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Detection of the photoactive protochlorophyllide–protein complex in the light during the greening of barley

Fabrice Franck[a] and Kazimierz Strzalka[b]

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

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A photoactive protochlorophyllide-protein complex with absorbance and fluorescence maxima at 648 and 653 nm was detected in greening barley leaves without any re-darkening. The variations of the amplitudes of the absorbance and the fluorescence of the photoactive protochlorophyllide with greening time at two different light intensities indicate a close relationship between the rate of chlorophyll synthesis and the amount of the complex during the first hours. The chlorophyllide resulting from photoreduction during greening has an absorbance maximum at 684 nm, which shifts towards a shorter wavelength within a few seconds, indicating rapid liberation of the pigment from the enzyme. We conclude that chlorophyll accumulation proceeds through continuous regeneration and phototransformation of the photoactive complex.

Chlorophyll synthesis; Protochlorophyllide reductase; Greening

1. INTRODUCTION

In angiosperms chlorophyll (Chl) synthesis depends on light. Although much effort has been undertaken in order to elucidate the mechanism governing Chl accumulation in the light, the role of the enzyme, protochlorophyllide (Pchlide) reductase, in this process is still controversial [1–3]. This enzyme is a major membrane protein of the etioplasts of etiolated plants where it catalyzes the photoreduction of Pchlide into chlorophyllide (Chlide). Upon continuous illumination of etiolated plants the amount and the activity of this enzyme, however, decrease to a low level within a short time (1–2 h) although the rate of Chl accumulation increases at the same time [1,2,4]. For this reason it was sometimes concluded that the function of Pchlide reductase in Chl synthesis is restricted to a short time after a sufficiently long period of darkness and that Chl synthesis in greening or green leaves might involve another, hypothetic enzymatic system [1,2].

In etioplasts, Pchlide reductase forms a stable complex with its substrate, Pchlide, and the cofactor, NADPH [5]. Upon illumination, Pchlide is rapidly reduced into Chlide in the complex which then dissociates within several minutes. The Pchlide–enzyme–NADPH complex of etiolated leaves, or 'photoactive Pchlide', has in vivo main absorbance and fluorescence bands at 650 and 657 nm, respectively [6,7]. These bands disappear after a short light flash and are replaced by Chlide bands. It is therefore in principal easy to detect photoactive Pchlide through in vivo spectroscopic measurements.

If Pchlide reductase remains the main light-requiring enzyme involved in Chl accumulation during greening, one expects the photoactive Pchlide–protein complex to be continuously regenerated and phototransformed in the light. If this is so, a small amount of the complex should be present at any time in the light, especially when light intensity is low. In this paper we show by in vivo spectroscopic measurements that this is indeed the case. Our results also indicate that Chlide dissociation from the enzyme is much more rapid in illuminated leaves than in etiolated ones, which can explain that rapid Chl accumulation is sustained even at relatively low enzyme levels.

2. MATERIALS AND METHODS

Barley seedlings (*Hordeum vulgare* cv. Avilion) were grown in darkness for 6 days on vermiculite and tap water at 23°C. Greening was performed with intact plants under cool white fluorescent tubes at two different intensities of 2 and 20 $W \cdot m^{-2}$ (low and medium intensities) at 25°C.

Fluorescence and fluorescence excitation spectra were recorded at 77 K using a Perkin-Elmer LS50 fluorimeter. Fast recordings of absorbance spectra were performed at room temperature with an optical multichannel analyzer (OMA II, Princeton Instruments). In this case greening was carried out in the experimental set-up with the measuring light (Sylvania 21 V projector lamp, type DKM) at the same time actinic and analytic, at an intensity which could be fixed by changing the voltage applied to the lamp. The flash used to saturate Pchlide photoreduction was provided by a Strobe Multiblitz flash lamp (duration of flash, 2 ms). Flash-induced absorbance variations at fixed

Correspondence address: F. Franck, Laboratory of Photobiology, Department of Botany B22, University of Liège, Sart-Tilman, B-4000 Liège, Belgium. Fax: (32)(41)562 926.

Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; Pchl, protochlorophyll; Pchlide, protochlorophyllide.

were obtained in the experimental set-up described in [9] with the same flash as above.

3. RESULTS

The 77 K fluorescence spectrum of a 6-day-old barley leaf exposed to low intensity illumination for 5 h shows the 3 emission bands of Chl at 685, 696 and 740 nm normally found in green leaves (Fig. 1, insert). This shows that the photosynthetic apparatus is already well developed at this stage under our conditions. No distinct emission can be seen in the region from 600 to 670 nm at the usual amplification. However, upon amplifying this region of the spectrum 2 emission bands at 635 and 653 nm appear, of which the latter is more intense (Fig. 1A, spectrum 1). When a short light flash was given immediately before freezing the leaf in the light, the 653 nm band almost disappeared but the 635 nm band was unchanged (Fig. 1A, spectrum 2). The 653 nm band was restored to its initial intensity if the leaf was kept under the same continuous light for 5 min after the flash and was then frozen (Fig. 1A, spectrum 3). Redarkening of the leaf for only 5 min after the 5 h greening period led to a large increase of the intensities of both 635 and 653 nm bands (Fig. 1A, spectrum 4). Similar results were obtained with leaves pre-illuminated with light of medium intensity, but in that case the amplitudes of the 635 and 653 nm bands were much lower and became difficult to detect in leaves frozen in the light (Fig. 1B).

In etiolated leaves, 2 fluorescence bands at 633 and 657 nm are always found [7]. The 657 nm band arises from the stable, ternary complex between Pchlide, the enzyme and the cofactor, NADPH (photoactive Pchlide). The 633 nm band arises from both protochlorophyll (Pchl) and Pchlide under inactive forms. The two bands at 635 and 653 nm that we found in illuminated leaves may arise from two equivalent Pchl(ide) complexes that would be continuously synthesized and transformed by light during greening. It is known, however, that Chl *b* emits fluorescence also around 655 nm in vivo [9]. In order to discriminate between Pchlide and Chl *b* emissions we recorded the excitation spectra of the 653 nm fluorescence.

The excitation spectrum of the 653 nm fluorescence in leaves frozen in the light after a 5 h period of greening under low light intensity shows 2 maxima at 452 and 470 nm (Fig. 2, spectrum a). The 470 nm band is due to Chl *b* whereas the 452 nm band should be ascribed to photoactive Pchlide [9]. When the leaves were darkened for 5 min prior to freezing, the 452 nm band emerged as the main one and a shoulder around 470 nm was also visible (Fig. 2, spectrum b). The large increase of the 452 nm band produced by the 5 min darkening confirms this band as the main excitation band of photoactive Pchlide in greening leaves. For comparison the excitation spectrum of the 657 nm fluorescence of photoactive Pchlide in etiolated leaves is shown (Fig. 2, spectrum d). It is characterized by a main band at 447 nm and a shoulder around 460 nm and is therefore slightly blue-shifted in comparison to greening leaves.

The intensity of the fluorescence of photoactive Pchlide was measured as a function of greening time. These measurements were done by calculating the difference between the spectrum of a leaf frozen in the light and the spectrum of a leaf frozen immediately after a saturating flash. Examples of difference spectra obtained after 3 h of greening under medium and low light intensity are shown in Fig. 3A. They exhibit a main band of photoactive Pchlide at 653 nm which varied in intensity during greening. Fig. 3B shows that at both intensities used, F653 increases for about 3 h and then decreases to a low level. On the other hand the calculated ratio of long-wavelength fluorescence (725–740 nm) from PSI to short-wavelength fluorescence (685–696 m) from PSII shows an increase during the entire

