Stabilization of the chlorophyll binding apoproteins, P700, CP47, CP43, D2, and D1, by synthesis of Zn-pheophytin *a* in intact etioplasts from barley

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Abstract Chlorophyll *a* was compared with Zn-pheophytin *a* for stabilization of chlorophyll binding apoproteins, P700, CP47, CP43, D2, and D1, in intact etioplasts from barley (*Hordeum vulgare* L.). Intact etioplasts were shown to effectively translate the chlorophyll apoproteins, to take up and esterify the exogenously added substrates, chlorophyllide *a* and Zn-pheophorbide *a*, with geranylgeraniolpyrophosphate. For stabilization of P700, CP47, D2, and D1, the product, Zn-pheophytin *a*, was shown to substitute for chlorophyll *a*. Stabilization of CP43 was selectively increased in the presence of Zn-pheophytin *a*. The degree of stabilization was shown to depend on the amount of newly synthesized Zn-pheophytin *a* and on the central atom of the chlorophyll molecule.

Key words: Chlorophyll; Reaction center protein; Stabilization; Etioplast; Greening

1. Introduction

Etioplasts isolated from 4-day-old dark-grown barley are an ideal in vitro system to study the chlorophyll *a* (Chl)-dependent accumulation of higher plant photosystems. In barley, etioplasts are formed from proplastids in the absence of light during the developmental phase of early primary leaf and plastid development [1].

Etioplasts accumulate protochlorophyllide *a* (Pchlide), a Chl precursor. In the light, Pchlide is reduced to chlorophyllide *a* (Chlide) by protochlorophyllide oxidoreductase in a NADPH-dependent reaction [2]. Chlide is then esterified with geranylgeranylpyrophosphate (GGPP) by Chl synthase in a light-independent step to yield Chl_{GG} [3]. Chl_{GG} is subsequently reduced to Chl_{phytol} [4]. In fully green plants Chlide is esterified directly with phytylpyrophosphate (PhPP) to form Chl_{Ph} [3,5]. In addition to prenylation of the natural substrates, Chlide *a* and *b*, chlorophyll synthase is also capable of prenylating modified tetrapyrrole derivatives [6]. Pentacoordinate metals (e.g. Mg or Zn) are accepted as central atom of the pheophorbide substrate, whereas metal-free pheophorbides or typical tetracoordinate central atoms (e.g. Cu, Co, Ni) are no substrate for the enzyme [7].

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Abbreviations: Chl, chlorophyll *a*; Chlide, chlorophyllide *a*; Chl *a*P, chlorophyll apoprotein P700, CP47, CP43, D1 and D2; GGPP, geranylgeranylpyrophosphate; LSU, large subunit of ribulose 1,5-bisphosphate carboxylase; Pchlide, protochlorophyllide *a*; PhPP, phytylpyrophosphate; Zn-phe, Zinc-pheophytin *a*; Zn-pheide, Zinc-pheophorbide *a*

In isolated etioplasts, Chl formation is paralleled by accumulation of the plastid-encoded Chl-binding apoproteins [8]. In the absence of Chl, etioplasts accumulate neither plastid-encoded Chl *a*-binding apoproteins, P700, CP47, CP43, D2 and D1 (Chl *a*P) [8,9], nor nuclear-encoded Chl *a*/*b*-binding apoproteins [10,11], although the plastid-encoded Chl *a*P are translated and degraded at high rates [12,13]. Via de novo synthesis of Chl from the precursors, Chlide and PhPP or GGPP, Chl *a*P were stabilized posttranslationally against proteolytic digestion and were shown to accumulate in the inner etioplast membranes during translation in a lysed etioplast expression system [14,15].

Here, we used Zn-pheophytin *a* (Zn-phe) as a tool, to show the impact of the central atom of Chl for stabilization of the newly synthesized Chl *a*P.

