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Biochimica et Biophysica Acta 1365 (1998) 531–540

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

The two spectroscopically different short wavelength protochlorophyllide forms in pea epicotyls are both monomeric

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Received 25 February 1998; revised 11 May 1998; accepted 13 May 1998

Abstract

The spectral properties of the protochlorophyllide forms in the epicotyls of dark-grown pea seedlings have been studied in a temperature range, from 10 to 293 K with conventional fluorescence emission and excitation spectroscopy as well as by fluorescence line narrowing (FLN) at cryogenic temperatures. The conventional fluorescence techniques at lower temperatures revealed separate bands at 628, 634–636, 644 and 655 nm. At room temperature (293 K) the 628 and 634–636 nm emission bands strongly overlapped and the band shape was almost independent of the excitation wavelength. Under FLN conditions, vibronically resolved fluorescence spectra could be measured for the 628 and 634–636 nm bands. The high resolution of this technique excluded the excitonic nature of respective excited states and made it possible to determine the pure electronic (0,0) range of the spectra of the two components. Thus it was concluded that the 628 and 634–636 nm (0,0) emission bands originate from two monomeric forms of protochlorophyllide and the spectral difference is interpreted as a consequence of environmental effects of the surrounding matrix. On the basis of earlier results and the data presented here, a model is discussed in which the 636 nm form is considered as an enzyme-bound protochlorophyllide and the 628 nm form as a protochlorophyllide pool from which the substrate is replaced when the epicotyl is illuminated with continuous light.

Keywords: Cryogenic temperature; Fluorescence; Fluorescence line narrowing; Inhomogeneous distribution function; Protochlorophyllide form

1. Introduction

The chlorophyll biosynthesis stops at the step of protochlorophyllide (Pchlide) formation in higher plants when they are kept in the dark because the NADPH-protochlorophyllide oxidoreductase (LPOR) is a light-dependent enzyme [1]. Recently, two isoenzymes of the LPOR have been identified: the LPOR-A which is accumulated in the dark and the LPOR-B which is thought to work in plants under light exposure [2,3]. The LPOR-A unit forms, together with Pchlide and NADPH, a ternary complex [4], which can assemble into multimolecular aggregates [5,6], making up the integral structures of prolamellar bodies (PLBs) [7]. Studies on dark-grown leaves or isolated etioplast inner membranes showed

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0005-2728/98/$19.00
PII: S0005-2728(98)00106-6

that Pchlide has a spectral multiplicity in vivo [8–10] which is due mainly to excitonic interactions between the Pchlide molecules [5] and to interactions with protein and NADPH [4] molecules located in the lipid environment of the etioplast inner membranes [11]. The Pchlide complexes, having different absorption and fluorescence bands are named 'spectral forms'. Circular dichroism (CD) measurements of isolated and purified membrane preparations proved that two forms, the Pchlide A_{638}-F_{644} and the Pchlide A_{650}-F_{657} (A_{xxx} indicates the position of the absorption maximum, F_{yyy} indicates the fluorescence emission maximum) contain Pchlide aggregates. The A_{628}-F_{633} form contains monomeric Pchlide or protochlorophyll [5]. The molecular organization of the A_{662}-F_{670} form has not been elucidated, but the fluorescence properties, i.e. the 463 nm excitation band [10], and the lack of a CD signal [5] indicate that it may be a large Pchlide aggregate of random geometry. Photoactivity measurements of etiolated wheat leaves showed that the aggregated Pchlide A_{638}-F_{644} and the Pchlide A_{650}-F_{657} have high phototransformation rates: they are transformed into chlorophyllide (Chlide) on a ms time scale and the reaction proceeds even at −70°C [12]. Moreover, at 77 K, photochemical intermediates are produced [13]. The monomeric Pchlide A_{628}-F_{633} form is transformed into Chlide very slowly, and only indirectly, after transforming into the above-mentioned longer wavelength forms [14].

Recent data obtained from epicotyls of dark grown pea seedlings indicates, that it may contain unusual structural forms of Pchlide connected with respective short-wavelength spectral bands in the 620–640 nm range of the fluorescence emission spectrum [15]. The experimental techniques used in the earlier works, however, did not make it possible to unambiguously correlate the spectral components with specific structural forms of Pchlide.

