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Spectroscopic characterization of protochlorophyllide photoreduction in the greening leaf

F. FRANCK, X. BARTHELEMY and K. STRZAŁKA*

Laboratory of Photobiology, Department of Botany B22, University of Liège, Sart-Tilman, B-4000 Liège, Belgium
*Institute of Molecular Biology, Jagiellonian University, Al. Mickiewicza 3, 31-120 Krakow, Poland**

Abstract

Room temperature absorbance and 77 K fluorescence measurements were used in order to identify Pchlide and Chlide spectral forms involved in protochlorophyllide photoreduction in greening leaves of barley. $Pchlide_{650}$ (the subscript refers to the *in vivo* absorbance maximum of the pigment) is the main photoactive Pchlide throughout the first 8 h of greening. Its photoreduction triggers a succession of Chl(ide) spectral forms that are identical to those normally found after photoreduction in unirradiated leaves. After an actinic radiation pulse, $Chlide_{684}$ appears within 2 s from an intermediate at shorter wavelength and is transformed to $Chlide_{672}$ in less than 2 min. The time-scale of the shifts is remarkably shorter than in unirradiated leaves, which is consistent with the acceleration of Chl accumulation during greening. $Pchlide_{630}$ and $Pchlide_{640}$ act as precursors of $Pchlide_{650}$ during its regeneration, which exhibits a marked inhibition at temperatures above 30 °C.

Introduction

The photoreduction of Pchlide in etiolated leaves takes place within pigment-protein complexes that accumulate during growth in darkness (for review see Hendrich and Bereza 1993). These complexes are constituents of prolamellar bodies and prothylakoids of the etioplasts. Spectroscopic studies established that three major Pchlide-protein complexes occurred in dark-grown leaves: two photoactive complexes ($Pchlide_{650}$ and $Pchlide_{638}$) and inactive $Pchlide_{630}$ (Sironval 1981, Böddi *et al.* 1992). Irradiation of an intact leaf by short radiation flash reduces photoactive

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Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; Chl(ide), chlorophyll and/or chlorophyllide; Pchlide, protochlorophyllide. *Subscript:* absorbance maximum of pigment in the red region.
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Pchlides to $Chlide_{678}$ which then shifts to $Chlide_{684}$ within seconds, and later to $Chlide_{672}$ within some minutes, this latter shift being known as the Shibata shift (Shibata 1957, Gassman *et al.* 1968, Bonner 1969). An interpretation of these major spectroscopic events, observed upon the first irradiation of an intact leaf, has emerged from *in vitro* studies: photoactive Pchlides consist of the stable ternary complexes of Pchlide, NADPH and the enzyme NADPH-Pchlide-reductase (El Hamouri *et al.* 1981, Oliver and Griffiths 1982). The rapid Chlide shift after a flash is due to replacement of $NADP^+$ by NADPH, followed by the release of Chlide from apoenzyme during the Shibata shift. Photoactive Pchlide complexes are most likely organized in multimeric structures, causing pigment-pigment interactions revealed by circular dichroism studies (Böddi *et al.* 1990). The disaggregation of these complexes is now proposed as the primary event in the Shibata shift, rapidly followed by Chlide release and esterification (Ryberg *et al.* 1992).

The events described above initiate the greening process which associates Chl accumulation and chloroplast differentiation during prolonged irradiation of etiolated leaves. We showed recently that photoactive and inactive Pchlide complexes, with spectroscopic properties close to those of etiolated leaves, are continuously regenerated and transformed under continuous irradiation (Franck and Strzałka 1992). In this paper we further characterize the process of photoreduction and the associated spectral shifts in etiolated barley leaves subjected to continuous greening.

Materials and methods

Barley seedlings (*Hordeum vulgare* cv. Avilion) were grown in darkness for 6 d on vermiculite and tap water at 23 °C. Intact plants were then placed under cool white fluorescent tubes for greening at an irradiance of 15 W m^{-2}, but for the experiments in Figs. 1 and 8 that were done at irradiances of 2 and 20 W m^{-2}, respectively.