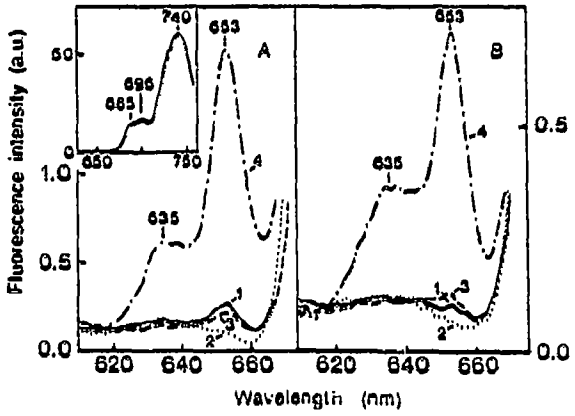

Fig. 1. 77 K fluorescence spectra of intact leaves in the Pchlide region after 5 h of greening at 2 (A) or 20 (B) W·m^{-2}. Excitation wavelength 440 nm. The leaves were frozen in the light (1), immediately after a flash (2), 5 min in the light after a flash (3) or after a 5 min dark period (4). Insert: fluorescence spectrum in the Chl region after 5 h of greening at 2 W·m^{-2}.

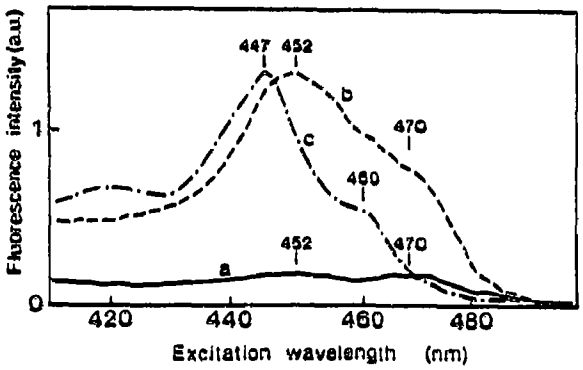

Fig. 2. 77 K excitation spectra of the 653 nm fluorescence in leaves frozen after 5 hours of greening at 2 W·m^{-2} (a) or after a further 5 min dark period (b), and 77 K excitation spectrum of the 657 nm fluorescence of an etiolated leaf (c). The amplitude of spectrum c has been adjusted to the one of spectrum b.

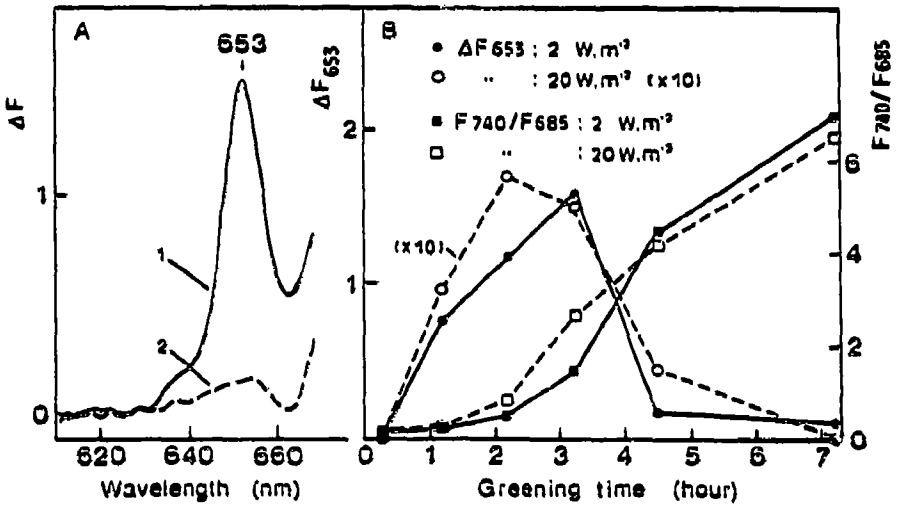

Fig. 3. (A) 77 K fluorescence spectra of the photoactive Pchlide under 452 nm excitation after 3 h 15 min of greening at 2 (1) or 20 (2) W·m⁻², obtained by calculating the difference spectra between a leaf frozen in the light and a leaf frozen after a flash (no normalization was used). (B) Time-course of the changes of ΔF653 and of the F740/F690 ratio during greening at both light intensities.

period of greening studied, which indicates normal development of the photosynthetic apparatus under both light intensities. After 7 h of greening, photoactive Pchlide could still be detected at low but not at medium light intensity. A general 10-fold decrease of ΔF653 was observed at medium light intensity (20 W·m⁻²) as compared to low light intensity (2 W·m⁻²) throughout the greening period investigated.