In this work, we took advantage of the high resolution of the fluorescence line narrowing (FLN) technique which yields fluorescence spectra resolved into vibronic components in the form of sharp lines [16]. The lines that are of purely electronic origin can be used to determine inhomogeneous distribution functions (IDF) corresponding to the pure (0,0) bands in the conventional spectra. The technique is applied at cryogenic temperature, consequently the temperature broadening of the spectral lines is reduced and the higher energy vibronic levels that normally complicate the spectra are not populated. A narrow bandwidth tunable laser is used to illuminate the sample and thus molecules with vibronic transitions equal to the photon energy of the laser light are selectively excited from the inhomogeneous population [17]. This provides high resolution to the technique, which allows detection of the (0,0) and vibronic transitions of the selected population in the form of sharp spectral lines comparable to those obtained by resonance Raman spectroscopy [18,19]. The same laser energy may excite more than one vibronic level if there are chromophores with distinct environmental perturbation within an inhomogeneous population with matching vibronic levels. In this case, the electrons from the different excited vibronic levels of the population will relax to different (1,0) levels and the emission will lead to multiple (0,0) lines with distinct (0,0) transition energies. When the laser is tuned, the same type of vibronic lines become excited in another molecule of the population and thus the corresponding (0,0) emission energy is also shifted. If the laser is tuned in the absorption band, a series of emission spectra can be recorded, where the integrated intensity of the intense (0,0) lines will follow the IDF of the molecular transition energies approximated by a gaussian curve [20]. The positions of the maxima in the IDF give the most probable (0,0) energies of the chromophore in the system, while the width gives information about the extent of conformational fluctuations in the environment at ambient temperatures [21–23]. The method requires that the excited state lifetime of the sample be in the range of 1–10 ns, or longer. Shorter excited state lifetimes characteristic of excitonic bands [18,24], lead to very broad spectral lines and lower resolution. The possibility of measuring in vivo etiolated leaves under fluorescence line narrowing conditions was first demonstrated by Avarmaa and coworkers [25]. They showed that vibrationally resolved sharp-line fluorescence spectra could be observed at 5 K in etiolated leaves in the 630 nm emission band, while in the 655 nm region sharp lines were absent due to fast energy migration within the set of pigment molecules, the components of the complex emitting here.

We show in this work that two monomeric forms of Pchlide in the pea epicotyls can be distinguished

by their IDF. We compare this result with earlier data and suggest a model concerning their molecular structures and molecular environments. This model explains not only the reasons for their spectral differences, but also the reasons for the slow rate of their recently observed phototransformation reaction [26].

2. Materials and methods

Eight-day-old dark-grown pea (*Pisum sativum L.* cv Kelvedon Wonder) seedlings were used in the experiments. At this age, the epicotyls were 10 cm long. The middle segments of the epicotyls were used for the spectroscopic studies. They were immersed in a glycerine/water (1:1, v/v) solution in a small cylindrical glass cuvette. This assured transparency and thermal contact. The cuvettes were immersed in liquid nitrogen in the dark, and held at 77 K to avoid phototransformation until placed into the cryostat for measurement.

The conventional fluorescence spectra were obtained with a FS900CD fluorometer (Edinburgh Analytical Instruments, UK) with a Xenon lamp light source. The resolution of the fluorometer was ~ 20 cm^{-1}. The high-resolution emission spectra were recorded with an FLN set-up: excitation was achieved using a Coherent 899-01 tunable dye laser with Rhodamine-590, pumped by a continuous wave Coherent Innova-307 argon ion laser (Palo Alto, CA). The power of the laser beam, measured with a Coherent FieldMaster LM-10 power meter, was attenuated to 2–3 mW by using neutral density filters (Schott Glaswerke, Mainz, Germany). The emission spectra were measured at 90° from the excitation light, using a THR-1000M monochromator (Jobin-Yvon, Longjumeau, France) with holographic grating. The detector was a cooled GaAs photomultiplier R943-02 (Hamamatsu Photonics, Japan). Fluorescence signals were collected with a photon counting unit C3866 (Hamamatsu Photonics, Japan) and Spectra link, (Jobin-Yvon, Longjumeau, France). The resolution of the FLN set-up was 2 cm^{-1}, in the measured spectral region (15 500–16 000 cm^{-1}). The sample was cooled to 10 K in a closed cycle He-refrigerator M22 (Cryophysics SA, Geneva, Switzerland), and the fluorescence line narrowing spectra were recorded at this temperature by vibronic excitation in the $Q_y(0,1)$ band. The excitation and emission spectra were corrected for the intensity variations of the excitation light and for the sensitivity of the photomultiplier. Light-scattering effects were not considered, because the registered wavelength region was short enough so as not to have significant distortion due to light scattering.

Conventionally measured fluorescence spectra and the IDF were analyzed by gaussian fitting (software SPSERV V3.14, copyright C. Bagyinka, Institute of Biophysics and Biology Research Center of the Hungarian Academy of Science). To determine starting parameters for the gaussian resolutions, the second and fourth derivatives of the spectra were evaluated. The fitting was performed on a wavenumber scale, but results of the conventional fluorescence spectra are shown re-converted into wavelength functions for convenience in the figures.

All measurements were repeated with five different plant samples. The spectra showed only insignificant variations. The spectra were reproducible and successive measurements on the same sample gave identical results. The figures of this work show representative spectra.

