 1661 cm^{-1}, respectively, in acetone solutions (Fig. 5F).

(Chlorophyll $b)_n$ oligomers in the solid state yield a broad asymmetric band with maximum intensity at 1631 cm^{-1}, which is attributed to formyl C = O groups bound to magnesium of other chlorophyll *b* molecules (Fig. 5B). The homologous band was observed at 1622 cm^{-1} for solutions in non-polar solvents at room temperature, a fact suggesting different structures for oligomers obtained by these two methods [15]. The band expected near 1650 cm^{-1} from stretching motion of ketone carbonyls bound to magnesium probably remains buried in the high frequency side

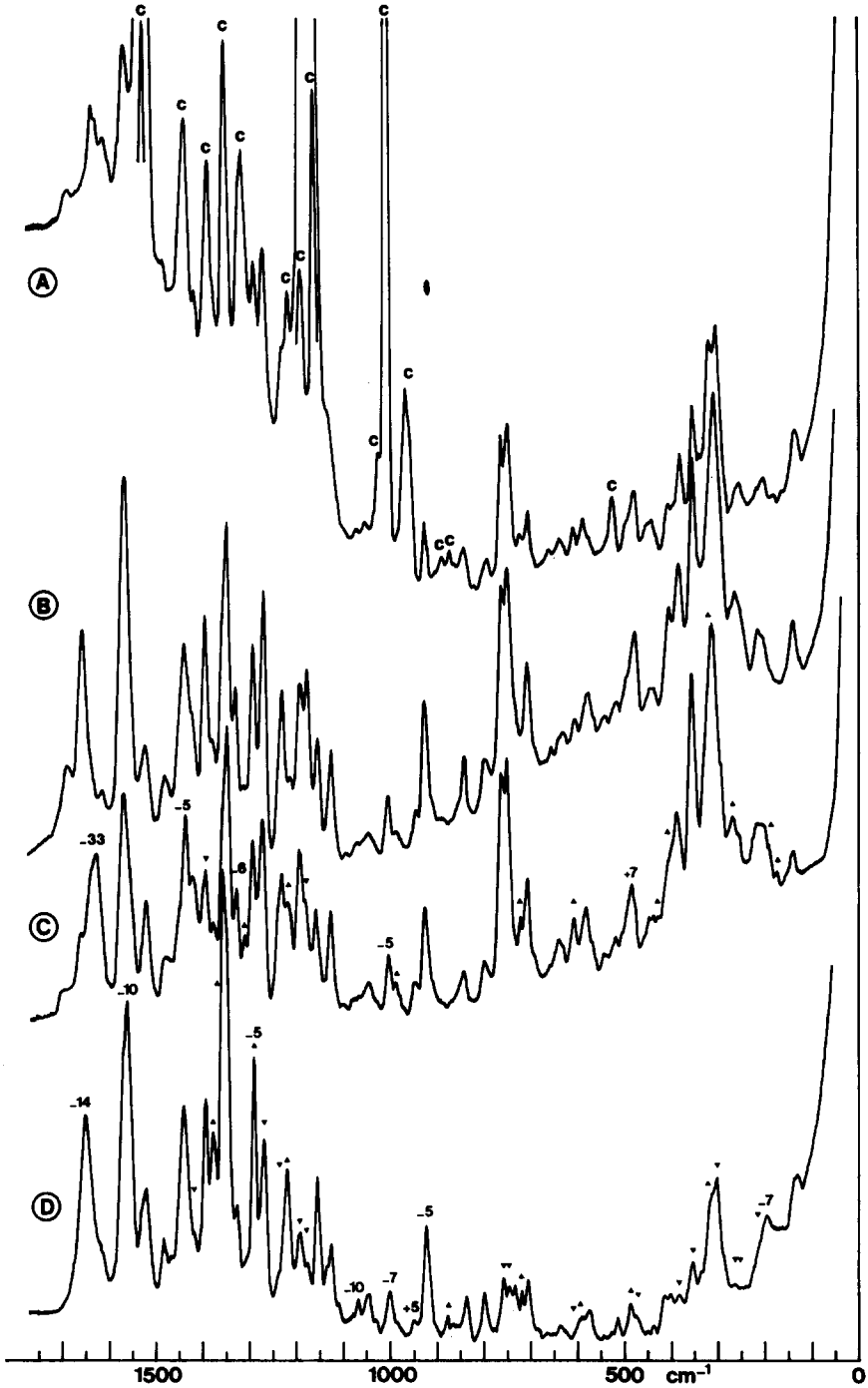
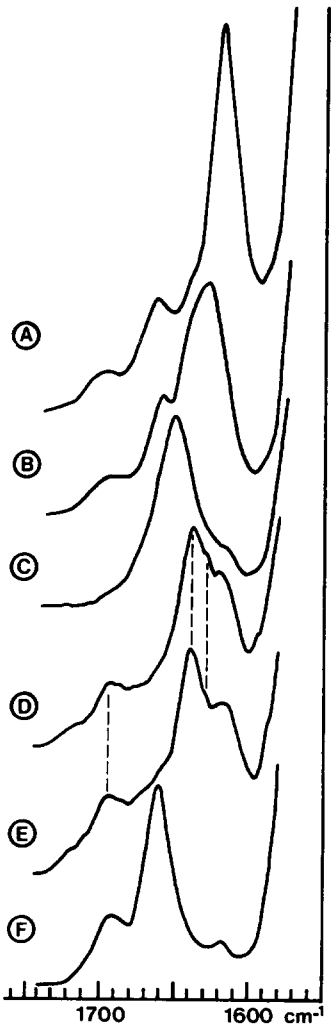


