Photosynthesis Research **60:** 87–98, 1999.

Regular paper

Protochlorophyllide forms and energy transfer in dark-grown wheat leaves. Studies by conventional and laser excited fluorescence spectroscopy between 10 K–100 K

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Received 10 November 1998; accepted in revised form 25 February 1999

Key words: 10 K-100 K temperature effects, energy transfer, fluorescence, fluorescence line narrowing (FLN), NADPH-protochlorophyllide-oxidoreductase (POR), prolamellar body (PLB), protochlorophyllide structures

Abstract

The fluorescence properties and role in energy transfer of protochlorophyllide (Pchlide) forms were studied in dark-grown wheat leaves by conventional and laser excited high resolution methods in the 10 K–100 K temperature range. The three major spectral bands, with emission maxima at 633, 657 (of highest intensity) and 670 nm as Bands I, II, and III were analyzed and interpreted as the contributions of six different structural forms. Band I is the envelope of three (0,0) emission bands with maxima at 628, 632 and 642 nm. Laser excitation studies in the range of Band II at 10 K reveal the presence of a spectrally close donor band besides the acceptor, Band II. The intensity in Band III originates mostly from being the vibronic satellite of Band II, but contains also a small (0,0) band with absorption maximum at 674 nm. Excitation spectra show that besides the Pchlides with absorption around 650 nm within Band II, another significant population of Band I with absorption around 640 nm is also coupled by energy transfer to the acceptor of Band II. The spectral difference between the two donor forms indicate different dipolar environments. Upon increasing the temperature, the intensity of Band II and its satellite, Band III decrease, while Band I remains unaffected. Band II shows also a broadening towards the blue side at higher temperatures. Both the quenching of fluorescence and the spectral change was explained by a thermally activated formation of a non-fluorescent intermediate state in the excited state of Pchlide acceptors.

Abbreviations: Chl – chlorophyll; Chlide – chlorophyllide; FLN – fluorescence line narrowing; Pchl – protochlorophyll, Pchlide – protochlorophyllide; PLB – prolamellar body; POR – NADPH-protochlorophyllide oxidoreductase; PT – prothylakoid

Introduction

The detailed knowledge of chlorophyll (Chl) biosynthesis has central interest in plant biochemistry. In this process, the transformation of protochlorophyllide (Pchlide) into chlorophyllide (Chlide) is a key step in higher plants because it is under strict regulation. The reaction is driven by NADPH-protochlorophyllide oxidoreductase (POR) which forms ternary complexes of the protein, Pchlide and NADPH (Griffiths 1991). These complexes are specifically arranged in the inner membranes of etioplasts if these plants are germinated or kept in the dark. The reduction of Pchlide into Chlide takes place only in light, the photosensitizer of the process is the substrate, Pchlide (Sundqvist and Dahlin 1997).

A combination of different spectroscopic methods can provide information about the native arrangement of Pchlide and its molecular environment, that has basic importance in the enzyme reaction. Detailed

spectral data of Pchlide or protochlorophyll (Pchl) are available based on *in vitro* studies: the pigments were dissolved in polar solvents, like diethyl ether or acetone (Renge et al. 1986; Kotzabasis et al. 1990). In these solutions the pigments were in monomeric state and the absorption and fluorescence spectra corresponded to transitions involving two split excited electronic states known as Soret and Q bands in the spectroscopy of porphyrins (Houssier and Sauer 1969). These spectra, however, are much more complex in the native structures. Model studies on the pigments dissolved in both polar and non-polar solvents (Brouers 1975), or contained in solid films (Böddi et al. 1980), and in micelles (Böddi et al. 1983) yielded information about solvent effects or spectral changes caused by the aggregation of the pigment molecules into dimers or oligomers. These studies were completed by the investigation of isolated etioplast inner membrane particles. Based on fluorescence spectroscopy at 77 K (Böddi et al. 1992), circular dichroism data (Böddi et al. 1989) and the results of experiments with cross-linked POR protein subunits (Wiktorsson et al. 1993), the following model has been suggested to describe the native arrangement of Pchlide complexes (Böddi 1994; Sundqvist and Dahlin 1997). The majority of the pigment is localized in the regular structure of the tubular network of prolamellar body (PLB) membranes which contains also most of the POR molecules (Wellburn 1984). The units of POR are arranged so close to each other that they interact and enclose Pchlide molecules. The pigments in these complexes that also contain NADPH show the properties of pigment aggregates (Apel et al. 1980; Reinbothe et al. 1996). These complexes were identified with the absorption maximum found at 650 nm and fluorescence emission maximum at 657 nm at 77 K. The absorption maximum at 638 nm and fluorescence emission maximum at 645 nm was identified with some smaller and looser aggregates on the edges of the PLBs. The prothylakoids (PT) are single membranes with little amount of POR (Böddi et al. 1989). The absorption maximum found in the spectra at 628 nm and fluorescence emission maximum at 633 nm was attributed to Pchlide and/or Pchl molecules in monomeric state, contained in the PTs. The existence of Pchlide pools was also suggested, and supposed that these would be localized in the lipid structures of PLBs and thus should have a red shifted absorption maximum. An absorption maximum was seen at 662 nm and the corresponding fluorescence emission maximum was supposed to be seen at 670 nm at 77 K.

The goal in the present work was to perform a detailed spectroscopic analysis to reinvestigate the suggested model for the separate structural forms of Pchlide in the etioplast of wheat leaves by taking advantage of the higher spectral resolution of low temperature spectroscopy and of the blocking of phototransformation into Chlide at cryogenic temperatures. Instead of absorption spectra, fluorescence excitation spectra were measured also at cryogenic temperatures and compared with the respective emission spectra. It was possible to distinguish between six different structural forms, and to determine the spectral bands of four forms in the Soret and Q range, and to characterize the major vibrational components in the vibronic envelope of fluorescence emission. Based on these further data, the previous model for the identification of the spectral components with Pchlide structural forms was verified and completed.

Materials and methods

Ten-day-old dark-grown wheat seedlings (Triticum aestivum, cult. MV 17) were used in the experiments. At this age the leaves were about 10 cm long. About 1 cm of the top segment was omitted and the next 1 cm was used in the measurements. These pieces of the intact etiolated wheat leaves were immersed into glycerin-water mixture (1:1, v/v) to assure thermal contact and transparency and placed into small cylindrical glass cuvettes, that were then kept immersed in liquid nitrogen. The samples were prepared and cooled to 77 K in the dark, to avoid accidental phototransformation.

