Sharp-line structure in the fluorescence and excitation spectra of greening etiolated leaves

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Vibrationally resolved sharp-line optical fluorescence and excitation spectra are observed for etiolated and greening barley leaves at T = 5 K. Conditions necessary for obtaining fine-structured spectra in the case of chromophores in biological cells are discussed. The observed fine-line spectra confirm earlier results that the inactive protochlorophyll F630 and initial chlorophyll(ide) forms are present as monomeric pigment molecules weakly bound to proteins, while the active holochrome F655 is an aggregated protochlorophyllide–protein complex.

Chlorophyll fluorescence *Etiolated leaf* *Low-temperature spectroscopy* *Vibrational structure* *Protochlorophyllide–protein complex* *(Barley)*

1. INTRODUCTION

Photosynthesizing cells and their fragments, such as reaction centres and antenna complexes, are widely studied by means of optical absorption, as well as fluorescence spectroscopy [1]. Several forms of chlorophyll (Chl) in vivo have been identified by the use of low-temperature spectra [1,2]. However, observed spectral bands are relatively broad and carry almost no information about the vibrational fine structure and the corresponding frequencies or linewidths. Resonance Raman spectroscopy has enabled one to measure the intramolecular vibrational frequencies for in vivo Chl and to distinguish some pigment–protein complexes [3]. Yet the activities of vibrations in Raman and optical spectra are different, and different electronic states are involved. Therefore, it is evident that resolution of vibrational fine structure in the S_1–S_0 electronic transitions would give valuable additional information on electron–vibrational interactions, especially in the S_1 excited state.

Abbreviations: Chl(-ide), chlorophyll(ide); PChl(-ide), protochlorophyll(ide)

For Chl *a* and its derivatives in solid solutions, sharp-line fluorescence spectra have been observed by means of monochromatic excitation at liquid helium temperature [4–9], and much information on vibrational frequencies, linewidths, etc. has been obtained. These studies are related to a rather general conclusion that the low-temperature spectra of most organic molecules in glassy solutions are inhomogeneously broadened, i.e., they consist of a great number of shifted homogeneous subspectra [10]. The latter may contain very narrow zero-phonon lines accompanied by broad phonon sidebands, as predicted theoretically [11].

The fluorescence bands of algae [12] as well as chloroplasts and their fragments [13–15] do not narrow essentially on cooling from nitrogen to liquid helium temperature. Attempts to obtain narrow-line spectra using monochromatic excitation of Chl in green leaves at 4.2 K [4,16] have also been unsuccessful. Some possible explanations of the lack of fine structure in the spectra of biological systems were suggested in [4,16]. First, zero-phonon lines may be absent owing to a strong electron–phonon (here 'phonons' conventionally designate low-frequency molecular vibrations) interaction involving large protein components

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bound to chromophores. Second, narrow lines, in principle present in homogeneous spectra, can be undetectable because of fast energy migration within the inhomogeneous manifold of pigment molecules (so-called spectral diffusion).

Recent observations of hole-burning (also based on the presence of zero-phonon lines) in the spectra of native phycoerythrin [17] and the quasi-line structure in the fluorescence spectra of an iron-free cytochrome *c* [18] have indicated that the pigment–protein interaction does not necessarily lead to the disappearance of zero-phonon lines.

We propose that the line structure should occur in the case of in vivo systems with low local concentration of chromophores, so that the distance between fluorescing molecules is much larger than the energy transfer radius – then spectral diffusion should be negligible. As a biological system with relatively low Chl concentration we have chosen for our study etiolated and greening leaves.

2. EXPERIMENTAL

The experimental set-up was essentially as in [9]. Fluorescence was recorded on a DFS-24 double grating spectrometer with 0.5 cm^{-1} resolution. Tunable excitation was performed with a Coherent CR-490 dye laser pumped by a CR-3 argon laser. The dye laser output was maintained constant by a feed-back to the pump laser current regulation. For exact dye laser wavelength calibration neon optogalvanic marker lines were recorded in parallel with the excitation spectra, as in [19].

Etiolated barley leaves were grown during 12–16 days at 25°C in darkness. Preillumination was performed at room temperature under diffuse daylight (≈1 $mW \cdot cm^{-2}$) immediately before cooling. For spectral measurements leaves were placed between quartz plates and suspended above the helium level, where a stable temperature of 5 K was achieved. Excitation densities of the order of 10 $mW \cdot cm^{-2}$ were used.

