CHLOROPHYLL ASSOCIATIONS IN THE CHLOROPLAST : RESONANCE RAMAN SPECTROSCOPY.

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SUMMARY. Resonance Raman (RR) spectra of spinach chloroplasts and grana are described. By varying the excitation wavelength, selective, individual enhancement of chlorophyll a, chlorophyll b and carotenoids contributions is evidenced. A comparison with RR spectra of chlorophyll solutions allows a preliminary description of chlorophyll a and b associations (*) with their environment.

INTRODUCTION. Selective excitation of Resonance Raman (RR) spectra of chlorophylls in their native environment was recently evidenced (1). Further, in vitro studies on chlorophylls and related molecules have shown that RR spectroscopy yields the same amount of information about their aggregation (*) states as IR spectroscopy does (2). We present here the first results on chlorophyll association states in spinach chloroplasts obtained from RR spectroscopy.

METHODS. Spectroscopic methods have been described elsewhere (1). Besides the argon laser excitation, an helium-cadmium continuous laser (4416 Å) was used for some experiments.

Whole or fragmented chloroplasts, extracted from spinach leaves (3), were suspended in water, or in aqueous solutions of polysaccharide (FICOLL), and carefully degassed.

RESULTS. Summary of in vitro studies. When excited in the 4400-5200 Å region, RR spectra of chlorophyll a and b exhibit about 40 frequencies; most of them arise from tetrapyrrolic macrocycle vibrations, this grouping being responsible for the visible electronic absorption. More precisely, the Soret band system rules the resonance process in that spectral region. A number of bands corresponding to some of the substituents bound to the tetrapyrrolic macrocycle

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(*) Throughout the present article, the word association will be used to indicate an interaction of a chlorophyll molecule with an undetermined partner, while the word aggregation will more precisely designate a chlorophyll-chlorophyll interaction.

and to the cyclopentanone ring have also been identified. Among these are the ca.1690 cm^{-1} carbonyl stretch of the ketone group of both chlorophylls a and b, and the ca. 1670 cm^{-1} carbonyl stretch of the aldehyde group of chlorophyll b (polar solvents). Upon aggregation of the chlorophylls with and without water, these vibrations undergo the same frequency shifts as in the I.R. (4). The magnesium bonded aldehyde of chlorophyll b constitutes an exception, however; the carbonyl stretch is seen at 1622 cm^{-1}, in contrast to the ca. 1610 cm^{-1} value given by Katz et al (4).

Aggregation of the chlorophylls also induces characteristic spectral changes in the low frequency region. Below 700 cm^{-1}, RR spectra of aggregated chlorophyll b, with and without water, yield a set of additional, weak bands, as compared to the monomer spectra, around 600 , 450, 380, 317, 215 and 180 cm^{-1}. On the other hand, pheophytinization of monomeric chlorophyll b reduces the intensity of bands at 580, 435, 390, 300, 205 and 135 cm^{-1}. The situation is similar for chlorophyll a, but less clear-cut, since in anhydrous apolar solvents, only the 310 cm^{-1} frequency can be unambiguously related to aggregation, possibly in connection with lower absolute intensity of the spectra. However, chlorophyll a-water aggregates yield a set of five characteristic frequencies at 627, 522, 307, 212 and 160 cm^{-1}. A reliable correlation of these frequencies with any association type on the central magnesium of chlorophylls rather than with another associated grouping is still impossible, since relevant vibrational data on magnesium complexes are scarce, and structures of chlorophylls-water aggregates remain uncertain (4,5).

Chloroplasts. Each of the 10 excitation wavelengths available, from 4416 to 5145 Å, permitted excitation of RR spectra of chloroplasts. Three noticeable contributions only build up these spectra, namely those from carotenoids, chlorophyll b and chlorophyll a. Moreover, by varying the excitation frequency, selective amplification of each of these three components, relatively to the other two, can be achieved. As a matter of fact, the non coincidence of the electronic absorptions allows a different wavelength zone to be optimum for resonant excitation of each molecular species. Thus excitation below 4500 Å relatively enhances the chlorophyll a spectrum (fig.1), between 4500 and 4750 Å, that of chlorophyll b (1), and above, that of carotenoids.

As a preliminary check, we ran RR spectra of the two fractions obtained through water-acetone treatment of grana. The insoluble, proteinic fraction only yielded, in the same conditions of examination as for intact chloroplasts, bands arising from traces of carotenoids and from the suspension medium. The soluble fraction, containing the pigments and lipids, yielded no other bands than those of carotenoids and chlorophylls in their mono-