2. Materials and methods

2.1. Plant growth and plastid isolation

Barley (*Hordeum vulgare* L., var. Steffi) seeds were planted in moist vermiculite and grown for 4.25 days at 25°C in a light-tight growth chamber located in a dark room. At this stage of development, seedlings were 4–5 cm tall and still within the coleoptile. Approximately 60 g of etiolated primary leaves were cut 1 cm above the seed and were ground in ice-cold buffer A (0.4 M Sorbit, 2 mM EDTA, 50 mM HEPES, pH 8.0) and cell organelles were concentrated by a brief centrifugation ($4097\times g$, 3 min). Intact etioplasts were isolated by Percoll step gradient centrifugation (35/65% Percoll, 0.4 M Sorbit, 2 mM EDTA, 50 mM HEPES, pH 8.0; $4097\times g$, 7.5 min). The Percoll interface was aspirated, diluted 10-fold in buffer B (0.4 M Sorbit, 50 mM HEPES, pH 8.0) and intact etioplasts were repelleted ($4097\times g$, 2 min). Plastids were resuspended in buffer B and quantitated in a hemocytometer utilizing a phase contrast microscope. Intactness was generally found to be about 95%. All manipulations of dark-grown seedlings and isolated etioplasts were performed under a dim green safety light (< 10 nE/m^{2}s), which was unable to photoconvert measurable amounts of Pchlide.

2.2. Synthesis of Chl and Chl aP

Conditions for Chl and Chl *a*P synthesis in etioplasts (1.4×10^7 plastids/assay) were obtained in a buffer mixture at 25°C containing 50 mM HEPES/KOH (pH 8.0), 2.0 mM ATP, 0.2 mM GTP, 7 mM magnesium acetate (pH 7.0), 118 mM potassium acetate (pH 7.0), 10 mM DTT, 100 μM of each amino acid (except methionine) and 2.96×10^6 Bq of [^{35}S]methionine (spec. act. $>2.96\times10^{13}$ Bq/mmol, Amersham and Buchler, Braunschweig, Germany). Synthesis of Chl and Zn-phe in etioplasts was either achieved through a 30 s illumination (500 μE/m^2 s) with red light >550 nm (OG 550 filter, Schott, Mainz, Germany) and addition of GGPP and 0.25 mM NADPH or in the dark by addition of the chemically prepared Zn-pheide (0–4.4 μM) and GGPP (0–0.13 mM). Assays for determination of Chl or Zn-phe were scaled 3-fold with respect to ingredients and volume over standard translation assays, to yield a final volume of 225 μl and to contain 4.2×10^7 plastids. For determination of the amount of radiolabeled Chl *a*P stabilized against proteolytic digestion, protein synthesis was terminated after a reaction time of 40 min by addition of

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chloramphenicol (100 μg/ml) and assays were incubated for an additional 40 min under translation conditions.

2.3. Quantitation of radiolabel incorporation in Chl aP

Radiolabeled proteins from soluble and membrane fractions were solubilized in SDS and separated on 12.5% SDS-PAGs containing 4 M urea [16]. Proteins were loaded onto SDS-PAGs (8×10^5 plastids or equal amount of Chl or Zn-phe synthesized). Gels were fixed in acetic acid (7%), fluorographed (Amplify, Amersham and Buchler) and exposed to X-ray film. Radiolabeling of Chl *a*P was quantitated by two-dimensional laser scanning densitometry (LKB-Pharmacia, Uppsala, Sweden) of fluorograms. Fluorograms were tested to be in a linear range of laser absorption.

2.4. Preparation and analysis of pigments

The insertion of zinc(II) into pheophorbide, electronic absorption spectra, ^{1}H-NMR spectra, thin layer chromatography, analytical HPLC and preparative separation of Zn-pheide were carried out as described [7,17].

For addition to plastid-based esterification and translation assays, the pigment was dissolved in peroxide-free ether, to a pigment concentration of 1 mM, and stored under argon at −20°C. For esterification of Chlide or Zn-pheide to Chl or Zn-phe, 10–15 nmol of the Zn-pheide was transferred to 50 mM HEPES-KOH pH 8.0 (standard: 0.1 nmol/μl). Diethylether was evaporated under an argon stream, the M-pheide/HEPES solution was sonified for 2 min. Undissolved pigment or pigment aggregates were separated by brief microcentrifugation and the supernatant saved. An aliquot of the clear supernatant was extracted in 80% acetone and the pigment concentration determined by spectroscopy using the molar absorption coefficients of 90.2×10^3 l M^{-1} cm^{-1} at 660 nm for Chl *a* [18] and of 90.3×10^3 l M^{-1} cm^{-1} at 654 nm for Zn-phe *a* [19].

