Magnesium Chelatase of *Hordeum vulgare* L. Is Not Activated by Light but Inhibited by Pheophorbide

Gerhard Pöpperl, Ulrike Oster, Inge Blos and Wolfhart Rüdiger

Botanisches Institut der Universität München, Menzingener Str. 67, D-80638 München

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The enzyme activity of magnesium chelatase was determined in intact etioplasts of barley (*Hordeum vulgare* L.) seedlings. Irradiation of isolated plastids with white light for 15 min does not lead to any activation of the enzyme but to a decrease in activity, especially in etioplasts. The enzyme was inhibited by chlorophyllide and zinc pheophorbide only to a certain extent. Strong inhibition was observed with the metal-free pheophorbide (Kj = 0.92 μM) but not with pheophytin or chlorophyll. Penetration of chlorophyllide through the envelope membrane was confirmed by the chlorophyll synthase reaction that occurs in the inner membranes of etioplasts and chloroplasts. The possible role of inhibition of magnesium chelatase by pheophorbide in senescent leaves and tetrapyrrole transport across the plastid envelope are discussed.

**Introduction**

Two branches of tetrapyrrole biosyntheses can be distinguished in plants, the iron branch leading to cyttochromes and the magnesium branch leading to chlorophylls. Branching occurs at the stage of protoporphyrin. Insertion of iron with ferrochelatase yields protoheme, the precursor of most cyttochromes. Insertion of magnesium with magnesium chelatase yields magnesium protoporphyrin, an intermediate in chlorophyll biosynthesis. Ferrochelatase consists of one peptide chain, the enzyme reaction requires only protoporphyrin and reduced iron ions without further cofactors. Magnesium insertion is more complicated, the reaction requires ATP besides protoporphyrin and magnesium ions. The enzyme magnesium chelatase consists of 3 subunits encoded by 3 genes named bchH, bchD and bchl in *Rhodobacter sphaeroides* (Gibson et al., 1995, Willows et al., 1996), chlJ, chlD and chlH in *Synechocystis* PC6803 (Jensen et al., 1996a) and Xan-f, Xan-g and Xan-h in *Hordeum vulgare* (Jensen et al., 1996b). Subunit BchH (and probably also the barley homology Xan-f) binds protoporphyrin (Gibson et al. 1995), the role of the other subunits is not yet clear.

The location of the enzyme has not yet been clearly established. Fuesler et al. (1984a) found inhibition with p-chloromercuribenzene sulfonate when they investigated the enzyme reaction in intact, developing cucumber chloroplasts. The authors concluded that the enzyme must be accessible from the outside of the chloroplast since the inhibitor does not readily penetrate through membranes and does not inhibit stromal enzymes. Experiments with broken plastids revealed a soluble and a membrane-bound fraction that were both needed for enzyme activity (Walker and Weinstein, 1991b, 1994). It was assumed by many authors without further proof that the insoluble fraction was localized in the envelope membrane. However, reexamination of the localization revealed that magnesium chelatase is a soluble enzyme located interior to the chloroplast inner envelope (Walker and Weinstein, 1995). Gibson et al. (1995) discussed the possibility that the known subunits that are soluble proteins might form a high-molecular complex that could be precipitated by centrifugation. This property could simulate membrane binding.

The ready accessibility by exogenous protoporphyrin (Fuesler et al., 1984a) cannot be taken as argument for localization of magnesium chelatase.
in the envelope membrane, because the product magnesium protoporphyrin is further metabolized in intact plastids to protochlorophyllide (in darkness) and chlorophyllide (in the light) (Fuesler et al., 1984b). Both compounds are accumulated in the inner membranes of plastids. On the other hand, lack of inhibition of the enzyme in intact plastids by exogenous protochlorophyllide and chlorophyllide (Walker and Weinstein, 1991a) could mean either that these compounds do indeed not inhibit the enzyme or that they do not penetrate to the site of enzyme location.

