Protochlorophyllide *b* does not occur in barley etioplasts

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Abstract Barley (*Hordeum vulgare* L.) etioplasts were isolated, and the pigments were extracted with acetone. The extract was analyzed by HPLC. Only protochlorophyllide *a* and no protochlorophyllide *b* was detected (limit of detection < 1% of protochlorophyllide *a*). Protochlorophyllide *b* was synthesized starting from chlorophyll *b* and incubated with etioplast membranes and NADPH. In the light, photoconversion to chlorophyllide *b* was observed, apparently catalyzed by NADPH:protochlorophyllide oxidoreductase. In darkness, reduction of the analogue zinc protopheophorbide *b* to zinc 7[1]-hydroxy-protopheophorbide *a* was observed, apparently catalyzed by chlorophyll *b* reductase. We conclude that protochlorophyllide *b* does not occur in detectable amounts in etioplasts, and even traces of it as the free pigment are metabolically unstable. Thus the direct experimental evidence contradicts the idea by Reinbothe et al. (Nature 397 (1999) 80–84) of a protochlorophyllide *b*-containing light-harvesting complex in barley etioplasts.

Key words: Chlorophyll biosynthesis; Chlorophyllid *b*; Etiolated; NADPH-protochlorophyllide oxidoreductase (POR); Photoconversion; *Hordeum*

1. Introduction

Chlorophyll (Chl) *b* which carries a formyl group at ring B where Chl *a* carries a methyl group, is found in Prochlorophyta, Euglenaceae, green algae and higher plants. As shown by isotope labeling, the formyl group arises from the methyl group present in Chl *a* and its precursor molecules by introduction of dioxygen [1,2]. The gene encoding the oxygenase for Chl *b* formation has recently been detected [3], but it is not yet clear at which stage the oxygenation occurs. Newly formed Chl *a*, chlorophyllide *a* and protochlorophyllide (Pchlide) *a* have been discussed as candidates (reviewed in [4]). The idea that Pchlide *a* is oxygenated to Pchlide *b* seems to be attractive because we showed already that the model compound zinc protopheophorbide *b* is photoconverted to zinc pheophorbide *b* by NADPH:protochlorophyllide oxidoreductase [5]. No data on Pchlide *b* photoreduction are available. However, to be photoreduced in etioplasts, Pchlide *b* must occur in this organelle. Early reports on the detection of Pchlide *b* were later withdrawn (reviewed in [6]). More recently, traces of Pchlide *b* were reported to occur in several green plants [7] and relatively high amounts of it (Pchlide *b*:Pchlide *a* = 5:1) in prolamellar bodies of dark-grown (etiolated) plants [8,9]. The latter report prompted us to reinvestigate the question of Pchlide *b* occurrence in barley etioplasts for three reasons: (1) Etiolated plants do not contain any Chl, thus misidentification with artifactual oxidation products of Chl can be excluded. (2) To exert its function in a light-harvesting complex for protection of dark-grown plants against photooxidative damage [8] Pchlide *b* must be present as a major pigment. (3) We have synthesized authentic Pchlide *b* (Fig. 1) which helps to identify this pigment in plant extracts. We show here that Pchlide *b* is indeed photoreduced upon incubation with etioplast membranes, but it does not occur in etiolated plants.

2. Materials and methods

Zn-Ppheide *b* was prepared according to [5]. Educt for the preparation of Pchlide *b* was Chlide *b* which was isolated from leaves of *Ailanthus altissima* taking advantage of the chlorophyllase reaction [10]. A solution of Chlide *b* in acetone was oxidized by adding a freshly prepared solution of DDQ in acetone drop by drop. The reaction was monitored by UV/VIS spectroscopy. After no further change in the absorption bands, the reaction mixture was quickly worked up with phosphate buffer and ethyl acetate [5]. Final purification on a self-packed polyethylene column (250×7.8 mm; isocratic separation at 60% aqueous 250 mM NH_4OAc buffer) yielded approximately 25% Pchlide *b*. UV/VIS spectrum is shown in Fig. 2. Mass spectrometry, using an electrospray atmospheric interphase and the positive ion mode (Finnigan LCQ) gave peak clusters at m/z 1253 (100%; $[2M+H]^+$) and 1879 (81%; $[3M+H]^+$); the molecular ion cluster at m/z 627 has only an abundance of 11%.