Absorbance difference spectra were calculated from absorbance variation kinetics recorded at room temperature with a *DW-2 Aminco* spectrophotometer in the dual-wavelength mode, the reference wavelength being set at 730 nm. A 2 s "white light" pulse (120 W m^{-2}) was used as actinic radiation. Rapid absorbance variations (in the second time-range) were obtained with the experimental set-up described by Michel and Sironval (1977), the actinic flash being provided by a *Strobe Multiblitz* flash lamp (duration of flash, 2 ms).

Fluorescence spectra were recorded at 77 K either with a *Perkin-Elmer LS50* fluorimeter (experiments in Figs. 1 and 8) or with an optical multichannel analyzer (*O.M.A. II, Princeton Instruments*) under the indicated excitation wavelength. No normalization was made, except where indicated.

Results

Photoactive protochlorophyllide in the light: If Chl accumulation during greening proceeds through continuous regeneration and phototransformation of photoactive

Pchlide-protein complexes, then small amounts of photoactive Pchlides should be continuously present in the light during greening of etiolated leaves. That this is indeed the case was shown in Franck and Strzałka (1992). An experiment which demonstrates occurrence of photoactive Pchlides in the light is presented in Fig. 1, comparing the 77 K fluorescence spectrum of a leaf frozen in the light after 7 h of continuous greening under fluorescent tubes with the spectrum of another leaf treated in the same way, except that it received an intense millisecond flash immediately before freezing. The band at 653 nm, which disappears after the flash, arises from photoactive Pchlide present under low irradiances. In order to detect its fluorescence, high amplification must be used since it represents (at this particular developmental stage) only about 0.1 % of the maximum Chl fluorescence intensity at 735 nm. The excitation wavelength is set around 452 nm, where the blue excitation maximum of photoactive Pchlide in green or greening leaves occurs (Lebedev *et al.* 1985, Franck and Strzałka 1992).

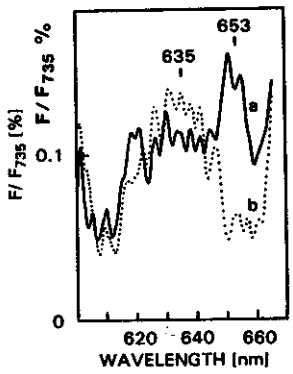

Fig. 1. 77 K fluorescence spectra of intact barley leaves in the Pchlide region after 7 h of greening. Excitation at 452 nm. The leaves were frozen in the light without flash (*a*) or immediately after a flash (*b*).

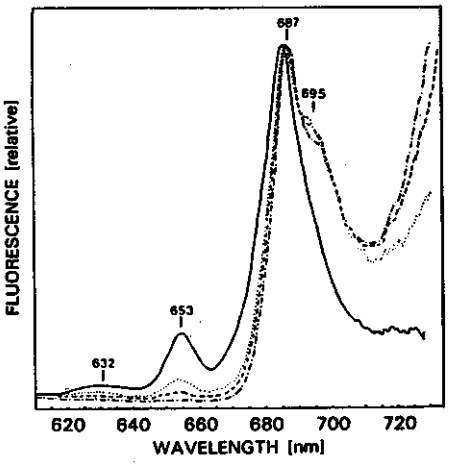

Fig. 2. 77 K fluorescence spectra of leaves frozen after a 15 min dark period following greening during 1 (—), 3 (···), 5 (-·-) or 7 (-··-) h. Excitation at 436 nm.

During a short period of darkness given after various greening times, photoactive Pchlides accumulate rapidly and become readily detectable in fluorescence spectra without special magnification. Fig. 2 shows 77 K fluorescence spectra obtained at the end of a 15 min dark period which followed greening during 1 to 7 h. Two bands at 632 and 653 nm were always found, corresponding to inactive and active Pchlides, respectively ($Pchlide_{628}$ and $Pchlide_{650}$). Their relative intensities decreased progressively with greening time. In fact, as previously demonstrated by Virgin (1984), a process of rapid regeneration of a small pool of photoactive Pchlide progressively develops during greening. Its half-time after interruption of irradiation is in the order of 1 min, as shown for example in the experiment of Fig. 3 where the room temperature absorbance of photoactive Pchlide at 650 nm has been measured as a function of time in darkness after 5 h greening.