Magnesium protoporphyrin, the product of the enzyme-reaction, and its monomethyl ester are believed to interact with transcription of several nuclear genes (Johanningmeier and Howell, 1984; Johanningmeier, 1988; Oster et al., 1997). Johanningmeier and Howell assumed localization of magnesium protoporphyrin in the envelope membrane for this interaction. However, Ryberg (1983) found this compound and its methyl ester accumulated in the inner membranes of wheat etioplasts after treatment with 5-aminolevulinate and 8-hydroxyquinoline. Both compounds were found in chlorophyll-protein complexes (Fradkin et al., 1988).

Magnesium chelatase as a key enzyme in the magnesium branch of tetrapyrrrole biosynthesis can be supposed to contribute to light regulation of chlorophyll biosynthesis. Transfer of etiolated barley seedlings from darkness to light resulted within 5–6 h in an about 50-fold increase in Xan-f mRNA and an about 4-fold increase in enzyme activity (Jensen et al., 1996b). The light effect was much smaller in green barley seedlings, it was superimposed by a circadian rhythm in this case. We found a rapid, transitory increase in magnesium protoporphyrin and its methyl ester after transfer of green barley seedlings from darkness to light, culminating at 30–60 min after the transfer (Pöpperl, Oster and Rüdiger, unpublished results). The level of protoporphyrin remained below the limit of detection all the time. Since the increase was significantly faster than the increase in the level of Xan-f mRNA, we asked the question whether light activation of magnesium chelatase was the reason for this increase. Besides the question of light activation, penetration of possible inhibitors of the enzyme will be described.

**Materials and Methods**

**Plastid isolation**

Barley seedlings (*Hordeum vulgare* L., cv. Steffi) were grown for 5 d at 26 °C on moistened vermiculite in total darkness for etioplast isolation or under 12 h light/12 h dark cycles for chloroplast isolation in a growth chamber under 80% relative humidity. All subsequent manipulations were performed under dim green safety-light keeping the plant material on ice and the solutions at 4 °C. The shoots (40 g fresh weight) were harvested, cut into small pieces with scissors immersed immediately into 400 ml buffer 1 (0.33 m sorbitol, 50 mM N-(2-hydroxyethyl)piperazine-N-(2-ethane-sulfonic acid) (Hepes)-KOH, pH 7.5) and homogenized with an ultra-thurax. The homogenate was filtered through a nylon mesh (22 μm). The residue was reextracted in the same way with the ultra-thurax and another 400 ml of the buffer 1. The resulting homogenate was filtered as before. Centrifugation of the combined filtrates at 3840×g for 1 min yielded a pellet consisting of intact and broken plastids. The pellet was resuspended in few ml of buffer 1 and filtered again through a nylon mesh (22 μm). This filtrate was applied to a Percoll step gradient consisting of 8 ml 85% Percoll and 10 μl 40% Percoll in buffer 2 (0.33 m sorbitol, 30 mM Hepes-KOH, pH 7.1, 1 mM EDTA). After centrifugation at 2600×g for 7.5 min, the intact plastids were in the interphase between the 2 Percoll concentrations. This layer was transferred into a new tube, diluted with 25 ml buffer 2 and centrifuged at 2,100×g for 3 min. The pellet was resuspended in few ml buffer 2. The protein concentration was determined and the concentration adjusted by dilution to 1 mg protein/ml. This suspension was immediately used for the enzyme reaction.

If indicated, the etioplasts or chloroplasts were irradiated for 15 min with 36 μmol · m⁻²·s⁻¹ white light (fluorescent tubes). They were kept on ice during irradiation.

For isolation of intact etioplast after the enzyme reaction on a microscale, the reaction mixture was applied to 40% Percoll in buffer 2 and centrifuged at 2100×g for 7.5 min. The intact etioplasts that formed a pellet under these condition were collected. The broken etioplasts were removed together with the supernatant containing the Percoll-buffer mixture.
Magnesium chelatase assay

The reaction was performed with intact etioplasts or chloroplasts according to Fuesler et al. (1984b) with modifications introduced by Walker and Weinstein (1991a). Each sample consisted of 250 μl buffer 2 (see above) containing 1.5 μM protoporphyrin IX, 5 mM MgCl₂, 8 mM ATP, 0.2% bovine serum albumin and plastids with 50 μg protein. The mixture was incubated in darkness at 30 °C for 60 min. The reaction was stopped with 750 μl acetone. The protein precipitate was removed by centrifugation. The content of magnesium protoporphyrin was determined in the clear supernatant by fluorescence spectroscopy (excitation at 415 nm, emission at 597 nm) in a fluorescence spectrometer (type F-2000, Hitachi). A calibration curve of authentic magnesium protoporphyrin was determinded in the clear acetone-buffer had been determined before.

