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A Light-Induced Protease from Barley Plastids Degrades NADPH:Protochlorophyllide Oxidoreductase Complexed with Chlorophyllide†

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The NADPH:protochlorophyllide oxidoreductase precursor protein (pPorA) of barley (*Hordeum vulgare* L. cv. Carina), synthesized from a full-length cDNA clone by coupling in vitro transcription and translation, is a catalytically active protein. It converts protochlorophyllide to chlorophyllide in a light- and NADPH-dependent manner. At least the pigment product of catalysis remains tightly bound to the precursor protein. The chlorophyllide-pPorA complex differs markedly from the protochlorophyllide-pPorA complex with respect to sensitivity to attack by a light-induced, nucleus-encoded, and energy-dependent protease activity of barley plastids. The pPorA-chlorophyllide complex is rapidly degraded, in contrast to pPorA-protochlorophyllide complexes containing or lacking NADPH, which are both resistant to protease treatment. Unexpectedly, pPorA devoid of its substrates or products was less sensitive to proteolysis than the pPorA-chlorophyllide complex, suggesting that both substrate binding and product formation during catalysis had caused differential changes in protein conformation.

The transition of etioplasts to chloroplasts in angiosperms, such as barley, is strictly dependent on light (2, 3, 15). Drastic changes occur in the ultrastructure of the plastid compartment when dark-grown seedlings are exposed to light (7–9). The prolamellar body of the etioplast disintegrates, and thylakoids are formed. Furthermore, light regulates the expression of plastidic and nuclear genes encoding plastid constituents, such as rRNAs, tRNAs, and proteins (36, 53), and induces chlorophyll formation (for a review, see reference 3).

The only known light-dependent step in the biosynthesis of chlorophyll is the reduction of protochlorophyllide to chlorophyllide, catalyzed by the enzyme NADPH:protochlorophyllide oxidoreductase (Por; EC 1.6.99.1) (2, 15). In angiosperms, this reaction requires both NADPH and light (14, 15, 49). Although light is needed for catalysis, it concomitantly inactivates Por (20, 49). This phenomenon has been observed not only for barley but also for diverse other plant species (4, 9). After inactivation, Por is rapidly proteolytically degraded (9, 17, 18, 20). The levels of other plastid proteins, such as the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO), remain constant, however (21, 22).

The molecular basis for the selective degradation of Por in etioplasts undergoing differentiation into chloroplasts has been only poorly understood to date. In this study, we addressed this problem by using an organelle-free assay with Por precursor molecules (pPor) synthesized by coupled in vitro transcription and translation of a *porA*-specific cDNA from barley and stromal protein extracts prepared from lysed barley plastids. We demonstrate that enzymatic product formation enhances degradation of pPorA by a light-induced, nucleus-encoded, ATP- and metal-dependent plastid protease activity.

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† Dedicated to Martin Luckner on the occasion of his 60th birthday.

MATERIALS AND METHODS

Plant material. Seeds of barley (*Hordeum vulgare* L. cv. Carina) were germinated on moist vermiculite at 25°C either in the dark or in light (ca. 30 W m^{-2}, provided by fluorescent bulbs) for 5 days. For inhibitor experiments, leaves were cut from the seedlings, and 5-cm segments beginning 1 cm from the leaf tip were placed in water or aqueous solutions containing either chloramphenicol or cycloheximide at final concentrations of 2 mg ml^{-1} (40) and 25 μg ml^{-1} (38), respectively.

Preparation of plastid lysates. Etioplasts and chloroplasts from dark- and light-grown seedlings, respectively, were isolated by Percoll (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) density gradient centrifugation (12). After a further purification step on Percoll cushions (16; as described in reference 41), the plastids were diluted 1:10 with buffer A (50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 8.0], 330 mM sorbitol) and sedimented by centrifugation at 5,000 rpm (2,702 × *g*) for 5 min at 2°C in a Sorvall RC5B centrifuge (DuPont de Nemours, Homburg, Germany) with a HB4 rotor. Plastids were resuspended in buffer B, which differed from buffer A in that it did not contain sorbitol. After a 10-min incubation on ice, the lysed plastids were centrifuged for 1 h at 60,000 rpm (246,000 × *g*, 2°C) in a Beckman Spinco L75 centrifuge with a Ti 70.1 rotor. The resulting supernatant fractions contained stromal proteins, such as the large and small subunits of RUBISCO, but were apparently devoid of thylakoid membrane proteins, such as the light-harvesting chlorophyll *a*- and *b*-binding proteins, as assessed by Western blot (immunoblot) analysis (54) with homologous polyclonal antisera (43).