3. Results

3.1. Conventional fluorescence emission and excitation spectra measured in the 10–293 K temperature region

The temperature dependence of the expected (0,0) range in the fluorescence emission spectra of the pea epicotyls have been studied and is shown in Fig. 1. The room temperature spectra were of low intensity and could be measured with 8 nm optical slits. The slight excitation wavelength dependence of the emission spectra shown at room temperature became more pronounced at low temperature. The intensity of the fluorescence increased and thus spectra with low signal-to-noise ratio could be recorded with 2 nm optical slits. At room temperature, emission maxima were observed at 636 and 641 nm when the sample was excited at 440 or 460 nm, respectively. Both bands were very broad (with over 20 nm total half-bandwidths) and unstructured, only their asymmetry indicated their complexity (Fig. 1A). At 10 K, how-

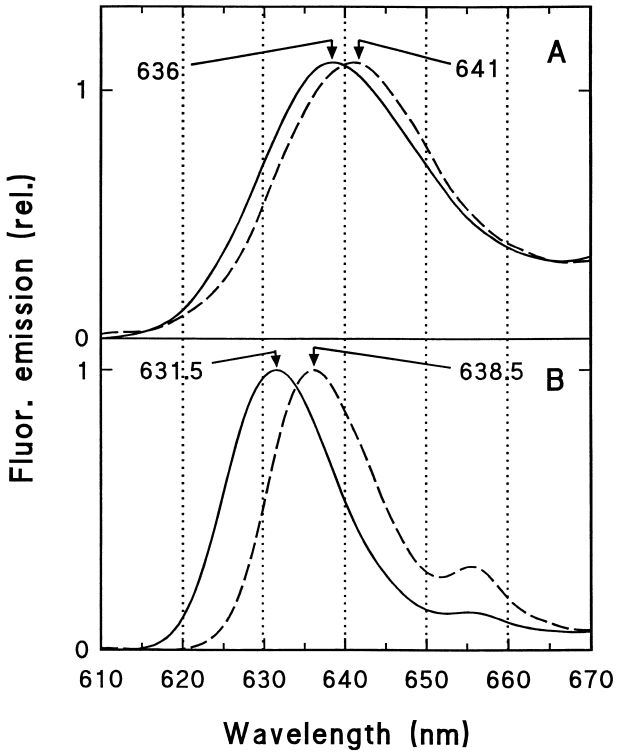

Fig. 1. Conventional fluorescence emission spectra of pea epicotyls measured at 293 K (A) and at 10 K (B). The excitation wavelength was 440 nm (solid lines) or 460 nm (dashed lines). The spectra were normalized at their maxima. The numbers in the figures indicate the positions of maxima in nm.

ever, the emission bands were narrow (with total half-bandwidths of 15 nm) and their maxima were at 631.5 and 638.5 nm with 440 and 460 nm excitation, respectively (Fig. 1B).

The gaussian deconvolution of the emission spectra measured at 10 K is indicative of the complexity of the emission bands. The fitting procedure shows that, at an excitation with 440 nm, the (0,0) range consists of two bands at 628 and 634 nm (Fig. 2A). The contribution from bands with maxima at 644 and 656.5 nm is also shown. The main emission band measured at an excitation of 460 nm contains a single (dominant) band at 636 nm and a contribution approximated by two additional low intensity bands at 646 and 656 nm (Fig. 2B). The vibronic envelope of the emission spectra of the two expected spectral components at 628 and 634 nm were measured conventionally in the longer wavelength range (above 670 nm) by exciting at 640 nm with 15 nm slitwidths. The spectrum could be resolved into two gaussian components located at 685 and 700 nm (Fig. 2C). The fitting parameters are listed in Table 1.

The emission spectra recorded at 10, 100, 200, 240, 273 and 293 K could be resolved into similar gaussian components, although, their characteristics changed in parallel with the increase of the temperature. The position of the components gradually shifted towards longer wavelengths, their half-bandwidth values increased and the integral under the components decreased (Table 2.)

Multiple Pchlide forms were also observed in the excitation spectra. The Soret regions of spectra recorded at 293 K at emission wavelengths of 625 and 640 nm were very similar, the maxima were at 444 and 446 nm, respectively (Fig. 3A). The spectra recorded at 10 K were more structured and were more dependent on the wavelength of observation. The excitation spectra at low temperature recorded at 625 and 640 nm had maxima at 440 and 446 nm, respectively (Fig. 3B).

Table 1
The positions of maxima and half-bandwidth values of gaussian components found in 10 K fluorescence emission spectra of dark-grown pea epicotyls[a]

	Maximum (nm)	Half-bandwidth (nm)
Excitation: 440 nm	628	10.2
	634	12.7
	644	13.2
	656	9.85
Excitation: 460 nm	636	12.4
	646	9.1
	656	9.5
Long-wavelength vibrational envelope bands	685	15.4
Excitation: 640 nm	700	23.0

[a]See Fig. 2.