3. RESULTS AND DISCUSSION

The fluorescence spectrum of an etiolated barley leaf excited near the Soret band region at 5 K is presented in fig.1a. The bands F630 and F655 (denoted by their fluorescence maxima) are attributed, respectively, to photochemically inactive

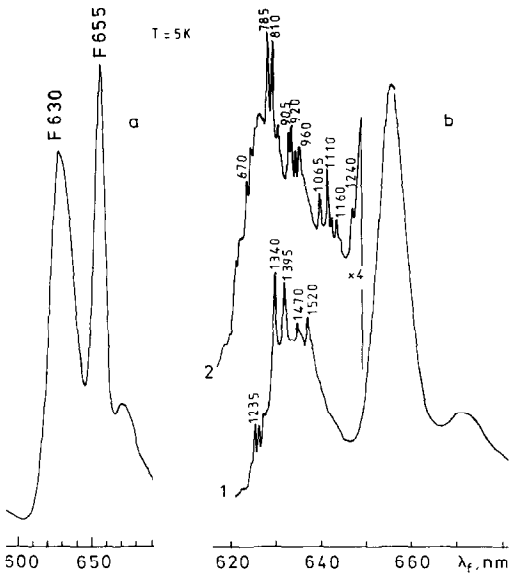

Fig.1. Fluorescence spectra of a 16-day-old barley leaf at T = 5 K under 441.6 nm Soret band excitation (a) and selective excitation within the vibrational sideband of the S_1–S_0 transition (b) at 580.9 (1) and 599.1 nm (2). Curve 2 coincides with curve 1 for $\lambda > 650$ nm. The vibrational frequencies (in cm^{-1}) in the S_1 electronic state are shown near the lines (see text). In fig.1–3, vertical scales represent fluorescence intensity in (linear) arbitrary units.

PChl(-ide) and photoactive PChl-ide holochrome (see [20] and references therein, e.g. [21,22]).

The non-convertible PChl F630 is thought to be a monomeric form weakly bound to the protein carrier [20,23]. For this band we found a sharp-line structure under monochromatic excitation within the vibrational side-band of the S_1–S_0 electronic transition (fig.1b). As is known, the excitation in the region of S_1–S_0 electron-vibrational (vibronic) transitions is a necessary condition for observing fine structure in the fluorescence spectra of inhomogeneous systems (see e.g. [9]). Besides, the distances from the excitation line to vibronic 0–0 fluorescence lines are equal to the intramolecular vibrational frequencies in the excited electronic state S_1, as indicated in fig.1. (The vibrational structure of the ground electronic state is difficult to observe owing to the overlapping emission from several Chl forms in the far-red region.) However, these frequencies are even more explicitly manifest in the monochromatically recorded fluorescence

excitation spectra (fig.2). As the samples were optically thin, the excitation spectra are to a good approximation proportional to the corresponding absorption coefficients. The relative line-to-background intensity ratio for the spectra in fig.2 is only slightly lower than in case of PChl in frozen solutions [9]. Therefore we can conclude that these spectra with their sharp-line structure are not any artefact belonging to some small amount of pigment deposited on the surface or to dissolved molecules. If the bulk of PChl were to give a structureless spectrum, the line contrast should have been very low, since fluorescence was recorded near the band maximum. Probably, similar to the case of Chl in vitro, the continuous background arises from the summation of the phonon wings (of ~100 cm^{-1} width) accompanying every sharp line.

Vibrational frequencies (± 5 cm^{-1} accuracy) as well as relative intensities of vibronic lines in the spectra of F630 are in surprisingly close agreement with those of monosolvated PChl in ether solution at 5 K [9]. Consequently, sharp-line vibronic spectra confirm the existence of F630 in etiolated leaves in the form of monomeric quasi-free molecules, probably monoligated to some protein site.

In contrast to F630, the band of photoactive PChl-ide F655 remained practically structureless even at selective excitation (fig.1). Only traces of vibrational line structure could be seen in the excitation spectra for recording wavelengths throughout this fluorescence band.

