Chlorophyll Degradation in a *Chlamydomonas reinhardtii* Mutant: An Accumulation of Pyropheophorbide a by Anaerobiosis

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Chlorophyll degradation was investigated in cells of a chlorophyll b-less mutant of *Chlamydomonas reinhardtii* under aerobic and anaerobic conditions. During degradation of chlorophyll under anaerobic conditions, chlorophyll catabolite P535, an open-tetrapyrrole, was not excreted, but pyropheophorbide a was accumulated as the end product with a transient accumulation of chlorophyllide a and pheophorbide a in cells, in contrast to the breakdown under aerobic conditions. It is likely that in the absence of oxygen, degradation of chlorophyll a proceeds to pyropheophorbide a by three consecutive reactions, dephtytylation, metal-releasing and demethoxycarbonylation, and then stops due to a limitation of the oxygen that the monooxygenase reaction requires for bilin formation. A novel enzyme catalyzing demethoxycarbonylation of pheophorbide a was partially purified. The enzyme activity increased dependent on the age of cells, and its increase was completely suppressed by cycloheximide. Production of P535 was also dependent on cytoplasmic protein synthesis.

**Key words**: *Chlamydomonas reinhardtii* mutant — Chlorophyll degradation — Green alga — Pyropheophorbide a accumulation.

**Introduction**

Recent advances in the study of chlorophyll (Chl) breakdown in higher plants and algae have revealed its biochemical process in detail (Brown et al. 1991, Gossauer and Engel 1996, Hörtensteiner 1999, Matile et al. 1999). Identification of breakdown products and studies on the subsequent catabolic pathway suggest that Chl breakdown proceeds in successive reactions, in each of which a specific enzyme is implicated. The first step in Chl breakdown, dephtytylation of Chl, is catalyzed by chlorophyllase (EC 3.1.1.14). Chlorophyllase is the best characterized enzyme in the degradation process of Chl. Recently, two chlorophyllase cDNAs have been cloned (Tschiya et al. 1999, Jacob-Wilk et al. 1999). One of the genes had a plastidic and the other had an extraplastidic signal sequence whose mRNA is inducible by coronatine and methyl jasmonate (Tschiya et al. 1999). However, it remains unknown which acts in the Chl degradation process or how. The removal of the central Mg ion from chlorophyllide is expected to follow chlorophyllase (Owens and Falkowski 1982, Ziegler et al. 1988, Shimokawa et al. 1990, Shioi et al. 1991). An enzyme (Vicentini et al. 1995, Azuma et al. 1999) and a smaller substance (Shioi et al. 1996a) are responsible for this metal-releasing process. There is, however, no consistent view regarding the function of these materials in the Mg-releasing reaction. The pheophorbide a formed is then enzymatically converted to pyropheophorbide a by a two-step reaction: hydrolysis and subsequent spontaneous decarboxylation (Shioi et al. 1996b). This reaction may be specific to certain plants because of a limited distribution of the enzyme. This enzyme is designated “pheophorbidase”.

The most characteristic change in the chemical structures and spectroscopic properties of the macrocycle in Chl breakdown, oxidative cleavage of the macrocycle, is suggested to be catalyzed by a monooxygenase in both higher plants and algae (Curty et al. 1995, Hörtensteiner et al. 1995). The enzyme is associated with the envelope of senescent chloroplasts and catalyzes the conversion of pheophorbide a to a linear tetrapyrrole product depending on oxygen and reductant (Hörtensteiner et al. 1995, Rodoni et al. 1997). The activity of monooxygenase that specifically recognizes pheophorbide a as a substrate is found only in senescent plants, while two previous enzymes, chlorophyllase and pheophorbidase, are constitutively present in pre-senescent plants and are also inducible by senescence (Tschiya et al. 1999).

Apart from these considerations, several catabolites other than chlorophyllide and pheophorbide are also found in natural habitats (Brown et al. 1991 and references cited therein). The implication of these derivatives in Chl breakdown remained largely unknown. Previously, we reported that a stable Chl catabolite, the open-tetrapyrrole P535 accumulated in a mutant of green alga, *Chlamydomonas reinhardtii* in association with the degradation of Chl during growth under aerobic conditions (Doi et al. 1997). Because of the lack of accumulation of intermediates possibly included in the breakdown of Chl, the biochemical sequencing of this process in vivo remains to be elucidated. As oxygen is likely to be one of the limiting factors on the regulation of Chl degradation, we investigated the Chl breakdown both under aerobic and anaerobic conditions. In this study, we confirmed that three successive reactions are
involved in the degradation of Chl to pyropheophorbide. A possible involvement of protein(s) encoded in the nuclear genome for P535 formation is also discussed.

