HPLC determination of photosynthetic pigments during greening of etiolated barley leaves

Evidence for the biosynthesis of chlorophyll *a*′

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Received 9 December 1997; revised version received 10 March 1998

Abstract The temporal evolution of pigment composition during greening of etiolated barley leaves was investigated by reversed-phase HPLC with particular attention to chlorophyll (Chl) *a*′ (C13^2 epimer of Chl *a*), which had been detected by ourselves in photosystem (PS) I particles. At early stages of greening, the Chl *a*′/Chl *a* molar ratio rapidly increased to a level more than twice that in mature leaves, then gradually leveled off, accompanied by a growth of the Chl *b*/Chl *a* ratio, to the mature level. After 3 h of illumination, the temporal evolution of the Chl *a*′/Chl *a* molar ratio nearly paralleled that of the P700/Chl *a* ratio with a stoichiometry Chl *a*′/P700 ≅ 2: this strongly suggests that Chl *a*′ is biosynthesized as a constituent of the PS I reaction center complex.

Key words: Photosystem I; Pigment biosynthesis; Chlorophyll *a*′; High performance liquid chromatography; Greening

1. Introduction

In oxygen-evolving photosynthesis, minor chlorophyll (Chl) derivatives play crucial roles in the light reaction. Our normal-phase HPLC analyses of pigment composition of higher plants, cyanobacteria and photosystem (PS) I and II particles [1–3] showed that two molecules of Chl *a*′ (C13^2 epimer of Chl *a*) are in close association with the PS I reaction center (RC), P700 [1], and suggested possible involvement of this minor pigment in the RC photochemistry. These analyses were, however, done exclusively for mature organisms. It is of much interest to examine how Chl *a*′ is synthesized in the dynamics of plant greening, where cofactor biosynthesis and photosystem assembly are taking place [4–6]. If this pigment is indeed an essential constituent of the PS I reaction center, its relative amount is expected to show a characteristic time course in association with the developmental stage of photosystems, and to take a kinetics paralleling that for the accumulation of P700. The experimental results suggest that two molecules of Chl *a*′ are biosynthesized as an ingredient of the PS I RC complex during greening.

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Abbreviations: Chl, chlorophyll; Pchlide, protochlorophyllide; Chlide, chlorophyllide; LHC, light-harvesting complex; PS, photosystem; RC, reaction center; GG, geranylgeranyl; DHGG, dihydro-GG; THGG, tetrahydro-GG; P, phytyl

2. Materials and methods

2.1. Plant growth

Seeds of barley (*Hordeum vulgare* L.) were planted in vermiculite, watered with a full-strength Hoagland's nutrient solution, and grown in a dark chamber for 5.75 days at 25°C. The greening was promoted by illumination with a white fluorescent tubes of an intensity 40 μW/cm^2 at 25°C. After prescribed periods of illumination, the leaves (2 cm from the top) were harvested and immediately frozen with liquid nitrogen and stored at −70°C until isolation of thylakoid membranes. This freezing treatment did not alter the pigment composition in samples (data not shown).

2.2. Isolation of thylakoid membrane

About 0.2 g of frozen leaves was homogenized in a Polytron type homogenizer with 2 ml of a grinding medium containing 0.33 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine-NaOH buffer of pH 7.8. The homogenate was filtered through four layers of a 50 μm nylon mesh and then centrifuged at 12 000 × *g* for 3 min. The resulting pellet was suspended in 30–150 ml of a hypotonic medium containing 10 mM NaCl, 5 mM $MgCl_2$ and 50 mM Tricine-NaOH (pH 7.8). For P700 measurement, 6–15 g of fresh leaves were homogenized with the grinding medium described above. After filtration, thylakoid membranes were pelleted by centrifugation at 4000 × *g* for 3 min and then washed once with a hypotonic medium at 8000 × *g* for 5 min. All the manipulations were done at 4°C under dim green light.