3. Results and discussion

3.1. Intact etioplasts are superior to lysed etioplasts with respect to the accumulation of Chl aP

Etioplasts isolated from 4-day-old dark-grown etiolated barley are an excellent source of Chl and protein synthesizing activity. When etioplasts were kept intact during the in vitro reaction, translation activity was high, resulting in radiolabeling of membrane (M) and stroma proteins (S2) (Fig. 1A; M and S2, lanes 1–4). During pulse labeling of intact etioplasts, accumulation of the membrane-bound Chl *a*P, P700, CP47, D2, and pD1 could already be observed in the dark (Fig. 1A, lane 1). After phototransformation and addition of GGPP to intact etioplasts, the membrane accumulation of P700, CP47, CP43, and D1 was increased over the level of Chl *a*P accumulation already observed in the dark (Fig. 1A; M, lane 2). The increased accumulation of membrane-bound Chl *a*P in the light could result from the selective induction of translation of membrane proteins or from stabilization of Chl *a*P against proteolytic digestion. However, upon illumination of etioplasts, no change in the abundance of translation initiation complexes or in the polysomal run off had been found, indicating that the light-stimulated synthesis of Chl led to a posttranslational stabilization of the Chl *a*P against proteolysis [15]. We therefore concluded that the light-induced Chl*a*P accumulation was achieved by uptake of GGPP into intact etioplasts and esterification with endogenous Chlide. Interestingly, no increase in the accumulation of the Chl *a*P, D2, could be observed, whereas accumulation of P700, CP47, CP43, and D1 was increased in an apoprotein-specific manner (Fig. 1A; M, lanes 1 and 2). This indicated that stabilization of Chl *a*P is regulated individually for every Chl *a*P. In parallel, no increased accumulation of the large subunit of ribulose 1,5-bisphosphate carboxylase (LSU) or the α and β subunit of the ATPase could be observed, indicating that in general translation of stroma proteins had not been affected through illumination and addition of GGPP (Fig. 1A; S2, lanes 1 and 2; LSU; α, β).

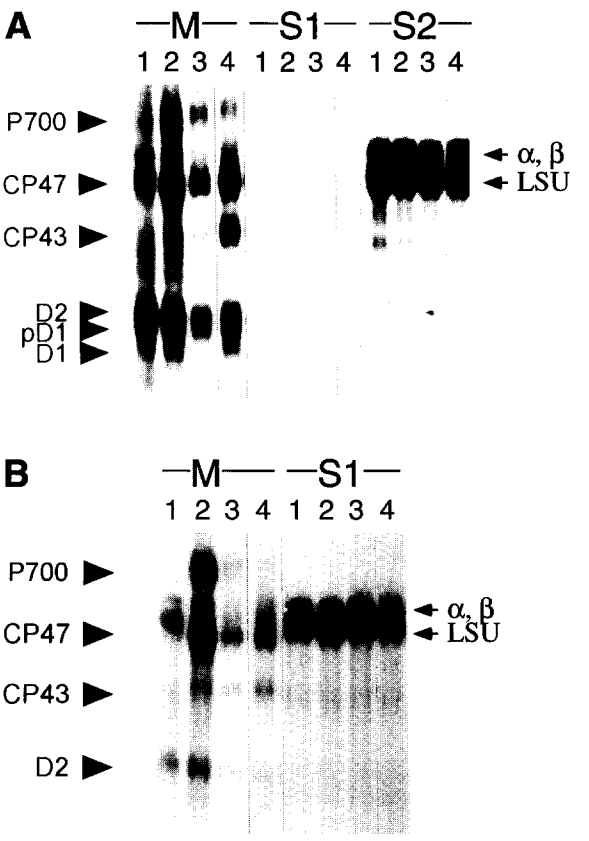

Fig. 1. Accumulation of Chl *a*P in intact or lysed etioplasts. Etioplasts (315 pmol Pchlide) were pulse-labeled for 40 min in the dark with [^{35}S]methionine under conditions allowing translation and synthesis of Chl (no pretreatment, minus GGPP (1); pretreatment (15 s illumination (500 μE/m^{2}s) on ice), plus GGPP (30 nmol) (2); addition of Chlide (900 pmol), plus GGPP (30 nmol) (3); addition of Zn-pheide (900 pmol), plus GGPP (30 nmol) (4)). The in organello translation assays (A) were microcentrifuged, the first supernatant saved (S1) and etioplasts fractionated into a membrane (M) and a soluble fraction (S2). The translation assays from etioplast lysates (B) were microcentrifuged and the membrane fraction (M) separated from the first supernatant (S1). Membranes from A or B were washed in CO_3/DTT (100 mM) and loaded on 12.5% SDS-PAG containing 4 M urea (equal Chl content (315 pmol) (M, lanes 2–4) or equal number of plastids (M, lanes 1 and 2; S1 and S2, lanes 1–4)). The migration of Chl *a*P, P700, CP47, CP43, D2, pD1, and D1 is indicated to the left, the migration of LSU and the α, β subunit of ATPase (α, β) to the right of the figure.

