Transport of Proteins into Chloroplasts

PARTIAL PURIFICATION OF A THYLAKOIDAL PROCESSING PEPTIDASE INVOLVED IN PLASTOCYANIN BIOGENESIS*

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Plastocyanin is synthesized in the cytoplasm as a larger precursor and transported across three membranes into the chloroplast thylakoid lumen. Processing to the mature size involves successive cleavages by a stromal and a thylakoidal peptidase. In this report we describe the partial purification and characterization of the thylakoidal peptidase involved. The enzyme has been purified 36-fold from Pisum sativum thylakoids after solubilization using Triton X-100. The peptidase processes the plastocyanin import intermediate to the mature size, but no further, and is capable of processing pre-plastocyanin to the mature size but at a lower rate. No detectable activity is displayed against non-chloroplast proteins or precursors of stromal proteins. The enzyme has a pH optimum of 6.5–7 and is activated by chelating agents such as EDTA and EGTA. No inhibitors of the peptidase have been found to date.

Chloroplast biogenesis involves the synthesis of proteins both in the organelle and in the cytoplasm. Many chloroplast proteins are encoded by nuclear genes, synthesized on cytoplasmic ribosomes, and transported into the chloroplast (1). Analysis of numerous imported proteins has shown that they are all synthesized initially as larger precursors containing amino-terminal pre-sequences. The precursors are thought to bind to receptors in the chloroplast envelope (2) before being transported into the chloroplast by an ATP-dependent, post-translational mechanism (3–5). Precursors of stromal proteins are processed to the mature size by a stromal peptidase during or shortly after transport across the two envelope membranes (6). This peptidase has been shown to be highly specific for chloroplast protein precursors (6, 7).

It is believed that the pre-sequences of imported chloroplast proteins carry some, if not all, of the information specifying transport into the organelle and subsequent suborganellar localization. For example, gene fusion experiments have shown that the pre-sequence of an imported stromal protein can direct a "foreign" protein into the stroma (8, 9). The nature of the targeting information is, however, poorly understood, since the pre-sequences of imported stromal proteins show little homology at the primary sequence level.

The biogenesis of cytoplasmically synthesized thylakoid lumen proteins is more complex than that of stromal proteins, since three membranes lie between the thylakoid lumen and the cytoplasm. One such protein is plastocyanin, a small, soluble photosynthetic electron carrier which is initially synthesized in precursor form on cytoplasmic ribosomes. The import of pre-plastocyanin can be divided into two stages. Initially, pre-plastocyanin is transported into the stroma and processed to an intermediate form, probably by the stromal peptidase involved in the maturation of imported stromal proteins. The import intermediate is subsequently transported into the thylakoid lumen and processed to the mature size by a second, thylakoidal peptidase (10, 11). The plastocyanin pre-sequence is thus apparently divided into two distinct domains, functioning in "envelope transfer" and "thylakoid transfer," respectively. Current evidence suggests that the first domain is entirely analogous to the pre-sequences of imported stromal proteins. Deletion of the thylakoid transfer domain does not affect transport of the mutant precursor into the stroma or cleavage by the thylakoidal peptidase, but blocks transport into the thylakoid lumen.1 Clearly, the envelope transfer domain promotes binding to import receptors, transport into the stroma, and processing by the stromal peptidase. In contrast, relatively little is known about the functions of the thylakoid transfer domain. It seems likely that this domain contains signals specifying transport into the thylakoid lumen and processing to the mature size. To date, however, very few details of these events have been reported. In particular, the energy dependence of the thylakoid protein transfer step and the roles (if any) played by receptor/transport proteins in the membrane have yet to be determined. Similarly, the role of the thylakoidal processing event and the mechanism by which it takes place, are presently unclear.

In order to obtain more precise information on the later stages of plastocyanin biogenesis, we have analyzed the thylakoidal processing event in greater detail. This report describes the partial purification and characterization of the peptidase involved.

EXPERIMENTAL PROCEDURES

Materials

Pean seedlings (Pisum sativum var. Feltham First) were grown under a 12-h photoperiod for 10 days as described (12). Most of the chemicals used were from Sigma. [35S]Methionine (specific activity > 800 Ci/mmol) and Amplify were obtained from Amersham International (United Kingdom). DEAE-Sephacel was from Pharmacia Biotechnology, Inc. and hydroxylapatite Bio-Gel HTP was from Bio-Rad. Sten clein protoplast cDNA encoding pre-plastocyanin was kindly provided by Dr. P. Weisbeek (Utrecht).

Purification of the Peptidase

The thylakoidal processing peptidase was prepared from pea chloroplasts as detailed below. All steps were carried out at 4°C.

Step 1. Thylakoid Preparation—Washed chloroplasts were prepared from 900 g (fresh weight) of pea leaves as described (12). The

1 P. Weisbeek, personal communication.
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final pellet was lysed with 100 ml of 1 mM Tricine2-NaOH, pH 7.0, 5 mM MgCl2, and left for 5 min. The thylakoid membranes were pelleted by centrifugation at 5000 × g for 5 min and washed twice with 160 ml of 10 mM Tricine-NaOH, pH 7.0, 300 mM sucrose, 5 mM MgCl2. This procedure removes the vast majority of ribulose bisphosphate carboxylase, the most abundant stromal protein.