Fig. 4. Resonance Raman spectra of chlorophyll *b* at 35 K. A, in intact *C. vulgaris*, excitation 465.8 nm. C labels bands involving significant contribution from carotenoids. B, monomer in acetone, excitation 457.9 nm. C, oligomers, desiccated solid deposit, excitation 488 nm. D, hydrated polymers in cyclohexane, excitation 496.5 nm. Resolution at 1000 cm^{-1}: A, B, 7 cm^{-1}; C, D, 6 cm^{-1}. Numbers and arrows as in Fig. 1C.



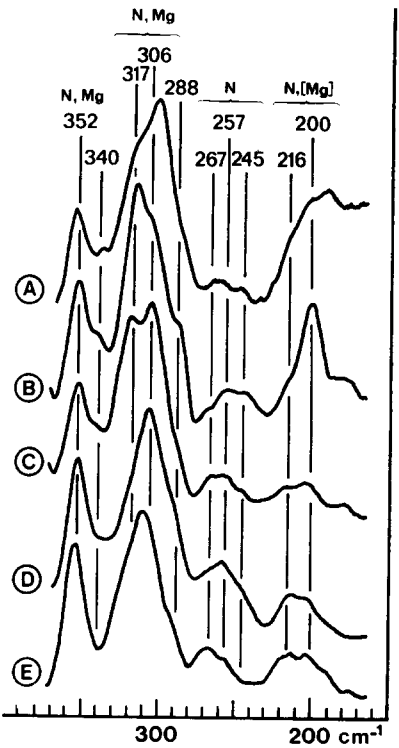


Fig. 5. Resonance Raman spectra of chlorophyll *b*, region of carbonyl stretching modes, averaged by summation. A, solution in carbon tetrachloride, 10^{-2} M, room temperature, excitation 488 nm. B, oligomers, desiccated, solid deposit, 35 K, excitation 488 nm. C, hydrated polymers in cyclohexane, 35 K, excitation 496.5 nm. D, in whole chloroplasts of spinach, 35 K, excitation 465.8 nm. E, in intact *C. vulgaris*, 35 K, excitation 465.8 nm. F, monomer in acetone, 35 K, excitation 457.9 nm. Resolution: A–C, 6 cm^{-1}; D–F, 7 cm^{-1}.

Fig. 6. Resonance Raman spectra of chlorophyll *b*, 200–400 cm^{-1}, averaged by summation, 35 K. A, hydrated polymers in cyclohexane, excitation 488 nm. B, in whole chloroplasts of *H. vulgare*, excitation 472.7 nm. C, in whole chloroplasts of *C. vulgaris*, excitation 465.8 nm. D, monomers in acetone, excitation 457.9 nm. E, oligomers, desiccated, solid deposit, excitation 488 nm. Resolution: A, B, E, 7 cm^{-1}; C, D, 8 cm^{-1}. Frequencies (cm^{-1}) indicated on top refer to spectra B and C. N, Mg: bands shifted on corresponding isotope substitution [16].

of the 1631 cm^{-1} band, which also includes a 1665 cm^{-1} shoulder arising from free formyl C = O groups. Comparing Figs. 5A and 5B suggests that a higher proportion of molecules may have their ketone C = O groups bound to magnesium in solid

samples at 35 K than in carbon tetrachloride solutions at room temperature.

Hydrated aggregates of chlorophyll *b* yield a broad, complex band peaking at 1650 cm^{-1}, in place of the 1645 cm^{-1} band observed at room temperature (Fig. 5C). Spectra obtained by illumination in the 482 nm electronic band of these aggregates show that no detectable amount of either ketone or formyl C = O groups remain free.

(1.2.2) 750–1620 cm^{-1} region: None of the several differences occurring in this region between resonance Raman spectra of chlorophyll *b* in its monomeric and self-associated states at low temperature can be reliably used in interpreting in vivo spectra (Figs. 4B and 4C). At 35 K, spectra of hydrated aggregates of chlorophyll *b* differ from those of the monomer by several features, including downshift of a band at 1572 cm^{-1} by 10 cm^{-1}, decrease in relative intensities of bands at 1420, 1235, 762 and 748 cm^{-1}, relative enhancement of bands at 1380, 1222, 735 and 720 cm^{-1} (Fig. 4D).

(1.2.3) 50–750 cm^{-1} region: Resonance Raman spectra of monomeric chlorophyll *b* dissolved in acetone, at 35 K, differ from those obtained at room temperature by the presence of a band at 215 cm^{-1} formerly thought to be characteristic of oligomeric samples [13], and by position of the Mg-N_4 band at 307 cm^{-1} in place of 300 cm^{-1}. Self-aggregated chlorophyll *b* in the solid state at 35 K yields additional bands at 430, 268 and 190 cm^{-1}, with respect to monomer at low temperature (Figs. 4 and 6E). The 300 cm^{-1} band of the monomer further shifts up to 311 cm^{-1}. This results in partial masking of a shoulder near 320 cm^{-1}. Some differences thus appear with the spectral characteristics previously attributed to chlorophyll *b* self aggregated in carbon tetrachloride at room temperature [15]. This again suggests different structures for these two types of aggregates, as did the observations in the C = O stretching region. Nevertheless, spectral variations with respect to monomers occur for both types near 310 and 190 cm^{-1}, most probably arising from pentacoordination of the magnesium atoms in both cases.