Chlorophyll synthase assay

The reaction was performed with intact etioplasts basically under the same conditions as the magnesium chelatase assay. Each sample consisted of 250 μl buffer 2 (see under plastid isolation) containing 8 mM ATP, 0.2% bovine serum albumin, etioplasts with 50 μg protein, and (if indicated) 12 nmol geranylgeranyl diphosphate. The reaction was started by addition of 0.5 or 1 nmol chlorophyllide that was dissolved in 10 μl dimethyl sulfoxide. The reaction was performed in total darkness for the indicated time (see Fig. 4) and then stopped by addition of 750 μl acetone. Mixing with about 100 mg anion exchange resin DE 52 (Whatman) resulted in binding of the non-reacted chlorophyllide. The reaction product chlorophyll was extracted into 500 μl n-hexane that was cleared by centrifugation. The chlorophyllide was determined in the hexane-phase by spectrophotometry according to Brouers and Michel-Wolwertz (1983).

Chemicals

Protoporphyrin was purified in analogy to a method for hemin purification (Weinstein and Beale, 1983). The disodium salt of protoporphyrin (150 mg, Sigma) was dissolved in 95% ethanol containing 40 μM KOH. The solution was applied to a small column (5 x 100 mm) filled with DEAE-sepharose-CL-6B (Sigma) which had been transformed into the acetate form with 1 M Na-acetate, pH 7.0, and washed with 95% ethanol. Some contaminants were removed from protoporphyrin by washing of the column with 95% ethanol and n-butanol/ethanol (1:1, v:v). The pigment was then eluted with 2 ml volumes of a step gradient ethanol/water acetic acid (67:31:2 to 67:16:17 v:v:v). Each step added 3 vol. of acetic acid. Protoporphyrin eluted at 11–17 vol. acetic acid. The fractions were mixed each with diethyl ether (2 ml)/water (3 ml). The acid was removed from the diethyl ether solution by repeated washing with water. The ether phase was collected. Residual water was then removed from the ether phase by freezing. The concentration of protoporphyrin was determined by spectrophotometry at 404 nm with ε₉₄₄ = 158. The diethyl ether was then removed by a stream of nitrogen and the residue dissolved in a known volume of dimethyl sulfoxide.

Pheophytin a, chlorophyllides a and b, pheophorbide a, zinc pheophorbide a were prepared according to Helfrich et al. (1994) and Helfrich (1995).

Results and Discussion

Magnesium chelatase is not activated by light

In order to study possible regulatory phenomena, magnesium chelatase activity was determined in intact plastids. The method was adopted from that of Fuesler et al. (1984b) with the modifications introduced by Walker and Weinstein (1991a). The first experiments were perfomed with etioplasts of barley seedlings of various age (Fig. 1). As expected, the activity declined with increasing age of the seedlings. The decline reflects the well-known symptom of aging during prolonged etiolomement. The symptom includes degradation of many enzymes. Surprising is the high enzyme activity in young (4 d old) seedlings. It is higher than in green plants if based on total protein (see Fig. 2). We explain the lower value in green plants by the dramatic accumulation of total protein during greening in the light. The increase in total protein is apparently larger than the increase in magnesium chelatase activity described by Jensen et al. (1996b).

