Envelope Membranes from Mature Spinach Chloroplasts Contain a NADPH:Protochlorophyllide Reductase on the Cytosolic Side of the Outer Membrane*

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Using fluorescence spectroscopy, we have demonstrated that isolated envelope membranes from mature spinach chloroplasts catalyze the phototransformation of endogenous protochlorophyllide into chlorophyllide in presence of NADPH, but not in presence of NADH. Protochlorophyllide reductase was characterized further using monospecific antibodies (anti-protochlorophyllide reductase) raised against the purified enzyme from oat. In mature spinach chloroplasts, protochlorophyllide reductase is present only in envelope membranes. We have demonstrated that the envelope protochlorophyllide reductase, a 37,000-dalton polypeptide, is only a minor envelope component and is present on the outer surface of the outer envelope membrane. This conclusion is supported by several lines of evidence: (a) the envelope polypeptide that was immunodecorated with anti-protochlorophyllide reductase can be distinguished from the major 37,000-dalton envelope polypeptide E37 (which was identified by monospecific antibodies) only after two-dimensional polyacrylamide gel electrophoresis; (b) the envelope protochlorophyllide reductase was hydrolyzed when isolated intact chloroplasts were incubated in presence of thermolysin; and (c) isolated intact chloroplasts strongly agglutinate when incubated in presence of antibodies raised against protochlorophyllide reductase. These results demonstrate that major differences exist between chloroplasts and etioplasts with respect to protochlorophyllide reductase levels and localization. The presence on the chloroplast envelope membrane of both the substrate (protochlorophyllide) and the enzyme (protochlorophyllide reductase) necessary for chlorophyll synthesis could have major implications for the understanding of chlorophyll biosynthesis in mature chloroplasts.

Higher plant chloroplasts are made up of three morphologically and functionally distinct compartments: envelope, stroma, and thylakoids. The envelope consists of two membranes, the inner and outer envelope membranes. The inner envelope membrane is also involved in the synthesis of plastid major lipid compounds such as glycerolipids (galactolipids, phosphatidylglycerol, sulfolipid) and prenylquinones (α-tocopherol, plastoquinone-9). The outer envelope membrane also is involved in lipid metabolism: an acyl-CoA synthetase, a galactolipid:galactolipid galactosyltransferase, and a galactolipid:galactolipid acyltransferase are localized on this membrane. Finally, the outer envelope membrane probably contains specific protein receptor(s) that mediates the post-translational uptake of cytoplasmically synthesized plastid polypeptides. These results demonstrate that major differences exist between chloroplasts and etioplasts with respect to protochlorophyllide reductase. Thus, we further investigated on the presence of active protochlorophyllide reductase in isolated envelope membranes and on its occurrence and localization within mature spinach chloroplasts using specific antibodies raised against oat protochlorophyllide reductase.

MATERIALS AND METHODS

Isolation of Purified Intact Spinach Chloroplasts—Chloroplasts were isolated from 3–4 kg of spinach (Spinacia oleracea L.) leaves obtained from local markets. Since Griffiths et al. (11) have found a higher level of protochlorophyllide reductase in plants which had been darkened before analysis, the leaves were stored overnight in the dark prior to the experiment in order to maintain the level of the enzyme to its maximum level. Deveined leaf sections were cut into chilled medium (2.5 liters/kg of leaves) containing 330 mM mannitol, 25 mM tetrasodium pyrophosphate, 0.1% bovine serum albumin, the pH was adjusted to 7.5 with HCl. The leaves were homogenized for 2–3 s in a 4-liter Waring Blender. All the operations were carried out at 0–5 °C. A crude chloroplast pellet was obtained as described by Douce et al. (12) and purified further by isopycnic centrifugation in Percoll gradients (13). The chloroplasts were recovered and used for purification of envelope membranes.