Determination of protease activities in stromal protein extracts. Three different approaches were used to determine protease activities in stromal protein extracts. First, the overall proteolytic activity contained in the various plastid stroma samples was determined by the Coomassie brilliant blue G-250 dye-binding technique described by Krauspe and Scheer (23). According to this method, autolytic degradation of stromal polypeptides can be measured by monitoring the loss of their Coomassie brilliant blue-binding capacities after various periods. Specific autolysis rates (ΔA_{578} mg^{-1} h^{-1}), where Δ indicates the change in A_{578}, were calculated from the determined ΔA_{578}, i.e., the difference of A_{578} at time zero and after 15 min. During this incubation time, the reaction proceeded linearly.

Azocaseinolytic activities in stromal protein extracts were measured in 200-μl assays at 30°C, as described by Krauspe and Scheer (23), using azocasein (with a 1% [wt/vol] final concentration) prepared by the method of Langner et al. (28). Specific azocaseinolysis rates were expressed as ΔA_{366} mg^{-1} h^{-1}; 10% (wt/vol) azocasein degradation gives an A_{366} of 1.47 (24).

For the determination of the protease activity specifically degrading pPorA, [^{35}S]methionine-labeled pPorA was produced by coupling in vitro transcription and translation (25) of the previously characterized barley cDNA designated A7 (50). pPorA-protochlorophyllide-NADPH complexes were prepared by incubating for 15 min in the dark 25 μl of the in vitro translation mixture containing the radioactively labeled pPorA (approximately 2 × 10^6 cpm, except for the experiments described in the legend to Fig. 2 and 6, in which 5 × 10^7 cpm was used),

2.5 μl of isolated protochlorophyllide (with a 15-μM final concentration), 20 μl of assay buffer consisting of 100 mM HEPES-KOH (pH 7.2), 660 mM sucrose, 5 mM ATP, 5 mM $MgCl_2$, 2 mM EDTA, 17 mM L-methionine, and 2.5 μl of an aqueous solution of NADPH (with a 0.5 mM final concentration) (44). Incubation mixtures to be used for the generation of pPorA-protochlorophyllide complexes contained doubly distilled water instead of NADPH. For the production of pPorA-chlorophyllide complexes, pPorA-protochlorophyllide-NADPH complexes formed during a dark preincubation were exposed to light for an additional 15-min period (44). The different pPorA-protochlorophyllide, pPorA-protochlorophyllide-NADPH, and pPorA-chlorophyllide complexes, as well as the naked pPorA lacking its substrates and products, were subsequently subjected to gel filtration on Sephadex G-25 equilibrated in the citrate-Na_2HPO_4 buffer of McIlvaine (34), which, if not stated otherwise, had been adjusted to pH 6.5. The identities of the pPorA-protochlorophyllide, pPorA-protochlorophyllide-NADPH, and pPorA-chlorophyllide complexes in the flowthrough after gel filtration were confirmed by assessing the fluorescence emissions of protochlorophyllide and chlorophyllide at 628 and 665 nm, respectively, at an excitation wavelength of 431 nm in a Perkin-Elmer spectrometer model LS50 (45).

The in vitro-synthesized pPorA was incubated for various periods with stromal protein extracts in 150-μl assays consisting of 50 μl of the gel-filtered pPorA-substrate or pPorA-product complexes, or of the naked pPorA lacking its substrates or products, 50 μl of dialyzed stromal proteins from the various plastid samples (equivalent to 50 μg of protein, determined according to Lowry et al. [30]), and 30 μl of fivefold-concentrated McIlvaine buffer (see above). As indicated in the text, the reaction mixtures were adjusted to the desired final pH value and supplemented with the following reagents (final concentrations): ATP or GTP (each, 0.2 mM) and Mg^{2+}, Mn^{2+}, Ca^{2+}, K^+, or Na^+ (each, 0.4 mM). Assays to be used for testing proteinase inhibitors additionally contained either leupeptin, antipain, chymostatin (in 1-mg-ml^{-1} stock solutions, each prepared in dimethylsulfoxide and used at a final concentration of 0.1 μg ml^{-1}), pepstatin A (in a 0.1-mg-ml^{-1} stock solution, prepared in dimethylsulfoxide, used at a final concentration of 0.1 μg ml^{-1}) diisopropyl fluorophosphate (in a 0.1-M stock solution, prepared in 50% [vol/vol] propan-2-ol shortly before use and added to the assays at a final concentration of 2.5 mM), L-*trans*-epoxysuccinyl-leucylamido (4-guanidino)butane (E-64) (in a 0.01-M stock solution, prepared in doubly distilled water, used at a final concentration of 5 μM), or phenylmethylsulfonyl fluoride (in a 0.1-M stock solution, prepared in 96% [vol/vol] ethanol, used at a final concentration of 25 μM). After 0.5, 1, 2, 5, 10, and 15 min, respectively, the incubation was stopped by the addition of 50 μl of a solution containing 20% (wt/vol) trichloroacetic acid (TCA). After precipitation overnight, TCA-insoluble material was sedimented by centrifugation and further processed by repeated washings with ethanol and ether (38). [^{35}S]methionine-labeled pPorA contained in the TCA-insoluble fractions was separated in denaturing 11 to 20% (wt/vol) polyacrylamide gradients (41) and detected by autoradiography. To identify low-molecular-weight degradation products of pPorA, one aliquot of the supernatant fraction obtained after sedimentation of the TCA-insoluble part of the incubation mixtures (see above) was diluted with equal volumes of a doubly concentrated sodium dodecyl sulfate sample buffer (27) and run in a 20% polyacrylamide gel.