The test for possible light activation of magnesium chelatase required irradiation of the isolated
etioplasts in vitro. The irradiation was performed while the etioplasts were kept on ice to avoid organelle lysis. Broken etioplasts do not exhibit magnesium chelatase activity under our experimental conditions. The time of irradiation was chosen as short as possible for the expected effect. Since the level of magnesium protoporphyrin increased already within 15 min irradiation of intact plants, this time should be sufficient for detection of a possible light activation. The result was convincingly negative (Fig. 1). There was no increase, but a small decrease in enzyme activity at every age of the seedlings.

Since etiolated plants might lack the full system for light protection that is present in green plants, we repeated the experiment with barley seedlings that were grown in 12 h dark/12 h light cycle for 5 days (Fig. 2). One batch of plants was harvested under dim-green safety light at the end of the dark period. The chloroplasts were prepared in the dark and the enzyme activity was determined without irradiation or with 15 min irradiation in vitro. A second batch of plants was irradiated in vivo for 10 min and a third batch of plants for 60 min before harvest. Any fast activation of the enzyme, if it would occur in intact plants, should be detectable at these time points. The chloroplasts from the second and third batch of plants were investigated before and after in vitro irradiation for 15 min. As shown in Fig. 2, no significant increase in activity was detectable after irradiation of intact plants. The previously described increase in activity after 5–6 h of irradiation (Jensen et al., 1996b) was not yet detectable after 10–60 min of irradiation. Irradiation in vitro resulted in a slight decrease of activity, similar to the results with etioplasts. Summarizing this part, no light activation of magnesium chelatase in barley seedlings was detectable.

**Metall-free pheophorbide inhibits magnesium chelatase activity**

An obvious effect of irradiation of etioplasts is the photoconversion of protochlorophyllide to chlorophyllide. To test whether chlorophyllide could be the compound that was responsible for the somewhat lower activity in irradiated etioplasts than in those that were kept in darkness, we incubated etioplasts with this compound in the dark. Several related pigments were applied for comparison. The results (Table I) can be summarized as follows: Chlorophyllide and the related zinc pheophorbide showed a small but significant inhibition of magnesium chelatase activity. However, inhibition by the metal-free pheophorbide was much stronger. The esterified pigments, chlorophyll a and pheophytin a, did not significantly inhibit magnesium chelatase activity.

Chlorophyllide looses very easily the central magnesium. Therefore, one has to consider the
Table I. Remaining magnesium chelatase activity in intact barley etioplasts after incubation with several pigments. The enzyme reaction was performed under standard conditions in 250 μl samples in total darkness (see Material and Methods). The pigments were added in 10 μl dimethyl sulfoxide. The reference sample contained only 10 μl dimethyl sulfoxide. The yield of magnesium protoporphyrin is given in percent based on that of the reference sample = 100%.

<table>
<thead>
<tr>
<th>Pigment added</th>
<th>Number of experiments</th>
<th>Product yield after addition of 0.5 nmol pigment [%]</th>
<th>Product yield after addition of 1.0 nmol pigment [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlide a,b</td>
<td>5</td>
<td>78 ± 9</td>
<td>63 ± 10</td>
</tr>
<tr>
<td>Zn-pheophorbide a</td>
<td>3</td>
<td>71 ± 6</td>
<td>59</td>
</tr>
<tr>
<td>Pheophorbide a</td>
<td>3</td>
<td>12 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>Chlorophyll a</td>
<td>4</td>
<td>85 ± 14</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>Pheophytin a</td>
<td>3</td>
<td>84 ± 15</td>
<td>83 ± 20</td>
</tr>
</tbody>
</table>

possibility that the inhibition detected after incubation with chlorophyllide was in reality the inhibition by pheophorbide formed during the incubation. We consider this possibility unlikely, because we did not detect more than traces of pheophorbide under the condition of incubation, and (2) the inhibition was nearly identical with that by zinc pheophorbide that does not loose its central metal unless heated with mineral acids. Zinc pheophorbide can substitute chlorophyllide in the esterification reaction with chlorophyll synthase (Helfrich and Rüdiger, 1992), and zinc protopheophorbides are equally good substrates as protochlorophyllide for light-dependent NADPH:protochlorophyllide oxidoreductase (Griffiths, 1980; Schoch et al., 1995). We assume that the nearly identical inhibition of magnesium chelatase activity by zinc pheophorbide and chlorophyllide indicates that these two compounds are exchangeable also here and have an identical binding site at magnesium chelatase.