The regeneration of photoactive Pchlide in the light was monitored quantitatively using room temperature absorbance measurements in vivo. Measurement of the in vivo Chl absorbance during the first 5 h of greening showed that the time-course of Chl accumulation was identical at both light intensities used (Fig. 4A). Quantification of the amount of photoactive Pchlide in the light was done by measuring sample absorbance before and after a flash given at various greening times and by calculating difference spectra (unflashed minus flashed) for each time. In order to minimize the regeneration of Pchlide during recording after flash, fast scanning (5 s per spectrum) was performed. Panel B of Fig. 4 compares difference spectra recorded In etiolated leaves (1)

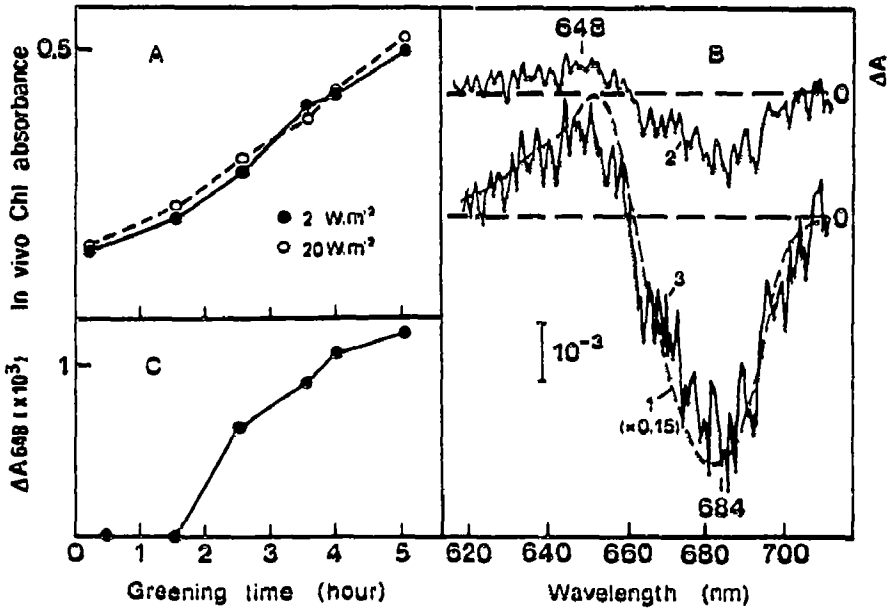

Fig. 4. (A) Time-course of the increase of the in vivo Chl absorbance (measured around 678 nm) at room temperature during greening. (B) Room temperature absorbance difference spectra (unflashed–flashed) obtained with etiolated leaves (1) or with the same leaves under greening light of 2 W·m⁻² after 3 h (2) or after a further 5 min dark period (3). Spectrum 1 has been normalized with spectrum 3. (C) Time-course of the increase of ΔA648 with greening at 2 W·m⁻².

and in the same leaves after 5 h of greening under low light intensity (2) or after a further 5 min re-darkening (3). They all show a positive part, around 648 nm corresponding to photoactive Pchlide, and a negative part, around 648 nm, corresponding to its photoreduction product, Chlide. The data show that the actually existing photoactive complex after 5 h of greening under low light intensity represents 6% of its amount in etiolated leaves. A 5 min re-darkening of the leaves raised this level to 15%. Under high light intensity the photoactive complex could not be detected using this technique due to background noise.

Changes of ΔA648 as a function of greening time under low light intensity are represented in Fig. 4C. The absorbance of photoactive Pchlide was clearly detected only after 2 h, and its amplitude then increased gradually in the course of greening.