2.3. Pigment extraction

Ten microliters of a thylakoid membrane suspension was added to 0.5 ml of chilled acetone/methanol (1/1, v/v) mixture, followed by sonication in ice-water for 10 s. Then the pigment solution was filtered through a 0.45 μm PTFE filter, Millipore Millex-FH_{13}, and evaporated under reduced pressure. The dried sample was redissolved in dichloromethane and evaporated again. All the manipulations were done as rapidly as possible under dim green light. The use of acetone/methanol (1/1) mixture ensured almost complete extraction of chlorophyllous pigments, as will be detailed elsewhere.

2.4. HPLC analysis

The dried pigment was dissolved in 30–150 μl of the HPLC eluent, acetone/acetonitrile/methanol/water (43/32/15/10, v/v). A 1–6 μl aliquot of the solution was injected into a polyetheretherketone column packed with Spherisorb-ODS2 (4.6 mm × 150 mm, Senshu Science, Tokyo). The column temperature was maintained at 20°C by circulating thermostatted water around the column. The pigments were eluted isocratically at a flow rate of 1.0 ml/min, and were monitored with two fluorescence detectors (JASCO FP-920 and Hitachi L-7480) in series, for preferential detection of Chl *a* or Chl *b* derivatives. The HPLC traces and peak areas were recorded on a Shimadzu C-R6A integrator.

The Chl *a*′/Chl *a* molar ratio was obtained directly from the peak areas because their fluorescence quantum yields are the same (Watanabe et al., unpublished result). The Chl *b*/Chl *a* molar ratio was deduced from calibration curves as described previously [7,8]. The amounts of loaded Chls *a* and *b* were always within the linear region of calibration curves (data not shown).

2.5. Determination of P700

The photooxidation of P700 was evaluated via the light-induced

PII S0014-5793(98)00344-5

absorbance change at 700 nm with a double-beam spectrophotometer JASCO V-560 modified for lateral illumination. The sample was illuminated with a blue-green light produced by passing white light from a 500 W Xe lamp (Ushio UXL 500 D-O) through a 10 cm water layer, a heat absorption filter (Hoya HA-50) and a broad band path filter (Corning 4-96). The actinic intensity was 9.5 mW/cm^2. The photomultiplier was protected from the actinic light by two sharp cut filters, Toshiba R-65.

The thylakoid membrane was solubilized by 0.2% dodecyl β-D-maltoside and the measurements were conducted in a reaction mixture containing 200 μM methyl viologen, 0.5 mM sodium ascorbate, 0.5 μM TMPD and the thylakoid membrane equivalent to 6–10 μM of Chl *a*. An extinction coefficient of 64 mM^{-1} cm^{-1} at 700 nm [9] was used to calculate the amount of P700. The photooxidation of P700 was in saturation under these conditions for any sample at different stages of greening. The concentration of a Chl *a* species was deduced from the respective peak area on the HPLC trace based on the calibration curve described above.

3. Results

3.1. Temporal evolution of pigments in early stages of greening

In greening leaves, several biosynthetic intermediates of Chls, in addition to the mature forms Chl a_P (Chlide *a* phytyl ester) and Chl b_P, are formed. These intermediates possess different isoprenoid side chains at the C17[3] moiety, such as Chl a_{GG}, Chl a_{DHGG} and Chl a_{THGG} [4], and reversed-phase HPLC has been a common choice for their separation [10–12]. Under the elution conditions developed in this work, all of these intermediates (and Chl a'_P) can be neatly separated.