The lacking inhibition by chlorophyll and pheophytin could indicate either that magnesium chelatase has no binding site for these compounds or that these compounds did not penetrate to the site of the enzyme. The poor solubility of these apolar pigments in the reaction buffer that resulted in differing results from experiment to experiment (see the large standard deviation in Table I) points to the second possibility. This conclusion is further supported by permeability studies (see below).

The nearly complete inhibition of magnesium chelatase by pheophorbide is remarkable, since the inhibitor concentration was in the same order of magnitude as the concentration of the substrate protoporphyrin. The inhibition was investigated in more detail. In a series of dilutions, 50% inhibition was found at 0.23 nmol pheophorbide per sample. This means that the $K_i$ value is at 0.92 μM, i.e. lower than the concentration (1.5 μM) of the substrate protoporphyrin. One obvious conclusion is the penetration of pheophorbide to the site of the enzyme. If magnesium chelatase is a stromal enzyme (Walker and Weinstein, 1995), the results of inhibition mean that pheophorbide either interferes with protoporphyrin uptake within the en-
velopes or partially pass through the envelope membrane and bind to magnesium chelatase.

Chlorophyllide penetrates the envelope membrane of plastids

The question which tetrapyrroles penetrate the envelope membrane of plastids was further investigated with chlorophyllide. Esterification of chlorophyllide is catalyzed by chlorophyll synthase, an enzyme that has been localized in the inner membranes of plastids, namely thylakoids of chloroplasts (Soll et al., 1983) and prothylakoids and prolamellar bodies of etioplasts (Lütz et al., 1981). No enzyme activity was found in the envelope membrane (Soll et al., 1983). Incubation of intact etioplasts with chlorophyllide and geranylgeranyl diphosphate should only yield esterified chlorophyll, if the substrates penetrate through the envelope membrane. Penetration of geranylgeranyl diphosphate through the envelope of etioplasts has been demonstrated before (Benz et al., 1981). Uptake of geranylgeranyl diphosphate and phytol diphosphate from the culture medium by tobacco cell cultures and incorporation into chlorophylls (Benz et al., 1984) means also penetration of these substrates through the envelope membrane of chloroplasts. Here we use the esterification reaction to demonstrate permeation of chlorophyllide through the etioplast envelope.

Intact etioplasts were incubated with chlorophyllide and geranylgeranyl diphosphate. We used the same buffer as for the magnesium chelatase assay so that the two sets of experiments can directly be compared to each other. We found a nearly linear rate of esterification during 60 min incubation at 20 °C and during at least 30 min incubation at 30 °C (Fig. 3). The decline of the esterification rate after 60 min at 30 °C is probably due to consumption of substrate, more than 60% of the added chlorophyllide were esterified at this time.

Fig. 4. HPLC chromatogram of the pigment extract of intact barley etioplasts that were isolated after the chlorophyll synthase reaction. The etioplasts were incubated for 60 min at 20 °C with 0.5 nmol chlorophyllide and 6 nmol geranylgeranyl diphosphate (A) or only with 0.5 nmol chlorophyllide (B). The separation of intact and broken etioplasts after the reaction was achieved with a Percoll gradient on a microscale (see Materials and Methods). The pigments of the intact etioplasts were extracted with acetone and applied to HPLC analysis. The conditions for chromatography were: 15 min 70% solution A (60% acetone) and 30% solution B (100% acetone), then a linear gradient to solution B within 10 min, further 10 min with solution B and within 15 min to solution A. For fluorescence detection, the excitation was set at 425 nm and the emission at 665 nm. Peak 1 = 13-hydroxychlorophyll a, peak 2 = chlorophyll a, peak 3 = 13-hydroxy-chlorophyllide a, peak 4 = chlorophyllide a.
To prove that the esterified pigment is present in intact etioplasts and not confined to those etioplasts that were broken during the incubation, we isolated the intact organelles via a Percoll gradient after the reaction. The pigments of these intact plastids were extracted and analyzed by HPLC (Fig. 4). When the etioplasts had been incubated with chlorophyllide and geranylgeranyl diphosphate, only esterified pigment was found in the isolated organelles (Fig. 4A). This is the clear evidence for penetration of exogenous chlorophyllide to the inner membranes of etioplasts. The pigments were identified by co-chromatography with authentic pigments (data not shown). The main product was chlorophyll $a_{GG}$ (peak 2). A small amount of the „allomerisation“ product, 132-hydroxy-chlorophyll, (peak 1) was formed under these conditions. The product of allomerization was also found when we investigated the esterified pigments obtained by infiltration of zinc pheophorbide into etiolated oat leaves (Scheumann et al., 1996). When the etioplasts had been incubated with chlorophyllide but without geranylgeranyl diphosphate, only chlorophyllide (peak 4) and traces of the 132-hydroxy deriative (peak 3) were found (Fig. 4B). This result confirms the previous finding (Benz et al., 1981) that intact plastids loose the endogenous geranylgeranyl diphosphate during the isolation procedure.