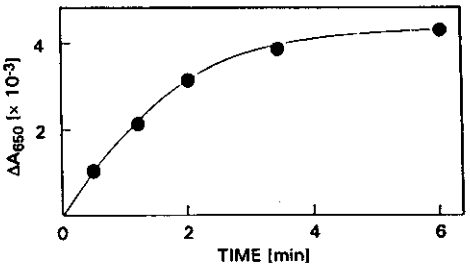

Fig. 3. Time-course of the regeneration of photoactive $Pchlide_{650}$ after greening during 5 h measured as the amplitude of the radiation-induced absorbance decrease at 650 nm as a function of time in darkness (all data obtained with a single leaf).

Transformations of Pchlide and Chlide spectral forms in greening leaves: The process of rapid regeneration of a pool of photoactive Pchlide after greening for some hours could be repeated several times with the same leaf during a series of short (2 s) actinic radiation pulses spaced apart by 4 min of darkness. In this case, always the same amount of photoactive Pchlide was reduced at each pulse. After having introduced a greening leaf in the spectrophotometer it was possible to measure light-induced absorbance variations at various selected wavelengths by changing the wavelength of the measuring beam before each successive actinic radiation pulse. Time-courses of light-induced absorbance variations were recorded in this way at wavelengths of every 5 nm between 630 and 705 nm. Actual traces obtained with a single leaf after 4 h of greening are shown in Fig. 4 at some wavelengths of interest. The traces at 655 and 635 nm show the photoreduction of photoactive Pchlide followed by its regeneration and the traces at 665 and 695 nm reflect a rapid Shibata shift after each actinic radiation pulse.

Whole difference spectra were calculated from the absorbance kinetics obtained after various greening times from 1 to 8 h. The absorbance difference spectra calculated at t = 10 s and 2 min after the actinic radiation pulse are shown in Fig. 5 for leaves taken after 1, 4 or 8 h of greening under fluorescent tubes. They exhibit constant features except for the 8 h spectra which are distorted in the Chl region due to a considerable flattening effect. In all cases the maximum of photoactive Pchlide is

located at 650 nm. A negative shoulder (or peak after 4 h) around 640 nm in the 2 min difference spectra indicates that Pchlide with an absorbance maximum in that region (probably $Pchlide_{638}$) is a precursor of $Pchlide_{650}$ during its rapid regeneration. Within 2 min after an actinic radiation pulse a Chl(ide) shift from 685 to 672 nm was observed. Difference spectra in the Chl(ide) region were identical to those measured in completely etiolated leaves during the Shibata shift from $Chlide_{684}$ to Chl_{672}. The shift is, however, much more rapid in greening leaves than in the etiolated ones (see also Franck and Strzałka 1992).

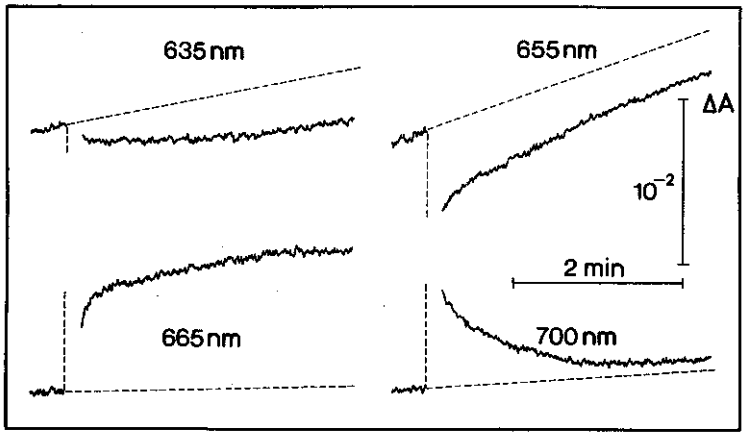

Fig. 4. Kinetics of the radiation-induced absorbance variations at indicated wavelengths measured at room temperature in a leaf after 4 h of greening. Each recording was done after a 4 min dark-adaptation period. *Dashed vertical lines* indicate the onset of the 2 s actinic radiation pulse.