Fig. 1 shows the HPLC traces of pigment extracts from thylakoid membranes at early stages of greening. On the trace for etiolated leaves (before illumination), peak 1 was identified as protochlorophyllide (Pchlide) based on its fluorescence property and absorption spectrum (data not shown). The trace for 15 min of illumination reveals the formation of several Chl *a*-type pigments. Peak 6 was identifiable as Chl a_P from its absorption spectrum and a retention time comparison with that of an authentic sample. Peaks 3, 4 and 5 were identified as Chl a_{GG}, Chl a_{DHGG} and Chl a_{THGG}, respectively, from their fluorescence properties and capacity factors (k'), the logarithm of which is known to depend linearly on the number of double bonds in the isoprenoid chain [11]. Peak 2 was identified as Chlide *a* from its absorption spectrum and the short retention time. The decrease in the relative heights of peaks 2–5 with prolonged illumination also supports the assignment of these components to the biosynthetic intermediates of Chl *a*.

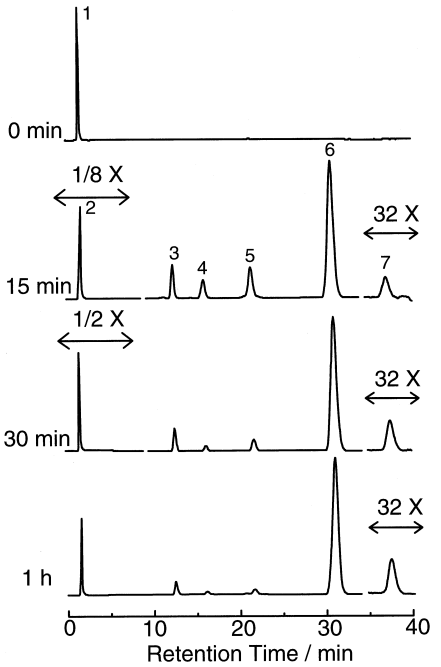

Fig. 1. HPLC traces of thylakoid membrane extracts at early stages of greening, detected with a fluorescence detector (excited at 425 nm, monitored at 670 nm). The Chl a_P peak areas are arbitrarily scaled to a common intensity.

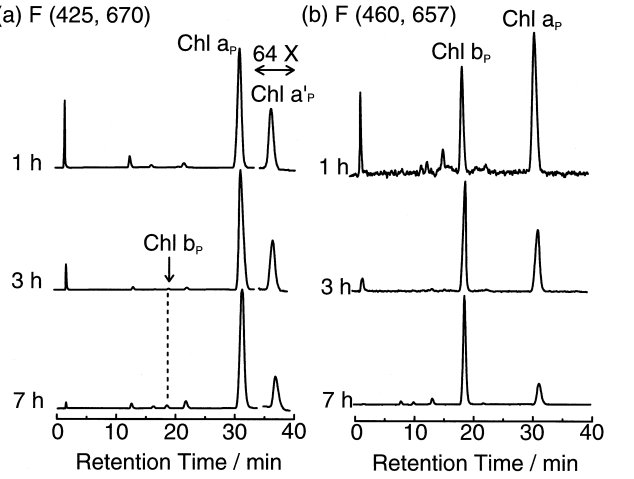

Fig. 2. HPLC traces, recorded simultaneously with two fluorescence detectors in series, of thylakoid membrane extracts at intermediate stages of greening. a: Chl *a*-enhancing detection (excited at 425 nm, monitored at 670 nm), where Chl a_P peak areas are arbitrarily scaled to a common intensity. b: Chl *b*-enhancing detection (excited at 460 nm, monitored at 657 nm), where Chl b_P peak areas are arbitrarily scaled to a common intensity. In b, the Chl a'_P peak was below the detection limit because of its low fluorescence intensity under the Chl *b*-enhancing detection.