Protease Treatment of Isolated Intact Spinach Chloroplasts—In some experiments, thermolysin, a protease isolated from Bacillus thermoproteolyticus (Calbiochem) was used to probe proteins on the cytosolic surface of the outer envelope membrane (14). Purified, intact

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chloroplasts (final concentration, 1 mg chlorophyll/ml) were incubated for 1 h at 4°C in the following medium: 0.3 M Tricine-NaOH, pH 7.8, 100 μg of thermolysin, 10 mM CaCl₂, and 1 mM CaCl₂. The digestion was stopped by the addition of 10 mM EGTA. Control experiments were carried out under the same conditions, except that the incubation medium contained at the same time thermolysin, CaCl₂, and EGTA. After digestion, chloroplasts were purified again through a Percoll gradient containing several protease inhibitors: 1 mM phenylmethylsulfonyl fluoride, 5 mM e-aminoacaproic acid, and 1 mM benzamidine HCl. This purification step was made in order to remove thermolysin and the broken chloroplasts. The treated and “control” intact chloroplasts were recovered and the envelope membranes separated as described below.

Purification of Envelope Membranes from Spinach Chloroplasts—Purified intact chloroplasts were lysed in hypotonic medium as described by Douce et al. (12). The stroma, envelope membranes, and thylakoids were purified from the lysate (swollen chloroplasts) by centrifugation through a sucrose step gradient as described by Pineau et al. (8). In such a preparation, the chlorophyll content of the envelope membrane fraction was less than 0.2 μg/mg of envelope protein. Envelope membranes were suspended in a medium containing 10 mM Tricine, pH 7.8, and, when not used immediately for enzymatic test, stored frozen with 40% (w/v) glycerol.

Spectrofluorometry—Fluorescence emission or excitation spectra were recorded either at room temperature or at liquid nitrogen temperature on a laboratory-made spectrofluorimeter, as previously described by Pineau et al. (8, 15). The envelope suspension sample (5 μl of protein ml⁻¹) was put on a filter paper disk at the bottom of a cylindrical microcell (diameter, 10 mm). The top of the cell was covered with a bifurcated fiber optics cable, the first arm coming from the exciting monochromator (M25, Jobin et Yvon, Longjumeau, France) and the second one going to the analyzing monochromator (same model as the exciting monochromator) connected to a red-sensitive photomultiplier (RTC XP2203 B, La Radiotechnique, Su- resnes, France). A linear relationship was observed between the intensity of fluorescence emission and the concentration of envelope membranes.

Assay for Protochlorophyllide Reductase—Purified envelope membranes (0.2 mg/ml) were first incubated in darkness for 1 h in a medium containing 10 mM Tricine-NaOH, pH 7.8, 10% glycerol, and 1 mM NADPH and further incubated for different times at room temperature under illumination with white light (0.15 watt/m²). Control experiments were performed in the dark or under illumination (in the absence of NADPH) or in the presence of 2 mM NADH and with envelope membranes purified from thermolysin-treated intact chloroplasts. After incubation, filter paper disks with 5 μl of the envelope sample were loaded at the bottom of the cylindrical microcell and fluorescence emission spectra were recorded at liquid nitrogen temperature under 440 nm light excitation.

Electrophoretic Analyses of Plastid Proteins—Polypeptides of the chloroplast subfractions (stroma, thylakoids, and envelope membranes) were separated by two-dimensional electrophoresis (PAGE). The samples were solubilized at room temperature in 50 mM dithiothreitol, 50 mM Na₂CO₃, 12% sucrose, and 2% sodium dodecyl sulfate (SDS). SDS-PAGE was performed at room temperature in slab gels containing a 7.5–15% acrylamide gradient. In some experiments, two-dimensional PAGE of envelope polypeptides was performed using two different polyacrylamide gel systems: in the first dimension, lithium dodecyl sulfate-PAGE was carried out (at 4°C) with a 7.5–15% acrylamide gradient, and, in the second dimension, SDS-PAGE was carried out (at room temperature) with a 12–18% acrylamide gradient (in the presence of 8 M urea). The experimental conditions for gel preparation, sample solubilization, electrophoresis, and gel staining have been described by Piccioni et al. (16). Polypeptides from the different fractions analyzed were transferred electrophoretically to nitrocellulose (Bio-Rad) or Immobilon (Millipore) sheets essentially according to Towbin et al. (17), using a Trans-Blot cell system (Bio-Rad). The electrophoresis was performed at 100 V and the following buffer: 25 mM Tris-HCl, 192 mM glycine, 20% (v/v) methanol, pH 8.3, the power was 60 V, 0.22 A. The transfer was complete after about 3 h (when using nitrocellulose) and 30 min (when using Immobilon). The sheets were then used for immunoblotting experiments. Immobilon sheets were first stained with Coomassie Blue for detection of envelope polypeptides that were transferred and destained with methanol prior to immunoblotting experiments for further characterization of immunodecorated envelope polypeptides.