In etioplasts lysed during the in vitro reaction, only accumulation of D2, membrane-bound LSU, and α and β subunit of ATPase could be detected in the membrane phase in the dark (Fig. 1B; M, lane 1). However, the amount of radiolabel accumulation in the D2 protein was low in the dark compared to the intact etioplast system (Fig. 1A,B, lanes 1 and 2). The accumulation of stromal proteins in the dark indicated that in general translation was not inhibited due to lysis of etioplasts (Fig. 1B; S1, lane 1). However, a comparison of radiolabel accumulation in LSU and α and β subunit of the ATPase revealed that the translation system based on intact etioplasts was superior to the lysed etioplast system for accumulation of radiolabeled apoproteins (Fig. 1A, S2, Fig. 1B, S1; lanes 1

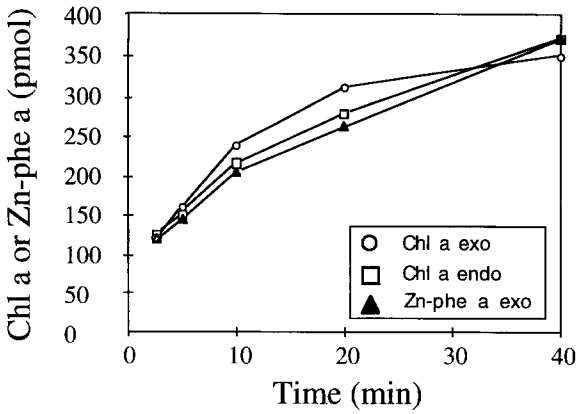

Fig. 2. Accumulation-kinetic of Chl and Zn-phe. Intact etioplasts (600 pmol Pchlide) were either illuminated for 15 s on ice (500 μE/m²s) and incubated at 25°C for the time indicated (Chl a endo, □); or Chl precursors, Chlide (350 pmol) (Chl exo, ○) or Zn-pheide (450 pmol) (Zn-phe exo, ▲), were added exogenously to intact etioplasts kept in darkness. The yield of Chl *a* or Zn-phe *a* (pmol) was determined after a reaction time of 2.5, 5, 10, 20 and 40 min under standard conditions (see Section 2).

and 2). Upon illumination, accumulation of stromal proteins was unchanged, whereas accumulation of P700 and CP47 was strongly induced in the lysed etioplast system and the accumulation of CP43, D2, and D1 was induced only slightly over the level of accumulation observed in the dark (Fig. 1B; M, lanes 1 and 2). Lysis of the etioplasts resulted in a loss of pD1 and D2 protein accumulation in the dark that could not be compensated by illumination of the etioplasts (Fig. 1A,B; M, lanes 1 and 2). These data could indicate that lysis of etioplasts activated proteolysis of Chl *a*P in the dark and that induction of chlorophyll synthesis through illumination and addition of GGPP selectively stabilized the Chl *a*P, CP47 and P700. In particular, the accumulation of pD1 and D2 was most sensitive for degradation after lysis of etioplasts (Fig. 1A,B; M, lane 1).

3.2. *Etioplasts take up exogenously added substrates for Chl synthesis*

In intact etioplasts, synthesis of Chl was achieved by exogenous addition of GGPP and either by phototransformation of endogenous Pchlide or by exogenous addition of Chl precursor molecules. The intactness of etioplasts was verified indirectly by the lack of radiolabel accumulation in the first supernatant S1 (Fig. 1A, S1). The S1 fraction was obtained from intact etioplasts by microcentrifugation of the translation system after completion of the translation reaction. Radiolabeled LSU or ATPase SU were only detectable in the S1 extracts of translation reactions conducted in lysed etioplasts, whereas intact etioplasts released radiolabeled LSU and ATPase SU only after resuspension of the microcentrifuged intact etioplasts in lysis buffer (S2) (Fig. 1A, S2, Fig. 1B, S1; lanes 1–4).