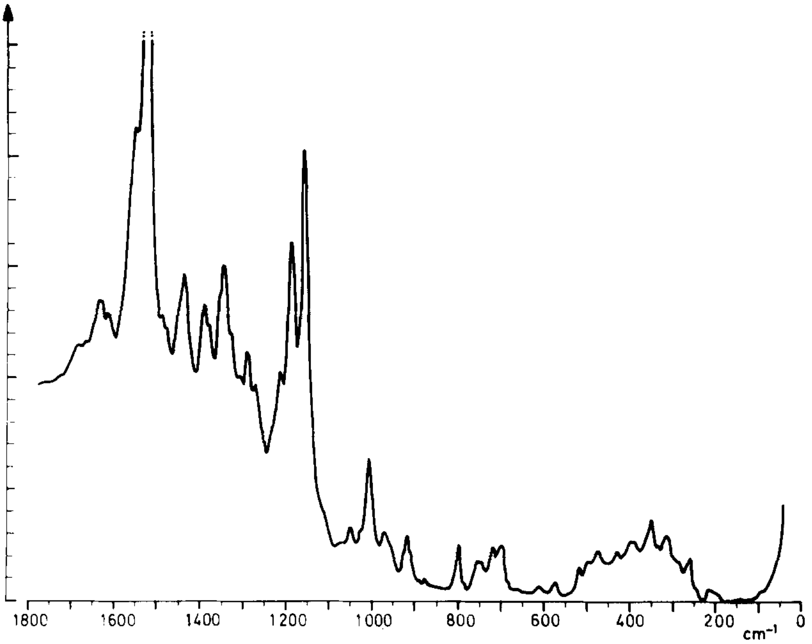

Figure 1. Resonance Raman spectrum of spinach chloroplasts under 4416 Å excitation. The chlorophyll a contribution is preponderant over that of chlorophyll b. Bands from carotenoids arise at 965, 1005, 1160, 1190 and 1527 cm^{-1}. Resolution at 1000 cm^{-1} : 8 cm^{-1}

meric states. We thus feel confident in unambiguously attributing all the vibrational frequencies here discussed to the corresponding species : chlorophyll a, chlorophyll b, or carotenoids.

Carotenoids. The spectra obtained are nearly identical to those observed elsewhere (6). Neither splitting nor significant band width increase are observed, as would be expected, since 4 different molecular species are likely to contribute significantly to the spectrum (7). This is explained by the fact that the nature of the end substituents and, a fortiori, the nature of their intermolecular associations, have a weak influence on the vibrational frequencies of the polyenic chain.

Chlorophyll b. By excitation in the 4500-4750 Å region the carotenoid spectrum is sufficiently lowered to allow clear observation of that of chlorophyll b. The two spectral regions obscured by the carotenoids cover a few planar skeletal vibrations of chlorophyll b. The chlorophyll a contribution is negligible. At room temperature, the C=O stretching region yields a broad complex band having its maximum at 1635 cm^{-1} (fig.2). In addition, a very weak band is seen at 1695 cm^{-1}, and a weak one at 1605 cm^{-1}. The latter is ascribed to a skeletal vibration, the former is attributed to free ketone stretching vibration. With the help of cooling, the main band system may be

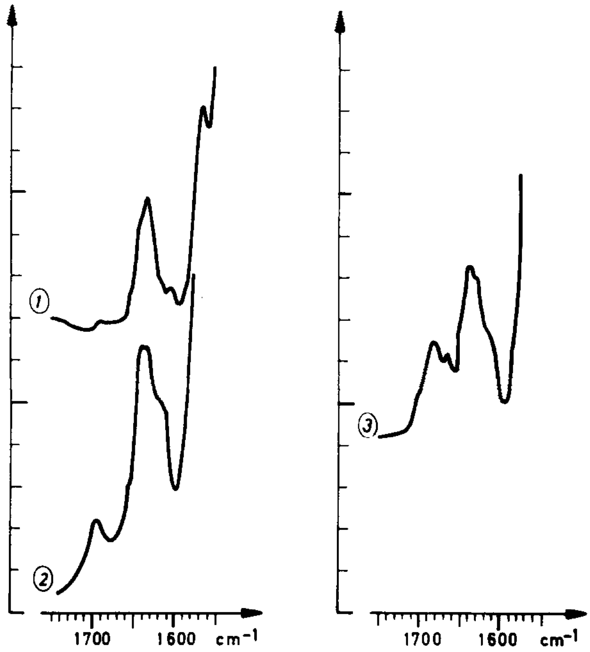

Figure 2. C = O stretching region of chloroplasts. 1. 4579 Å excitation (chlorophyll b), room temperature. Resolution 7 cm^{-1}. 2. The same, 100° K. 3. 4416 Å excitation (chlorophyll a), room temperature. Resolution 7,5 cm^{-1}.

decomposed into a weak 1650-5 cm^{-1} shoulder, attributable to an associated ketone carbonyl; another shoulder is apparent at ∼ 1645 cm^{-1}, and may correspond either to an associated ketone or to an associated aldehyde carbonyl; its relative intensity increases by cooling. The main component at 1635 cm^{-1} is attributed to an associated aldehyde. A weak shoulder, finally, is seen at ∼ 1620 cm^{-1}, and may arise, in part, from an associated aldehyde fraction. Upon heating at 80°C, the 1635 cm^{-1} cluster of bands decreases while a strong 1662 cm^{-1} free aldehyde carbonyl band appears.