Immunoblotting Studies of Plastid Proteins—We used polyclonal and monoclonal antibodies raised against E37, a major polypeptide from spinach chloroplast envelope (14). We also used polyclonal antibody raised against purified protochlorophyllide reductase from oat etioplasts (18, 19). Immunoblotting experiments were performed essentially according to Burnette (20). Antigen-antibody complexes were revealed using goat antibody IgG-horseradish peroxidase conjugate and 4-chloro-1-naphthol (Bio-Rad).

Immunocytochemical Studies of Intact Chloroplasts—Antibodies raised against E37 and protochlorophyllide reductase were used to probe the outer surface of intact chloroplasts. For agglutination assays, 18 μl of a chloroplast suspension (0.1 mg of chlorophyll/ml) were incubated on a glass slide with 50 μl of the following medium (incubation medium): 0.3 M sucrose, 10 mM MOPS-NaOH, pH 7.8, and antibodies (diluted to 1/10). The slides were shaken for 5 min on a gyratory shaker and the suspensions were then examined under light (or phase contrast) microscopy to follow agglutination.

Protein and Chlorophyll Determination—Protein concentration was determined according to Lowry et al. (21) using bovine serum albumin as a standard. Chlorophyll concentration was measured according to Arnon (22).

RESULTS

Phototransformation of Fluorescent Signals in Isolated Envelope Membranes—Purified envelope membranes, illuminated at 440 nm, gave two broad fluorescence emission peaks at 639 and 680 nm at room temperature (Fig. 1). At liquid nitrogen temperature, a strong enhancement of the fluorescence intensity of these emissions was observed with a small blue shift of their maxima (633 and 678 nm) (Fig. 1). A third additional peak appeared at 735 nm (Fig. 1) and was due to a very low contamination of envelope membranes by discrete thylakoidal pieces (8). We have previously demonstrated that the two major fluorescence emission peaks at 633 and 678 nm were due to protochlorophyllide and chlorophyllide, respectively (8). Protochlorophyllide was detected by fluorescence only in envelope membranes and apparently not in the stroma nor in thylakoids (8). Since this compound is a substrate for protochlorophyllide reductase, we looked for the occurrence of this activity in purified envelope membranes.

![Figure 1](http://www.jbc.org/) Room (22°C) and liquid nitrogen (−196°C or 77 K) temperature fluorescence emission spectra of envelope membranes isolated from intact spinach chloroplasts. The mean emission levels of 5 μl of envelope membrane suspensions (2 μg of protein/ml) were recorded at different temperatures. Excitation (exc) light, 440 nm. Fluorescence emission intensity is expressed as arbitrary units.
Illumination of an envelope suspension under white light (0.15 watt/m²), at room temperature and in the presence of NADPH, induced a decrease of the 633 nm fluorescence emission peak together with a parallel increase of the 678 nm fluorescence emission peak (Fig. 2). Dark incubation of envelope membranes for 2 h in the presence of NADPH did not result in any significant modification of level of the fluorescence emission peaks (not shown). Light incubation of envelope membranes in absence of NADPH induced only a limited decrease of all fluorescence emission peaks at low (0.15 watt/m²) irradiance (Fig. 3), but at higher irradiance photodegradation of the pigments was rather quick (not shown). Finally, NADH was unable to replace NADPH in the reaction (Fig. 3A). Therefore, the light-dependent modifications of the levels of fluorescence emission peaks at 633 and 678 nm shown in Fig. 2 were not due to a nonspecific photodegradation but were dependent on the presence of both NADPH and light.

In addition, the excitation spectrum of the 678 nm fluorescence emission at liquid nitrogen temperature did not change during the reaction (Fig. 4), indicating no further modification in the nature of the fluorescing pigment during illumination in presence of NADPH. Therefore, the increase in the amplitude of the 678 nm fluorescence emission peak was due to an increase in the amount of pigment (chlorophyllide). Moreover, this activity was lost when envelope membranes were stored in absence of glycerol.