Conventional fluorescence spectra were obtained with a FS900CD luminometer (Edinburgh Analytical Instruments, UK) equipped with a Xenon light source, and a cooled Hamamatsu R955 photomultiplier tube as a detector. The resolution of the fluorimeter was 0.5 nm (12 cm^{-1} at 650 nm). All excitation spectra were corrected for the emission spectrum of the Xenon lamp. The fluorescence excitation spectra were not corrected for the intense light scattering towards shorter wavelengths that reduces the apparent intensity in the Soret range. The fluorescence emission spectra were corrected for the spectral sensitivity of detection.

Fluorescence line narrowing (FLN) spectroscopy at cryogenic temperatures was used to demonstrate energy transfer in the system of chromophores of a high level of organization. In case of energy transfer between the species corresponding to a wide spectral

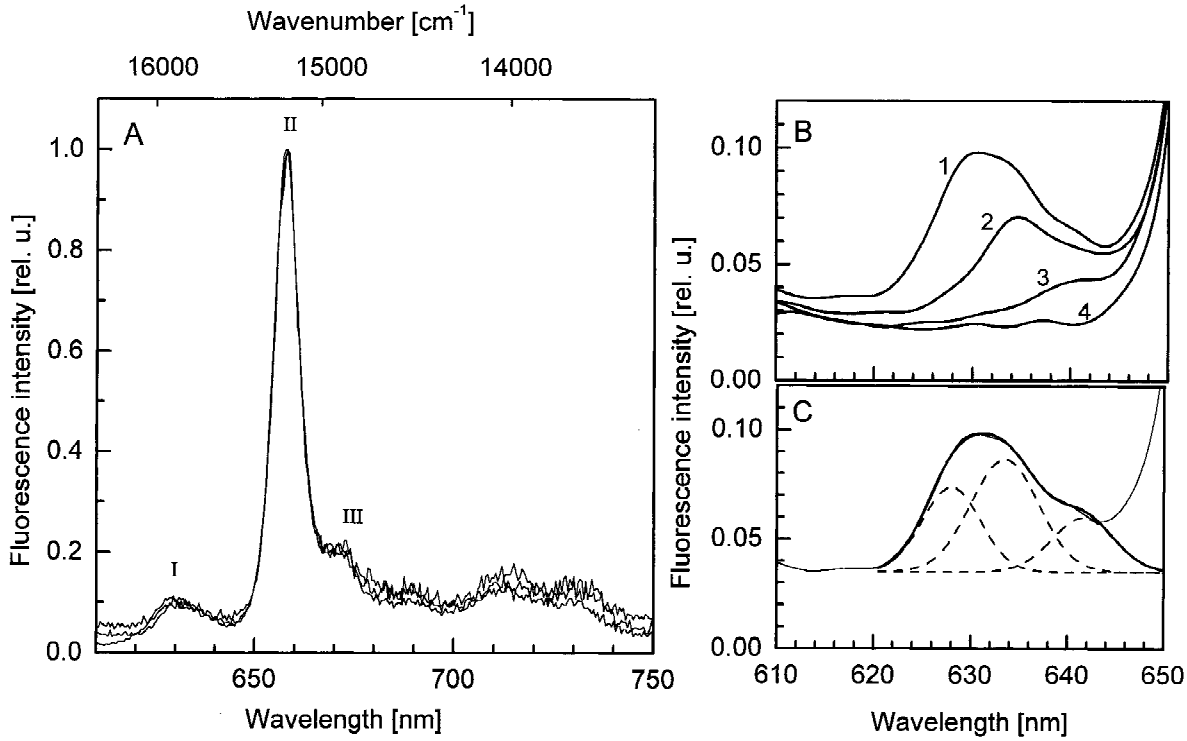

Figure 1. Conventional fluorescence emission spectra of wheat leaves measured at 10 K. The excitation wavelengths were: A: 440, 535, 580 nm; B: 580, 585, 590, 595 nm for curves 1, 2, 3, 4 respectively; C: 580 nm. Spectra were normalized at their maxima (Band II). The experimental curves are drawn with solid lines, and Gaussian components with dashed lines. Maximum positions and respective half-bandwidth values (in parentheses) are: 628 (6), 633 (7) and 642 (7) nm.

band, emission will take place from the acceptor type of chromophores independently from the energy of excitation. If, however, the broad spectral band represents an inhomogeneous distribution of independent chromophores, laser excitation at low temperature is able to select a subpopulation with characteristic emission spectrum. In such a case, site selection is observed, that is, the emission spectrum shifts with the excitation energy on the energy scale (Personov 1992). In our studies, this effect was used to differentiate between monomeric and aggregated forms of Pchlides. The high-resolution emission spectra were recorded with the FLN set-up: excitation was achieved by a Coherent 899-01 tunable dye laser with DCM dye, pumped by a cw Coherent Innova-307 argon ion laser (Palo Alto, CA). The power of the laser beam was attenuated to 1–2 mW by using neutral density filters. The spectral width of the laser line was 0.5 cm^{-1} (corresponding to 0.02 nm at 600 nm). The emission spectra were measured at 90 degrees from the excitation light, using a THR-1000M monochromator with holographic grating (Jobin-Yvon, Longjumeau, France, linear dispersion of 0.8 nm/mm). The detector was a cooled GaAs photomultiplier R943-02 (Hamamatsu Photonics K.K., Japan). Fluorescence signals were collected with a photon counting unit C3866 (Hamamatsu Photonics K.K., Japan) and controlled by Spectra link (Jobin-Yvon, Longjumeau, France). The resolution of the spectrophotometer was 2–3 cm^{-1}. The sample was cooled to 10 K by a closed cycle He refrigerator Cryophysics SA M22 (Geneva, Switzerland).