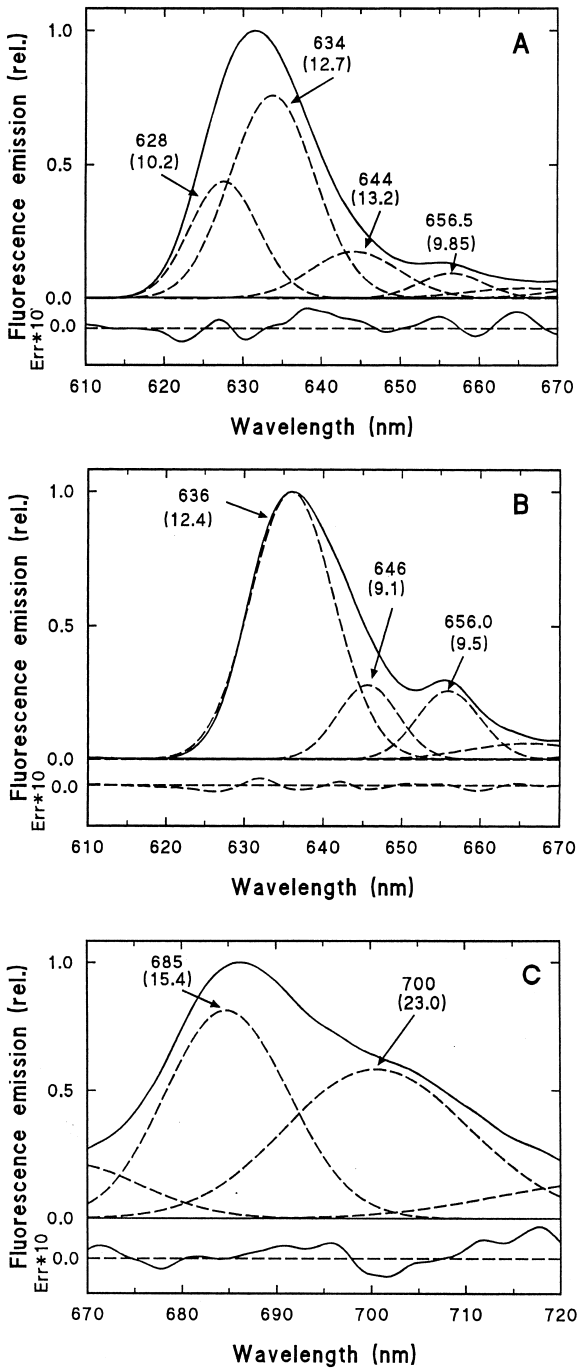

Fig. 2. Gaussian deconvolution of the 10 K fluorescence emission spectra of pea epicotyls. Excitation wavelengths 440 (A), 460 (B) and 640 (C) nm. The experimental curves are drawn with solid lines, the gaussian components with dashed lines. The maximum positions and the half-bandwidth values (in parentheses) of the gaussian components are indicated in the figures in nm. The bottom panels of the figures show the error of the fit of the sum of gaussian components to the experimental spectrum.

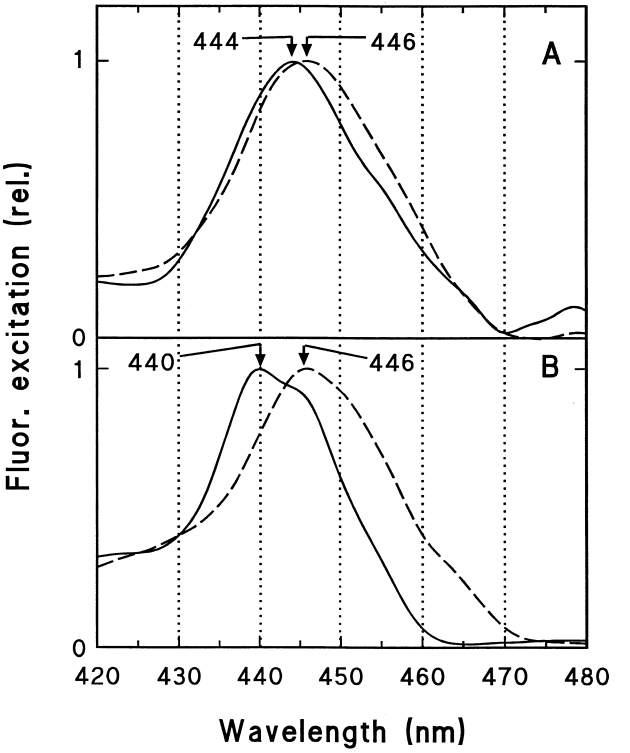

Fig. 3. The Soret region of the fluorescence excitation spectra of pea epicotyls. The spectra were measured at 293 K (A) and at 10 K (B) at 625 nm (solid lines) or 640 nm (broken lines). The spectra were normalized at their maxima. The numbers in the figures indicate the positions of maxima in nm.