Materials and Methods

Algal culture
A Chl b-less mutant NL-105 of C. reinhardtii (Nakamura et al. 1981) was used throughout this study. The algal cells were grown as described earlier (Doi et al. 1997).

Determination of Chl and its derivatives by HPLC
Analysis of Chl a and its derivatives including chlorophyllide a, pheophorbide a and pyropheophorbide a was carried out according to the method described below. HPLC was performed using a Mightysil RP-18 column (4.6×150 mm) (Merck, Darmstadt, Germany). Pigments were eluted by using a linear gradient from methanol-500 mM ammonium acetate (80/20, v/v) to methanol-2-propanol (80/20, v/v) for 15 min at a flow rate of 1.0 mL min⁻¹ at room temperature. The pigments were monitored at 665 nm using a SPD-6AV spectrophotometer (Shimadzu, Kyoto) and quantified by an integrator, Chromatopac C-R5A (Shimadzu).

Identification and quantification of pigments
The concentration of Chl a and P535 were determined as described previously (Doi et al. 1997). The product of the pyrolytic enzyme, pyropheophorbide a, was identified by comparison with an authentic sample and also coelution in HPLC analysis.

Assay for pyrolytic (pheophorbide demethoxycarboxylation) enzyme
For routine analysis and monitoring of activity in the purification steps, activity was assayed using pheophorbide a as a substrate as follows. The reaction mixture consisted of 20 mM MOPS-NaOH, pH 7.2, 40 μM pheophorbide a, 0.1% Triton X-100 and the enzyme preparation in a total volume of 40 μL. The reactions were carried out in darkness at 35°C for 1 to 3 h and terminated by the addition of 160 μL acetone. After centrifugation to remove insoluble materials, amounts of pheophorbide a and pyropheophorbide a in the supernatant were assayed using the HPLC system described above.

Partial purification of pyrolytic enzyme
Algal cells grown for 3 d under continuous light and 4 d in darkness were used as starting materials for purification of pyrolytic enzyme. After harvesting, the cells were washed once with 25 mM NaCl containing 150 mM NaCl. The protein was eluted with the same buffer at a flow rate of 30 mL h⁻¹. Fractions (2 mL per fraction) were collected and assayed for pyrolytic activity with pheophorbide a as a substrate. The fractions eluted around 0.15-0.2 M NaCl were pooled (DEAE fraction), and ammonium sulfate was added to 1 M before loading onto a column (1.6×5 cm) of phenyl-Tosylxeryl (Tosoh, Tokyo) previously equilibrated with the 20 mM MOPS buffer, pH 7.2, containing 1 M ammonium sulfate. The protein was eluted with a reverse-linear gradient from 1 to 0 M ammonium sulfate in buffer B. The active fractions that had been eluted around 0.2 M ammonium sulfate were pooled and concentrated by Centriflo CF-50 (Amicon, Beverly, MA, U.S.A.) (phenyl pool). The concentrated protein was chromatographed on a column (2.2×60 cm) of Sephacryl S-300 Fine (Amersham Pharmacia Biotech) equilibrated with buffer B containing 150 mM NaCl. The protein was eluted with the same buffer at a flow rate of 30 mL h⁻¹. Fractions (2 mL per fraction) were collected and assayed for the activity. Active fractions were pooled, dialyzed against for 10 mM MOPS buffer, pH 7.2, and concentrated by Centriflo CF-50 (Sephacryl fraction). This fraction was used as a partially purified enzyme for enzymatic analyses.

The molecular mass of the enzyme was estimated using the Sephacryl S-300 column described above by comparison with standard proteins. The standards used were β-amylose (200 kDa), γ-globulin (170 kDa), bovine serum albumin (66 kDa) and cytochrome c (12.4 kDa).

Chlorophyll and its derivatives
Chl a, pheophorbide a and pyropheophorbide a were purchased from Wako Pure Chemical Industries (Osaka). Chlorophyllide a was prepared from Chl a by enzymatic reaction using a chlorophyllase prepared from C. reinhardtii (unpublished data). Pyropheophorbide a was also prepared from pheophorbide a according to a modified method of Pennington et al. (1964), if necessary. Chl and its derivatives were further purified by the HPLC described above and used as standards for identification in HPLC assay.

SDS-PAGE
SDS-PAGE was carried out with a RAPIDAS AE-6500 apparatus (ATTO, Tokyo) using 1-mm thick slab gels containing 10% acrylamide according to the procedure of Laemmli (1970). After electrophoresis, the gel was silver-stained (Nacalai Tesque, Kyoto). Molecular-mass standards (Pharmacia) were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Protein estimation
Protein was determined using a Bio-Rad Protein Assay Kit based on the method of a Bradford (1976) with bovine serum albumin as a standard.