Results

Conventional fluorescence excitation and emission spectra at 10 K

In Figure 1A the fluorescence emission spectrum of etiolated wheat leaves is shown by Soret excitation at 10 K. As it is expected based on previous studies, there are three major emission bands in the (0,0) range denoted as Band I, Band II and Band III in the Figure. Band II is an intense emission band at 657 nm that dominates the spectrum. Band I appears as

Table 1. Band positions in the fluorescence excitation spectra of etiolated wheat leaf at 10 K

Emission (0,0)		Excitation band positions: Soret (0,0)		$Q_x(0,1)$		$Q_y(0,1)$ and $Q_x(0,0)$[a]		$Q_y(0,0)$[b]	
nm	cm^{-1}	nm	cm^{-1}	nm	cm^{-1}	nm	cm^{-1}	nm	cm^{-1}
628	15924	440	22750	536	18653	577	17343	625	16000
633	15798	442	22620	539	18566	581	17224		
645	15504	443	22568	543	18413	588	17004	639	15649
657	15221	447	22360	547	18295	594	16830	650	15385
670	14925	447	22360	547	18295	594	16830		

[a] The two Q bands have a large overlap, the sharp maxima at the listed wavelengths refer to $Q_x(0,0)$ that is superimposed on a broad envelope of band $Q_y(0,1)$.

[b] The emission was detected at wavelengths longer than 685 nm (at energies below 14600 cm^{-1}).

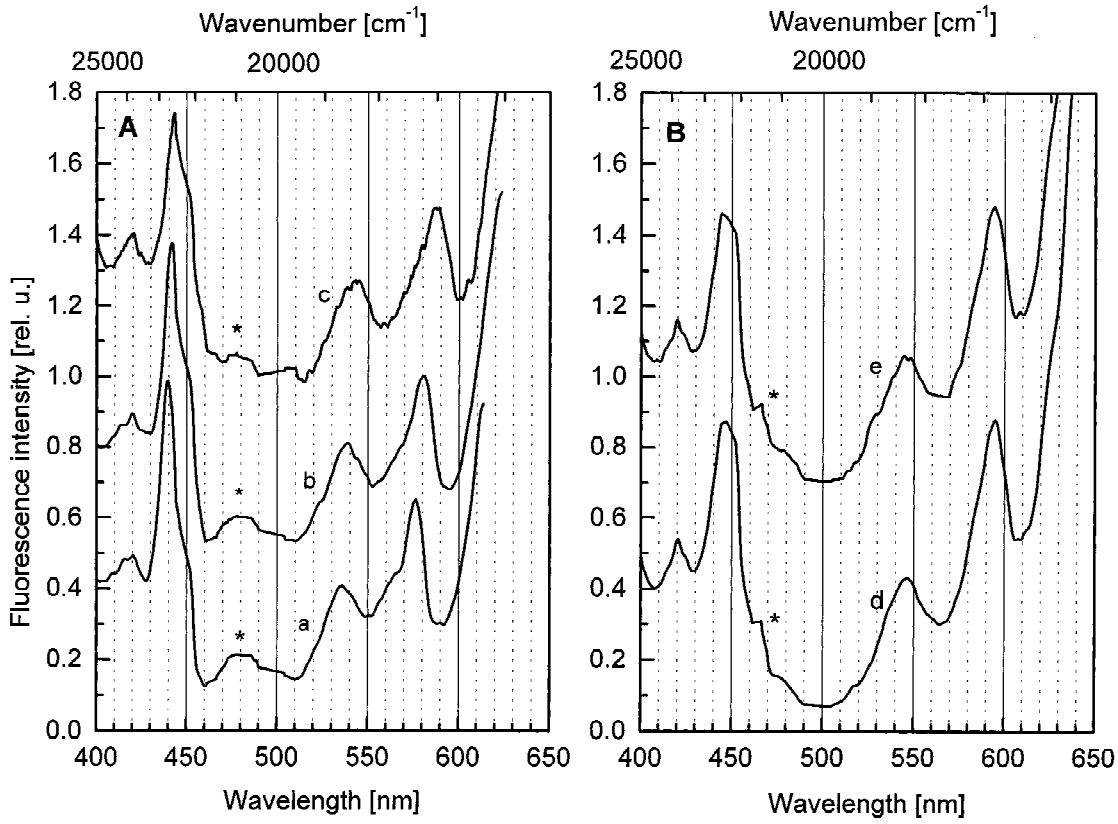

Figure 2. Conventional fluorescence excitation spectra of etiolated wheat leaves at 10 K. Emission was detected at emission Band I 628, 635, 645 (A), at Band II 657, and at Band III 670 nm (B) for the spectra a, b, c, d, e respectively. Spectra were corrected for the Xenon-lamp spectrum, however some artifacts still remained in the spectra around 480 nm marked by asterix.

a contribution of low intensity with maximum around 630 nm at the higher energy side of Band II. On the lower energy side, Band III forms a shoulder at 670 nm. The bands at even lower energies we attribute to overlapping vibrational bands.

Previous studies by emission spectroscopy with Soret excitation (Böddi et al. 1992), and our studies by the present methods on pea epicotyl (Böddi et al. 1998) suggested the composite nature of Band I. We performed a systematic parallel fluorescence excita-