When endogenous Pchlide was phototransformed, addition of GGPP to intact etioplasts induced accumulation of Chl *a*P in the membrane (Fig. 1A, lane 2). This indicated that endogenously formed Chlide had been esterified with exogenously added GGPP and Chl *a*P had been stabilized by newly synthesized Chl. Most remarkably, when GGPP and the Chl derivative, Zn-pheide, were added to intact etioplasts in the dark, accumulation of the Chl*a*P, CP47 and CP43 was induced over the level of apoprotein accumulation obtained in the dark, and accumulation of CP43 could even be induced over the level obtained by illumination of etioplasts (Fig. 1A; M, lanes 1, 2 and 4). However, while CP47 and CP43 were stabilized with a high yield, the stabilization of P700 and D2 was decreased (Fig. 1A; M, lanes 2 and 4). In the control reaction, exogenous addition of Chlide and GGPP, accumulation of Chl *a*P was not increased over the level obtained in the dark (Fig. 1A, lanes 1 and 3). Thus, results indicated that newly synthesized Zn-phe stabilized the Chl *a*P within intact

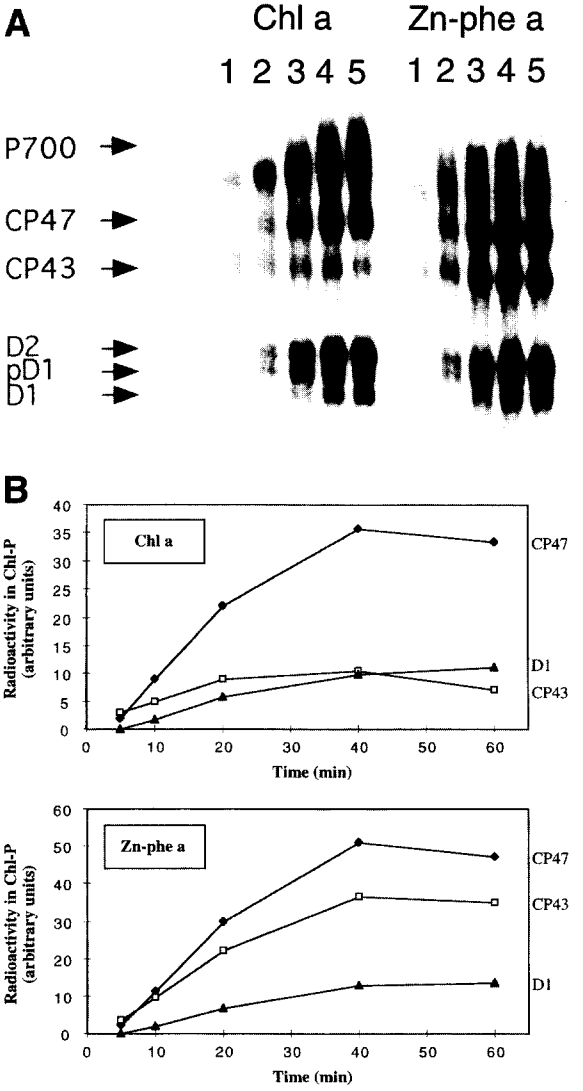

Fig. 3. A: Accumulation-kinetic of Chl *a*P in intact etioplasts. Intact etioplasts (600 pmol Pchlide) were labeled with [^{35}S]methionine for 5, 10, 20, 40 and 60 min (lanes 1–5) in the dark, either after a 15 s preillumination (500 μE/m²s, on ice) (Chl a) or in the presence of Zn-pheide *a* (450 pmol) (Zn-phe a). Etioplasts were fractionated into a membrane and a soluble phase (see Section 2) and membranes loaded on 12.5% SDS-PAG containing 4 M urea, on the basis of an equal plastid number (lanes 1–5). The migration of Chl *a*P, P700, CP47, CP43, D2, pD1, and D1 is indicated to the left of the figure. B: Quantitation of Chl *a*P accumulation. In organello synthesis of Chl *a*, Zn-phe *a*, and of Chl *a*P were as described (Figs. 2 and 3A). Quantitation of radioactivity in Chl *a*P (CP47, CP43 and D1; ◆, □, ▲) was achieved through laser scanning densitometry of autoradiograms (Chl *a* and Zn-phe *a*) and is given in arbitrary units of radioactivity per reaction time.