To provide an overview of the corresponding excitation and emission bands and their vibrational satellites, a matrix correlation chart was done. A set of fluorescence emission spectra was recorded at 10 K, with excitation wavelengths varied from 400 to 615 nm in steps of 5 nm. The spectra were collected into a spreadsheet and were summed as a topological projection (Fig. 4). The complex character of the areas corresponding to Soret and Q bands are clearly seen. Three sets of contours are evident in the projection. The most pronounced set is localized in the 410–470/620–650 region. This set corresponds to the most intense Soret excited (0,0) emission bands. The oval shape of the contour sets shows the complexity of bands, the focal centers of the contours indicate the positions of the main excitation/emission band pairs. Such a center was found at 443/632 and 448/636 nm. A second set can be identified in the 440–460/675–705 nm region, corresponding to the Soret excited vibrational emission bands. The centers of the contours in this set was found at 684 and 697

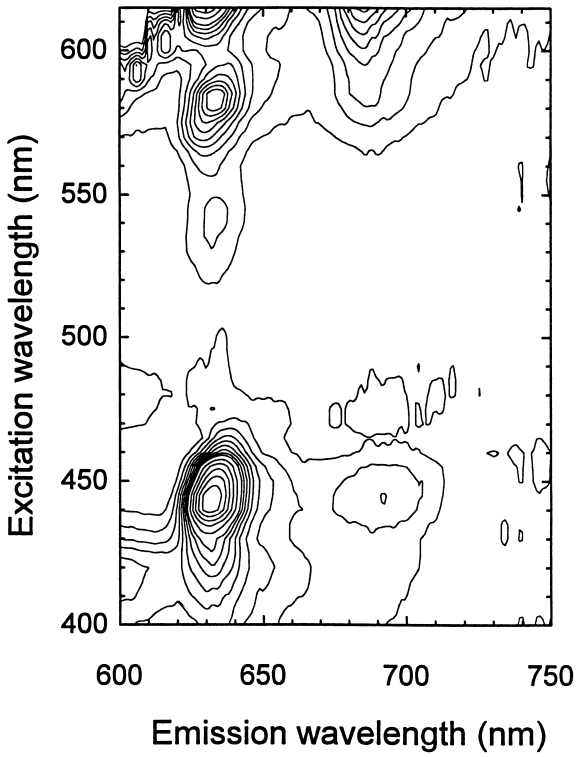

Fig. 4. Matrix correlation chart of the fluorescence of pea epicotyl at 10 K. The excitation wavelength was varied from 400 to 615 nm by 5 nm increments. The spectra were collected into a spreadsheet and were summed as a topological projection of the set of spectra. The shapes and symmetries of the iso-intensity lines were used to identified the excitation/emission band pairs (see text).

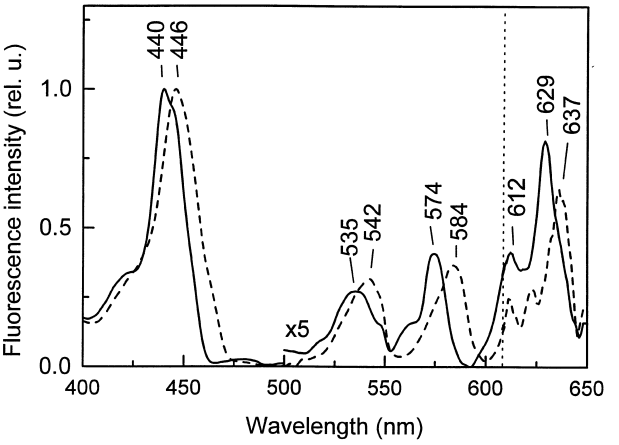

Fig. 5. Excitation spectra of pea epicotyls at 10 K. The spectrum drawn with solid line was measured at 625 nm in the 400–610 nm region (on the left of the dotted line across the panel) and at 695 nm between 610 and 650 nm (on the right of the dotted line across the panel). The spectrum drawn with a dashed line was recorded at 640 nm in the 400–610 nm region (on the left of the dotted line across the panel) and at 710 nm between 610 and 650 nm (on the right of the dotted line across the panel).

nm. The third set of contours is in the 570–590/620–640 nm region with centers at 575/628 and 584/636 nm. These are vibronically, i.e. $Q_y(0,1)$ excited (0,0) emission peaks. These latter data show potential laser excitation positions. Due to the overlap of the iso-intensity contour lines, the center positions cannot precisely show the exact positions of the fluorescence bands, rather they are indicative of their existence.

For further characterization of the two spectral forms and to find excitation bands appropriate for laser excitation, excitation spectra were scanned at 10 K from 400 nm to 610 nm with the emission monitored at 625 or 650 nm and from 610 to 650 nm with the emission at 695 or 710 nm (Fig. 5). The four spectra were plotted in two pairs to show all excitation bands in the visible region, i.e. the spectrum recorded at 625 nm was combined with the spectrum

Table 2
The effect of temperature on spectral characteristics of the two short-wavelength emission bands in the emission spectra of pea epicotyls