Step 2. Triton X-100 Extraction—The washed thylakoid pellet was resuspended in 50 mM Tricine-NaOH, pH 7.0, 15 mM NaCl, 5 mM MgCl2 at a final concentration of 1 mg/ml chlorophyll. Triton X-100 (25%) was added slowly with stirring to give a ratio of 2.5 mg of Triton/mg of chlorophyll. The mixture was stirred in the dark for 30 min and then centrifuged at 30,000 × g for 30 min.

Step 3. Hydroxyapatite Chromatography—The supernatant from step 2 was loaded onto a column (20 × 2.5 cm) of hydroxyapatite (Bio-Gel HTP) pre-equilibrated in 20 mM Tris-HCl, pH 7.0, 0.15% (v/v) Triton X-100 (buffer A). The column was washed with 50 ml of the same buffer and eluted with a 200-ml linear gradient of buffer A/50 mM K2PO4. Fractions of 5 ml were collected.

Step 4. DEAE-Sephacel Chromatography—Pooled fractions from step 3 were loaded onto a column (10 × 1.5 cm) of DEAE-Sephacel pre-equilibrated in buffer A. The column was washed with 50 ml of buffer A and eluted with a 60-ml linear gradient of buffer A/100 mM NaCl. Fractions of 2 ml were collected.

Preparation of Pre-plastocyanin Processing Intermediate

An artificial pre-plastocyanin processing intermediate (PCI) was synthesized from transcripts of Silene pratensis pre-plastocyanin cDNA in which the initiation codon has been deleted (11). The transcripts were prepared by SP6 RNA polymerase transcription of the cDNA as described (11). Translation of the transcripts in a wheat germ system in the presence of [35S]methionine results in the synthesis of an artificial processing intermediate which is marginally larger than the true intermediate (11). After incubation, the wheat germ extract containing the translation product was diluted 20-fold with 20 mM Tris-HCl, pH 7.0, and stored at −80°C.

Assay of the Thylakoidal Processing Peptidase

Thylakoid extracts were assayed for processing of PCI by incubation with the artificial intermediate prepared as above. Incubation mixtures contained 2 μl of diluted translation product, 18 μl of thylakoid extract, and EDTA added to a final concentration of 10 mM. After incubation for 90 min at 27°C, the reaction was stopped by the addition of 1 volume of sample buffer (125 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 5% (w/v) sucrose, 5% (v/v) 2-mercaptoethanol) followed by boiling for 2 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (13) followed by fluorography using Amplify. When necessary, processing of plastocyanin processing intermediate was monitored by excision of the labeled bands from the dried gel and measurement of radioactivity (14). One unit of activity is defined as the amount of enzyme required to produce mature-sized plastocyanin containing 20% of the radioactivity initially present in the processing intermediate. Mature-sized Silene pratensis plastocyanin was prepared by incubation of pre-plastocyanin with isolated chloroplasts as described (11).

Other Methods

Published methods were used for the determination of protein (15), for silver staining polyacrylamide gels (16), for the determination of chlorophyll (17), and for partial purification of the stromal processing peptidase (6).

RESULTS

Extraction of the Thylakoidal Processing Peptidase from Pea Chloroplasts—Plastocyanin is transported into the thylakoid lumen by a 2-step import mechanism which involves the generation of a stromal processing intermediate. Maturation is completed within the thylakoids by a second peptidase which recognizes the intermediate form of plastocyanin (11). This processing activity is released by Triton X-100 treatment of washed, stacked thylakoid vesicles and remains in the supernatant when the extract is centrifuged at 30,000 × g (Fig. 1). This centrifugation step pellets unsolubilized and partially solubilized stacked vesicles which are enriched in photosystem II. Consequently, essentially all of the light-harvesting chlorophyll binding protein (LHCP), the major membrane protein of photosystem II, is removed at this stage. Most, but not all, of the processing activity is released by this method, and it is possible to release some of the remaining activity by re-extraction of the pellet vesicles.

Partial Purification of the Thylakoidal Peptidase—The peptidase was partially purified from pea thylakoids by a protocol detailed under "Experimental Procedures." The peptidase was assayed using PCI, which is slightly larger than the true intermediate (described in Ref. 11). The most highly purified preparations of peptidase process this intermediate to the mature size, apparently in a single step (Fig. 2). The markers for mature and intermediate forms of plastocyanin were generated by incubation of pre-plastocyanin with isolated pea chloroplasts (the intermediate form is more apparent in Fig. 5, lane M). Fractionation of the chloroplasts after incubation has shown that the intermediate form is located in the stroma (10). All of the purification steps were carried out in the presence of Triton X-100 because the peptidase is irreversibly inactivated in the absence of nonionic detergents (not shown).