For partial purification of the pPorA-degrading protease, stromal proteins were prepared from chloroplasts and precipitated with ammonium sulfate (at a 90% [wt/vol] saturation, pH 6.5). After dialysis overnight with the McIlvaine's buffer (see above), stromal proteins were subjected to gel filtration on Sephadex G-25.

Miscellaneous. Protochlorophyllide was isolated from dark-grown barley seedlings according to the procedure of Griffiths (15).

RESULTS

Enzymatic product formation renders pPorA susceptible to degradation by a protease activity of isolated barley chloroplasts. pPorA was synthesized by coupled in vitro transcription and translation of the barley cDNA A7 (see Materials and Methods). After the incubation, the assays were supplemented with either protochlorophyllide or protochlorophyllide plus NADPH. Parts of the latter assays were kept in the dark, while other parts were exposed to light for an additional 15-min period to induce chlorophyllide formation. The resulting pPorA-protochlorophyllide, pPorA-protochlorophyllide-NADPH, and pPorA-chlorophyllide complexes were subsequently subjected to gel filtration on Sephadex G-25 to remove the excess of nonbound protochlorophyllide. The different binary and ternary pPorA-substrate and pPorA-product complexes recovered in the flowthrough after gel filtration were detected by their blue light-induced fluorescence emissions at 628 and 665 nm, respectively (data not shown, but see references 44 and 45). The pPorA-protochlorophyllide, pPorA-protochlorophyllide-NADPH, and pPorA-chlorophyllide complexes were then incubated in the dark in the standard assay buffer (pH 6.5) with a protein extract prepared from lysed barley chloroplasts (see Materials and Methods). As a control, pPorA devoid of its substrates or products was included. After various time intervals, the reaction was stopped by the addition of TCA. TCA-insoluble [^{35}S]methionine-labeled pPorA was recovered by centrifugation, separated electrophoretically, and detected by autoradiography. As demonstrated in Fig. 1A, the naked pPorA, which did not contain either its substrates or products, was rapidly proteolytically degraded in the presence of the chloroplast protein extract. In contrast, the levels of pPorA remained constant in assays containing either the pPorA-protochlorophyllide (Fig. 1B) or pPorA-protochlorophyllide-NADPH complexes (Fig. 1C). If protochlorophyllide and NADPH were bound to the precursor protein during a dark preincubation, followed by illumination of the resulting pPorA-protochlorophyllide-NADPH complex, a change in protease sensitivity could be observed: pPorA was more rapidly degraded than the naked polypeptide (Fig. 1D). Presumably, the enzymatically produced chlorophyllide, which has recently been shown to remain tightly bound to the precursor protein (44), had destabilized pPorA.