Spectra of (chlorophyll $b \cdot nH_2O)_m$ aggregates are readily differentiated from those of monomeric samples, at low temperature, by additional components at 600, 488, 400, 340 and 190 cm^{-1}, as well as by relative weakening of bands at 475, 385 and 245–260 cm^{-1} (Figs. 4 and 6A). These aggregates also yield a prominent shoulder at 317 cm^{-1} together with a band at 306 cm^{-1}.

(2) Resonance Raman spectra of antenna chlorophylls

At 35 K, the highest selectivity in producing resonance Raman spectra of chlorophyll *a* included in chloroplast membranes with respect to the contributions of chlorophyll *b* and of carotenoids is obtained at 441.6 nm, while 465.8 and 472.7 nm were the best wavelengths for selecting for chlorophyll *b* (Figs. 1 and 4). Both series of spectra include strong contributions from resonating modes of carotenoids, however. In spectra of antenna chlorophyll *a*, the carotenoid resonances are limited to a few bands in the 950–1550 cm^{-1} region, and have the sole disadvantage of occulting chlorophyll *a* bands with high C···N bond stretching participation in the 1100–1200 cm^{-1} region. Spectra of chlorophyll *b* include carotenoid bands unobserved at room temperature, which is possibly related to modifications in the electronic structure of the carotenoids occurring on cooling [27]. Most of these bands are observed in the 950–1500 cm^{-1} region and closely correlate with lattice or group modes previously observed in vitamin A-type molecules [28]. Some of them, however, particularly in

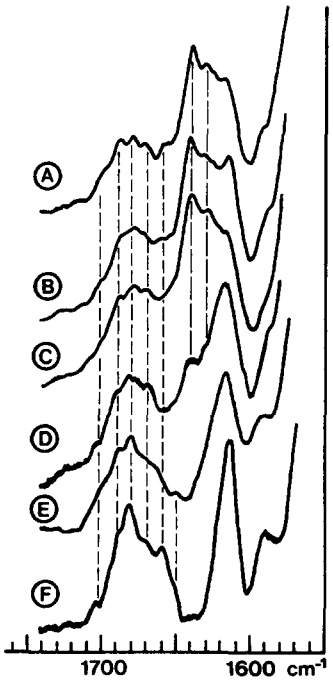


Fig. 7. Resonance Raman spectra of antenna chlorophyll *a*, region of carbonyl stretching modes, averaged by summation, 35 K, in intact chloroplasts of: A, spinach; B, *C. vulgaris* (whole cells); C, normal barley; D, greening maize; E, mutant barley lacking chlorophyll *b*; F, *B. alpina* (whole cells). Excitation wavelength: 441.6 nm. Resolution: A, B, D, 5 cm^{-1}; C, E, F, 7 cm^{-1}. Long dashed lines refer to stretching modes of 3-C = O groups of chlorophyll *b*.

the low frequency region, depend on the nature of the carotenoids present and may contain information on carotenoid states in vivo.

(2.1) *Antenna chlorophyll a.* A resonance Raman spectrum representative of those obtained from chloroplasts of green plants and of monocellular algae at 35 K under 441.6 nm illumination is reproduced in Fig. 1A [14]. Comparing these spectra with those of chlorophyll *a* and *b* in vitro shows that in addition to carotenoid bands, bands of chlorophyll *a* alone occur in the regions of phorbin vibrations.

(2.1.1) Carbonyl stretching region (1550–1750 cm^{-1}): Fig. 7 reproduces the carbonyl stretching regions of resonance Raman spectra of six different organisms at 35 K under 441.6 nm illumination.

Spectra of spinach chloroplasts and of *Chlorella* present two medium intensity components at 1630 and 1640 cm^{-1}, which are reduced to two weak shoulders at the same positions in spectra of maize mesophyll chloroplasts having the chlorophyll *a*/chlorophyll *b* ratio increased to over 6 by growth under flashing light. Furthermore, spectra of chlorophyll *b*-free materials, namely *Botrydiopsis* and the barley mutant, do not contain any band of significant intensity either at 1630 or at 1640 cm^{-1}. These two bands present in the spectra of the chlorophyll *b*-containing species are thus unlikely to involve any significant participation from 9-C = O groups of chlorophyll *a*, but only, as confirmed in section 2.2.1, from carbonyl groups of chlorophyll

*b**. Bands near 1585 and 1615 cm^{-1} correlate with bands arising from C = C stretching motions of the phorbin skeleton in spectra of chlorophyll *a* in vitro.

Thus, the stretching modes of the 9-C = O groups of all of the antenna chlorophyll *a* of all the materials studied give rise to bands confined in a cluster between 1650 and 1705 cm^{-1}. In particular, no significant number of these groups vibrate at frequencies characteristic of hydrated aggregates of chlorophyll *a*, i.e. 1639–1641 cm^{-1}.