In order to explain the rapid Chl accumulation at relatively low levels of enzyme [1–4], we hypothesized that the turn-over rate of the photoactive complex is much faster in greening plants than in etiolated ones. To verify this we followed the kinetics of the transformation of Chlide 684 to Chlide 672 (Shibata shift, [10]), which reflects the liberation of Chlide from the enzyme and its esterification to Chl *a* [11,12]. Original traces of flash-induced absorbance variations at 665 and 695 nm during this shift are reproduced in Fig. 5 for leaves illuminated for 5 h at medium intensity. The measured half-time of the process (8 s) proved to be several orders of magnitude shorter than the corresponding half-time observed in 6-day-old etiolated barley leaves after a short flash (6 min, [12], and data not shown).

4. DISCUSSION

Up to now the photoactive Pchlide complex has been detected in green or greening plants only after transferring them to darkness [9,13–16]. The results presented in this paper demonstrate that this complex exists in the light. This finding strongly suggests that Chl accumulation proceeds through continuous regeneration and phototransformation of a Pchlide–enzyme–NADPH complex with a red absorbance band at 648 nm, and is in agreement with a previous study showing that the action spectrum of Chl accumulation during greening has a red maximum around 650 nm [17]. According to our measurements, the complex has slightly different emission and excitation maxima in greening leaves than in etiolated ones (653 and 452 nm instead of 657 and 457 nm). Such a shift was also observed in re-darkened green leaves [9], and its nature is not well understood. The Chlide which results from Pchlide photoreduction during greening has a red absorbance maximum at 684 nm, which is found also upon Pchlide photoreduction in etiolated leaves [6,8,10].

The amount of the complex depends on greening time and on light intensity. Shortly (=15 min) after the onset of illumination the complex could not be detected, but after 1 h its fluorescence at 653 nm could be easily measured. The intensity of this fluorescence increased then up to about 3 h and then sharply declined. On the other hand detection of the complex by measuring its absorbance was possible only after 2 h. This apparent discrepency can be explained by different sensitivities of both methods. Unlike its fluorescence intensity, the absorbance of the complex exhibits a continuous increase approaching a plateau around 5 h of illumination. The different behaviour of fluorescence intensity and absorbance during the period 3–5 h after the onset of illumination is most probably related to an increase of energy transfer efficiency from Pchlide to Chl, as it exists in re-darkened green leaves [14]. An increase of the amount of photoactive complex during the first hours of greening is consistent with the acceleration of Chl synthesis and with the increase of the amount of Pchlide observed in this period [18].

The actual amount of the photoactive complex depends on light intensity. This is clearly evidenced by our fluorescence data (Fig. 3B) which show an inverse proportionality with light intensity in an intensity range at which Chl accumulation still occurs at the same rate (Fig. 4A). This observation can be explained by the linear dependence of the rate constant of Pchlide photoreduction on light intensity [19], if one assumes that Pchlide and NADPH availability is not limited. In other words, the turn-over of the complex increases with light intensity, which ensures efficient synthesis of Chl at low steady-state levels of the photoactive complex. Such rapid turn-over would not be possible if the release time of Chlide was long, as observed for the classical Shibata shift in etiolated leaves [10,12]. A shortening of this shift would be a necessary condition for the observed rate of Chl synthesis. Our kinetics measurements indeed revealed a considerable acceleration of the process.

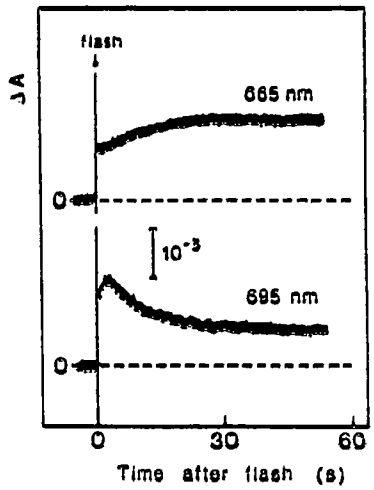

Fig. 5. Kinetics of the flash-induced absorbance variations at 665 and 695 nm after 3 h of greening at 20 $W \cdot m^{-2}$ followed by a 1 min dark period.

In conclusion, the presented results clearly indicate that Pchlide photoreduction in greening leaves is operated by the enzyme Pchlide reductase as it is in etiolated leaves, but that the turn-over of the photoactive complex is greatly accelerated.

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