Together, these results strongly suggest that isolated envelope membranes contain the enzyme NADPH-protochlorophyllide reductase and were able to phototransform the small endogenous protochlorophyllide pool into chlorophyllide. To characterize further (using a different technique) the presence of this enzyme in chloroplast envelope membranes, we employed anti-protochlorophyllide reductase, a monospecific polyclonal antibody raised against oat protochlorophyllide reductase. The specificity of this antibody for protochlorophyllide reductase was previously analyzed by Kay (18). In addition, Darragh et al. (19) further characterized this antibody; digestion products obtained after staphylococcal V8 proteinase treatment of purified protochlorophyllide reductase and of immunoprecipitated protein were demonstrated to be very close. Finally, this antibody was also used for selection of cDNA clones corresponding to protochlorophyllide reductase (19); the amino acid composition of protochlorophyllide reductase as derived from the cDNA sequence was shown to be almost identical to that obtained by chemical analyses of the purified protein (19).

**Immunochemical Analyses of Envelope Polypeptides**—Stroma (S), thylakoid (T), and envelope (E) polypeptides separated by polyacrylamide gel electrophoresis (Fig. 5A) were transferred to nitrocellulose sheets and analyzed using anti-protochlorophyllide reductase antibody. The antibody reacted only with a single envelope polypeptide having a molecular weight of about 37,000 (Fig. 5B). No protochlorophyllide reductase could be detected in the other chloroplast compartments, i.e., stroma or thylakoids (Fig. 5B). The envelope polypeptide which reacted with anti protochlorophyllide reductase antibody was apparently identical to E37, a major envelope polypeptide (23). Indeed, using a polyclonal (anti-E37) and a monoclonal (28-7-1) antibody raised against E37, we found that these two types of antibodies reacted with a single polypeptide having the same electrophoretic mobility as the polypeptide labeled after incubation with anti protochlorophyllide reductase (Fig. 5B). This experiment demonstrates first that chloroplast envelope membranes, in contrast with thylakoids and stroma, contain a polypeptide which reacts with a nonspecific antibody raised against protochlorophyllide reductase. This experiment also suggests that...
either (a) protochlorophyllide reductase is a major chloroplast envelope polypeptide (E37) or (b) that the Coomassie Blue-stained emission peak of envelope membranes isolated from intact spinach chloroplasts and incubated in the presence or absence of NADPH. NADPH, the curve corresponds to the sample illuminated 1 h in the presence of 1 mM NADPH; the experimental conditions for the incubation are identical to those of Fig. 2. no NADPH, the curve corresponds to the control sample illuminated 1 h in the absence of NADPH; the experimental conditions for the incubation are identical to those of Fig. 3A.

To solve this problem, we first separated envelope polypeptides by electrophoresis using a two-dimensional polyacrylamide gel system (23); the polypeptides were then transferred electrophoretically to Immobilon sheets and probed using our amide gel system (23); the polypeptides were then transferred electrophoretically to Immobilon sheets and probed using our anti-PCR antibodies against protochlorophyllide reductase (anti-PCR), polyclonal (ANTI-E37), and monoclonal (28-7-Z) antibodies raised against the different plastid compartments were separated by polyacrylamide gel electrophoresis at room temperature with a 7.5-15% acrylamide gradient. Protein loaded in each slot was equivalent to 100 mg of total protein. A, polypeptides were revealed with Coomassie Blue staining; B, immunostaining of polypeptides using polyclonal antibodies raised against protochlorophyllide reductase (ANTI-PCR), polyclonal (ANTI-E37), and monoclonal (28-7-Z) antibodies against the major envelope polypeptide E37, the antibody-antigen complexes were revealed as described under "Materials and Methods." Lanes 1 and 4, analyses of stroma polypeptides; lanes 2 and 5, analyses of thylakoid polypeptides; lanes 3, 6, 7, and 8, analyses of envelope polypeptides. Note that only envelope membranes contain a polypeptide that reacts with anti-protochlorophyllide reductase (lanes 4-6) and that this polypeptide has apparently the same electrophoretic mobility as E37, a major inner envelope polypeptide, as shown with specific antibodies to E37 (lanes 7 and 8).