Cooling brings partial resolution of these clusters for spinach and *Botrydiopsis*, and the observed frequencies correlate one by one within experimental uncertainty (Fig. 7 and Table I). In the less resolved spectra of *Chlorella*, barley and greening maize, shoulders on the main 1680–1682 cm^{-1} component appear to match closely with the preceding features.

(2.1.2) 700–1600 cm^{-1} region: All of the organisms studied yield resonance Raman spectra which, in this region of stretching and angular motions of the phorbin skeleton of chlorophyll *a*, are virtually indistinguishable from those of monomeric chlorophyll *a* at low temperature (Fig. 1). Neither splitting nor broadening occur for any of these bands, indicating very similar environments for all chlorophyll *a* molecules in antenna.

In particular, they present none of the spectral characteristics found for hydrated aggregates. Relative intensities and frequencies of bands at 1587, 1560, 1392–1380, 1293, 1270, 1050, 924 cm^{-1}, as well as in the 695–805 cm^{-1} region do not coincide with those of the hydrated aggregates.

In vivo spectra also depart from those of $(\text{chlorophyll } a)_n$ oligomers at room and low temperatures, having a higher frequency and a lower relative intensity of a band at 1293 cm^{-1}, the absence of any component near 1230 cm^{-1}, as well as of a medium band at 750 cm^{-1}, and a higher relative intensity of the 695 cm^{-1} band relatively to the 705 cm^{-1} component (Fig. 1).

Differences with respect to resonance Raman spectra of chlorophyll *a* in polar solvents at room temperature occur in the relative intensities of the 1380–1392 and 1331–1350 cm^{-1} pairs of bands, and in the presence of a 747–755 cm^{-1} doublet in vivo. This suggests that the electronic intermolecular interactions assumed by antenna chlorophyll *a* are closer to those encountered for monomers in solid samples than for monomers in concentrated solutions at room temperature.

(2.1.3) 50–700 cm^{-1} region: resonance Raman spectra of antenna chlorophyll *a* do not coincide as a whole, in this particular region, with any of the resonance Raman spectra of chlorophyll *a* in vitro. Aggregation-sensitive bands may be separated into two classes. The first class includes bands at 572, 474, 445, 395, 292 and 261 cm^{-1}, which occur in vivo at positions and with relative intensities very close or identical to those found for monomeric chlorophyll *a* at low temperature. The second class includes bands at 375, 309–318 and 212 cm^{-1} (Fig. 3). The 375 cm^{-1} band occurs at the same position as found in microcrystals spectra, 5 cm^{-1} lower than in the spectra of $(\text{chlorophyll } a)_n$ and of monomeric chlorophyll *a*. The 318 cm^{-1} band

* There is the possibility that vibrational forms of chlorophyll *a* are specific to antenna structures containing chlorophyll *b* and absent from organisms devoid of chlorophyll *b* [29, 30]. Absorption spectra indicate that no electronic spectral form of antenna chlorophyll *a* is actually lacking in these spectra [2]. Thus, a fortiori, no vibrational form of chlorophyll *a* should be lacking in chlorophyll *b*-free material.

TABLE I

RESONANCE RAMAN FREQUENCIES OBSERVED IN THE CARBONYL STRETCHING REGION FOR CHLOROPHYLL *a* AND CHLOROPHYLL *b* IN INTACT CHLOROPLASTS AT 35 K

Frequencies in cm^{-1} · m, medium relative intensity; w, weak; v, very; e, extremely; sh, shoulder; b, broad; exc, excitation wavelength.

Spinach		*Chlorella*		Barley	Greening maize	Barley mutant chlorophyll *b* free	*Botrydiopsis*	Attribution
exc. 465.8 nm	441.6 nm	465.8 nm	441.6 nm	441.6 nm	441.6 nm	441.6 nm	441.6 nm	
1618 m	1618 nm	1616 m	1615 nm	1616 m	1616 m	1616 m	1614 m	phorbin, ν (C_a-C_m)
	1625 vwsh		1625 vwsh	1625 swsh				phorbin
1629 sh	1631 sh	1630 wsh	1630 sh	1629 sh	1629 wsh			Chl *b*, ν (3-C = O)
							1638? ew	
1639 m	1640 m	1640 m	1641 m	1641 m	1641 sh			Chl *b*, ν (3-C = O)
		1655 bsh						Chl *b*, ν (9-C = O)
			1654? ew		1655 vwsh	1651 vw	1651 vwsh	Chl *a*, ν (9-C = O)
	1660 sh		1662 vw			1663 wsh	1660 m	Chl *a*, ν (9-C = O)
	1671 w		1670 wsh	1670 w	1669 sh	1679 vw	1670 sh	Chl *a*, ν (9-C = O)
	1680 w		1680 w	1679 w	1682 w	1681 m	1683 m	Chl *a*, ν (9-C = O)
1686? ew		1685? ew						Chl *b*, ν (9 C = O)
	1689 w		1688 sh	1688 w	1691 sh	1688 sh	1691 sh	Chl *a*, ν (9-C = O)
1694 w		1695 w						Chl *b*, ν (9-C = O)
	1700 sh		1700 sh	1701 vwsh	1703 sh	1700 wsh	1704 sh	Chl *a*, ν (9-C = O)

is broader than observed for monomeric chlorophyll *a* and is clearly bipartite, containing a shoulder at 309 cm^{-1}. A sharp band occurs in vivo at 212 cm^{-1}, close to a 218 cm^{-1} band of the microcrystals. Dry (chlorophyll *a*)$_n$ oligomers do not yield this band, and monomeric chlorophyll *a* presents a broad, weaker component in this region. This latter class includes bands with recognised (309–318 cm^{-1}) or suspected (212 cm^{-1}) participation of Mg-N_4 modes. The other band known to involve such modes, at 355 cm^{-1}, is only weakly sensitive to interaction state of chlorophyll *a*.