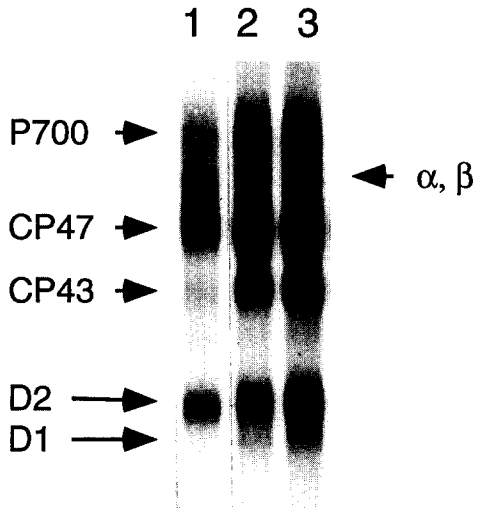

Fig. 4. Stabilization of Chl *a*P by Chl and Zn-phe. Intact plastids (400 pmol Pchlide) were incubated in a standard translation and Chl synthesis assay for 40 min in the dark and then incubated in the presence of chloramphenicol for an additional 40 min. Assays were either pretreated with light (500 μE/m^{2}s, on ice) to photoconvert endogenous Pchlide to Chlide (300 pmol Chlide) (lane 1) or were pretreated with light and incubated in the presence of Zn-pheide (pmol), lanes 2 (180), 3 (300). Following the translation reaction, intact plastids were lysed and separated in a membrane and soluble phase and membranes loaded on a SDS-PAG containing 4 M urea (equal plastid basis; 8×10^5 pt/lane). The migration of the Chl *a*P, P700, CP47, CP43, D2, pD1, and D1 is indicated to the left; migration of the α, β subunit of the ATPase (α, β) to the right of the figure.

etioplasts. Synthesis of Zn-phe or Chl in lysed etioplasts stimulated the formation of the Chl *a*P, CP47 and CP43, over the dark control; however, with respect to light-induced formation of CP47 and P700, no stimulation of Chl *a*P could be obtained (Fig. 1B; M, lanes 1–3). From these data we concluded that newly formed Chl and Zn-phe could not be delivered equally well to the Chl *a*P or altered the stabilization of the Chl *a*P.

In general, the degree of esterification of Chlide or Zn-pheide was unchanged between intact and lysed etioplasts. Within 20 min a mean of 50% of the substrates were esterified with GGPP. Hence, the low amount of apoprotein stabilization in lysed etioplasts could indicate that lysis of the etioplasts separated the site of esterification from the site of stabilization of the Chl *a*P.

In chloroplasts, esterification and also translation have been localized exclusively to the thylakoid membrane [5,20]. In etioplast subfractions, esterification of Chlide has been localized to the inner etioplast membrane system, consisting of the prolamellar body and prothylakoid membranes and not to the stroma fraction [21]. Translation of Chl *a*P has been detected on membrane-bound polysomes only; however, no localization of a translationally active membrane fraction has been undertaken within the etioplast [15]. Substrates for chlorophyll synthesis may have passed the envelope membranes and after esterification in the prothylakoid or prolamellar membrane stabilized the Chl *a*P in the inner etioplast membranes. Therefore, lysis of the etioplasts should not result in a separation of the site of esterification and translation; however, the Chlide or Zn-pheide delivery system could have been affected by removal of the envelope membranes during lysis.

3.3. *Accumulation of CP43 is increased at least 4-fold through synthesis of Zn-phe*

The selective stabilization of Chl *a*P through synthesis of Chl or Zn-phe could be a function of the amount of Chl or Zn-phe synthesized. When 600 pmol of Pchlide was phototransformed to yield 450 pmol of endogenous Chlide, or 450 pmol of Zn-pheide was added exogenously to intact etioplasts, in the presence of a 30-fold molar surplus of GGPP over Zn-pheide, no difference could be observed in the esterification kinetics of both substrates (Fig. 2, Chl endo and Zn-phe exo).