To localize more precisely the envelope protochlorophyllide reductase within chloroplast envelope membranes, we used first thermolysin digestion of isolated intact chloroplasts (14, 24) to probe whether the envelope protochlorophyllide reductase was localized on the cytosolic side of the outer membrane. We analyzed envelope polypeptides from thermolysin-treated and nontreated intact chloroplasts. As shown in Fig. 7, and in agreement with our previous observations (14), few envelope polypeptides were hydrolyzed during treatment of intact chloroplasts with thermolysin (100 mg/ml); at this thermolysin concentration, E24, a specific marker polypeptide for the outer surface of the outer envelope membrane (14), was partly hydrolyzed. In contrast, the major inner envelope polypeptides such as E30 or E37 were not digested (Fig. 7), because thermolysin was unable to pass through the outer envelope membrane (14, 24). Using anti-PCR antibodies, we analyzed the envelope polypeptides present in the different fractions by immunoblotting: in contrast with the control experiments, protochlorophyllide reductase was barely detectable in envelope membranes from thermolysin-treated chloroplasts (Fig. 7). These data suggest that in spinach chloroplasts, protochlorophyllide reductase is probably located on the cytosolic side of outer envelope membrane.

To provide further evidence for this conclusion, we incubated isolated intact chloroplasts with antibodies to protochlorophyllide reductase. We have previously demonstrated that incubation of isolated intact chloroplasts with antibodies (which do not penetrate membranes) to envelope polypeptides lead to chloroplast agglutination if the antigenic domains of polypeptides are accessible to antibodies (14). Fig. 8 shows that strong agglutination indeed occurred with anti-PCR (diluted to 1/10), indicating that the envelope protochlorophyllide reductase present antigenic domains which are exposed to the outer surface of the outer envelope membrane. Only intact chloroplasts (which are highly reflective and present a bright halo under phase contrast microscopy) but not the broken chloroplasts (which are dark and granular under phase contrast microscopy) were agglutinated (not shown). In con-
was SDS-PAGE performed at room temperature with a 12-18% acrylamide gradient in presence of 8 M urea. Protein load for each gel was equivalent to 100 µg of total envelope proteins. Thermolysin treatment of intact chloroplasts was not performed as described under "Materials and Methods" using a final thermolysin concentration of 100 µg/ml. Envelope polypeptides were revealed with Coomassie Blue staining. Anti-PCR immunostaining with anti protochlorophyllide reductase (PCR) (B1), anti-E37 (B2), and 28-7-1 (B3) of polypeptides after electrophoretic transfer on Immobilon sheets of the envelope polypeptides separated by two-dimensional PAGE. The envelope polypeptides were first localized by Coomassie Blue staining, and then (after destaining) the antibody-antigen complexes were revealed as described under "Materials and Methods." Note that in each condition the polypeptide that reacted with antibody was not drawn for a more clear observation; the other polypeptides were drawn as in A.

FIG. 6. Electrophoretic and immunochemical analyses of envelope polypeptides from intact spinach chloroplasts. Envelope polypeptides were separated by a two-dimensional PAGE. The first dimension was lithium dodecyl sulfate (LDS)-PAGE performed at 4°C with a 7.5-15% acrylamide gradient. The second dimension was SDS-PAGE performed at room temperature with a 12-18% acrylamide gradient in presence of 8 M urea. Protein load for each gel was equivalent to 100 µg of total envelope proteins. Thermolysin treatment of intact chloroplasts was performed as described under "Materials and Methods" using a final thermolysin concentration of 100 µg/ml. Envelope polypeptides were revealed with Coomassie Blue staining. Anti-PCR immunostaining with anti protochlorophyllide reductase. The antibody-antigen complexes were revealed as described under "Materials and Methods." Note that in each condition the polypeptide that reacted with antibody was not drawn for a more clear observation; the other polypeptides were drawn as in A.

FIG. 7. Electrophoretic and immunochemical analyses of envelope polypeptides from intact spinach chloroplasts treated with thermolysin. Envelope polypeptides were separated by polyacrylamide gel electrophoresis at room temperature with a 7.5-15% acrylamide gradient. Protein load in each slot was equivalent to 30 µg of total envelope proteins. Thermolysin treatment of intact chloroplasts was performed as described under "Materials and Methods" using a final thermolysin concentration of 100 µg/ml. Envelope polypeptides were revealed with Coomassie Blue staining. Anti-PCR immunostaining with anti protochlorophyllide reductase. The antibody-antigen complexes were revealed as described under "Materials and Methods." Note that in each condition the polypeptide that reacted with antibody was not drawn for a more clear observation; the other polypeptides were drawn as in A.