The presence of a 309 cm^{-1} component and of a medium intensity band at 212 cm^{-1} are taken as evidence that the magnesium atoms of many antenna chlorophyll *a* molecules are in pentacoordinated states. Moreover, similarities with room and low temperature spectra of hydrated aggregates of chlorophyll *a* (bands at 375, 292 and 212 cm^{-1}) rather than with those of dry oligomers suggest that the coordination characteristics of the magnesium atoms of antenna chlorophyll *a* are closer to that occurring for hydrated aggregates. As in these assemblies, the fifth, external ligand bound to magnesium could be water for many molecules.

However, some differences in the structure of the 309–318 cm^{-1} band and in the relative intensity and frequency of the band near 215 cm^{-1} were observed between the resonance Raman spectra of the different organisms [14]. These might indicate that different proportions of antenna chlorophyll *a* in these organisms could present differences in characteristics of bonding of the fifth ligand on magnesium, and, possibly, in its nature.

Bands of the first class defined above, which do not involve Mg-N_4 modes, except perhaps for the band at 292 cm^{-1}, indicate that the degree of interaction between antenna chlorophyll *a* molecules is close to that encountered in samples of concentrated monomeric chlorophyll *a* at low temperature. This reinforces the conclusion based on the interaction-sensitive bands of the 700–1600 cm^{-1} region.

(2.2) *Antenna chlorophyll b.* (2.2.1) Carbonyl stretching region (1550–1750 cm^{-1}): The 1550–1750 cm^{-1} regions of the resonance Raman spectra of spinach and *chlorella* under 465.8 nm illumination are reproduced in Fig. 5, and the frequencies observed are listed in Table I. In spectra of spinach at room temperature, we previously attributed the strongest bipartite band of this region, at 1635–1645 cm^{-1}, chiefly to stretching motion of the 3-formyl carbonyl group, because this group gave rise, in spectra of monomeric chlorophyll *b*, to a band about four times more intense than that attributed to stretching of the ketonic 9-C = O group [13]. On the same basis, we attribute the 1630 and 1640 cm^{-1} components of the 35 °K spectra to stretching modes of the 3-formyl C = O groups of chlorophyll *b*, downshifted from the 1668 cm^{-1} frequency of their free state by extramolecular bonding. The relative intensities of these two components are in reverse order with respect to their room temperature values, possibly because of changes in electronic absorption properties of chlorophyll *b* in vivo consequent upon cooling. A relative enhancement of the 1617 cm^{-1} skeletal band also observed on cooling probably results from enhanced contribution of a weakly active mode of carotenoids, recognized at this position in vitro. The 1695 cm^{-1} bands present in spectra of both samples are unambiguously attributed to stretching vibrations of the free 9-ketone C = O groups of chlorophyll *b*, while the weak features at about 1655 and 1685 cm^{-1} could arise from other 9-C = 0 groups interacting with undetermined partners.

As observed for 9-C = O stretching frequencies of antenna chlorophyll *a*, a

remarkable correlation is found between the carbonyl stretching frequencies of chlorophyll *b* in spinach chloroplasts and those of chlorophyll *b* in *Chlorella.*

(2.2.2) 760–1620 cm^{-1} region: As noted above, resonance Raman spectra of chlorophyll *b* in vivo obtained at low temperature are partly obscurred in this region by bands from carotenoids and, thus, cannot easily be compared to spectra of chlorophyll *b* in vitro. Differences are seen, however, upon comparison with spectra of hydrated aggregates. These include the position of a band at 1572 cm^{-1}, presence of bands of chlorophyll *b* at 1425 and at 1235 cm^{-1}, and high relative intensity of bands at 749 and 762 cm^{-1} in in vivo spectra (Fig. 4). Room temperature spectra of chlorophyll *b* in spinach present some similarities with spectra of monomeric samples. These include the weak relative intensity of a shoulder at 1425 cm^{-1} with respect to those of bands at 1397 and 1440 cm^{-1}.