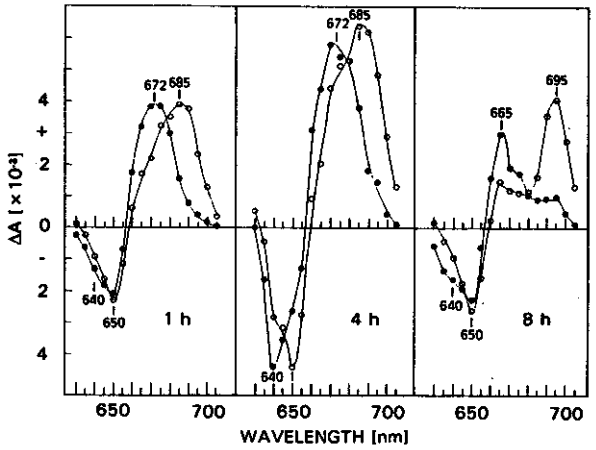

Fig. 5. Difference spectra of radiation-induced absorbance changes calculated at 10 (○) or 120 s (●) after onset of the actinic radiation pulse. The measurements were done after greening times of 1, 4 or 8 h as indicated. The dark period between two consecutive recordings was 4 min.

In etiolated leaves, $Chlide_{684}$ is not the "primary" Chlide spectral form resulting from Pchlide photoreduction. It arises from a rapid transformation of a precursor at shorter wavelength, $Chlide_{678}$. This transformation (or rapid Chlide shift) preceeds the Shibata shift and takes place within some seconds after a short millisecond flash (Gassman *et al.* 1968, Bonner 1969). In order to verify that this particular shift also exists in greening leaves, we have compared room temperature absorbance variations at 705 nm in the second time-range after a millisecond flash in etiolated leaves and in leaves after 3 h of greening. The rapid shift from $Chlide_{678}$ to $Chlide_{684}$ resulted in an increase of the 705 nm absorbance after the flash which was observed in greening as well as in completely etiolated leaves (Fig. 6). Again, this shift is markedly accelerated in greening leaves (half-time close to 1 s instead of 4 s).

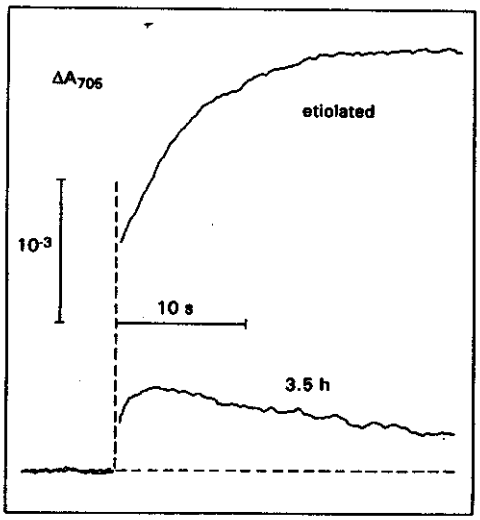

Fig. 6. Room temperature absorbance variations at 705 nm induced by a ms flash in an unirradiated leaf (*upper trace*) and in a leaf after 3.5 h of greening followed by a 5 min dark period (*lower trace*).