The weakness of zero-phonon lines in the spectra of F655 is consistent with models [21,22] where the photoactive holochrome is shown to consist of several PChl-ide molecules in a complex with large protein parts. Rapid energy migration in such an aggregate may cause spectral diffusion; also, the presence of hydrogen bonds [23] can result in an increased electron–phonon interaction and a related decrease of line intensities.

At initial stages of illumination F655 is converted into F674 [23] and later into several forms near 680–690 nm. In fig.3 we show the spectra of a relatively long preilluminated leaf, where the main fluorescence band is at 680 nm. As in case of F630, the excitation spectrum reveals a distinct vibronic line structure. The vibrational frequencies

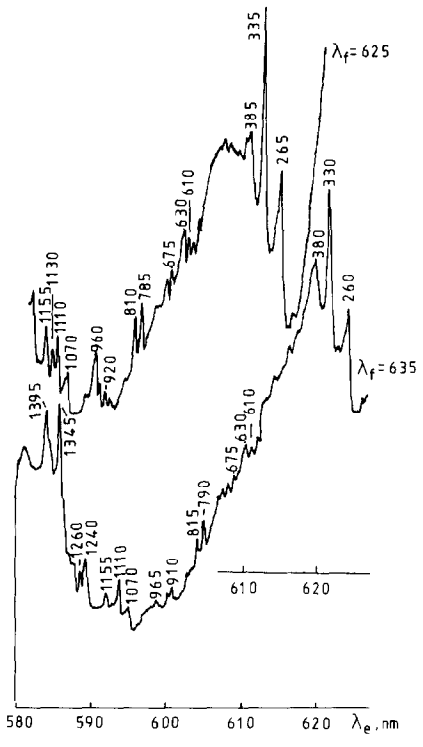

Fig.2. Fluorescence excitation spectra of the same sample as in fig.1 (T = 5 K) for narrow-band fluorescence recording at 635 and 625 nm (the latter is upshifted, as shown by the upper λ scale). Again, the S_1 state vibrational frequencies are indicated.

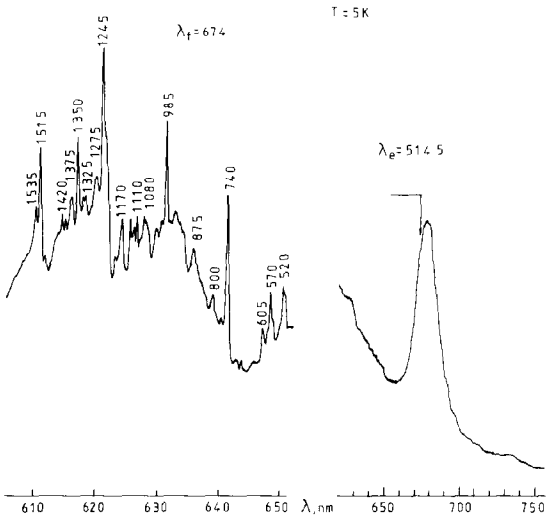

Fig.3. Spectra of a 12-day-old etiolated barley leaf measured after 3 h continuous illumination with white light of 10 $W \cdot m^{-2}$: fluorescence spectrum at 514.5 nm excitation (right) and fluorescence excitation spectrum (left) for recording at 674 nm, as indicated by the arrow.

agree with those of the Chl *a* monoligated form in solid solutions. Therefore, in agreement with [20,23], the in vivo Chl form F680 can be described as a monomer of Chl *a* ligated to lipoprotein carrier via an Mg atom. Similar spectra were obtained in different stages of greening, but as the Chl concentration increases, the lines gradually disappear. No detailed vibrational analysis was performed within this preliminary work.

The main inference of the present study is that fluorescence and excitation spectra with resolved vibrational line structure can be obtained for chromophores in plant tissue (at least in the early greening stage) by means of selective laser excitation at liquid helium temperature. Such measurements may usefully complement Raman spectroscopy results, as data concerning vibronic interactions in the excited electronic state become available. Similar to a recent coherent anti-Stokes Raman investigation [24], high spectral resolution in vibronic spectra is combined with the selectivity by S_1–S_0 electronic transition energies.

As modern laser wavelength calibration techniques enable exact measurements of frequencies, one possible extension of this work could be a detailed comparison of vibrational frequencies of different Chl forms with those in vitro depending on the degree of ligation. Also, the relaxation rates of excited state vibrational sublevels can be estimated from the corresponding homogeneous widths using the hole-burning method [9,25]. Photochemical hole-burning has already been observed on reaction centre preparations [26], but measuring of only absorption spectra restricted the usage of the technique.