(2.2.3) 50–700 cm^{-1} region: As for chlorophyll *a*, aggregation-sensitive bands of chlorophyll *b* in vivo found in this region may be divided into two classes. The first class contains bands which present the same characteristics as their homologues in spectra of monomeric chlorophyll *b* at low temperature. These are located at 606, 476, 450, 405–380 and 352 cm^{-1}. Bands at 606 and 450 cm^{-1} are absent from spectra of chlorophyll *b* in polar solvents at room temperature [15]. The second class consists in bands with characteristics close to those found in spectra of hydrated aggregates at low temperature. These are bands at 340, 317–303 and 200 cm^{-1}. As for chlorophyll *a*, the 303 and 317 cm^{-1} bands involve modes of the Mg-N_4 grouping, and the 200 cm^{-1} band is likely to involve such modes [16]. Bands of this second class are indicative of pentacoordination of the magnesium atoms of many of antenna chlorophyll *b* molecules. As for antenna chlorophyll *a*, the structural state of these Mg-N_4 groupings should be closely similar to that encountered in hydrated aggregates of chlorophyll *b*.

The resonance Raman spectra of spinach, barley and *Chlorella* obtained at 472.7 and 476.5 nm show relative enhancement of bands at 317, 245 and 200 cm^{-1} with respect to bands at 303 and 257 cm^{-1}, when compared to spectra obtained at 457.9 and at 465.8 nm (Figs. 6B and 6C). This suggests that, in addition to the pool of chlorophyll *b* molecules with pentacoordinated magnesium atoms, another pool, characterized by a strong band at 306 cm^{-1} and a weak or absent 317 cm^{-1} component, as well as by a Soret band slightly blue shifted with respect to the former, could have hexacoordinated magnesium atoms. Molecules of this latter pool could have their 3-C = O groups vibrating at 1630 cm^{-1}.

Finally, significant differences are found in the 50–700 cm^{-1} region between spectra of chlorophyll *b* in vivo and those of $(\text{chlorophyll } b)_n$ oligomers at low temperature. These are the positions of bands at 476 cm^{-1} (oligomers: 485 cm^{-1}), 380 cm^{-1} (387) and 134 cm^{-1} (140), as well as a different structure for the 200 cm^{-1} band. It thus appears probable that the presence of a nitrogen-sensitive, aggregation-sensitive band at 720 cm^{-1} merely arises from pentacoordination of magnesium atoms (Fig. 4).

DISCUSSION

Environmental heterogeneity of antenna chlorophyll a and chlorophyll b

Stretching modes of the 9-C = O groups of chlorophyll *a* included in photo-

synthetic membranes give rise to a complex cluster of Raman bands. This cluster is about 50 cm^{-1} wide at both room and low temperature and is resolved into five to six components for some organisms at 35 K (Fig. 7). Two distinct components are also assigned to stretching modes of the formyl carbonyl of chlorophyll *b*. In vitro studies at both room and low temperature have shown that resonance Raman bands arising from stretching of carbonyl groups of chlorophyll *a* and of chlorophyll *b* do not exhibit any observable structure at 5 cm^{-1} resolution, provided that a monodisperse pool of these molecules is examined. Thus, the complexity observed in vivo cannot be ascribed to an intrinsic complexity of the $\nu(C=O)$ bands of chlorophyll, either at room or at low temperature. Splitting of bands by interaction coupling effects is also unlikely, because no such phenomena were observed in vitro, even for the strongly interacting chlorophyll molecules involved in microcrystalline aggregation, and because no skeletal band of chlorophyll in vivo presented an increased complexity.

Resonance Raman spectra obtained at 35 K from chloroplasts of various origins are thus demonstrative of the existence in vivo of a limited number of different and discrete interaction states assumed by the 3- and 9-C = O groups of chlorophyll *b* and *a*, respectively. Five to six distinct environmental subspecies of chlorophyll *a* are thus evidenced in vivo. These numbers clearly are minimum values. However, the actual number of subspecies should not be much higher, because the carbonyl cluster actually could be resolved in some cases, and because the frequencies of its components closely matched for different organisms, despite variations in relative intensities.

These results bring qualitative, independent confirmation to spectroscopic studies of the Q_y electronic bands of chlorophyll *a* in vivo, which generally have been interpreted as indicative of several interaction subspecies among these molecules [1–4]. Four to six major forms of chlorophyll *a* and one or possibly two forms of chlorophyll *b* have been distinguished from electronic spectra [2–4, 31]. Although these numbers are close to those of the vibrational forms, the existence of one by one correlations between absorption subspecies and vibrational subspecies is by no means necessary, since differentiation between forms in each set occurs because of interactions of different origin. Even the chlorophyll *a* molecules having their 9-C = O groups vibrating around 1700 cm^{-1} cannot be identified with the "free" 662 nm form. For example, one electronic form of chlorophyll *b* in vivo may absorb at 640 nm, a wavelength characteristic of a "free state", but no sizable amount of chlorophyll *b* molecules observed in resonance Raman spectroscopy appears to have their 3-C = O groups free from interaction.

Universality of forms of environmental interactions in vivo

Resonance Raman spectra of chloroplasts of five different green plants and algae yield identical values, within experimental uncertainty, for the stretching frequencies of each class of 9-C = O groups of antenna chlorophyll *a*, as well as for those of the 9-C = O and 3-C = O groups of chlorophyll *b*. This strongly suggests that the same sets of environmental interactions differentiate the various classes of antenna chlorophyll in all of these organisms. Differences in relative intensities of the $\nu(C=O)$ components are also observed among spectra of the different organisms. These most likely arise from different proportions of chlorophyll molecules assuming a given set

of interactions. Several universal forms of chlorophyll in vivo were also evidenced by their electronic spectra [2, 4]. The existence of such universal electronic forms thus finds further support at the level of bonding interactions; although, in line with remarks of the preceding section, it is not a necessary consequence of the universality of vibrational forms.