Etioplasts were isolated and purified from barley as described by Eichacker et al. [11] with modifications described by Scheumann et al. [12]. Pigments were extracted in 80% acetone. After centrifugation, the carotenoids and lipids of the supernatant were extracted into hexane, and the Pchlides were extracted into ethyl acetate. The pigments were transferred into acetone and subjected to HPLC analysis [13] (see Fig. 4). A column (250×4.0 mm) filled with C-18 reverse phase silica gel (Shandon, Hypersil ODS 5 μm) was used at a flow rate of 1.0 ml/min with a step gradient starting with 34% 25 mM aqueous NH_4OAc, 15% acetone and 51% methanol, increasing to 16% H_2O, 60% acetone and 24% methanol within 20 min and finally to 100% acetone another 4 min later. The gradient was hold at 100% acetone for another 10 min. Each second, an absorption spectrum of the eluted pigments was recorded from 350 nm to 750 nm with a diode-array-spectrophotometer. The spectrofluorimetric detector was set at 449 nm (excitation) and 631 nm (emission), the relatively highest sensitivity for Pchlide *b*. The data were evaluated with the LabControl software Spectra Chrom version 1.5. Pigments were identified by their absorption spectrum and their retention time by comparison with a standard.

The photoreduction of exogenous added Pchlide *b* to etioplasts, which were preirradiated before to photoreduce endogenous Pchlide *a*, was monitored by UV/VIS spectroscopy. The spectra were recorded on a double beam spectrophotometer (UV 2401 PC, Shimadzu), equipped with an Ulbricht globe.

1×10^8 etioplasts were resuspended in 1 ml buffer (1 mM $MgCl_2$, 1 mM EDTA, 10 mM Tricine, 10 mM HEPES/KOH, pH 7.2, 15 mM *n*-octyl-β-D-glucoside, 30% glycerol). After the addition of 1 mM NADPH, the sample was shaken in darkness at 0°C for 15 min.

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Abbreviations: Chl, chlorophyll; Chlide, chlorophyllide; DDQ, 2,3-dichloro-5,6-dicyano-benzoquinone; Pchl, protochlorophyll; Pchlide, protochlorophyllide; Ppheide, protopheophorbide; POR, NADPH:protochlorophyllide oxidoreductase

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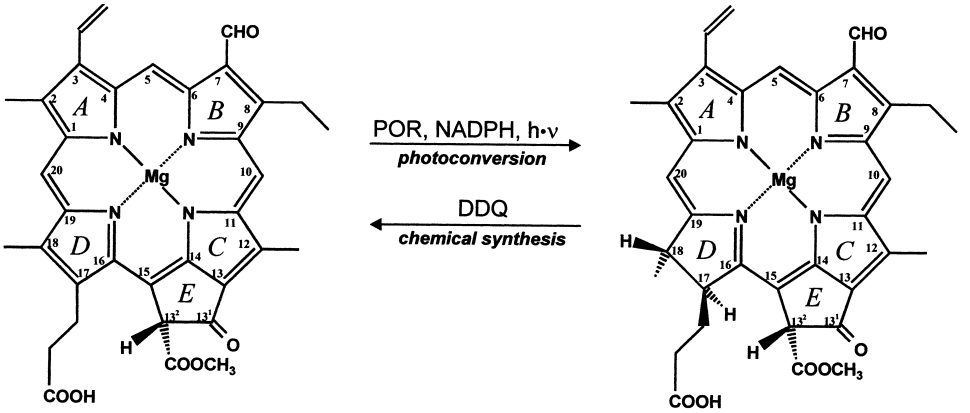

Fig. 1. Structures of protochlorophyllide *b* and chlorophyllide *b*. Pchlide *b* was prepared by chemical synthesis from Chlide *b*, enzymatic photoconversion leads back to Chlide *b*.

The following irradiation was performed with white light (approximately 3.6 $\mu Em^{-2}s^{-1}$ at the sample) for a 15 min period. Light source was a cold light lamp (KL 2500; Schott, Germany). To verify that all photoreducible endogenous Pchlide *a* was reduced to Chlide *a*, the sample was kept in darkness for another 15 min and irradiated afterwards with a light flux of 120 $\mu Em^{-2}s^{-1}$ for 30 s. The difference absorption spectrum showed that no further photoreduction of Pchlide *a* occurred. Then, 2 μl of a solution of Pchlide *b* in DMSO was added to the sample and after 5 min incubation in darkness, irradiation was performed in the same way as described for the endogenous pigment.