Peak 7 was identified as Chl a'_P from retention time comparison with that of an authentic sample. The peak 7/peak 6 ratio remained unchanged at other excitation/emission wavelength combinations, namely under pheophytin *a*-enhancing conditions (excited at 410 nm and detected at 680 nm) and Chl *b*-enhancing conditions (see caption of Fig. 2). This indicates that peak 7 has the same fluorescence property as Chl a_P, and further supports the identification of peak 7. The pigment is detectable already after 15 min of illumination, and its amount increased through the early stage of greening. Detection of Chl a'_P before 15 min of illumination was hampered by its extremely low content. At early stages of greening, Pchls carrying side chains (Pchl$_{GG-P}$) also exist in the sample. According to Shioi et al. [13], Pchl$_{DHGG-P}$ are eluted after Chl a_P. It is possible that Pchl$_{DHGG-P}$ exhibit similar retention times as Chl a'_P and a trace amount of Pchl$_P$ overlaps with the Chl a'_P peak. However, in our HPLC conditions, Pchl$_{DHGG-P}$ were clearly separated from Chl a'_P (data not shown).

Fig. 2 depicts HPLC traces of a common thylakoid membrane extract after 1 h, 3 h and 7 h of illumination, recorded on two fluorescence detectors in series, namely a Chl *a*-enhancing detector F(425, 670) and a Chl *b*-enhancing one F(460, 657). The former detector neatly shows that the

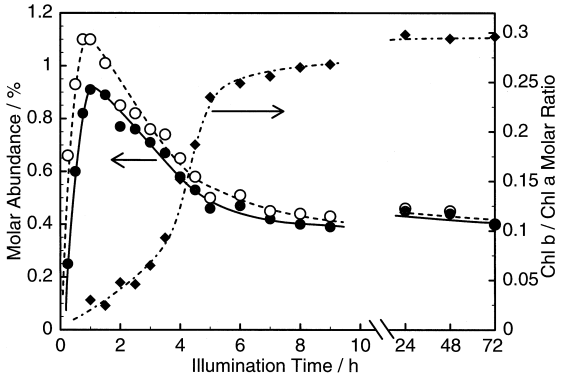

Fig. 3. Time courses of Chl a'_P/Chl a_{total} (●), Chl a'_P/Chl a_P (○) and Chl b_{total}/Chl a_{total} (♦) molar ratios in the course of greening. Each point represents the mean of three independent runs.

amount of Chl a'_P relative to Chl a_P decreases, in sharp contrast to the previous stages (Fig. 1), as the greening proceeds. This point will be discussed below.

Chl b_P was clearly detected already after 1 h of illumination, and the Chl b_P/Chl a_P ratio tended to increase in the progress of illumination. Three intermediates, Chl $b_{GG-THGG}$, which were identified from the log k' values of the respective peaks as in the identification of Chl $a_{GG-THGG}$, were observed after 3 h of illumination, and the molar abundance of these species slightly increased from 3 h to 7 h of illumination but remained below 10% of the mature Chl b_P throughout the greening. The abundance of Chl $a_{GG-THGG}$ decreased from 1 h to 3 h, then showed a slight increase toward 7 h of illumination (Fig. 2a).

3.2. Temporal evolution of the Chl a'/Chl a ratio

A series of HPLC analytical results are summarized in Fig. 3 in the forms of Chl a'_P/Chl a_P, Chl a'_P/Chl a_{total} and Chl b_{total}/Chl a_{total} molar ratios as a function of illumination time. The Chl a'_P/Chl a_P or Chl a'_P/Chl a_{total} molar ratio exhibited a characteristic temporal evolution: it increased abruptly from 15 min to 1 h of illumination, and reached a maximum at around 1 h at a level more than twice that in leaves after 72 h of illumination. The higher Chl a'_P/Chl a_P ratio than the Chl a'_P/Chl a_{total} ratio at very early stages of greening reflects the coexistence of a large amount of Chlide *a* (ca. 60% at 15 min, see Fig. 1). From 1 h to 6 h of illumination, the Chl a'_P/Chl a_{total} ratio gradually decreased, with a concomitant increase in the Chl b_{total}/Chl a_{total} ratio (Figs. 2 and 3). The increase in the Chl b_{total}/Chl a_{total} ratio is in line with previous reports [18–20]. After 9 h of illumination, the Chl a'_P/Chl a_P ratio reached a constant level close to that in samples after 72 h of illumination. At this stage, the Chl a'_P/Chl a_P molar ratio agrees well with the figure obtained for mature plants by means of normal-phase HPLC analysis [1].