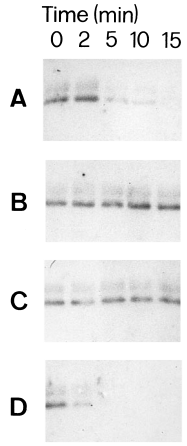

FIG. 1. Effects of protochlorophyllide, protochlorophyllide plus NADPH, and enzymatically-produced chlorophyllide on the sensitivity of pPorA in a treatment with a protease activity from barley chloroplasts. [^{35}S]methionine-labeled pPorA molecules (approximately 2×10^6 cpm) were synthesized by coupled in vitro transcription and translation of a *porA*-specific cDNA. After synthesis, the assays were supplemented with either protochlorophyllide (B) or protochlorophyllide plus NADPH (C and D) or were left unsupplemented (A). After a 15-min incubation in the dark (B and C) or in light (D), the assays were subjected to gel filtration (see Materials and Methods). A stromal protein extract prepared from lysed barley chloroplasts was added to proteins eluting in the flowthrough, and the assays were incubated in the presence of 0.5 mM ATP at pH 6.5 in the dark for the indicated periods. Then, TCA was added to terminate the reaction. TCA-insoluble material was recovered by centrifugation and separated in a denaturing 11 to 20% polyacrylamide gradient. Radiolabeled pPorA remaining after protease treatment of the naked, pigment-free pPorA (A) or of the pPorA-protochlorophyllide (B), pPorA-protochlorophyllide-NADPH (C), and pPorA-chlorophyllide (D) complexes was detected by autoradiography.

The apparent lack of degradation products in the TCA-insoluble fraction suggested that pPorA contained in the pPorA-chlorophyllide complex was completely proteolytically degraded. However, we could not exclude that some low-molecular-weight degradation products of pPorA might have escaped detection because of their solubility in TCA. To trace such degradation products, a 12.5-fold-higher amount of the radioactively labeled pPorA (5×10^7 cpm) was incubated with the chloroplast stromal protein extract, and the reaction tem-

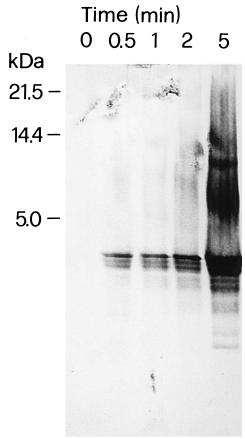

FIG. 2. Detection of pPorA degradation products. pPorA-chlorophyllide complexes containing approximately 5×10^7 cpm of the radioactively labeled precursor protein were produced and incubated at 4°C in the dark with chloroplast protein extracts containing the pPorA-specific protease, as described in the legend to Fig. 1. After terminating the reaction at the indicated time points by the addition of TCA, the incubation mixtures were centrifuged. The supernatant was carefully aspirated, and TCA-soluble material contained in this fraction was analyzed electrophoretically. The autoradiogram shows degradation products of pPorA depending on the duration of protease treatment. The positions of soybean trypsin inhibitor (21.5 kDa), hen egg white lysozyme (14.4 kDa), and a barley leaf thionin (5 kDa), used as molecular mass standards, are indicated.

perature during protease treatment was lowered to 4°C. After different lengths of incubation, TCA-insoluble material was sedimented by centrifugation and an aliquot of the supernatant fraction was analyzed electrophoretically. As shown in Fig. 2, at least three low-molecular-weight degradation products of pPorA could be detected in the TCA-soluble fraction. Their levels increased during the first 2 min of the incubation but then seemed to decline. The simultaneous drastic increase in the amount of smaller fragments and presumably also in free radioactivity, as seen by the smear in the lane of the autoradiogram after 5 min (Fig. 2), suggested that proteolytic degradation of pPorA and its low-molecular-mass products was completed. The large extent of radioactivity in the high-molecular-mass range after 5 min of incubation may be explained by assuming that the chlorophyllide released during the degradation of pPorA might have negatively affected subsequent electrophoretic separation of the sample.

Light induction of the pPorA-degrading protease activity. We next investigated whether etioplasts present in dark-grown seedlings contain a pPorA-degrading protease activity similar to that present in chloroplasts. Stromal protein extracts were prepared from etioplasts and incubated with the pPorA-chlorophyllide complex. As demonstrated by Fig. 3, the level of pPorA remained constant in the incubation mixture. Although this result seemed to indicate that etioplasts did not contain pPorA-degrading protease activity, one could not exclude that protochlorophyllide present in the etioplast protein extract might have displaced chlorophyllide from the pPorA-product complex and thus stabilized the precursor protein. If protochlorophyllide and chlorophyllide were indeed interchangeable in the pPorA-substrate and pPorA-product complexes, it should conversely then be possible to displace chlorophyllide by an excess of exogenously added protochlorophyllide from the pPorA-chlorophyllide complex and thus to stabilize pPorA in the chloroplast protein extract.