Reduction of Zn-Ppheide *b* was carried out in a dark-room under dim-green safety light as described previously [12]. The reaction mixture contained HEPES/KOH (50 mM, pH 7.5), $MgCl_2$ (10 mM), NADPH (2 mM), glucose-6-phosphate (16 mM), glucose-6-phosphate dehydrogenase (0.01 unit) and dithiothreitol (10 mM). Zn-Ppheide *b* (1.55 nmol) was dissolved in 1 μl DMSO and added to the sample containing 8.5×10^7 etioplasts in a total volume of 100 μl. The reaction was carried out at 28°C for 90 min and was stopped by adding acetone to a final concentration of 80%. The pigments were extracted and applied to HPLC as described above. The analytical column used here (Fig. 5) was filled with reverse phase material (Rosil C-18, size 5 μm) and the pigments were eluted at a flow rate of 1.5 ml/min with the following solvent system: the gradient started with 50% acetone/water, adjusted to pH 3.5 with acetic acid, and increased to 63% acetone within 23 min. The final concentration of 100% acetone was reached after another 6 min and held for 10 more min. In this case, the spectrofluorimetric detector was set at 435 nm (excitation) and 630 nm (emission) which is optimal for the detection of Zn-7^1-OH-Ppheide *a*.

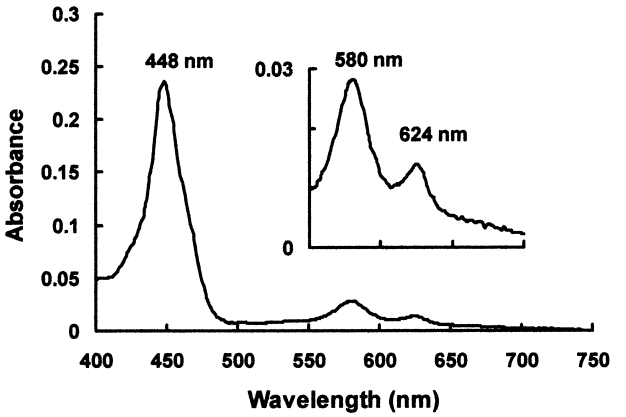

Fig. 2. Absorption spectrum of protochlorophyllide *b* in 80% acetone. Insert: wavelength range 550–700 nm at higher magnification.

3. Results and discussion

The preparation of Pchlide *b* by dehydrogenation of Chlide *b* with DDQ required the same type of precaution as the preparation of Pchl *b* from Chl *b* [5]. The relatively slow reaction with the molar ratio DDQ/Pchlide *b* = 1:1 yielded several by-products, but the rapid reaction with an excess of DDQ yielded one main product. The absorption spectrum

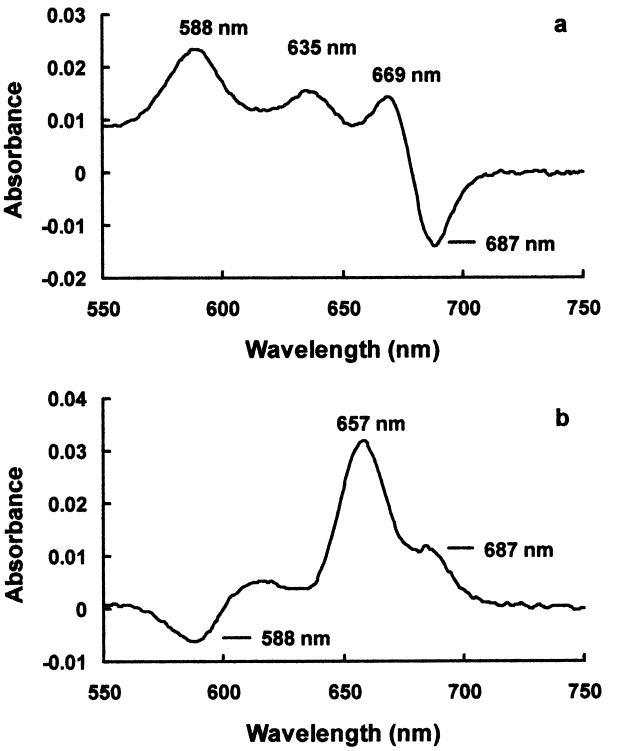

Fig. 3. Photoconversion of Pchlide *b* with preirradiated membranes of barley etioplasts. a: Difference absorption spectrum obtained by addition of Pchlide *b* (positive peaks at 588 and 635 nm) to the membranes in darkness. The negative peak at 687 nm indicates release of endogenous Chlide *a* from the enzyme with formation of free Chlide *a* absorbing at 669 nm. b: Difference absorption spectrum (spectrum of the irradiated sample minus spectrum a). Negative peaks at 588 and 635 nm signify disappearance of Pchlide *b*, the positive peak at 657 nm results from formation of Chlide *b*, and the shoulder at about 687 nm is due to formation of Chlide *a* from residual endogenous Pchlide *a*.