Results

P535 formation during chlorophyll degradation under aerobic conditions
As previously reported (Doi et al. 1997), a breakdown of Chl a was observed during the growth of cells under aerobic conditions in a Chl b-less mutant of C. reinhardtii. Typically it begins at the late exponential growth phase and is accompanied by the excretion of a red pigment, P535, into the culture medium. P535 was previously identified as a stable Chl catabolite that has an open-tetrapyrrrole structure. Chl concentration and P535 formation were analyzed under aerobic conditions after the transition from light to dark (Fig. 1). Throughout this study, to prevent breakdown of Chl by light and to accelerate the degradation of Chl, the cells were shifted to the dark after reaching the stationary phase at 4 d, although Chl degradation occurs in the light incubation. Chl concentration increased in the light growth conditions, but gradually decreased during dark incubation. Conversely, the Chl degradation product, P535, began to accumulate after transition to the dark. The
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amounts of Chl degraded and P535 produced were almost comparable. Together with P535, a quite small amount of degradative intermediates of Chl to P535 such as pheophorbide *a* was also found in the medium.

The activities of the enzymes, chlorophyllase and pyrolytic enzyme, were also determined at the different ages of the cells under aerobic conditions. Chlorophyllase activity, estimated on a fresh weight basis, slightly increased during light incubation and then sharply decreased with increasing age of the cells during Chl degradation. No induction of chlorophyllase activity was observed during the dark incubation (data not shown). Pyrolytic enzyme activity was not detected in up to 3-day-cultured cells, but then it gradually increased and reached maximum in 7-day-cultured cells. This enzyme was induced by dark incubation as described below (see Fig. 4).

**Accumulation of pyropheophorbide *a* during chlorophyll degradation in the absence of oxygen**

In the absence of oxygen, instead of excretion of P535 into the medium, a progressive accumulation of chlorophyllide *a*, pheophorbide *a* and pyropheophorbide *a* in the cells was observed during Chl breakdown (Fig. 2). After cells were transferred to anaerobiosis, a characteristic lag time was found before Chl begins to degrade. The major end product accumulated during Chl breakdown under anaerobic conditions was largely dependent on the age of the cells. When young cells at an early exponential phase of growth were kept under anaerobic conditions, pheophorbide *a* was accumulated as the main product during degradation of Chl *a* (Fig. 2A). The accumulation of pyropheophorbide *a* was also apparent, but to a much smaller extent than pheophorbide *a*. In contrast, when the cells were transferred at the early stationary phase of growth to anaerobiosis, where Chl had already begun to bleach, degradation of Chl *a* occurred after a fairly long lag period (Fig. 2B).

First, chlorophyllide *a* and pheophorbide *a* were transiently accumulated, and then pyropheophorbide *a* was accumulated as a stable product. The sum of the amounts of each derivative was almost the same as that of Chl *a* degraded at any stage throughout the incubation under anaerobic conditions. The levels of both pheophorbide *a* (Fig. 2A) and pyropheophorbide *a* (Fig. 2B) were unchanged for several weeks when the cells were kept in the dark under anaerobic conditions. Thus, oxygen exclusion enables the accumulation of pyropheophorbide *a* as an end product during Chl breakdown.

**Partial purification and properties of pyrolytic enzyme**

To confirm the presence of pyrolytic enzyme in this alga, we partially purified the protein having pyrolytic activity and characterized it. Most activity was retained in the supernatant fractions after ultracentrifugation of cell-free extracts. Purification of proteins was performed through three chromatography steps: ion exchange, hydrophobic and gel filtration chromatography (see Materials and Methods). The protein was purified 76-fold with a 23% yield of the initial activity through these
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steps of purification. At present, a further purification of the enzyme is not practical because of a great decrease in the yield with a concomitant drop in the specific activity.

The elution profiles of the activity and separation of proteins on SDS-PAGE in the fractions on gel filtration were examined (data not shown). Although several proteins on the gel were obvious in the fractions containing activity, distribution of two major bands corresponding to the respective molecular masses of 87 kDa and 83 kDa coincided only with that of activity. A similar correlation between activity and proteins was found in the preceding chromatography steps. The molecular mass of the native enzyme was estimated by Sephacryl gel filtration to be 170 kDa, indicating that the enzyme is possibly a heterodimer.