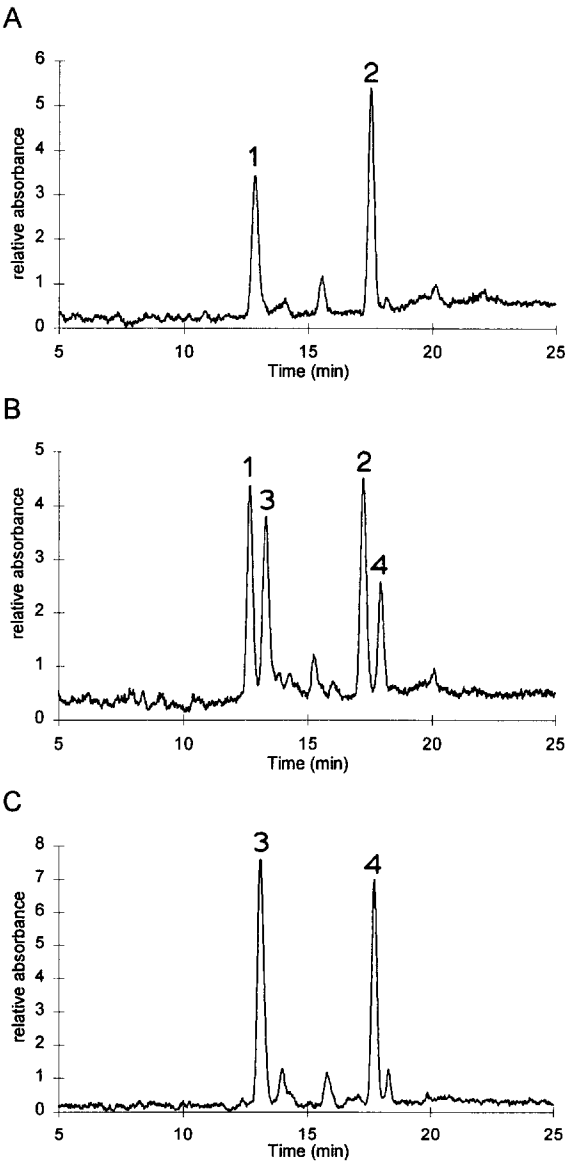

Fig. 5. HPLC analysis of in organello esterification products. Intact plastids (350 pmol Pchlide) were incubated for 80 min (see Section 2): either after illumination, in the absence of Zn-pheide (A); after illumination, in the presence of Zn-pheide (117 pmol) (B); or in darkness, in the presence of Zn-pheide (190 pmol) (C). Reaction products were separated by HPLC on silica gel (see Section 2). The ultraviolet-visible detection of zinc complexes was achieved at 652 nm and magnesium complexes at 659 nm. In the presence of both compounds, the ultraviolet-visible detection was set at 659 nm. Reaction products 1, 2, 3, and 4 are Chl-GG, Chl-Ph, Zn-phe-GG, and Zn-phe-Ph, respectively.

Within 10 min about 40% and within 40 min about 80% of the substrates were esterified. However, the addition of 350 pmol of Chlide to intact etioplasts resulted in an increased rate of esterification. Within 10 min 65% and within 40 min about 100% of the substrates were esterified (Fig. 2, Chl exo).

The influence of the central atom of Chl or Zn-phe on the stabilization of Chl*a*P had to be compared on the basis of an equal pigment content. In our system, endogenous Chlide and exogenously added Zn-pheide were esterified at an equal rate. We therefore investigated the stabilization of Chl*a*P during esterification of Chl from endogenous Chlide, whereas Zn-phe was synthesized from exogenously added precursor. This allowed us to compare the kinetic accumulation of Chl *a*P in the presence of Chl and Zn-phe (Fig. 3A,B). In the presence of Zn-phe, accumulation of the Chl *a*P, CP47 and CP43, was slightly faster, whereas in the presence of Chl, more P700 protein accumulated within a reaction time of 40 min (Fig. 3A, Chl *a* or Zn-phe *a*, lanes 1–4). Compared to an equal amount of Chl, synthesis of Zn-phe selectively increased the accumulation of CP43 and CP47 about 4- and 1.4-fold, respectively (Fig. 3A, lane 4; Fig. 3B Chl *a* or Zn-phe *a*, CP43 or CP47). Accumulation of the Chl *a*P ceased after a reaction time of 40 min in the presence of Zn-phe and Chl; however, in the presence of Chl synthesis, CP43 was even degraded after 40 min of the pulse kinetic (Fig. 3A; Zn-phe *a* or Chl *a*, lanes 4 and 5). This indicated that newly formed CP43 could not be fully stabilized through formation of Chl, whereas synthesis of the same amount of Zn-phe increased the amount of CP47 and D1 in parallel to the increase in CP43 stabilization (Fig. 3B; Zn-phe *a*: CP47, CP43, D1). Therefore, the individual stabilization of the Chl *a*P could be dependent on the central atom in Chl or Zn-phe.