3.3. Pigment integrity during analysis

To examine whether or not the low level of Chl a'_P detected in the present work arises from spontaneous epimerization of Chl a_P during sample treatment, a standard addition method was applied, where a solution of 99.9% pure Chl a_P (with 0.1% Chl a'_P as the sole impurity) was added to the extraction solvent which was then used for the thylakoid membrane extraction/HPLC analysis. The amount of pure Chl a_P added was changed stepwise, and the leaves after 1 h illumination (with maxima Chl a'_P/Chl a_P ratio, see Fig. 3) and after 24 h of illumination were employed as samples. Chl b_P was used as internal standard to evaluate the added Chl a_P. The results are plotted in Fig. 4 as the Chl a'_P/Chl a_P against the Chl b_P/Chl a_P ratio. The solid lines, which tend to the Chl a'_P/Chl a_P molar ratio (0.1%) for the 99.9% pure Chl a_P added at the left-hand extreme, represent the situations where no epimerization has taken place throughout the extraction/analysis procedures. The plots in Fig. 4 hence ensure that the analytical results displayed in Fig. 3 (and Figs. 1 and 2) well reflect the temporal evolution of pigment composition, or specifically the biosynthesis of Chl a'_P, during greening of etiolated barley leaves.

3.4. Accumulation of P700

A series of measurements, separate from those given in Fig. 3, were conducted for simultaneous determination of the Chl a'_P/Chl a_{total} ratio and P700/Chl a_{total} ratios at 1–8 h of illumination, and the results are displayed in Fig. 5. No photooxidation of P700 was detected before 1 h of illumination, despite the clear accumulation of Chl a'_P. The chemical oxidation method unraveled no spectroscopic characteristics of

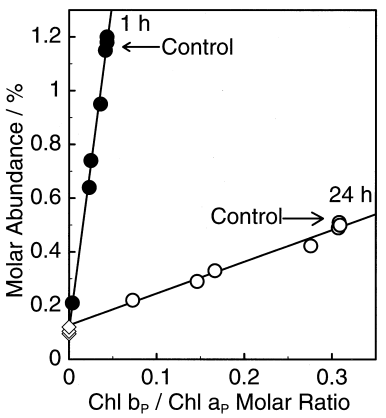

Fig. 4. Results of standard addition experiments for thylakoid membrane extracts at two greening stages (see text for details).

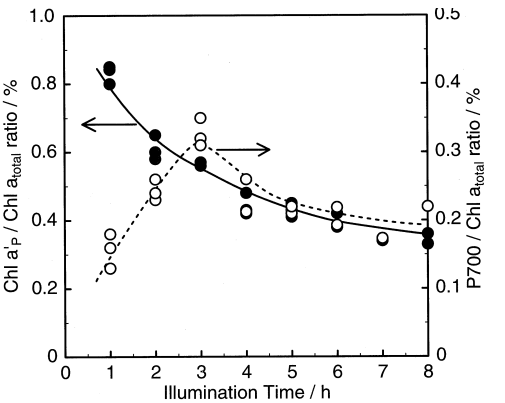

Fig. 5. Time courses of Chl a'_P/Chl a_{total} (●) and P700/Chl a_{total} (○) molar ratios during greening. Since the ordinate for P700/Chl a_{total} is scaled to half that for Chl a'P/Chl a_{total}, the relationship Chl a'_P/P700 = 2 holds if the solid and open circles take a common value.