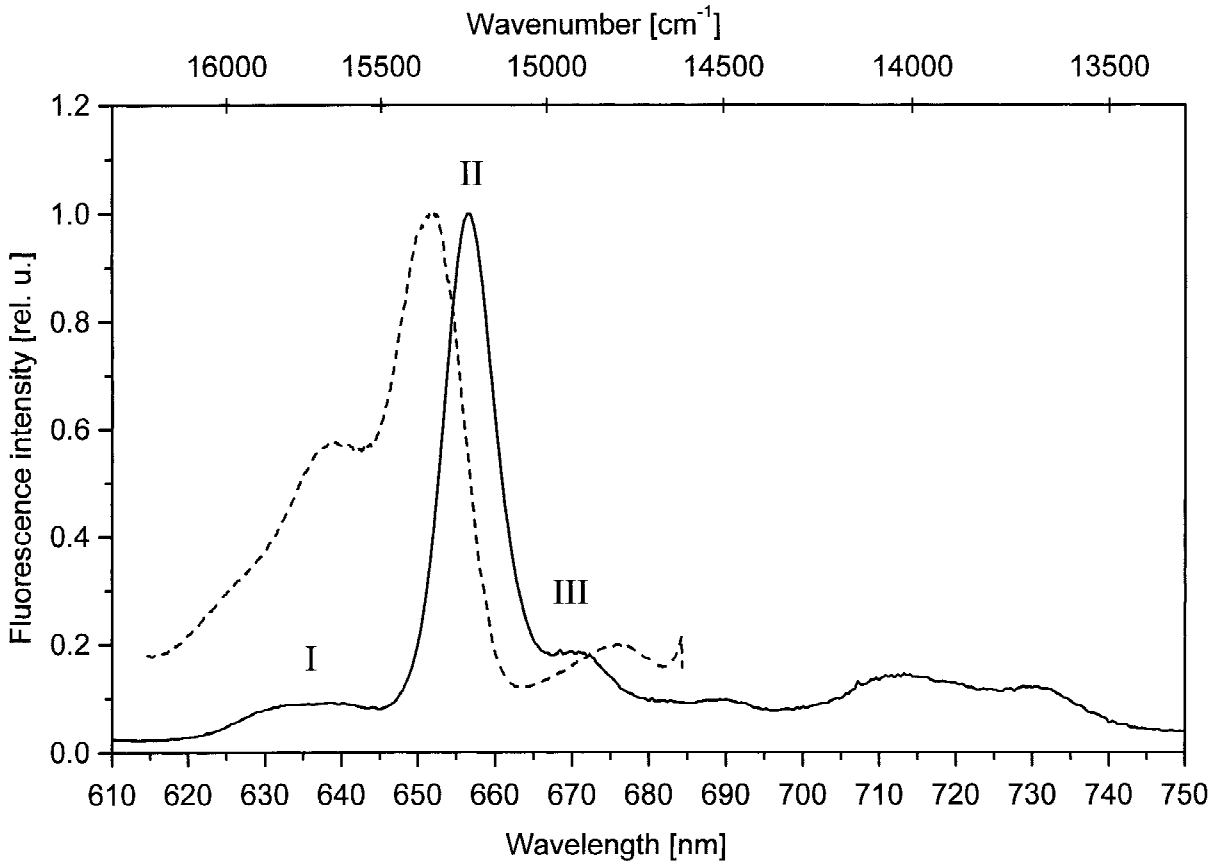

Figure 3. Fluorescence emission (solid line) and the $Q_y(0,0)$ range of the fluorescence excitation spectrum (dashed line) of etiolated wheat leave at 10 K. The emission spectrum was excited at 442 nm. The excitation spectrum was measured by detection at the whole vibrational region, through a cut-off filter, above 685 nm.

tion and emission spectroscopic study to unravel (0,0) components within this range. The studies resulted in the conclusion that three (0,0) bands can be differentiated: when the emission wavelength was set to 628 nm, to 635 nm and to 645 nm, the respective fluorescence excitation spectra showed different features mainly in the Q range as shown in Figure 2A. The band positions are given in Table 1.

The reverse experiment was to adjust the excitation wavelength according to the maxima in the excitation spectra and detect the emission spectra. The result of such an experiment is seen in Figure 1A: the emission spectra recorded with excitations at 440, 535 and 580 nm were clearly identical, indicating that these excitation wavelengths belong to the same Pchlide complexes representing their Soret and Q excitation bands respectively. Similar results were obtained for the two other complexes. The spectral difference between the Pchlide forms was the most prominent in the lowest energy Q band of the excitation spectra (Figure 2A). These band maxima were used to excite characteristic emission spectra of the components as shown in Figure 1B. The different emission maxima in curves 1, 2 and 3 can be interpreted as contributions of (0,0) bands at 628, 633 and 642 nm respectively. A corresponding Gaussian resolution of curve 1 is shown in Figure 1C. Curve 4 in Figure 1B is shown as a reference: in this case, no contributions of Band I could be excited.

The systematic studies by conventional low temperature spectroscopy yielded important information concerning also Band III. The fluorescence excitation spectra with detection at 657 and 670 nm, are shown in Figure 2B. The similarity of these two spectra suggests, that Band III may mostly be of vibrational origin, a satellite band of Band II. The low intensity of Band III makes it very unprobable that it would be coupled to Band II by energy transfer. To verify the vibronic nature of Band III, the excitation spectrum

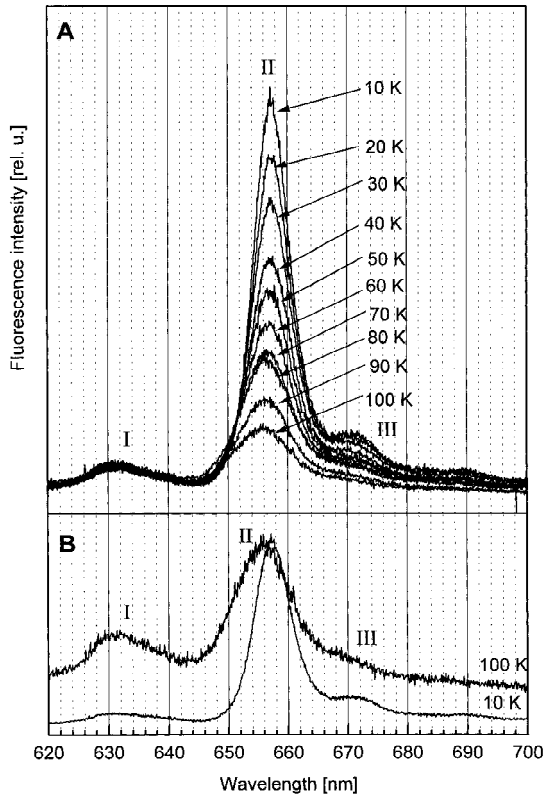

Figure 4. Temperature dependence of the fluorescence emission spectrum. Temperatures are indicated next to the curves (A). Emission spectra corresponding to 10 and 100 K normalized to the Band II are compared (B). Excitation wavelength was at 559 nm.

was detected in the vibrational region above 685 nm by using a cut off filter. (The vibrational energy range can be estimated based on previous literature data (Böddi et al. 1992)). The excitation spectrum was detected at wavelengths above 615 nm and is shown compared with the emission spectrum by Soret excitation in Figure 3. In the range of Band III, the excitation spectrum shows some (0,0) intensity, but this contribution has a different maximum than that in the emission spectrum. The conclusion is that the band labeled in the emission spectrum as Band III is mostly a vibronic satellite of Band II, however, some contribution of (0,0) origin can also be identified in this range.