The reaction product of this enzyme was determined to be pyropheophorbide *a* by both spectroscopic and HPLC analyses with comparison of the authentic sample. The conversion of pheophorbide *a* to pyropheophorbide *a* by a partially purified enzyme proceeded linearly for 3 h and gradually decreased in rate in the subsequent incubation time (Fig. 3). The stoichiometry of pheophorbide *a* consumed and pyropheophorbide *a* formed was roughly equal. In addition, boiling the protein for several min resulted in a total loss of the activity. The enzyme showed Michaelis-Menten kinetics at concentrations up to 200 μM pheophorbide *a*, with a *K*ₘ value of 283 μM. Characterization of this enzyme, including substrate specificity for Chl derivatives, is now in progress.

Effect of protein synthesis inhibitors on the formation of P535 and the level of pyrolytic enzyme in cells under aerobic growth conditions

In the absence of inhibitors (control), Chl was converted almost completely into P535 with a trace of Chl derivatives (Table 1). Addition of cycloheximide, which interferes with cytoplasmic protein synthesis, however, completely inhibited the accumulation of P535. Instead, large amounts of green pigments were excreted into the medium. HPLC and spectral anal-

![Fig. 3](image1)

**Fig. 3** Time-dependent pyrolysis of pheophorbide *a* by a partially purified enzyme (12.5 μg protein ml⁻¹). After incubation at 35°C in the dark for the indicated periods of time, the amounts of both pheophorbide *a* and pyropheophorbide *a* were determined by HPLC as described in the text. The result of a typical experiment is shown.

![Fig. 4](image2)

**Fig. 4** Effect of protein synthesis inhibitors on the increase in pyrolytic enzyme during aerobic incubation in the dark. The cells were grown in light for 4 d and then shifted to the dark as shown at the top. Cycloheximide (10 μg ml⁻¹) and chloramphenicol (200 μM) were added at the time as indicated by the arrow. The enzyme activity was determined as described in the text. The result of a typical experiment is shown. “Control” means the experiments without addition of inhibitor. + CHI, cycloheximide added; + CAP, chloramphenicol added.

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<th>Chl decreased a</th>
<th>P535 formed</th>
<th>Chl deriv. b</th>
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<tr>
<td>Control</td>
<td>0.97</td>
<td>0.85</td>
<td>0.04</td>
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<tr>
<td>Cycloheximide</td>
<td>0.97</td>
<td>0.10</td>
<td>0.86</td>
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<tr>
<td>Chloramphenicol</td>
<td>1.49</td>
<td>1.33</td>
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Cycloheximide (10 μg ml⁻¹) or chloramphenicol (200 μM) was added to 3-d light-grown cells. The cells with or without inhibitor were cultured for 5 further days in the dark with shaking. Data represent the mean of three experiments, the standard deviations not exceeding 11.3% for P535 formation.

a Chl decrease during incubation in the dark.

b Determined as pheophorbide *a* using mM absorption coefficient of 55.5 at 667 nm (Rüdiger and Schoch 1988).
yses showed that the pigments were a mixture of pheophorbide \textit{a} as the main component with several minor Chl derivatives such as chlorophyllide \textit{a} and unidentified species probably including hydroxy derivatives (data not shown). However, pyropheophorbide \textit{a} could not be detected. In contrast to the case of cycloheximide, application of chloramphenicol, which arrests the plastidic protein synthesis, rather stimulated the Chl degradation and did not influence on the accumulation of P535. A small amount of Chl derivatives was also excreted as in the case of the control.

The change in the level of pyrolytic enzyme observed depended on the age of the cells and presence of the inhibitor of protein synthesis (Fig. 4). The specific activity of the enzyme held fairly constant at low levels during the first 4 d of growth, then gradually increased in the following 3 to 4 d and reached maximal levels corresponding to about 7-fold of the initial activity. During the first 4 d of growth, neither breakdown of Chl nor accumulation of P535 was observed (see Fig. 1). The level of activity increased in a similar fashion as P535, a Chl catabolite in this alga, accumulated in the medium. In order to test whether the increase in activity depended on newly synthesized protein, a protein inhibitor was supplied to the growth medium. Addition of cycloheximide totally inhibited the increase in enzymatic activity and rather reduced the level to less than 85% of the initial activity. \textalpha-Amanitin also suppressed the increase in the level of enzymatic activity. On the other hand, chloramphenicol had only a mild effect on the increase of the enzyme. Thus, we conclude that pyrolytic enzyme is newly synthesized in the cells under aerobic incubation.