P700 in this early phase, either (data not shown). At 1 h of illumination, the P700/Chl a_{total} ratio was lower than that in leaves after 8 h of illumination, in contrast to the fact that the Chl a'_P/Chl a_{total} ratio took a maximum value in the course of greening. The P700/Chl a_{total} ratio rapidly increased between 1 h and 3 h of illumination and the Chl a'_P/P700 ratio reached around 2, which is the value previously observed in mature thylakoid membranes and PS I particles [1]. In this period, the Chl a'_P/Chl a_{total} still showed a higher value than that in leaves after 8 h of illumination. After 3 h of illumination, the P700/Chl a_{total} and Chl a'_P/Chl a_{total} ratios decreased toward the values in mature leaves, with the Chl a'_P/P700 ratio being kept at about 2.

4. Discussion

The temporal evolution of pigment composition during greening (Fig. 3) shows that the rate of Chl a'_P biosynthesis is different from those of Chl a_P and Chl b_P. The rapid increase in the Chl a'_P/Chl a_P ratio from 15 min to 1 h of illumination, and the high Chl a'_P/Chl a_P ratio at early stages of greening, indicate that the Chl a'_P here is synthesized faster than Chl a_P, as compared with the later phase of greening. This suggests that Chl a'_P is located in a core part, which naturally is formed in early stages, of the photosynthetic apparatus [5]. In a later phase, formation and assembly of light-harvesting complexes begin to proceed, taking up Chl *b* and about half the amount of Chl a_P constituting the whole photosynthetic system [14]. The gradual increase in the Chl b_{total}/Chl a_{total} ratio with concomitant decrease in the Chl a'_P/Chl a_P ratio down to a 0.4% level from 1 to 9 h of illumination, could be rationalized by these considerations.

The nearly parallel decrease in the P700/Chl a_{total} and Chl a'_P/Chl a_{total} ratios after 3 h of illumination (Fig. 5) and the constant Chl a'_P/P700 ratio around 2 suggest that two molecules of Chl a'_P are biosynthesized as a constituent of the PS I RC complex. To date, a few studies have been published on the determination of the P700/Chl *a* ratio during greening [15–17]. Earlier works confirmed the PS I-mediated electron transfer from 2,6-dichlorophenol indophenol to methyl viologen [18–20], and the formation of Psa A/B apoprotein [15,21] within 15 min of illumination. However, no report is available which describes spectroscopic observation of P700 photooxidation for such an early stage of greening as in this study [6,15–17].

The temporal evolution of the Chl a'_P/Chl a_{total} ratio is neatly different from that of the P700/Chl a_{total} ratio at a very early stage of greening (Fig. 5). Though the cause for this is still unclear, a possible reason may be the degradation or inactivation of P700 during isolation of thylakoid membranes. The Psa A/B is already assembled with Chl *a* and a'_P at the very early stage, where co-translational binding of cofactors and apoproteins of RC complex is taking place [22,23]. However, the structure and activity of P700 is relatively unstable against thylakoid membrane isolation, due to incomplete assembly of nucleus-encoded subunits such as Psa D and E, that are accumulated with some delay during greening [24]. It was reported that a Psa D-deficient mutant of *Synechocystis* PCC6803 showed 60% lower P700 activity as compared to the wild type [25] and was suggested that Psa D and E subunits play a protective role for electron transfer activity of PS I [26]. Further examination is still needed to confirm the relationship between biosynthesis of Chl a'_P and the appearance of P700 activity.

Recently, the substrate specificity of enzymes participating in the Chl biosynthesis has been extensively studied. It was shown that the naturally occurring Pchlides have a 13^2 (*R*) configuration, and Pchlide a'-type (*S* configuration) derivatives are not photoreduced to Chlide a' [27]. In addition, chlorophyll synthetase does not catalyze the prenylation of a'-type Chlides. Helfrich et al. proposed that Chl a' would be formed after prenylation of Chlide *a* via an action of possible epimerase or the RC apoprotein itself [28]. Further work is in progress to establish the HPLC conditions that can separate Chl a'_{GG}, Chl a'_{DHGG} and Chl a'_{THGG} from possibly overlapping pigments, toward the goal of elucidating the biosynthetic pathway(s) of Chl a'.

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