(Fig. 2) is virtually indistinguishable from that of Pchl *b*. Compared with zinc Ppheide *b* [5] the maximum of the Soret band of Pchlide *b* is blue-shifted by 2 nm and that of the Q_x and Q_y band red-shifted by 3–5 nm. The Q_x band has a higher absorbance than the Q_y band, this is a typical feature of all investigated pigments of the proto *b* series: Pchlide *b* (this paper), Pchl *b* and zinc Ppheide *b* [5], Chl c_3 [14].

Pchlide *b* was further characterized by its photoconversion with POR to Chlide *b*.

At first, the etioplast membranes were solubilized with octyl-glucoside, incubated with NADPH, and irradiated to transform most of the endogenous Pchlide *a* into Chlide *a*. Addition of Pchlide *b* in darkness resulted in the expected increase in the typical Pchlide *b* absorption (Fig. 3a), the shift from 580 nm to 588 nm and from 624 nm to 635 nm results most likely from solvent effects and protein binding of the added Pchlide *b*. The protein binding effect is corroborated by the negative peak in the absorption difference spectrum at 687 nm which indicated replacement of POR-bound endogenous Chlide *a* by the added pigment, the resulting free Chlide *a* shows then a positive peak at 669 nm. The analogous replacement of Chlide *a* by Pchlide *a* had been described before [15]. Irradiation of this sample leads to immediate formation of Chlide *b*, visible at 657 nm in the difference spectrum (Fig. 3b). This photoconversion confirms the binding of the added Pchlide *b* to the active center of POR. Furthermore, it proves the intactness of our Pchlide *b* preparation because allomerization products are no substrates for POR. Residual endogenous Pchlide *a* was phototransformed to Chlide *a* at the same time (Fig. 3b). If a less complete preirradiation had left more endogenous Pchlide *a* untransformed, photoconversion of both endogenous Pchlide *a* and exogenous Pchlide *b* was easily detected at the same time in analogous experiments (data not shown). Thus we conclude that the presence of Pchlide *b* should be visible not only by direct analysis but also by appearance of the typical Chlide *b* absorption upon phototransformation in solubilized etioplast membranes.

Etioplasts, isolated from dark-grown barley seedlings, were used for pigment extraction with acetone. The bulk of carotenoids and lipids was transferred into *n*-hexane, and the more polar pigments including Pchlide(s) were then extracted into ethyl acetate. This extract was applied to HPLC. There was no peak detectable at 9.55 min, the retention time of Pchlide *b* (Fig. 4a). The retention time was determined by addition of a defined amount of authentic Pchlide *b* to the pigment mixture (Fig. 4b). Allomerization products of Pchlide *a* have retention times not far away from that of Pchlide *b* (see Fig. 4a). However, such products have the typical absorption spectrum of Pchlide *a*. The limit of detection for Pchlide *b* besides Pchlide *a* is well below 1% Pchlide *b* in the presence of 99% Pchlide *a*. We used also a fluorescence detector for HPLC but did not detect any Pchlide *b* either (data not shown). Due to the low yield of fluorescence emission of Pchlide *b*, about the same limit of detection was observed. Thus we conclude that etioplasts do not contain any significant amounts of Pchlide *b*.

Neither Pchlide *b* nor chlorophyllide (Chlide) *b* are metabolically stable in etioplasts as the free pigments. We showed previously that Chlide *b* is reduced to Chlide *a* via 7^1-OH-Chlide *a* in intact and lysed etioplasts, and that the same reaction occurs with its analogue zinc pheophorbide *b* [12]. The reaction is catalyzed by chlorophyll *b* reductase, it requires NADPH for the first reduction step and reduced fer-