Changes in the 77 K fluorescence of Pchlide associated with Pchlide phototransformation and regeneration in greening leaves are shown in Fig. 7 for greening times of 3 and 5 h. Leaves frozen in the light showed always three Pchlide emission bands around 632, 643 and 653 nm (the 643 nm component was seen only as a shoulder). These maxima are slightly blue-shifted in comparison with those of three major Pchlide forms usually found at 633 (inactive Pchlide), 645 and 657 nm (active Pchlides) in dark-grown leaves before irradiation. When the greening leaves were kept for 5 min in darkness before freezing, a large increase of Pchlide fluorescence was observed, reflecting the rapid accumulation of the three Pchlide forms. Flash irradiation of 5 min redarkened leaves strongly reduced the 653 nm peak but did not alter the 632 nm one, thus confirming the 632 nm emitting Pchlide of greening leaves as an inactive form of the pigment equivalent to the $Pchlide_{630}$ of unirradiated leaves. After the flash a rapid conversion of this inactive Pchlide to 643 and 653 nm emitting, active Pchlides occurred, as seen in the spectra of leaves frozen at increasing times after the flash. Identical events were observed at the two selected

greening times of 3 and 5 h. Changes in the 77 K Chl(ide) fluorescence during the above experiment were more difficult to appreciate than changes in Pchlide fluorescence because of the large background of Chl fluorescence that made comparison between different leaves less reliable. We observed, however, a transient increase of the Chl(ide) fluorescence around 695 nm after a flash (not shown), thus confirming the formation of $Chlide_{684}$ upon Pchlide photoreduction in greening leaves ($Chlide_{684}$ has a 77 K fluorescence maximum at 696 nm in dark-grown leaves after a flash, Sironval 1981).

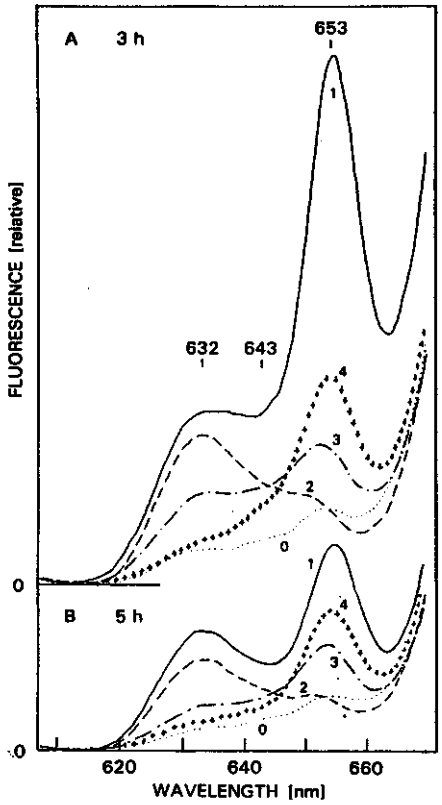

Fig. 7. 77 K fluorescence spectra in the Pchlide region after greening for 3 (*A*) or 5 (*B*) hours. Excitation at 450 nm. Pre-treatment was as follows: none (frozen under the greening radiation) (*0*), 5 min of darkness (*1*) followed by a 1 ms flash (*2*) and a further 10 s (*3*) or 2 min (*4*) dark period. Spectra were not normalized.

Temperature effect on the regeneration of photoactive Pchlide in greening leaves: Photoactive Pchlide complexes of dark-grown leaves are very sensitive to temperature. They are inactivated when the temperature is kept above 40 °C (Dujardin and Sironval 1970). We have attempted to evaluate the temperature sensitivity of photoactive Pchlides and their regeneration in greening leaves by measuring the modification of the Pchlide 77 K fluorescence properties produced by an elevation of the temperature during a short time after a 5 h greening period. The

leaves were heated during 10 min to various temperatures between 25 and 55 °C in a water bath and were then placed in darkness for 5 additional min at 25 °C before freezing. The 77 K fluorescence intensities at 653 and 632 nm (emission maxima of photoactive and inactive Pchlides) were then measured.

As shown in Fig. 8, the 653 nm fluorescence intensity declined rapidly at temperatures above 35 °C and reached almost zero around 45 °C. The large increase of the 653 nm fluorescence intensity at temperatures above 45 °C was due to an increase of Chl *b* fluorescence, as revealed by the excitation spectra of this emission showing a maximum at 470 nm (not shown). The 632 nm fluorescence intensity was practically unchanged at temperatures up to 40 °C but showed a very large increase at higher temperatures, with a maximum around 50 °C. The complex temperature dependence of the 653 and 632 nm fluorescence can be explained by considering the effects of two independent phenomena: the inhibition of photoactive Pchlide assembly (being complete around 45 °C) and the inhibition of energy transfers from Pchlide and Chl *b* to Chl *a* which takes place at higher temperatures. The drop of the 632 nm fluorescence intensity between 50 and 55 °C may be due to an additional alteration of Pchlide at high temperature.