Nature of environmental forms of antenna chlorophyll

Antenna chlorophyll molecules most probably assume certain properties of mutual orientation [1, 32–34]. These properties must be determined by sets of bonding interactions with surrounding molecules which, as shown by resonance Raman spectroscopy, involve the magnesium atoms and carbonyl group(s) of chlorophyll [12]. Hypotheses on the nature of the partners chemically bound to chlorophyll in vivo may be divided into two main categories. A first series of hypotheses attribute other chlorophyll molecules as principal binding partners to chlorophyll [6, 8–10]. The second series attribute foreign molecules as binding partners to chlorophyll, generally proteins [7, 30, 35]. A comparison of resonance Raman spectra of chlorophyll in vivo and in vitro permits discrimination between these two groups of proposals.

Chlorophyll a. Similarities in the absorption spectra of antenna chlorophyll *a* imbedded in photosynthetic membranes, where it occurs with high local concentration, and of chlorophyll *a* self associated in vitro have led to the assumption that antenna chlorophyll *a*, in part [10, 33] or in totality [6], should be aggregated into dimers or oligomers with a structure identical to that of (chlorophyll *a*)$_n$ oligomers in vitro, involving 9-C = O . . .Mg extramolecular bonds. Resonance Raman spectra of (chlorophyll *a*)$_n$ dimers and oligomers in various solvents at room temperature and in the solid state at low temperature showed that the 9-C = O groups bound to magnesium have stretching frequencies ranging between 1650 and 1655 cm^{-1} [13, 15]. Taking into account infrared data [24], it appears that the cumulative effects of oligomer size and of environment do not affect the stretching frequency of 9-C = O groups bound to magnesium by more than ± 10 cm^{-1} around an average 1655 cm^{-1} value. Two components of the 9-C = O cluster in vivo, at 1650 and 1660 cm^{-1}, could thus be related to the presence of (chlorophyll *a*)$_n$ oligomers in antenna structures (Fig. 2). If dimers or short size oligomers were present, 9-C = O groups unbound to magnesium should contribute to components of higher frequencies. In spectra of *Botrydiopsis* obtained at 441.6 nm, the 1665 cm^{-1} component constitutes over 25 % of the total integrated intensity of the 9-C = O cluster (Fig. 2D). Despite this fact, however, no evidence is found in the region of skeletal motions, for any of the spectral characteristics of (chlorophyll *a*)$_n$ oligomers (Results, section 2.1.2). Furthermore, excitation of spectra at wavelengths longer than 441.6 nm results in an increase of the relative intensity of the 1683 cm^{-1} component with respect to the 1665 cm^{-1} component (Fig. 2E). As observed in vitro [15], the opposite should occur if the latter component alone arises from (chlorophyll *a*)$_n$.

Hence, resonance Raman spectra of antenna chlorophyll *a* exclude the possibility that any sizable proportion of these molecules should be arranged as (chlorophyll *a*)$_n$ oligomers.

It has been recently proposed, on the basis of structural data on crystals of ethylchlorophyllide dihydrates, that antenna chlorophyll *a* could consist in (chloro-

phyll $a \cdot 2H_2O)_n$ oligomers with association numbers ranging from 2 to 8 [8]. The proposed model has a structure closely akin to that attributed to the hydrated polymers that we have studied, in line with their electronic spectra being close to those of chlorophyllide crystals [36]. Thus the properties of internal modes of vibration of these short oligomers should be close to those of the hydrated polymers. In particular, the important shift, more than 60 cm^{-1}, observed for the ν (9-C = O) frequency of chlorophyll *a* so polymerized has been attributed to H-bonding with water, modified by further bonding of the water to the magnesium atom of another chlorophyll *a* molecule [37, 38]. This shift is thus related to the structure of the basic molecular assembly and should be insensitive to aggregate size, as also indicated by the sharpness of the ν (9-C = O) band yielded by our preparations, most probably heterogeneous in association number. Short size oligomers should then present ν (9-C = O) frequencies close to 1640 cm^{-1} as well, which actually were not observed in resonance Raman spectra of antenna chlorophyll *a*.

Moreover, stronger electronic interactions are expected between partners of the hydrated, planar dimer than between partners of the dimer in carbon tetrachloride, as indicated by differences in energies of their respective Q_y transitions [8, 19]. These should lead to greater perturbations in resonating modes of the phorbin skeleton in the Raman spectra of the hydrated than in those of the dry dimer. Actually, resonance Raman spectra of antenna chlorophyll *a* in these regions are closer to those of monomeric chlorophyll *a* at low temperature than to those of dimers of chlorophyll *a* in carbon tetrachloride (Results, section 2.1).

We thus consider that the presence of significant amounts of (chlorophyll $a \cdot nH_2O)_m$ oligomers in antenna structures may be rejected on the basis of resonance Raman data.

Hence, in antenna, chlorophyll *a* molecules are not interconnected by their magnesium atoms and keto carbonyl groups, with or without interposition of water. Then, except for a small "free" fraction possibly not involved in light gathering structures, most chlorophyll *a* molecules must be bound to foreign molecules by their 9-C = O groups. Their magnesium atoms are also most likely bound to one fifth, foreign ligand, which could be water in many cases. (Results, section 2.1.3).