The excitation spectrum in Figure 3 can be considered as the continuation of those in Figure 2. In this way one can have information concerning the spectral band feature in the whole Soret-Q range. In the interpretation of the spectral bands one has to note that the excitation spectra may have been distorted due to light scattering effects, manifested in the fact that the Soret band seemed to be unusually small relative to the Q bands shown in Figure 2. The general feature is that there are three major bands in the Q range and one (split) Soret band. There are two important points that should be emphasized here. In the range of the excitation spectrum shown in Figure 3, there is an increased intensity around 639 nm relative to the emission spectrum. Such a great difference most probably cannot be attributed solely to vibrational levels, but is rather an indication for a strong energy transfer between the form at 642 nm in Band I and the species of Band II. The other observation is that Band II is characterized with a significant Stokes shift of $\sim 140\ \mathrm{cm}^{-1}$.

Temperature dependence of fluorescence emission spectra

The spectra when studied in function of the temperature revealed specific characteristics of the intense band seen in fluorescence at 657 nm (Band II). The fluorescence emission spectra were measured in the temperature range from 10 K to 100 K. The spectra were identical under excitations at different wavelengths as: 451 nm (Soret band), 559, and 585 nm (Q_y(1,0)). One representative series of spectra is shown in Figure 4A. Temperature affected the spectra very much, the intensity of the main peak at 657 nm decreased rapidly with temperature increase and the band became wider with a maximum position at higher energies (see Figure 4B). After reaching 100 K, the sample was recooled to 10 K, and the original fluorescence spectrum could be retrieved with less than 5% difference (data not shown). To exclude that the observed changes were due to photochemical processes, the measurement was repeated at various laser light intensities. The same effect could be detected in all cases. Possible photoproduct generation at higher temperatures would be expected to cause spectral bands at the lower energy side of the spectra (Dobek et al. 1981), but no such effect could be observed. The intensity of the spectrum did not change significantly at the higher energy side (within Band I) with increasing temperature. The intensity of this band, however, is too low for analyzing this fact in details. The shoulder at the lower energy side, Band III at 670 nm, changed in parallel with the main peak.

Site-selected fluorescence spectra

High resolution laser excited fluorescence spectroscopy was applied to differentiate between isolated/monomeric and interacting/aggregated forms of the Pchlide chromophores based on the site selection

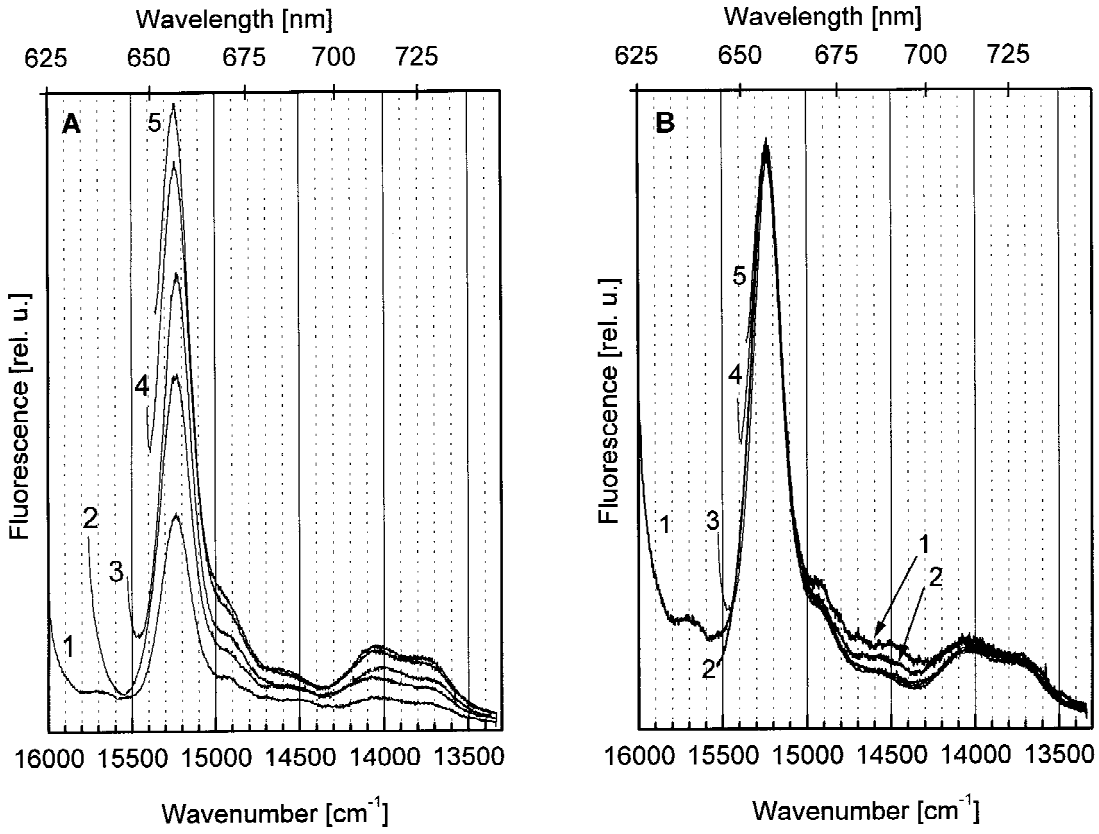

Figure 5. Fluorescence emission spectra, excited by laser selectively at the blue side of the $Q_y(0,0)$ absorption band of the etiolated wheat leaves at 10 K. Excitation wavenumbers are 16 038, 15 787, 15 555, 15 545 and 15 383 cm^{-1} (623.5, 633.4, 642.9, 643.3 and 650 nm) for curves 1, 2, 3, 4, and 5 respectively (A). Spectra were normalized for Band II (B).

effect. A series of fluorescence spectra was measured with 4 cm^{-1} resolution with selective excitation in the $Q_y(0,0)$ range of the different Pchlide forms at 10 K. Excitation in Band I (633 nm, 15 798 cm^{-1}), or at frequencies in the blue wing of Band II (below 650 nm, above 15 385 cm^{-1}) lead to very similar emission spectra with main fluorescence emission peak at 657 nm (15220 cm^{-1}) as shown in Figure 5A. The same effect is even better seen in Figure 5B, where the spectra are overlaid after normalization. In the measurement leading to curves 1 and 2, the excitation was within Band I. The corresponding emission spectra in Figure 5B show the vibronic features of curves 4 and 5 that originate from Band II excitations. Some additional intensity that is also seen in the vibronic range of curves 1 and 2 is most probably the vibrational contribution of (0,0) excitations (in Band I), that do not lead to energy transfer. The effect shows that in most excitations, the excitation energy migrates from Band I also to the emitting state of the molecules corresponding to Band II. The fact that excitation with laser light of narrow spectral range did not lead to emission spectra with resolved narrow vibronic lines also indicated that the species excited in the studied range are of very short lifetime (of broad spectral lines), which is an indication of coupling by energy transfer.