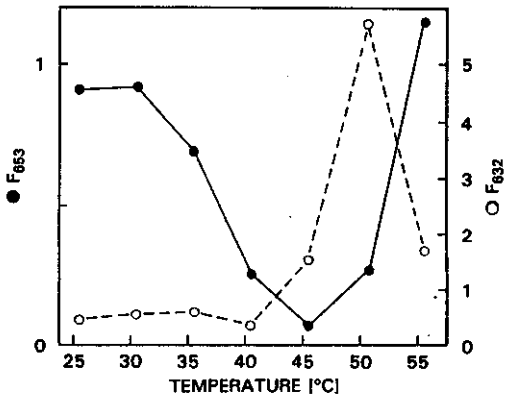

Fig. 8. 77 K fluorescence intensities at 653 (●) and 632 (○) nm as a function of temperature during a 10 min heat treatment applied in the light after 5 h of greening. The leaves were transferred in darkness at room temperature after heat treatment during 5 min before freezing. Note the different scales used for the two wavelengths.

Discussion

Photoactive Pchlide exists not only in the leaves of etiolated plants. It was also found in greening and in green leaves after some period of darkness (Cohen and Rebeiz 1981, Lebedev *et al.* 1985, Minkov *et al.* 1988). Our recent findings that photoactive Pchlide can even be detected in light in greening leaves and that its actual amount depends directly on irradiance (Franck and Strzałka 1992) demonstrate that radiation-dependent accumulation of Chl involves continuous formation and photoreduction of a Pchlide-protein complex very similar to the $Pchlide_{650}$ of dark-grown leaves.

The main evidence arising from the results in this paper is that Pchlide photoreduction in greening leaves involves a succession of spectral forms of Pchlide and Chlide similar to the one found previously in dark-grown leaves. Inactive $Pchlide_{630}$ is a precursor of the photoactive $Pchlide_{650}$ which upon radiation excitation produces $Chlide_{684}$ from an intermediate at shorter wavelength (probably identical to $Chlide_{678}$). $Chlide_{684}$ is then rapidly transformed to $Chlide_{672}$ (Shibata shift). $Pchlide_{638}$ is identified here as a precursor of $Pchlide_{650}$. This was not reported in studies on unirradiated leaves but it might also be true in that case if one considers that $Pchlide_{638}$ and $Pchlide_{650}$ most probably represent different states of pigment aggregation in the complex (Ryberg *et al.* 1992).

In etiolated barley, the spectral shifts leading to Chl_{672} (the end product of the Shibata shift) are completed within some 15 min after a single flash *in vivo* (Henningsen and Thorne 1974). They are followed by a slower red shift corresponding to the formation of photosynthetically active Chl-protein complexes (Shibata 1957, Fradkin *et al.* 1982, Franck 1993). Under continuous irradiation these processes are markedly accelerated as shown in this paper by the rapid formation of $Chlide_{684}$ and $Chlide_{672}$ from the photoactive Pchlide which was regenerated within 5 min of darkness after some hours of greening. They might even be faster when they occur without this short dark-adaptation time, as suggested by the decrease of the half-time of the Shibata shift with shorter dark-adaptation times (only 8 s for a dark-adaptation time of 1 min, Franck and Strzałka 1992).

The very slow time-course of the Shibata shift in etiolated leaves is probably due to the structural constraints imposed by the association of Pchlide-protein complexes with the prolamellar bodies, which dissociate at the beginning of greening in continuous radiation (Henningsen and Boynton 1969). When this step is achieved, a fast release of Chl(ide) from phototransformed Pchlide-protein complexes would be possible and would in turn favour the rapid accumulation of Chl in the light.

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