Proteolytic protection of the Chl *a/b* binding protein, LHC II, has been shown by means of in vitro reconstitution studies to depend on the binding of pigments [22]. The proteolytic protection of the Chl-binding Chl *a*P by Chl or Zn-phe may be considered in two different ways. Stability may be conveyed directly to the apoproteins as the result of binding between the central atom and an electron donor from an amino acid group, or indirectly by Chl or Zn-phe dependent inhibition of a Chl *a*P specific protease. The selective accumulation of CP43 in the presence of Zn-phe could then be explained through a more effective binding of Zn-phe than of Chl to CP43, or a more effective inhibition of a CP43 specific protease by Zn-phe. However, the necessary assumption of several apoprotein-specific proteases which all would have to be inhibited in a Chl or Zn-phe concentration-dependent fashion makes it unlikely that accumulation of Chl *a*P is regulated indirectly. Instead, the selective accumulation of CP43 by synthesis of Zn-phe favors a direct control of apoprotein stabilization which is dependent on the chemical nature of the central metal of Chl. We therefore tested the effect of the different central metals in Zn-phe and Chl for stabilization of CP43 in a competition study (Fig. 4).

3.4. The central atom of Chl is a major determinant of Chl *a*P stabilization

Etioplasts were phototransformed in vitro and subjected to a pulse labeling of 40 min. Hereby, 280 pmol of Chl were synthesized. The stability of the Chl *a*P, P700, CP47, CP43, and D2 was then tested during chloramphenicol treatment for an additional 40 min (Fig. 4, lane 1). In parallel assays, synthesis of 150 and 240 pmol of Zn-phe was induced by exogenous addition of Zn-pheide (Fig. 4, lanes 2 and 3). The stabilization of CP43 was increased in a non-linear fashion, whereas the amount of stabilized P700, CP47, D2 and D1 increased linearly, when the ratios of Zn-phe/Chl were increased from 0 to about 1/2 and about 1/1, respectively (Fig. 4, lanes 1–3). In lysed etioplasts, the accumulation of all Chl *a*P stopped at a Chl concentration that corresponded to about 50% of the endogenous Pchlide content [14]. However, by esterification of Zn-phe, we show here that the amount of Chl *a*P can be increased (Fig. 4). This could indicate that Chl *a*P not stabilized by Chl was protected from degradation by a higher binding affinity of Zn-phe for Chl *a*P. Alternatively, the increased stabilization of Chl *a*P obtained for the mixture of Zn-phe and Chl could indicate the presence of a higher amount of stabilizing pigment per protein. Furthermore, the selectively increased stabilization of CP43 at low Zn-phe concentrations could indicate that the degree of stabilization of an individual apoprotein is dependent on the affinity of the apoprotein for binding of Chl or Zn-phe. These results clearly show that the central atom of the Chl molecule is a major determinant for the selective stabilization of the individual Chl *a*P.

We next investigated whether the selective stabilization of the Chl *a*P was brought about by differences between Chl and Zn-phe in the degree of reduction of the geranylgeraniol moiety to phytol (Fig. 5). When Chlide or Zn-phe were esterified with GGPP during a reaction time of 80 min, about 60% of Chl-GG was transformed into Chl-Ph, whereas only about 45% of Zn-phe was completely reduced to the phytol containing form (Fig. 5A,C). During esterification of a mixture of Chlide and Zn-pheide (3:1) with GGPP, the efficiency of reduction of the geranylgeraniol moiety was reduced by about 10% for both pigments to about 50 and 35%, respectively (Fig. 5B).

To find out whether the small differences found for the degree of reduction of Chl-GG and Zn-phe-GG could account for the selective stabilization of the Chl *a*P and in particular for the increased stabilization of CP43, we then tested by direct esterification of Zn-phe with phytylpyrophosphate. However, no difference could be found for the stabilization of the Chl *a*P between esterification of Zn-phe with geranylgeraniol- or with phytyl-pyrophosphate. In particular the degree of stabilization of CP43 was increased in the presence of $Zn\text{-}phe_{Ph}$, but not in the presence of Chl_{Ph} (data not shown). We therefore concluded that the central Zn(II) atom in Zn-phe was responsible for the selectively increased stabilization of the Chl *a*P, possibly via an increased affinity of the central Zn atom for direct binding of a ligand of the Chl *a*P.

We demonstrate that the chlorophyll derivative, Zn-phe, successfully substitutes for Chl during stabilization of the Chl *a*P. Through chemical derivatization of Zn-phe side groups, further investigations about the substrate specificity of Chl *a*P for binding of Chl are now possible. This work is in progress. Furthermore, the successful uptake and esterification of Zn-pheide in intact etioplast and the successful stabilization of the Chl *a*P allows us now to follow the Chl-dependent stabilization and assembly of the chloroplast- and nuclear-encoded Chl-binding apoproteins in greening etioplast membranes. With Zn-phe, we provide a most useful tool to reveal the impact of Chl for stabilization and assembly of Chl *a*P during the greening process.

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