To address these points, etioplast and chloroplast protein extracts were incubated with the pPorA-chlorophyllide complex either in the absence or presence of a 10-fold excess of

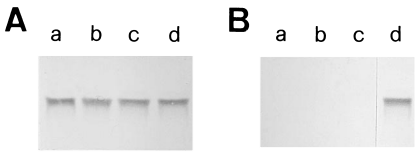

FIG. 3. Effects of exogenously added chlorophyllide and protochlorophyllide on the degradation of pPorA-substrate and pPorA-product complexes in etioplast (A) and chloroplast (B) protein extracts. Etioplast and chloroplast stromal protein extracts were prepared from dark- and light-grown seedlings, respectively, as described in the legend to Fig. 1. pPorA-chlorophyllide (lanes a to c) and pPorA-protochlorophyllide (lanes d) complexes formed during a preincubation (see Fig. 1) were then added to the different stromal extracts, and the incubation mixtures were supplemented with either additional chlorophyllide (lanes b and d) or protochlorophyllide (lanes c). Control assays did not contain either pigment (lanes a). The autoradiograms show the levels of pPorA in the TCA-insoluble fractions at the end of a 15-min dark incubation, as determined by the procedure shown in Fig. 1.

exogenously added chlorophyllide or protochlorophyllide. As shown in Fig. 3A (lane b), chlorophyllide could not destabilize pPorA in the etioplast protein extract. On the other hand, exogenously added protochlorophyllide was not able to stabilize pPorA in the chloroplast protein extract (Fig. 3B, lane c). Furthermore, exogenously administered chlorophyllide could not destabilize pPorA, if the pPorA-protochlorophyllide complex was offered as the substrate for the protease assay with either the chloroplast or etioplast protein extract (Fig. 3A and B, lanes d). Collectively, these results showed that the lack of protease activity degrading pPorA in the etioplast protein extract was not due to stabilization of the precursor protein by endogenously occurring protochlorophyllide. Rather, the results suggested that the appearance of the pPorA-specific protease might have been a light-dependent process correlated to chloroplast development.

To test this possibility and to distinguish whether the appearance of the pPorA-specific protease activity was either due to light induction or light activation of the enzyme, the following experiment was performed. Either purified intact etioplasts or etioplast protein extracts were exposed to light for various periods, and their protease activities were determined with the pPorA-chlorophyllide complex as the substrate. As shown in Fig. 4, light did not cause the activation of the pPorA-degrading protease activity. Neither in the etioplasts (Fig. 4) nor in the protein extracts prepared therefrom (data not shown) could pPorA-degrading protease activity be detected after various periods of light treatment (data not shown). In chloroplast protein extracts, however, the pPorA-degrading protease was easily detectable, as seen by the rapid degradation of the PorA

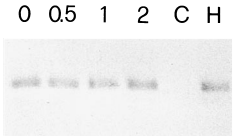

FIG. 4. pPorA-specific protease is not activated but induced by light in barley plastids. Etioplasts were prepared from dark-grown barley seedlings, as described in the legend to Fig. 2, and were then exposed to light for the indicated time intervals in incubation mixtures containing 330 mM sorbitol and 5 mM ATP. As a control, chloroplasts were prepared from light-grown seedlings and incubated similarly. Stromal polypeptides were prepared from the various plastid samples and added to incubation mixtures containing the pPorA-chlorophyllide complex enriched by gel filtration (cf. Fig. 1). Prior to the incubation, one aliquot of the chloroplast protein extract was boiled for 3 min (lane H), while another aliquot was left untreated (lane C). After a 15-min dark incubation, the amount of pPorA was determined as described in the legend to Fig. 1.

TABLE 1. Proteolytic activities of barley plastids during light-induced chloroplast development[a]

Plastid type[b]	Light exposure time (h)	Rate of autolysis (ΔA_{578} mg^{-1} h^{-1})	Rate of azocaseinolysis (ΔA_{366} mg^{-1} h^{-1})
Etioplast	0	0.05	0.12
	0.5	0.04	0.11
	1	0.07	0.12
	2	0.05	0.10
	8	0.08	0.13
Chloroplast	NA[c]	0.06	0.12

[a] After gentle lysis of the plastids, stromal protease activities were determined as described in Materials and Methods. Rates of autolysis and azocaseinolysis were calculated as indicated in Materials and Methods.

[b] Etioplast stromal protein extracts were isolated from dark-grown seedlings and seedlings that had in addition been illuminated for the indicated periods, and chloroplast stromal protein extracts were isolated from light-grown seedlings.

[c] NA, not applicable.

precursor protein (Fig. 4, lane C). Heat-denatured chloroplast protein extract was inactive (Fig. 4, lane H).

We next addressed the question of whether the appearance of the pPorA-degrading protease activity reflected a general developmental regulation of the proteolytic machinery of the plastids by light. Two different approaches were chosen to answer this question. First, the autolytic degradation of stromal polypeptides was measured with the Coomassie brilliant blue-binding technique described by Krauspe and Scheer (23). According to this method, the proteolytic activity can be measured by monitoring the autolytic loss of proteins in a given sample. Proteins present in the stromal protein extract were incubated with the Coomassie brilliant blue G-250 dye in the absence of additional proteases. As shown in Table 1, the autolytic activities were similar in both etioplasts and chloroplasts and also did not change in the course of chloroplast development.