In the low frequency region (fig.3), evidence is again found for associated states of chlorophyll b : by heating the chloroplasts, a set of weak bands around 605, 450, 317 and 225 cm^{-1} are reduced in intensity. As indicated above, present in vitro data do not permit to investigate Mg association state from these spectral features.

Thus RR spectra indicate that in spinach chloroplasts, most if not all chlorophyll b molecules are bonded by their C_3 aldehy$\underset{H}{d}$e group, in one or two different ways, which are both different from the C = O...Mg links in apolar solvents. A part of them have their C_9 ketone group free, and another part have it associated. The latter have their C_9 carbonyl vibrating at the same frequency as that observed for Mg-bonded C_9 keto in apolar

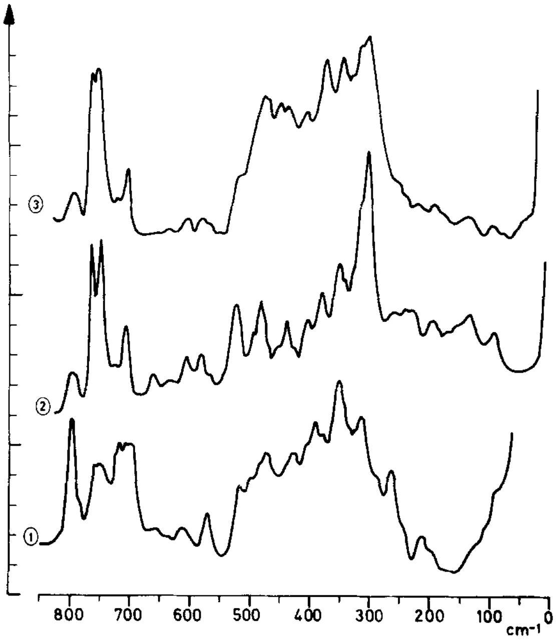

Figure 3. Lower frequencies region of chloroplasts. 1. 4416 Å excitation (chlorophyll a), room temperature. Resolution 8,5 cm^{-1}. 2. 4579 Å excitation (chlorophyll b), 50°K. Resolution 8 cm^{-1}. 3. The same, room temperature.

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solvents. This, and the presence of the low frequency association bands mentioned above, could indicate the presence of $C_9 = O...Mg$ bonds in vivo. Finally, if the 1642 cm^{-1} shoulder arises from bonded $C_9 = O$ and not from $\overset{H}{-C}=O$, another population of chlorophyll b could be associated in a way similar, but not identical, to that encountered in water-chlorophyll b aggregates in apolar solvents. At this point, it must be emphasized that, in RR spectroscopy, relative concentration evaluations from relative intensities, even between bands of the same vibrator in different association states, must be made with extreme care, since the intensity parameter prevailing here is not the concentration, but the conditions of resonance, which depend on electronic spectral changes.

Chlorophyll a. When excited at 4416 Å, chloroplasts yield a RR spectrum in which the chlorophyll a contribution is preponderant with respect to that of chlorophyll b. The C=O stretch region looks more complex than for chlorophyll b (fig.2). Two clusters of bands contain at least 7 components. A ca. 1700 cm^{-1} shoulder arises from free ketone carbonyl of chlorophyll a. Two or three frequencies at 1685, (1675) and 1660 cm^{-1} may be ascribed to H-bonded ketone of chlorophyll a. We note, however, the attribution by Katz and coll. of ca. 1660-1670 cm^{-1} IR frequencies to Mg-bonded $C_9 = O$ for concentrated chlorophylls in certain hydrocarbon solvents. The 1660 cm^{-1} is not likely to arise from the free aldehyde of chlorophyll b, since it is not seen, or extremely weak, under 4579 Å excitation. A 1650 cm^{-1} shoulder is present, on the side of a strong 1640 cm^{-1} band. Both may again be attributed to associated $C_9 = O$ of chlorophyll a, at least in part : the 1632 cm^{-1} component, which is attributed to associated aldehyde of chlorophyll b, is no longer predominant, as it was under preferential excitation of chlorophyll b. That argument must be considered with the note of caution indicated above. Finally, two shoulders at ∼1620 and 1605 cm^{-1} are attributed to skeletal vibrations of chlorophyll a. In the low frequency region, similarly as for chlorophyll b, disorganization of the chloroplast structure by mild heating lowers the intensity of bands at 610, 520, ∼ 460, 315 and 215 cm^{-1} (fig. 3). Again, the possibility of C = O...Mg bonding is suggested by the couple of 1650 and 310 cm^{-1} bands, as well as that of structures analogous to those of water-chlorophyll a adducts is suggested by the 1640 cm^{-1} band and the low frequency set. Thus in the chloroplast, together with a possible "free" fraction, chlorophyll a assumes a variety of associated states, through its C_9 ketonic function. This may likely be related to the variety of chlorophylls a evidenced by other techniques (8).

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