Our results confirm the viewpoint that the main factors hindering the observation of vibronic sharp-line spectra for pigment molecules in biological systems are fast excitation energy migration at high concentration and, in some cases, strong complexation with the protein carrier. In view of the promising data presented here, it is quite probable that high resolution spectra can be obtained in a similar manner for other biologically important chromophores in situ.

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REFERENCES

[1] Lavorel, J. and Etienne, A.-L. (1977) in: Primary Processes of Photosynthesis (Barber, J. ed) Top. Photosynth. vol.2, pp.203–268, Elsevier, Amsterdam, New York.
[2] Litvin, F.F., Rikhireva, G.T. and Krasnovskii, A.A. (1962) Biofizika 7, 578–581.
[3] Lutz, M. and Breton, J. (1973) Biochem. Biophys. Res. Commun. 53, 413–418.
[4] Avarmaa, R. and Rebane, K. (1975) Stud. Biophys. (Berlin) 48, 209–218.
[5] Fünfschilling, J. and Williams, D.F. (1977) Photochem. Photobiol. 26, 109–113.
[6] Bykovskaya, L.A., Litvin, F.F., Personov, R.I. and Romanovski, Yu.V. (1980) Biofizika 25, 13–20.
[7] Platenkamp, R.J., Den Blanken, M.J. and Hoff, A.J. (1980) Chem. Phys. Lett. 76, 35–41.
[8] Hála, J., Pelant, I., Parma, L. and Vacek, K. (1981) J. Luminescence 24/25, 803–806.
[9] Rebane, K.K. and Avarmaa, R.A. (1982) Chem. Phys. 68, 191–200.
[10] Personov, R.I., Al'shits, E.I., Bykovskaya, L.A. and Kharlamov, B.M. (1973) JETP 65, 1825–1836.
[11] Rebane, K.K. (1970) Impurity Spectra of Solids, Plenum Press, New York.
[12] Cho, F. and Govindjee (1970) Biochim. Biophys. Acta 216, 139–150.
[13] Kochubey, S.M. (1970) Dokl. Akad. Nauk SSSR 192, 446–449.
[14] Rijgersberg, C.P., Amesz, J., Thielen, A.P.G.M. and Swager, J.A. (1979) Biochim. Biophys. Acta 545, 473–482.
[15] Kramer, H.J.M., Amesz, J. and Rijgersberg, C.P. (1981) Biochim. Biophys. Acta 637, 272–277.
[16] Rebane, K.K. and Avarmaa, R.A. (1976) in: Molecular Spectroscopy of Dense Phases – Proceedings of the 12th European Congress of Molecular Spectroscopy (Grossmann, M. et al. eds) pp.459–462, Elsevier, Amsterdam, New York.
[17] Friedrich, J., Scheer, H., Zickendraht-Wendelstadt, B. and Haarer, D. (1981) J. Chem. Phys. 74, 2260–2266.
[18] Angiolillo, P.J., Leigh, J.S. and Vanderkooi, J.M. (1982) Photochem. Photobiol. 36, 133–137.
[19] Avarmaa, R. and Suisalu, A. (1983) Eesti NSV Tead. Akad. Toimet., Füüs. Matem., in press.
[20] Raskin, V.I. (1981) Photoreduction of Protochlorophyllide (in Russian), Nauka i Tehnika, Minsk.
[21] Boardman, N.K. and Anderson, J.M. (1978) in: Chloroplast Development (Akoyunoglou, G. et al. eds) pp.1–14, Elsevier, Amsterdam, New York.
[22] Canaani, O.D. and Sauer, K. (1977) Plant Physiol. 60, 422–429.

[23] Van der Crammen, J.C.J.M. and Goedheer, J. (1981) Photobiochem. Photobiophys. 3, 159–165.
[24] Höxtermann, E., Werncke, W., Stadnichuk, I.N., Lau, A. and Hoffmann, P. (1982) Stud. Biophys. 92, 147–175.
[25] Gorokhovski, A.A. and Kikas, J. (1977) Opt. Commun. 21, 272–274.
[26] Maslov, V.G., Chunaev, A.S. and Tugarinov, V.V. (1980) Biofizika 25, 925–927.