Second, azocasein was used as an artificial substrate to determine the overall protease activity in the various stromal protein extracts. As shown in Table 1, plastid stroma prepared from dark- and light-grown seedlings contained similar proteolytic activities. Together, these results strongly suggested that the appearance of the pPorA-specific protease in light did not reflect a general developmental regulation of the proteolytic machinery of the plastids.

The pPorA-degrading protease activity is likely nucleus encoded. We next attempted to determine the site of synthesis of the pPorA-degrading plastid protease. Inhibitor experiments were performed with cycloheximide and chloramphenicol, which specifically block protein synthesis at 80S and 70S ribosomes, respectively (38, 40). Leaves were cut from dark-grown barley seedlings and placed into aqueous solutions containing either of the inhibitors. After a 2-h light incubation, the plastids were isolated from the leaf tissues and their protease activities were determined as described previously. As shown in Fig. 5A, cycloheximide but not chloramphenicol inhibited the light-induced appearance of the pPorA-degrading plastid protease activity. To assure that these differential effects were not caused by differences in the rates of uptake of the inhibitors, the labeling of the plastid-encoded large subunit of RUBISCO was determined. Leaf proteins were labeled with [^{35}S]methionine during incubation with the inhibitors, and plastid protein extracts were subjected to immunoprecipitation (42). As shown in Fig. 5B, chloramphenicol but not cycloheximide strongly depressed the labeling of the large subunit of RUBISCO, compared with that of leaf tissue not treated with either inhibitor.

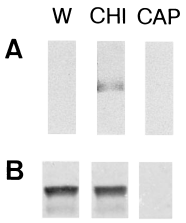

FIG. 5. Attempts to determine the site of synthesis of the pPorA-degrading protease. Leaves were cut from dark-grown barley seedlings and placed into water (lane W) or aqueous solutions containing either cycloheximide (lane CHI) or chloramphenicol (lane CAP) and incubated for 2 h in the light. During the incubation, parts of the leaf samples were labeled with [^{35}S]methionine, whereas other parts were left untreated. Stromal proteins were extracted from the various plastid samples and tested for either the pPorA-degrading protease activity (A) or the presence of the [^{35}S]methionine-labeled large subunit of RUBISCO (B).

Partial characterization of the pPorA-degrading protease. Stromal proteins were recovered from lysed chloroplasts by precipitation with ammonium sulfate (see Materials and Methods). After dialysis, stromal proteins were subjected to gel filtration on Sephadex G-25. As demonstrated in Fig. 6A, the activity of the pPorA-specific plastid protease was completely lost during these few steps of purification. Because either the protein itself or a cofactor specifically required for protease activity might have been removed during this procedure, we attempted to reconstitute the protease activity by adding different monovalent and divalent metal ions together with ATP to the assays. As shown in Fig. 6B, divalent cations, such as Mg^{2+} or Mn^{2+}, restored the protease activity, but neither monovalent ions, such as Na^+ or K^+, nor the divalent Ca^{2+} did so. The pH optimum of the protease activity was found to be around 6.5 (Fig. 6C), which was very close to the pH value used

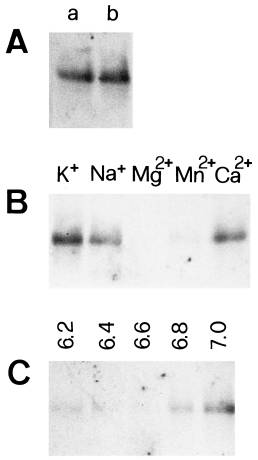

FIG. 6. Partial characterization of the pPorA-degrading protease. Radioactively labeled pPorA-chlorophyllide complexes were prepared as described in the legend to Fig. 2 and added to chloroplast stromal protein extract that had been subjected to gel filtration on Sephadex G-25 (A). Lanes a and b show the levels of pPorA before and after a 15-min dark incubation with the gel-filtered chloroplast protein extract. (B) Attempts to reconstitute the activity of the gel-filtered pPorA-degrading protease by adding monovalent and divalent metal ions to assays containing ATP (at a 0.5-mM final concentration). (C) pH optimum of the Mg^{2+}-dependent activation of the pPorA-degrading protease. Numbers indicate the final pH values of the incubation mixtures. Further details are as given in the legend to Fig. 1.