When the laser excitation was tuned in the red wing of Band II (above 650 nm, below 15 385 cm^{-1}), the emission spectra shifted parallel with the energy of excitation, that is, a site selection effect was observed. The spectra as obtained are shown with four major vibronic maxima marked in Figure 6A. In Figure 6B, the result of a transformation is shown; the spectra are plotted in function of the difference of excitation and emission energies. This latter presentation demonstrates that at each excitation, molecules with similar Frank-Condon overlaps are excited, and the photon emission happens independently from each other's presence. The vibronic features with maxima at 350, 750, 1250 and 1550 cm^{-1} agree very well with

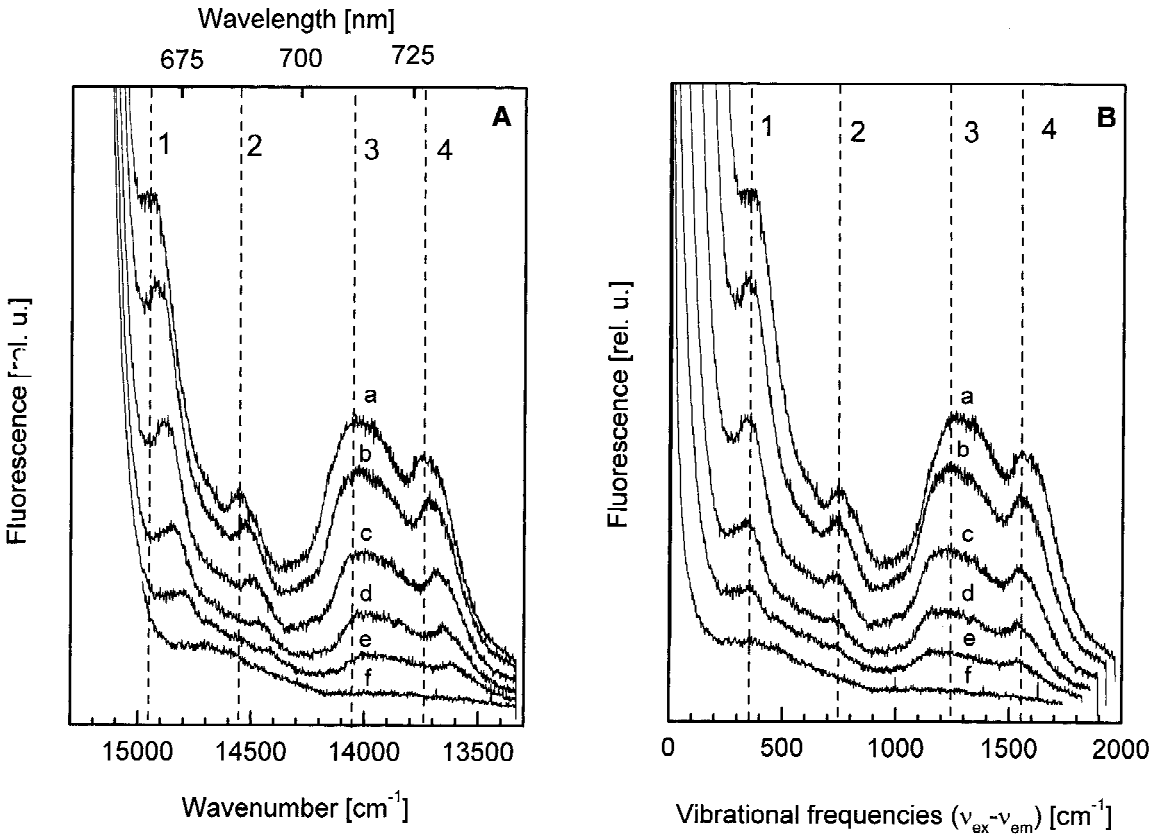

Figure 6. Fluorescence emission spectra, excited by laser selectively at the red side of the $Q_y(0,0)$ absorption band of the etiolated wheat leaves at 10 K. Excitation wavenumbers are 15303, 15264, 15228, 15191, 15156 and 15069 cm^{-1} (653.5, 655.1, 656.7, 658.3, 659.8 and 663.6 nm) for curves a, b, c, d, e, and f respectively (A). Spectra are converted into the vibrational frequency scale (B). Characteristic vibrational regions are indicated by vertical dashed lines at 350, 750, 1250 and 1550 cm^{-1}, indicated as 1, 2, 3 and 4 respectively.

literature data concerning protochlorophyll (Pchl) in solution (Renge et al. 1986). It is interesting to note that the vibronic satellite by 350 cm^{-1} below the energy of excitation appears in the emission spectra at a position that overlaps with that of Band III. This fact supports the identification suggested above.

Discussion

The spectra of the Pchlide forms

The pigment in the studies we identify with the monovinyl form of Pchlide based on HPLC results under comparable conditions (Shioi et al. 1992). Based on previous results (Böddi et al. 1989), we accounted for the fact that a small amount of Pchl may also be present, bound to the PTs, with a slight contribution in Band I. The absorption spectra of Pchl and Pchlide correspond to that of other metal-porphyrin derivatives: in the UV-VIS region, two electronic transitions can be excited, leading to the Soret (B), and the Q spectral bands. The respective energy levels (excited states) that are degenerate in porphyrins of D_{4h} symmetry are split in both Pchl and Pchlide (leading to bands B_x-B_y and Q_x-Q_y), because of the lower (D_{2h}) symmetry of the porphyrin ring in chlorophylls (Gouterman 1978). Pchlide in solution has three intense bands in the Q range. One interpretation is given as $Q_y(0,0)$, $Q_y(0,1)$ and $Q_y(0,2)$ (Houssier and Sauer 1969) in consecutive order according to their energies. Concerning this identification we feel it a problem that the frequency difference between $Q_y(0,0)$ and $Q_y(0,2)$ as $\sim$ 3000 cm^{-1} is too high to account it for vibronic excitation. Thus it is more probable that the three bands are formed as a result of overlapping (0,0) and vibronic bands of the Q_x-Q_y split energy pair. Comparison with present data and those in the