**Discussion**

Protochlorophyllide reductase is an enzyme which catalyzes the phototransformation of protochlorophyllide into chlorophyllide in presence of both light and NADPH (9, 10). The data reported in this article confirm and extend our previous observations (8) and present evidence for the presence of protochlorophyllide reductase in envelope membranes from mature spinach chloroplasts. First, envelope membranes are able to catalyze the NADPH-dependent phototransformation of protochlorophyllide into chlorophyllide. In our experimental conditions, the reaction proceeded until almost complete disappearance of the endogenous protochlorophyllide pool, but only at a slow rate. Second, envelope membranes contain a 37,000 dalton polypeptide which cross-react with an antibody raised against oat protochlorophyllide reductase. The molecular mass value for the envelope polypeptide is very similar to that of protochlorophyllide reductase (35,000-38,000) from etioplasts (25-31). From these results and our previous data (8), we can conclude that envelope membranes from spinach chloroplasts contain both the substrate (protochlorophyllide) and the enzyme for chlorophyllide synthesis. The low level of both the enzyme and protochlorophyllide is consistent with the observations that all the protochlorophyllide present in etioplasts is associated with the enzyme (32), two or three protochlorophyllide molecules being bound to each polypeptide molecule (33). However, this remains to be demonstrated in the case of chloroplast envelope membranes.

To date, protochlorophyllide reductase is the second envelope protein to be identified among the different envelope polypeptides separated by polyacrylamide gel electrophoresis. The first envelope polypeptide to be characterized was E30, the phosphate/triose phosphate translocator (6). In addition, envelope preparations contain ribulose-1,5-bisphosphate carboxylase, but probably as a contaminant, and its two subunits (E54 and E14) have been characterized by Pineau and Douce (34), Pineau et al. (35), and Joyard et al. (23).

Interestingly, Hinterstoisser et al. (36) and Peschek et al. (37) have found that the chlorophyll-free plasma membrane...
of a cyanobacterium, Anacystis nidulans, contains chlorophyll precursors, i.e. protochlorophyllide and chlorophyllide, and probably protochlorophyllide reductase. These observations have to be compared with our previous data (8) and provide further evidence for a possible analogy between the limiting membranes of cyanobacteria and the outer envelope membranes from chloroplasts (38). However, in cyanobacteria, the protochlorophyllide reductase could use both NADH and NADPH whereas the chloroplast envelope enzyme is specific for NADPH.

The results presented in this article are consistent with observations that in green leaves the level of protochlorophyllide reductase is very low (11, 31). Immunoblotting experiments, after separation of envelope polypeptides by two-dimensional PAGE and immunoadjugation experiments of intact chloroplasts, clearly demonstrate that protochlorophyllide reductase is not E37, one of the major envelope polypeptides, but only a minor protein. From the Coomassie Blue staining of envelope polypeptides (Fig. 6), one can estimate that this minor polypeptide represents less than 1% of the total envelope proteins. Since envelope membranes only represent a few percent of total chloroplast proteins, the low level of protochlorophyllide reductase is probably (38) explained by the results of immunoelectrophoretic experiments, where it was demonstrated that protochlorophyllide reductase is present in the envelope membranes at least as a minor contaminant (47). In contrast to intact plastids, do not contain protochlorophyllide reductase (30). Further experiments, the envelope protochlorophyllide reductase is present in the outer face of the outer envelope membrane, as demonstrated by two types of experiments: (a) the 37,000-dalton polypeptide is hydrolyzed when isolated intact chloroplasts are incubated in presence of thermolysin (Fig. 7) and (b) isolated intact chloroplasts strongly agglutinate when incubated with anti-protochlorophyllide reductase (Fig. 8). However, the possibility that some additional, but low, amounts of protochlorophyllide reductase could be localized within the chloroplast (in the inner envelope membrane, in the stroma or in thylakoids) cannot be entirely ruled out, due to the sensitivity of the methods used. These conclusions demonstrate major differences between etioplasts and chloroplasts with respect to protochlorophyllide reductase levels and localization. However, the dramatic decrease of enzyme observed after illumination of etioplasts can explain these differences: upon illumination almost all (~99%) of the enzyme present inside the etioplast is destroyed, probably owing to a specific protease (28), and it is possible to suggest that only the envelope protochlorophyllide reductase remains in the plastid after greening (the enzyme being protected from the proteolytic digestion inside plastids by its localization on the outer surface of the outer membrane). It is quite difficult to probe this hypothesis since one of the major problems in purifying envelope membranes from etioplasts is to get rid of any contaminating internal membranes (pieces of prolamellar body and prothylakoids), which contain much larger amounts of protochlorophyllide reductase than the envelope. In fact, the low levels of protochlorophyllide reductase found in etioplast envelope membranes are usually considered to reflect the contamination of the envelope fraction by internal membranes (47).