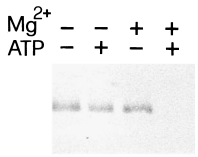

FIG. 7. Energy dependence of the pPorA-degrading protease. Gel-filtered pPorA-degrading protease, prepared as described in the legend to Fig. 6, was added to the pPorA-chlorophyllide complex and incubated with ATP (0.2 mM) and/or Mg^{2+} (0.4 mM) as indicated. The autoradiogram shows the levels of pPorA in each lane at the end of a 15-min dark incubation.

in the standard assay. EDTA reversed the effect of added Mg^{2+} (data not shown).

To address the question of whether the pPorA-degrading protease is energy dependent, the effects of ATP and GTP were determined. As shown in Fig. 7, neither compound could stimulate the pPorA-degrading protease, if tested without additional Mg^{2+} ions. ATP plus Mg^{2+}, however, restored the plastid protease to full activity (Fig. 7). Together with Mg^{2+}, GTP was less effective than ATP (data not shown).

The effects of proteinase inhibitors on the pPorA-degrading protease was investigated in subsequent experiments. As shown in Fig. 8, both antipain and pepstatin A inhibited the pPorA-degrading protease, suggesting that both cysteine- and aspartic acid-type proteinases were involved in degrading pPorA. In accordance with this interpretation, E-64 strongly reduced the activity of the pPorA-specific protease. In contrast, inhibitors of serine proteinases, such as leupeptin and phenylmethylsulfonyl fluoride (Fig. 8), as well as diisopropyl fluorophosphate (data not shown), did not inhibit the pPorA-degrading protease activity.

DISCUSSION

It is well established that intracellular protein degradation is an important and integral part of cell growth and differentiation (13, 35, 47). However, only a very few protease activities have been identified in plants thus far (31, 55). In particular, little is known about plastid-specific proteases. With chromogenic substrates, such as succinyl-$(ala)_3$-nitroaniline, several aminopeptidases could be purified from the plastids of pea (29) and *Chlamydomonas reinhardtii* (19). These plastid proteases differed in their pH optima, requirements for divalent cations, and/or sensitivities to proteinase inhibitors (19, 29). Their natural (native) substrates remained largely unknown, however. Previous findings suggested that thylakoid membrane proteins, such as the D1 protein of photosystem II (51), which has an extraordinarily high rate of turnover in light (26, 32), chlorophyll *a*- and *b*-binding proteins not incorporated into the photosynthetic membrane complexes (1), or protomers not assembled into their holocomplexes (10), might be substrates for the different proteinases. Obviously different from these proteinases are the signal peptidases, which specifically cleave off the N-terminal transit peptides of nuclear-encoded, post-translationally imported chloroplast proteins (39, 48).

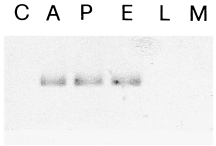

FIG. 8. Effects of proteinase inhibitors on the gel-filtered pPorA-degrading protease. pPorA-chlorophyllide complexes were incubated with the gel-filtered pPorA-degrading protease in incubation mixtures supplemented with ATP and Mg^{2+}, as described in the legend to Fig. 7. The autoradiogram shows the levels of pPorA remaining after a 15-min protease treatment in assays containing antipain (lane A), pepstatin (lane P), E-64 (lane E), leupeptin (lane L), or phenylmethylsulfonyl fluoride (lane M) (see Materials and Methods), in comparison with the protease inhibitor-free assay (lane C).

In this study, we have identified a plastid protease activity that specifically degrades pPorA in vitro and presumably also the mature PorA of barley in vivo. Using a homologous in vitro system, we demonstrated that proteolysis of pPorA is controlled at both the target protein and the proteinase levels. A likely catalytically induced change in protein conformation destines pPorA for degradation by a light-induced, nuclear-encoded, metal- and ATP-dependent plastid protease activity. Although pPorA is also susceptible to proteolysis in the absence of its substrates, product formation enhances the rate of its proteolytic degradation. The product of catalysis, chlorophyllide, remains tightly bound to the precursor protein (44, 45) and cannot be displaced by protochlorophyllide in either etioplast or chloroplast protein extracts. Although in mitochondria enzymes such as cytochrome *c*:heme lyase catalyze the covalent binding of tetrapyrrole pigments, such as heme, to the various cytochromes (37), no evidence has been obtained thus far that similar enzymes might also occur in plastids. The attachment of protochlorophyllide to pPorA seems to be a spontaneous process, whereas the release of the pigment is likely to be due to proteolytic degradation of the protein. By analogy to pPorA used in the in vitro protease assays described in this study, the mature PorA enzyme imported into δ-aminolevulinic acid-fed and thus protochlorophyllide-containing chloroplasts displays protease sensitivities in the dark different from those produced in the light. Upon import, a ternary PorA-protochlorophyllide-NADPH complex is likely to be produced, which is stable in the dark but is rapidly degraded when the organelles are shifted from the dark to the light (44). Presumably, the enzymatically produced chlorophyllide destabilizes the mature PorA enzyme in organello. On the basis of the coincidence between the results obtained in vitro and in organello, we propose that the degradation of PorA may also follow a similar route in vivo and in planta. However, final proof of this proposal is still lacking.