Temperature (K)	Band position (nm)	Half-bandwidth (nm)	Integral %	Band position (nm)	Half-bandwidth (nm)	Integral %
10	627.6	10.19	24.1	633.7	12.70	52.1
100	628.1	10.60	18.6	634.3	13.05	32.6
200	629.4	11.76	17.9	635.9	13.13	19.6
240	629.6	12.13	12.7	636.3	14.23	19.3
273	630.2	14.77	8.6	638.6	15.30	19.4
293	630.2	14.80	7.9	638.8	15.30	19.7

The spectra of the same sample were registered at different temperatures with 440 nm excitation. The spectra were resolved into gaussian components the characteristics of which are shown. The integral under the whole spectrum (600–780 nm) measured at 10 K was taken to 100%.

recorded at 695 nm, and the spectrum recorded at 650 nm was combined with the spectrum measured at 710 nm. The two combined spectra show bands at 440, 535, 574, 612 and 629 nm and at 446, 542, 584, 621, and 637 nm, respectively. In both spectra, these bands correspond to the overlapped $B_y(0,0)-B_x(0,0)$ transition and to the $Q_y(0,2)$, $Q_y(0,1)$, $Q_x(0,0)$ and $Q_y(0,0)$ electronic transitions as previously determined for protochlorophyll in diethyl ether solution [27].

3.2. High resolution fluorescence spectra

The conventional optical spectra of the sample consists of rather broad bands due to a distribution of environmental perturbation (inhomogeneous broadening) and temperature broadening. The temperature broadening becomes less significant upon cooling; thus at cryogenic temperatures, the inhomogeneous broadening can be studied which represents the effect of the environment on the chromophore. The determination of IDF of the molecular transition energies, however, is suitable to distinguish the true (0,0) bands from bands of vibronic origin in a conventional spectrum. To determine the IDF, a series of emission spectra were measured with the laser tuned in the $Q_y(0,1)$ vibronic band of the absorption spectrum (Fig. 6). Vibrational excitation energies corresponding to the observed sharp lines are indicated in the figure. The low signal intensity of the sample required a slit width of 3.5 cm^{-1} to detect the emission; thus in the spectra, the sharp FLN lines are superimposed on a structureless background and are somewhat broadened. The appearance of a significant background is also indicative of significant phonon contribution in both the excitation and the emission process. A baseline correction was applied for non-resonant excitations via phonons. The sharp lines were considered as pure electronic origins and their integrated intensities were used to determine the IDF in the 15 400–16 100 cm^{-1} region. The IDFs of the distinct chromophores were identified by a gaussian fitting procedure (Fig. 7). The curve connecting the calculated data points was fitted with a different

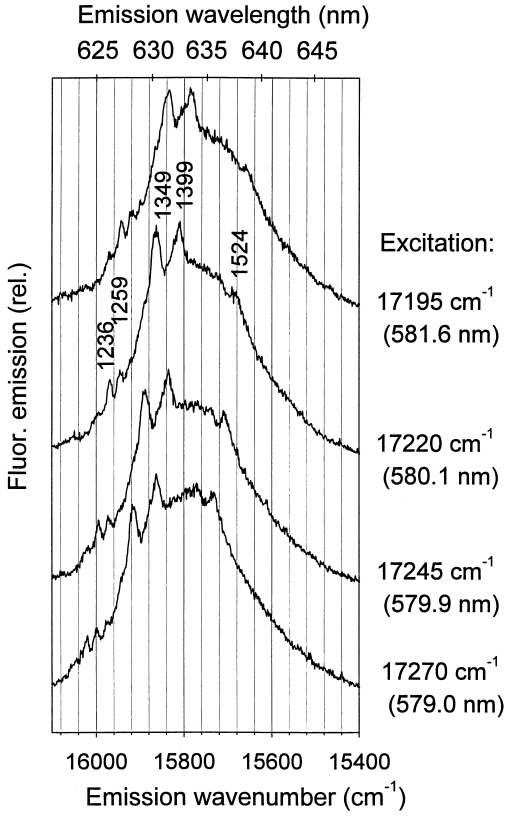

Fig. 6. Variation of the (0,0) emission lines as a function of excitation wavenumber in fluorescence line narrowing (FLN) spectra of dark-germinated pea epicotyl recorded at 10 K. Excitation wavenumbers (and the corresponding wavelength values in parentheses) are given on the right of the panel. The excited state vibrational energies are labeled on the spectra.

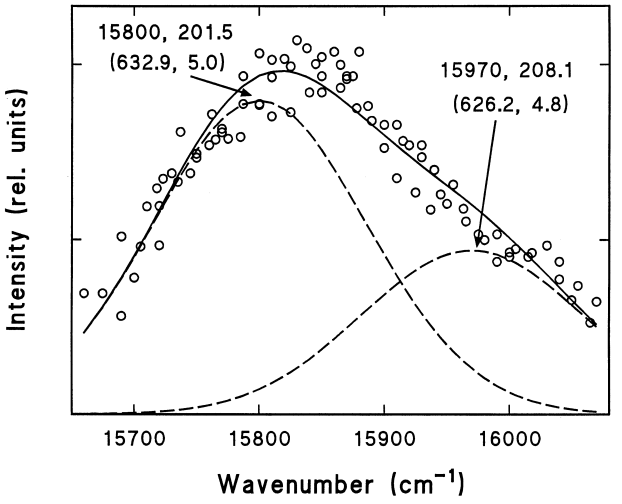

Fig. 7. The inhomogeneous distribution function (IDF) of protochlorophyllide forms in dark-germinated pea epicotyls. The data for the IDF were calculated from the intensity values of the sharp lines in FLN spectra (see Fig. 6), corresponding to the following excited state vibrations: 1236, 1259, 1349, 1399 and 1524 cm^{-1}. The data were fitted with the gaussian curves (dashed lines), their sum is drawn with a continuous line.