The presence of a small pool of protochlorophyllide reductase on the cytosolic side of the outer chloroplast envelope membrane, of a very large pool of enzyme in the prolamellar body from etioplasts, and of cytosolic enzyme in etiolated and
greening leaves also raises the question of the existence of distinct isoforms specifically associated with a given com-
part ment. Ikemichi and Murakami (27) first reported that the
M, 36,000 polypeptide of protochlorophyllide reductase from
squeezed plastids could be resolved, by isoelectric focusing,
to at least four different polypeptides (a, b, y, and d) of
identical M, but of different isoelectric points: 9.1, 8.5, 8.2,
and 8.2, respectively. Dehes et al. (30) demonstrated that all
four polypeptides reacted with an antibody to protochloro-
phyllide reductase. Unfortunately, it is not yet known whether
the cytosolic protochlorophyllide reductase (44) presents the
same or additional isoforms. Studies on the nature of envelope
isoforms are presently under investigation in our laboratory.
The possible existence of protochlorophyllide reductase iso-
forms also question the number of genes involved in the
synthesis of this protein and their regulation. Answer to this
question will probably be given soon since Darrah et al. (19)
have recently cloned and sequenced cDNA corresponding to
protochlorophyllide reductase.

The localization of the envelope protochlorophyllide reduc-
tase on the outer face of the outer envelope membrane raises
also the question of the physiological significance of this
enzyme. One can question whether the presence of protochlo-
rophyllide reductase on the outer envelope membrane is
indeed relevant to the participation of this membrane system
to chlorophyll biosynthesis. We first should keep in mind that
mature chloroplasts need chlorophyll to replace the molecules
which are continuously destroyed by photooxidation. The
sequence of the reactions involved in chlorophyll biosynthesis
had been thoroughly studied (for a review, see Ref. 10), but
little is known about compartmentalization within chloro-
plasts of the enzymes involved. The first steps in chlorophyll
biosynthesis, namely from (2)α-aminolevulinic acid to proto-
porphyrin IX, occur in the soluble phase of chloroplasts (48),
but all the subsequent steps in protoporphyrin IX transfor-
mation are catalyzed by membrane-bound enzymes (10). A
complex heterogeneity of the chlorophyll precursors, probably
due to an heterogeneity of their biosynthetic origin, has been
demonstrated by Rebez and coworkers (see, for instance, Ref.
49). The final step of chlorophyll formation involves addition
of the phytol moiety and is specifically associated with thyl-
lakoids (50). Therefore, chlorophyllide transfer to thylakoids
could modify the equilibrium of the reaction catalyzed by
protochlorophyllide reductase by modifying the dynamic equi-
librium between protochlorophyllide and chlorophyllide. Our
results suggest that at least some of the enzymes involved in
the transformation of protoporphyrin IX to chlorophyllide
could be associated with envelope membranes. If this hypo-
thesis is true, the pathway for chlorophyll biosynthesis would
involve a very complex cooperation between all chloroplast
compartments. Until now, there has been only limited evi-
dence for this, except for the data presented in this article.
However, in support for this hypothesis, Fuesler et al. (51)
have shown that in intact chloroplasts, Mg-chelatase was
accessible to molecules unable to pass through the inner
envelope membranes, thus suggesting that Mg-chelatase could
be present in envelope membranes (outer membrane?). Fur-
thermore, Johannimager and Howell (52) have demonstra-
ted that the level of Mg-protoporphyrin methyl esters is
able to regulate the light induced cytosolic light-harvesting
chlorophyll a/b binding protein mRNA accumulation. Con-
sequently, these authors have postulated that such interme-
diates in chlorophyll synthesis could be located in the chlo-
roplast envelope. The localization of the components of the
Mg-protoporphyrin IX monomethyl ester oxidative cyclase
activity (53) remains to be elucidated, but the involvement of
the plastid envelope membranes cannot be excluded (54).

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Photochlorophyllide Reductase of Chloroplast Envelope Membranes

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