In contrast to chloroplasts, etioplasts do not contain the protease activity which degrades the PorA enzyme. By analogy to the PorA precursor protein used in this study, the post-translationally imported, mature enzyme is stable both in the dark and in the light in etioplasts (40a). The pPorA-degrading protease is induced by light, and its appearance requires protein synthesis de novo at cytoplasmic ribosomes. No evidence could be obtained that the pPorA-degrading protease is a plastid gene product. However, other chloroplast proteases may contain plastid-encoded constituents. At least the existence of one plastid-encoded protease activity has previously been proposed on the basis of the finding that a homolog of *clpP*, the *Escherichia coli* gene for the proteolytic activity of the ATP-dependent protease Clp (33), occurs in the plastid genomes of tobacco, *Marchantia polymorpha*, rice, and wheat (52).

The pPorA-degrading protease is active only in the presence of both ATP (or GTP) and Mg^{2+} and thus is similar to one of the previously purified protease activities from pea plastids (29). Despite this fact, the plastid proteases from barley and pea, as well as that from *C. reinhardtii*, differ in their localizations and sensitivities to proteinase inhibitors. While the barley enzyme could easily be extracted from the plastid stroma, as shown here, the enzymes from pea and *C. reinhardtii* were found to be associated with the thylakoid membranes (19, 29). The barley and pea (29) enzymes are both insensitive to leupeptin, in contrast to the algal protease (19). However, it is still

undetermined whether leupeptin inhibits specifically serine-type or cysteine-type proteinases (23, 24). The lack of inhibition of the pPorA-degrading protease by leupeptin, phenylmethylsulfonyl fluoride, and diisopropyl fluorophosphate, which are serine-type proteinase inhibitors (6, 11), and the strong coincidence between the inhibitory effects of antipain and E-64 appear to favor the explanation that the barley enzyme is a cysteine-type proteinase. In contrast, the plastid protease activity from *C. reinhardtii* was found to be insensitive to cysteine-type protease inhibitors, such as E-64 and antipain (19). As the barley protease activity could also be inhibited by pepstatin A, an aspartic acid-type proteinase inhibitor (46), it is likely that this enzyme is composed of several constituents. Attempts to further purify the pPorA-degrading protease have failed thus far, likely because of the high lability of the enzyme (data not shown).

The results of this study have two interesting implications. First, they provide further evidence that the light-induced reduction of protochlorophyllide to chlorophyllide catalyzed by PorA might be the cause of the rapid disintegration of the prolamellar body occurring in etioplasts upon illumination (7, 8). Although the appearance of the PorA-specific protease is delayed, compared with the rapid ultrastructural changes taking place during the breakdown of the prolamellar body, it is tempting to speculate that the degradation of PorA might be an essential step in the formation of the photosynthetic apparatus. Particularly interesting in this context is the observation that three low-molecular-mass fragments of pPorA could be detected in vitro. Although we do not yet know whether these fragments contain chlorophyllide and whether they are normally also produced in vivo and in planta, one may assume that such chlorophyllide-binding peptides might operate as vehicles for the transport and subsequent integration of freshly formed chlorophyllide into the developing thylakoid membranes. While similar proposals have already been made in previous studies (20, 49), no experimental approach has yet been available to address this important point.

Second, our results support the notion that the recently discovered protochlorophyllide-dependent transport pathway of pPorA into the plastids (45) might be a powerful mechanism to prevent the seemingly wasteful degradation of the enzyme in fully developed chlorolasts. By limiting its uptake, pPorA presumably escapes rapid proteolytic degradation in light.

Just the opposite mechanism seems to operate in the case of fructose-1,6-bisphosphatase from *Saccharomyces cerevisiae*. This enzyme of gluconeogenesis is transported to the vacuole and subjected to proteolytic degradation through glucose (5). Although these few known examples are remarkably different, they show that the metabolite-dependent transport of proteins into distinct subcellular compartments may be a powerful mechanism to regulate intracellular protein degradation.

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