literature (Renge et al. 1986) concerning the vibrational energies of these molecules lead us to conclude about the three major Q bands, listed according to their energies, as follows. The band with lowest energy is the $Q_y(0,0)$; the next contains partially its vibronic satellite, $Q_y(0,1)$, and $Q_x(0,0)$; the third band is the vibronic satellite of $Q_x(0,0)$, that is, $Q_x(0,1)$. In the second band, the contribution of $Q_x(0,0)$ usually appears as a sharper peak on top of the broader $Q_y(0,1)$, as seen in Figure 2A, e.g. curve a. In fluorescence emission, the (0,0) purely electronic transition is seen only from the lowest energy level, $Q_y(0,0)$, and corresponding vibronic transitions to the ground state vibrational levels appear in the spectrum (0,1) (Gouterman 1978). In the present work, we used this outlined concept to identify the spectral bands with separate Pchlide species.

The Pchlide forms with contribution in Band I

Based on previous measurements at 77 K and Gaussian resolution, it was suggested that Band I is the envelope of two spectral forms (Böddi et al. 1992). At 10 K, a composite, small band is seen in this region (Figure 1A). The detailed analysis of series of fluorescence excitation and emission spectra lead to the conclusion that (0,0) emission bands of three independent Pchlide structures contribute to this emission (Figures 1, 2). The three $Q_y(0,0)$ bands are at 628 nm (15 923 cm^{-1}), 633 nm (15 797 cm^{-1}) and at around 642 nm (15 576 cm^{-1}).

We identify the first component (F628) with Pchlide molecules which are not connected to the protein, as the band position is similar to Pchlide in solution (Kotzabasis 1990). This Pchlide form resembles the (0,0) band attributed to the Pchlide pool, in pea epicotyl (Böddi et al. 1998). The absorption bands of this form could be obtained from the fluorescence excitation spectra, and given in Table 1. In the Soret range a shoulder on the long wavelength side of the band (Figure 2A) suggests that the band is with a splitting of $\sim$ 500 cm^{-1}. In the Q range, the Q (0,0) split pair $Q_x(0,0)$ and $Q_y(0,0)$ was found with a splitting of 1300 cm^{-1}. As it is expected, the splitting effect becomes weaker in the B band than in the Q transition (Gouterman 1978). The envelope of the vibronic contribution has a maximum by $\sim$1300 cm^{-1} above both the $Q_x(0,0)$ and $Q_y(0,0)$ bands that agree with the emission spectra (e.g. Figure 6B), where the envelope of the vibronic transitions shows a pronounced maximum around 1300 cm^{-1}. In wheat leaves, only very small amount of unbound Pchlide is present, thus the F628 form is very weak.

The second component of the fluorescence emission (F633) is the dominating part in the blue range of the spectra (Figure 1C). This form is present especially in young leaves (Wellburn et al. 1982), and previous studies show that it is located in the PTs (Böddi et al. 1989). It was reported as photochemically inactive at flash illumination (Franck et al. 1993). The F633 form was attributed to monomeric Pchlide or Pchl molecules possibly bound to a protein of PT (Böddi et al. 1993). The selectively detected fluorescence excitation spectra yielded split Soret bands at positions very similar to those of F628. The Q bands (Table 1) show a difference compared to those of F628, what proves the existence of component F633 as the signature of a separate structural form. We have to note, however, that in this latter case it is hard to exactly determine the position of the $Q_y(0,0)$ band, because the fluorescence excitation spectrum becomes very intense in the range of the contribution from the F645 component of Band I (seen in Figure 3), and due to this dominating contribution it is impossible to determine the absorption band position of F633.

Based on previous studies by fluorescence emission spectroscopy of etiolated wheat leaves at 77 K a Gaussian component around 645 nm (F645) was suggested (Böddi et al. 1989; Böddi et al. 1992). This component was also found in the spectra of holochrome preparations and after detergent treatments (Wiktorsson et al. 1992). A strong energy migration to the F657 form was reported as a characteristic property of this band (Kahn et al. 1970). Therefore it was supposed that this spectral form corresponds to the enzyme bound Pchlide, situated in close proximity to the F657 form, on the edges of the PLBs (Böddi et al. 1992, Böddi 1994). In our fluorescence emission measurements at 10 K a small contribution from this form was found around 642 nm (15 575 cm^{-1}) (Figure 1). However, in the excitation spectra (detected in the vibrational region), the $Q_y(0,0)$ band at 640 nm (15 625 cm^{-1}) was rather intense (Figure 3), as expected if the emission of this component was weakened because of energy migration in fluorescence measurements. Thus our results support the previous identification of this structural form. The high emission intensity in the excitation spectrum shows that there is a significant amount of molecules, organized in this structural form.

The spectral band positions given in Table 1 for the three components of Band I being different show

the existence of three (0,0) components in this range. The estimated B and Q splittings in all three forms are very similar, what suggests that the reason for this splitting is mostly the asymmetric structure of the molecule itself and not the electric field of the embedding matrix.

The Pchlide forms with contribution in Band II

It was known from literature that the emission spectrum of etiolated leaves is usually dominated by one intense emission spectrum (Goedher and Verhülsdonk 1970). In wheat leaves, in experiments at 77 K, the intense (0,0) band was found at 657 nm (F657), the maximum of the corresponding vibrational envelope was at 726 nm (Böddi et al. 1992). Our measurements at 10 K made possible to directly determine the position of the (0-0) emission as 657,5 nm (15 209 cm^{-1}) of 7 nm (167 cm^{-1}) width, and vibrational satellites with maxima at 669, 687, 714 and 730 nm, the corresponding vibrational frequencies are: 350, 750, 1250 and 1550 cm^{-1} (see Figure 6). We believe, that to approximate such a composite range by fitting one or two Gaussians may be misleading, because the vibronic range is the envelope of many vibronic lines of specific pattern, and the envelope may change dramatically with structural deformations of the pigment. By comparison with data obtained at 77 K before, one can identify the published position of the vibrational satellite as that of the maximum at 1550 cm^{-1}, in this work. The composite nature of the emission spectrum may also be a problem in identifying the vibronic contribution of a single species because of overlaps with some (0-0) bands. The absorption bands could be estimated by fluorescence excitation spectra (Table 1). The width of the Q_y(0,0) band (of a fitted Gaussian) in the excitation spectrum was 190 cm^{-1}, by 23 cm^{-1} wider, than in the emission spectrum (Figure 3). This suggests the composite character of this form, since it shows that emission occurs only from a component in the red side of the absorption band. The situation resembles that in chromophore dimers, where both donor and acceptor parts of the molecule are seen in the excitation spectrum, in emission, however, only the acceptor fluorescence is present (Mauring et al. 1995). The significant Stokes shift of 140 cm^{-1} (Figure 3) is an indication of a distorted molecular structure of the chromophore.