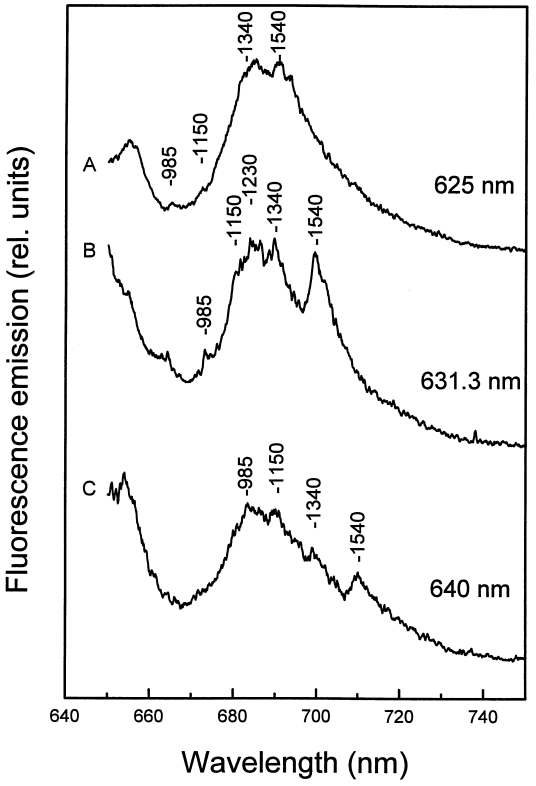

Fig. 8. Fluorescence emission spectra of dark-germinated pea epicotyl at 10 K. The samples were selectively excited with 625 (A), 631.3 (B) and 640 (C) laser lines, i.e. in the $Q_y(0,0)$ region of protochlorophyllide. The ground state vibrational energies are labeled on the spectra.

number of gaussian components. These calculations showed that at least two components are needed to describe the curve. These gaussians had maxima located at 15 800 and 15 970 cm^{-1} (632.9 and 626.2 nm), their half-bandwidth values were 201.5 and 208.1 cm^{-1} (5.0 and 4.8 nm), respectively. The insertion of a third component resulted in an unjustifiable narrowing of the components. Resolved lines were found only in the 622–642 nm region, no such lines appeared at longer wavelengths.

To study the ground state vibrational energies of the Pchlide forms, laser excitation was applied at 625, 631.3 and 640 nm which is the $Q_y(0,0)$ region of Pchlide forms, at 10 K and the emission spectra were recorded between 650 and 750 nm. The spectra showed FLN lines (widths < 5 nm, i.e. 100 cm^{-1}) which shifted as a function of excitation wavelength (Fig. 8). They appeared at 985, 1150, 1230, 1340 and 1540 cm^{-1} from the laser wavelength. Intensive ground state vibrational frequencies are indicated in the figure. This pattern shifts with the excitation wavelength and becomes better resolved at longer wavelength excitations due to the lower contribution of phonons (Fig. 8).

4. Discussion

The conventional fluorescence emission spectra of pea epicotyls are complex [15]. The complexity was less conspicuous in the 293 K spectra, where only the broadness and the slight dependence of the spectra on the excitation wavelength (Figs. 1A and 3A) referred to the simultaneous existence of several chromophores. In the low-temperature spectra, however, the complexity became obvious. The emission spectra contained two main short-wavelength bands with maxima between 627.6 and 630.2 nm (denoted here as P1), and between 633.7 and 638.8 nm (denoted here as P2), respectively. The variation in wavelength depended on the temperature of the sample. Further components approximated by bands at 644 and 656 nm were also found. Vibronic bands of the P1 and P2 emission bands are present in this region too, but they are strongly overlapped by the 644 and 656 nm bands emitted by the well-known Pchlide forms active in the chlorophyll biosynthesis. These bands at wavelengths longer than 640 nm were described earlier in detail [9,10] therefore they are not discussed in this work.

The temperature-dependence of the P1 and P2 bands were similar: the positions of their maxima shifted towards shorter wavelengths in parallel with the decrease of their half-bandwidth values and increase of their integrals when the samples were cooled from 293 to 10 K (Table 2). However, there were differences in the extent of the changes, the blue-shift was 2.6 and 5.1 nm, the decrease of the half-bandwidths was 4.61 and 2.60 nm and the increase of the integral was 8.11 and 16.2 (relative values) for P1 and P2, respectively. The blue-shift upon cooling and the narrowing half-bandwidth are consequences of the weaker contribution of the coupling to phonon vibrations of the surrounding matrix [16].