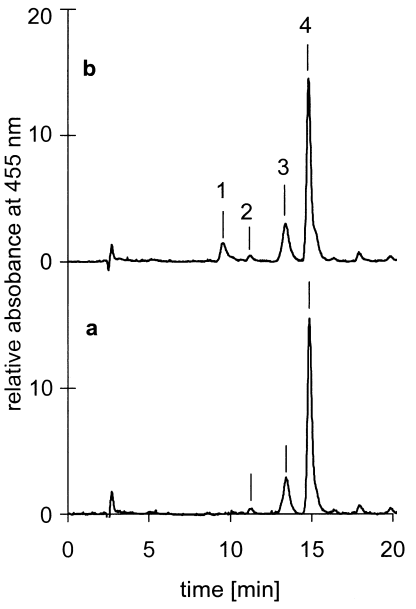

Fig. 4. Protochlorophyllide *b* is not detectable in barley etioplasts. a: Pigment analysis by HPLC of an extract shows only Pchlide *a* (peak 4) and allomerization products of Pchlide *a* (peaks 2 and 3). b: To the extract of a, containing 3.5 nmol Pchlide *a*, 100 pmol Pchlide *b* were added (peak 1). The absorbance of 455 nm given here corresponds to the Soret band of Pchlide *b*. The extinction of the same amount of Pchlide *a* at this wavelength is five times lower. Detection limit is at 10–20% of the Pchlide *b* amount added here, corresponding to $<1\%$ of the Pchlide *a* content.

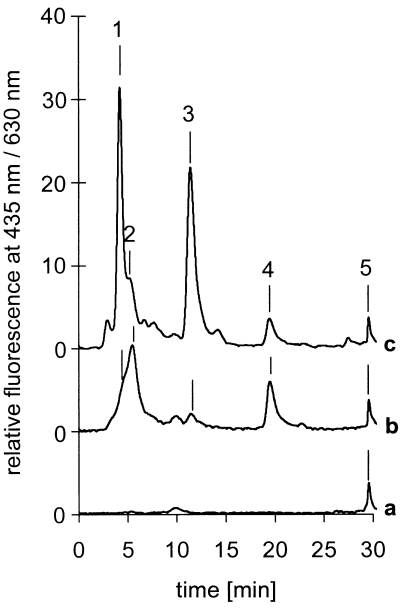

Fig. 5. Chlorophyll *b* reductase of barley etioplasts reduces Zn-protopheophorbide *b*. The reaction was carried out in darkness with 8.5×10^7 etioplasts in 100 μl buffer at 28°C for 90 min. Pigments were extracted with acetone and analyzed by HPLC. a: Endogenous pigments of the etioplasts. b: The same as a but incubated with Zn-Ppheide *b*. c: Incubation with Zn-Ppheide *b* and the NADPH reducing system. Peaks 1 and 2 are dithiothreitol addition products of Zn-Ppheide *b*; peak 3, Zn-7^1-OH-Ppheide *a*; peak 4, Zn-Ppheide *b*; peak 5, Ppheide *a*.

redoxin for the second reduction step. Here we show that zinc protopheophorbide (Ppheide) *b* is likewise reduced by chlorophyll *b* reductase (Fig. 5). When we incubated zinc Ppheide *b* with lysed etioplasts and NADPH in darkness to avoid phototransformation, we observed reduction of the formyl group to a hydroxy group. The resulting zinc 7^1-OH-Ppheide *a* (peak 3, Fig. 4c) makes up about 40–45% of the original zinc Ppheide *b* (peak 4, Fig. 4b, c) under the experimental conditions. Under identical conditions, 65–70% of added zinc Ppheide *b* were reduced (data not shown). To keep chlorophyll *b* reductase active, the lysed etioplasts had to be treated with dithiothreitol. This reagent gives addition products (peak 1 and 2, Fig. 4b, c) in a non-enzymatic reaction (control experiments not shown). Even if we do not find the complete reduction of the added pigment in vitro within 90 min, the capacity of chlorophyll *b* reductase in etioplasts is large enough to reduce as much Pchlide *b* during the long time of etiolation as accumulates in the form of Pchlide *a*. We cannot exclude the remote possibility that traces of Pchlide *b*, below the limit of detection, are bound to an (unknown) protein and therefore not accessible for chlorophyll *b* reductase.

During recent years, we performed numerous experiments with pigment analysis of etioplasts from barley, wheat, oat, and tobacco. We did not find any Pchlide *b* in these experiments, nor did we detect the typical peak of Chlide *b* upon phototransformation of endogenous pigments with solubilized membranes. The absence of Pchlide *b* from etioplasts is most probably a general property among flowering plants.

The idea that etiolated barley plants are protected against photodamage when exposed to light by a Pchlide-oxidoreductase-NADPH complex containing Pchlide *b*/Pchlide *a* in a ratio 5:1 [8,9] requires Pchlide *b* as a major pigment of etioplast membranes. Our analytical and biochemical data show that this is not the case.

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