Site selective excitation was performed to reveal the composite nature of Band II. We found different phenomena when the excitation was in the blue or in the red side of the Q_y(0,0) band. In case of excitation in the blue (<652 nm, >15 340 cm^{-1}) was that the emission spectrum was independent of the energy of excitation and the emission spectrum had its (0,0) position at a well observable red shifted position relative to the energy of excitation (Figure 5A). The interpretation is that in case of these excitations the emitting electronic state is not the one that is directly excited, but the energy is transferred to always the same, one specific type of molecules with an emitting state of lower energy. In case of excitation in the red side of the band, the emission spectrum shifted with the tuning of the exciting laser frequency. This shows that in each case, the excitation selects certain groups of molecules from an inhomogeneous population of transition energies, with very similar vibrational features but slightly different (0,0) transitions. In these emission spectra, the intense (0,0) could not be measured being too close in energy to the site of excitation. The process of photon emission in these molecules is not affected by the presence of other members of the population. Thus the FLN studies at 10 K in Band II reveal two kinds of structural organizations with (0,0) band positions in close proximity, which suggests that their environment can not be very much different with respect to polarity. The experimental data can be interpreted as the effect of energy migration (transfer) in case of excitation in the blue side of the absorption band to the group of emitting chromophores that act as acceptors. When the excitation is in the red side, the acceptor molecules are directly excited. The site selection effect proves that these excited states are not coupled by energy transfer. Very similar results were obtained for the PS II reaction center complex and interpreted by ‘intraband’ energy transfer (Kwa et al. 1994).

The effect of the temperature can elucidate the biological functioning of the structural forms found by the FLN studies at low temperatures (below 100 K). The temperature seems to affect mostly those phenomena that are related to Band II, as the components of Band I do not significantly change. There were two consequences of increasing the temperature: the emission intensity decreased remarkably, and the shape of Band II changed by broadening towards higher frequencies. The decrease of the fluorescence yield of photoconvertible Pchlide was described earlier for bean leaves when the temperature was increased from 77 K to 183 K (Goedher and Verhülsdonk 1970). In experiments at room temperature by flash light excitation, short lived intermediates of Pchlide phototransformation with absorption bands at wavelengths longer than

that of the excitation were detected in bean leaves (Franck and Mathis 1980). These forms were also detected at 77 K and were found to be non-fluorescing (Dujardin and Correla 1979). The proportion of these intermediates was suggested to increase with the temperature (Ignatov and Litvin 1995). A temperature activated intermediate formation explains the decrease of the emission intensity in our experiments also. There can be a set of structural conditions that is required for the formation of these species in the excited state of Pchlide. An excited state reaction is supported by the fact, that the temperature effect was reversible below 100 K.

When inhomogeneously broadened spectra are measured at different temperatures, in the absence of interaction between chromophores, red shift of the fluorescence emission band is observed with increasing temperature, as the increased probability of the generation of phonon vibrations means a loss in the energy of emitted photons (Pullerits et al. 1995). In our system, however, the effect did not correspond to this prediction. We believe that the spectral change can also be interpreted as the consequence of the temperature activation of one (or more) non-fluorescent intermediate state competing with the fluorescent state seen at low temperature as Band II in emission with high intensity. The appearance of additional emitted photon energies at the blue side of Band II at higher temperatures may be simply the contribution of those Pchlide molecules that are in an environment characteristic for the donor type structural form, but not properly located for energy transfer to the acceptors. This contribution may even be always present in this band, just overwhelmed by the intense emission from the acceptors at low temperature. In PS I, isolated from spinach, an intense emission band was observed at 735 nm, at 77 K. Upon increasing the temperature, the intensity of this band decreased and the maximum shifted toward shorter wavelengths. The effect was attributed to a temperature activated energy transfer from an antenna complex to the reaction center (Mukerji and Sauer 1989), similarly to our experience. The literature data thus support our interpretation of the results.

The interpretation of Band III, vibronic contributions

Band III was separately analyzed because it was identified by Gaussian fitting as a spectral component in the emission spectra in measurements at 77 K (Böddi et al. 1989). In the present studies it became evident that most of the intensity in this range arises through vibronic transitions. The most clear evidence came from FLN studies: when the fluorescence was excited selectively in the red wing of Band II, the shoulder at 670 nm (14 925 cm^{-1}) shifted with the excitation wavelength, as vibrational bands do (Figure 6). We can identify this contribution with the band in the vibronic envelope at a vibrational energy of 350 cm^{-1}. The fluorescence excitation measurements when detected at longer wavelengths (Figure 3) reveal, however, that there is a (0,0) contribution at the long wavelength side of Band II, with a maximum at 674 nm. Such a small contribution may arise from the presence of random aggregates of Pchlide, since Pchlide aggregates in solution had strongly red shifted bands (Kotzabasis 1990).

Conclusions

Low temperature comparative fluorescence emission and excitation spectroscopy revealed several structural forms of Pchlide in etiolated wheat leaves. The data suggest the presence of two monomeric structural forms, a significant amount of small size aggregate (possibly dimeric) structures in close contact with the main structural form of higher order protein aggregates and a small amount of random Pchlide aggregates. The main structural form was found to be organized in Pchlide donor and acceptor structures. When the temperature is raised from 10 K to 100 K the energy absorbed in the small size aggregates and in the donor-acceptor complexes of the main structural form, is transferred to non fluorescent intermediates. The formation of intermediates is a reversible, thermally activated excited state reaction in the studied temperature range.

Acknowledgements

The authors are grateful for the helpful assistance by Rózsa Markács-Árpádi at the Institute of Biophysics. Support from the Hungarian grants MKM/173, OTKA T25545, MKM-FKFP 11911/1997 and ETT 425/1996 is greatly appreciated.

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