Further differences were observed by studying the vibrational satellite bands of P1 and P2. P1 had a satellite band at 685 nm with a 13.4 nm half-bandwidth and the P2 had one at 700 nm with 23 nm

half-bandwidth in the 10 K spectra. The distance between the P1 and its satellite, (i.e. the maximum of the envelope of the most intense vibrational bands) was 57 nm (1325 cm^{-1}) and between the P2 and its satellite 66 nm (1487 cm^{-1}).

The spectra in this work account for the presence of Pchlide or its esterified derivatives. The vibrational frequencies measured here (Fig. 8) correspond to those described for Pchl in ether, since the presence of the phytol chain does not influence the spectral properties of the porphyrin ring [18]. However, a second vinyl side group on the pyrrole rings, like in divinyl Pchlide, modifies the electronic transitions in the visible region [28], thus the appearance of the two short-wavelength bands could be explained by the simultaneous presence of monovinyl and divinyl Pchlide. Earlier HPLC studies however, showed that the pea epicotyls predominantly contain monovinyl Pchlide [15]. Consequently, the spectral heterogeneity of the pea epicotyls cannot be explained by chemical heterogeneity of the pigments. The spectral differences of forms representing P1 and P2 refer to differences in their molecular environments, molecular distortions and/or different electric properties (polar groups etc.) of the surrounding matrix can cause such differences.

In the experiment in which the temperature dependence of the spectral properties were studied, there was a possibility, however, that the observed differences were, at least partially, due to photochemical transformations caused by the excitation light source [13]. The calculation of the total integrals of the spectra measured at different temperatures showed that no photochemical reactions proceeded under 100 K and they were not significant under experimental conditions up to 200 K either.

The possible different molecular environments of the chromophores can be more clearly studied with high resolution techniques. Both forms with the P1 and P2 emission bands had resolved lines in the FLN spectra (Fig. 6). The appearance of these lines depends on the appropriate fluorescence lifetime and the absence of energy migration or transfer – which can decrease the fluorescence lifetime. Such energy migration or transfer can occur between distinct monomeric molecules or between molecules of aggregates of chromophores. Thus the detection of resolved FLN spectra of (0,0) lines in the region of the P1 and P2 bands and their decomposition into two IDF maxima being in agreement with the conventional spectra, provide direct evidence that the corresponding structural forms are not aggregates of Pchlide molecules, but two monomeric species. On the other hand, it is worth mentioning that no resolved FLN lines were found in the region of the 645 and 656 nm emission bands. The loss of resolution can be interpreted either by the vibronic origin of these bands or by the presence of aggregates. The latter conclusion is supported by earlier results [5] showing that the emission maxima belong to aggregated Pchlide forms in this range [18,25].

The IDF in Fig. 7. reveals pure electronic (0,0) band components that are comparable with the conventionally determined P1 and P2 bands. The components in the IDF have mean values at somewhat shorter wavelengths than those given in Table 1, i.e. 626.2 nm as compared to 628 and 632.9 nm as compared to 636 nm, respectively. It is known, however, that the conventional measurement also contains matrix phonon contributions which adds a contribution to the long wavelength side in the emission measurement [16]. In the calculated band shape given by the IDF, only the electronic transitions are considered. In the IDF, the two spectral forms had 208.1 and 201.5 cm^{-1} widths which correspond to those reported for chlorophyllide substituted into apomyoglobin [29]. In heme proteins with a centrally located heme such as horseradish peroxidase [21] or cytochrome *c* [30] or in well-ordered protein pockets, much narrower widths are observed, in the range of 30–70 cm^{-1}. However, in the case of porphyrins in glassy matrices (frozen solutions) the width is of several 100 cm^{-1} [20,22]. Comparing these data with our observations allows the conclusion that, in pea epicotyls, the Pchlide molecules do not have a uniform, strictly packed structure with respect to their intermediate surroundings. In the IDF, the dominating band was at 632.9 nm. Our data indicate, that it corresponds to a monomeric form of the Pchlide–enzyme complex, which had the most intensive band in the conventional emission spectra. We associate this species with the majority of the Pchlide molecules that are bound to the LPOR protein. The width of the band was 5 nm, suggesting that the Pchlide is not enclosed in a well ordered protein pocket, it can be bound rather close to the surface

and in a variety of conformations. If the enzyme does not hold its substrate in a well ordered way, that may explain the slow kinetics of the phototransformation described in the pea epicotyl [26]. The properties of the other band at 626.2 nm may correspond to a Pchlide pool, containing Pchlide molecules, which are not bound to the protein.

Acknowledgements

This work was supported by Grant 173 of the Hungarian Ministry of Education and Hungarian Grants FKFP 11911/1997 and ETT 425/1996.

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