These results suggest further that cycloheximide inhibits the degradation of Chl due to the suppression of cytoplasmic protein synthesis in the dark. Accordingly, P535 formation does not depend on the protein(s) synthesized in the plastid, but on the protein(s) synthesized in the nuclear genome, at least including the pyrolytic enzyme apart from its localization in the cells.

**Discussion**

Keeping the cells in anoxic conditions caused transient accumulation of chlorophyllide \textit{a} and pheophorbide \textit{a}, followed by pyropheophorbide \textit{a} as an end product during Chl breakdown (Fig. 2). Our results thus showed oxygen participation in the formation of P535. The degradation pathway in this alga is, therefore, likely to follow the sequence from Chl to chlorophyllide, pheophorbide, and pyropheophorbide. Under oxygenic conditions, subsequently, ring cleavage of formed pheophorbide or pyropheophorbide takes place due to the action of monooxygenase to form the open-tetrapyrrole, P535. Considering a more specific substrate specificity of the monooxygenase for pheophorbide \textit{a} (Hörtensteiner et al. 1995), however, the possibility that pyropheophorbide formation is an auxiliary reaction in the Chl degradation is not ruled out. The amounts of accumulation of these products were dependent on the growth conditions of cells including age probably due to the different levels of enzymes such as chlorophyllase and pyrolytic enzyme (see Fig. 4), although there is no information concerning the Mg-dechelation reaction and monooxygenase of this alga.

Formation and/or accumulation of pyropheophorbide has been reported in plants, algae and photosynthetic bacteria under appropriate conditions (Owens and Falkowski 1982, Haidl et al. 1985, Ziegler et al. 1988, Shimokawa et al. 1990, Louda et al. 1998). In a \textit{Chlorella} mutant, enzymatic formation of pyropheophorbide was demonstrated in starving cells cultured in the dark, but the enzyme was not adequately characterized (Ziegler et al. 1988). More recently, we reported the enzymatic conversion of pheophorbide to pyropheophorbide in a higher plant, \textit{Chenopodium album} (Shioi et al. 1991), and characterized it thoroughly (Shioi et al. 1996b, Watanabe et al. 1999). The enzyme, pheophorbidase, of this plant is an esterase and actually catalyzes the conversion of pheophorbide to an intermediate, C-13'-carboxylpyropheophorbide, followed by the chemical decarboxylation of C-13'-carboxylpyropheophorbide to pyropheophorbide. This enzyme has a molecular mass of 105 kDa, consisting of 28 and 29 kDa subunits. In this study, we also confirmed enzymatic conversion of pheophorbide to pyropheophorbide in a \textit{Chlamydomonas} mutant by partial purification and characterization of the protein that can catalyze the reaction (Fig. 3). We could not, however, detect the intermediate in the reaction by pheophorbidase using this algal extract. In addition, its catalytic and molecular properties apparently differ from those of the pheophorbidase described previously. Thus, this enzyme appears to be a novel type of enzyme, although further study is needed to analyze the mode of reaction of this enzyme. Purification and characterization of the enzyme from the \textit{Chlamydomonas} mutant will be published elsewhere.

Formation of P535 and increase in the pyrolytic enzyme activity were inhibited by cycloheximide, which is well known to cause the retardation of Chl bleaching in several higher plants (Adachi and Shimokawa 1998) (Table 1 and Fig. 4). On the other hand, treatment with chloramphenicol caused a partial suppression of the increase in the level of the pyrolytic enzyme and had practically no effect on the accumulation of P535. The formation of P535 is obviously dependent on the induction of some protein(s) including at least the pyrolytic enzyme that is encoded in the nuclear genome. Similar results were obtained in degreening cells of \textit{Chlorella protothecoides} grown in nitrogen-free medium (Hörtensteiner et al. 2000). In the culture medium of the cells incubated with cycloheximide, no pyropheophorbide was detected, but pheophorbide and other Chl derivatives were instead of P535. This clearly supports the inhibition of synthesis of pyrolytic enzyme by cycloheximide (Table 1 and Fig. 4). Our results, however, do not rule out the possibility that concomitant inhibition of monooxygenase leads to the formation of P535. Accordingly, whether pyropheophor-
bide acts as a substrate for the monooxygenase is unclear at present. We are now investigating monooxygenase to solve this problem.

A recent study shows that the induced type of chlorophyllase has an extraplastidic signal (Tschiya et al. 1999). This suggests Chl degradation in the cytosol or vacuole, which is where the degradative enzymes seem to exist. The localization of the pyrolytic enzyme as well as monooxygenase in the cell is still unknown, but it would be very interesting to explore the site where Chl degradation takes place. To elucidate this, it will be necessary to determine the expression of these enzymes during Chl degradation at the molecular level.

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References


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