We used the penetration of chlorophyllide and subsequent esterification to check whether the different effect of chlorophyllide and chlorophyll upon magnesium chelatase activity (Table I) was also found when the chlorophyll was formed within intact plastids. As shown in Fig. 5, prolonged preincubation of etioplasts with chlorophyllide resulted in stronger inhibition of chelatase activity compared to shorter preincubation (Table I). Contrary to the results with exogenous chlorophyll, the activity with endogenous chlorophyll, i.e. incubation together with geranylgeranyl diphosphate, was not higher than that with chlorophyllide alone. This could either mean that chlorophyll inhibits magnesium chelatase to the same extent (or even somewhat more than) chlorophyllide and that exogenous chlorophyll does not penetrate to the site of the enzyme, or the esterification that is not complete under these conditions (see Fig. 4). does not remove enough of the inhibiting chlorophyllide from the magnesium chelatase. We cannot distinguish between these alternatives at the moment.

Conclusions

The strong inhibition of magnesium chelatase activity by pheophorbide can be physiologically significant. Pheophorbide is an early intermediate of chlorophyll breakdown (Langmeier et al., 1993; Vicentini et al., 1995). Magnesium dechelatase that removes magnesium from chlorophyllide is activated i.e. transformed from a latent to an active form in senescent leaves (Vicentini et al., 1995). Inhibition of magnesium chelatase seems to be of advantage under the conditions of senescence. As soon as chlorophyll breakdown starts (with formation of pheophorbide), synthesis of chlorophyll is inhibited (by pheophorbide) at the stage of the key enzyme magnesium chelatase.

The permeability of the plastid envelope for chlorophyllide (this paper) seems to be surprising at the first view. However, it is in line with a number of scattered observations which point to permeation of different tetrapyrroles through the...
plastid envelope. If we accept the localization of magnesium chelatase in the plastid stroma, then both the substrate protoporphyrin and probably also the inhibitor pheophorbide (this paper) must penetrate the envelope. A transport system for protoporphyrinogen or protoporphyrin from plastids to mitochondria has been postulated (Jacobs and Jacobs, 1993) based on the finding that earlier enzymes of tetrapyrrole biosynthesis, e.g. porphobilinogen deaminase (Witty et al., 1996) and coproporphyrinogen oxidase (Smith et al., 1993) are confined to the plastids but protoporphyrinogen oxidase and ferrochelatase have been found in both plastids and mitochondria. The phytochrome chromophore is synthesized within the plastids and exported into the cytoplasm for incorporation into the apophytochrome protein (Terry and La-
garias, 1991, Terry et al., 1993). Magnesium protoporphyrin and its methylester have been discussed to interfere with cytoplasmic proteins or acting as possible effectors for expression of nuclear genes (Johanningmeier and Howell, 1984; Johanningmeier, 1988; Oster et al., 1997). At least for some of these observations, a specific tetrapyrrole trans-
port system in the plastid envelope must be postulated.

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