Chlorophyll b. Data derived from resonance Raman spectra of chlorophyll *b* are not so clearly discriminating as for chlorophyll *a* with respect to their possible interaction states in vivo. The problem arises from the presence of the additional functional carbonyl group, which increases the number of possible oligomers of chlorophyll *b*, as well as the complexity of their resonance Raman spectra in the ν(C = O) region. Moreover, the information content of the in vivo spectra is lowered by the interference of carotenoid bands. Although keto carbonyl groups of many chlorophyll *b* molecules remain free from bonding in vivo (Results, section 2.2.1), others may give rise to unresolved components in the ν(3-C = O) cluster. All chlorophyll *b* molecules appear to have their 3-C = O groups bound and vibrating at frequencies close to those observed for oligomers in vitro. Nevertheless we can exclude the presence of important amounts of (chlorophyll $b)_n$ oligomers in vivo from the discrepancies observed between in vivo and in vitro spectra in the 1000–1600 cm^{-1} region at room temperature, and in the region of lower frequencies at low temperature.

The presence of (chlorophyll $b \cdot 2H_2O)_2$ dimers in vivo, recently proposed by Serlin et al. [9], cannot be rejected unambiguously on the basis of the present spectra,

inasmuch as some 9-C = O groups may vibrate at frequencies close to 1640 cm^{-1}, and because magnesium-sensitive bands in vivo bear similarities to those observed in (chlorophyll $b \cdot nH_2O)_m$ polymers. However, the proposed model leaves the 3-C = O groups free from bonding, in disagreement with their actual states in vivo.

Still, oligomers of chlorophyll *b* are not needed in vivo to understand the resonance Raman data. Indeed, most of the observable skeletal bands of chlorophyll *b* in vivo present the same characteristics as those of monomers at low temperature. Moreover, the characteristics of the magnesium-sensitive bands are also those found for antenna chlorophyll *a*. Finally, the shifts assumed by the ν(3-C = O) frequencies of chlorophyll *b* in vivo from their values in the free state are 25–35 cm^{-1}, and thus fall in the range of the shifts displayed by ν(9-C = O) frequencies of antenna chlorophyll *a*.

Thus, the chlorophyll *b* molecules of antenna structures are most probably not aggregated into dry nor hydrated oligomers, but are bound to foreign molecules by their 3-C = O groups. Some of them may also have their 9-C = O groups similarly bonded. A population of these molecules, which may be the 1640 cm^{-1} vibrational form, most likely have their magnesium atoms coordinated to one-fifth external ligand, which could be water, while another population could have their magnesium atoms hexacoordinated.

Nature of molecules bound to antenna chlorophyll. The nature of the foreign molecules interacting with carbonyl groups of antenna chlorophyll *a* and *b* cannot be deduced from the present Raman spectra alone. However, the 0–40 cm^{-1} range observed for the in vivo shifts with respect to the free stretching frequencies suggests an origin in hydrogen bonding [39]. The highest values might represent double H-bonding, of common occurrence for carbonyl groups [39]. Chlorophyll *a* in pure methanol indeed yields two resonance Raman bands at 1643 and 1625 cm^{-1}, that we assign to the stretching modes of 9-C = O groups H-bonded to one and two methanol molecules, respectively.

Increasing experimental evidence is becoming available that chlorophyll in photosynthetic membranes is bound to proteic moieties (Review: ref. 29). The present Raman data are entirely consistent with antenna chlorophyll being involved in such complexes. Hydrogen-bonding of carbonyl groups with different amino acids of polypeptide chains may give rise to the different vibrational subspecies evidenced in Raman spectra. Magnesium atoms may also participate by binding to amino acids either directly or with interposition of water. These interactions, together with others involving atomic groups inactive in resonance Raman spectra, as ester carbonyls and the phytil chains, should insure multibonding of antenna chlorophyll molecules to polypeptidic chains, and thus determine their relative orientations and distances.

Very similar conclusions were recently drawn from an X-ray diffraction study of a soluble antenna bacteriochlorophyll-protein complex [7]. The present work on intact light-harvesting structures provides strong support to the suggestion of Fenna and Matthews [7] that the structure of this particular complex should be representative of the general bonding state of antenna (bacterio) chlorophyll in green bacteria and in green plants and algae.

CONCLUSION

Resonance Raman spectroscopy of intact chloroplasts of green plants and

algae shows that the arrangement of chlorophyll molecules in antenna structures is more complex than assumed by most of the current models drawn from vibrational, electronic and structural data obtained on in vitro assemblies of chlorophylls. It indeed demonstrates the multiplicity of the interaction sets established between antenna chlorophyll molecules and their surroundings, and suggests the universality of these sets in higher plants and algae. Moreover, it was shown that the bonding interactions assumed by these molecules through their magnesium and carbonyl functional groups are not used in establishing chlorophyll-chlorophyll bonding but rather in binding them to foreign molecules, which may be proteins. The relative positioning of chlorophyll molecules in antenna structures is thus insured by anchoring each of them to host sites of the photosynthetic membranes, rather than by interlocking.

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