The elution of the peptidase activity during ion-exchange chromatography and hydroxylapatite chromatography is displayed in Fig. 3. The peptidase activity elutes as a single peak from each of these procedures. Details of the purification factors, and the polypeptides present after each step, are given

2 The abbreviations used are: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PCI, artificial processing intermediate of plastocyanin; SDS, sodium dodecyl sulfate; EGTA, [ethylenbis(oxyethylenenitrilo)]tetraacetic acid.

Fig. 1. Extraction of PCI processing activity from pea thylakoids. Pea thylakoids were incubated in the presence of Triton X-100 and centrifuged at 30,000 × g as described under "Experimental Procedures." The resulting pellet was resuspended in the original volume of extraction buffer containing Triton A, Coomassie-stained SDS-polyacrylamide gel of total thylakoid protein (lane 1), supernatant (lane 2), and resuspended pellet (lane 3). Lane M, molecular weight markers. B, assay for processing of PCI by the Triton extract before centrifugation (lane 2). PCI was separated (lane 3) and resuspended pellet (lane 4). Lane 1, PCI translation product. Marker tracks of mature-size PC are shown in Figs. 2, 5, and 7. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography. LHCP, light-harvesting chlorophyll binding proteins.
FIG. 2. Processing of PCi by the partially purified thylakoidal peptidase. Time course analysis of processing is shown. 100 μl of partially purified peptidase and 10 μl of PCi were incubated at 27 °C. At the times indicated above the lanes, 10-μl aliquots were removed, mixed with sample buffer, and boiled for 2 min. Lane M, authentic intermediate (I) and mature (M) plastocyanin produced by incubation of pre-plastocyanin with isolated pea chloroplasts. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

in Table I and Fig. 4, respectively. The purification protocol results in a 36-fold purification of the peptidase with a 7% recovery of activity. 10–15 bands are visible on silver-stained gels of the most highly purified preparations of peptidase; we have not yet been able to identify a band representing the peptidase.

Characteristics of the Processing Peptidase—Several types of protease inhibitor have been tested for their effect on the thylakoidal peptidase. Fig. 5 shows that leupeptin, an inhibitor of some vacuole proteases, has no effect on the peptidase (lane 3). Similarly, the serine protease inhibitor, phenylmethylsulfonyl fluoride, does not inhibit the peptidase (lane 4). A third category of potential inhibitor, chelating agents, have emerged instead as activators of the peptidase. EDTA and EGTA, inhibitors of metalloproteases containing catalytically essential Zn<sup>2+</sup> and Ca<sup>2+</sup> ions, respectively, both stimulate processing activity markedly (lanes 5 and 6). The basis for this stimulation is presently unclear. One possibility is that these compounds are chelating free divalent ions which otherwise inhibit the peptidase. However, no such ions should be present after several purification steps, and no change in enzyme activity is observed if the peptidase is dialyzed against a buffer containing only Tris-HCl, pH 7.0, and Triton X-100 (not shown).

The thiol protease inhibitor, iodoacetic acid, has no effect on the processing of PCi (lanes 7 and 8). We have also tested pepstatin and bestatin (microbial protease inhibitors), N-ethylmaleimide and iodoacetamide (thiol protease inhibitors), and benzamidine hydrochloride, an inhibitor of trypsin. None of the compounds tested affects processing of PCi, and we cannot therefore assign the thylakoidal peptidase to a particular class of protease.

The effect of pH on the processing of PCi has been investigated. The enzyme is active over a broad pH range, with optimal processing of PCi occurring at pH 6.5–7 (not shown).

Specificity of the Peptidase—The data shown in Fig. 2 demonstrate that the partially purified peptidase is capable of cleaving the plastocyanin processing intermediate to the mature size, but that no further cleavages are observed even under conditions of enzyme excess. Almost certainly, cleavage is taking place at the amino terminus, since both the authentic and artificial processing intermediates are processed to the mature size by the crude thylakoid extract (11). The two substrates have clearly different mobilities on SDS-polyacrylamide gels, and cleavage at a site near the carboxyl terminus would therefore yield correspondingly different products. These results suggest that the isolated enzyme is a processing peptidase involved in the maturation of plastocyanin (and perhaps other imported proteins) rather than a protease functioning in chloroplast protein turnover. To further test this
massie-stained SDS-polyacrylamide gel showing samples of thylako-
lone staining. I). Idal processing activity at different stages of purification (see Table 1). Lane 2, total thylakoid protein; lane 3, 30,000 X g supernatant: lane 4, hydroxylapatite eluate; lane 5, DEAE-Sephacel eluate. Lanes 1 and 6, molecular weight markers. Lane 7, as lane 5 but silver-stained.

FIG. 4. Purity of the thylakoidal processing activity. Coomassie-stained SDS-polyacrylamide gel showing samples of thylako-
idal processing activity at different stages of purification (see Table 1). Lane 2, total thylakoid protein; lane 3, 30,000 X g supernatant: lane 4, hydroxylapatite eluate; lane 5, DEAE-Sephacel eluate. Lanes 1 and 6, molecular weight markers. Lane 7, as lane 5 but silver-stained.

hypothosis, the peptidase was incubated with a variety of polypeptide substrates from diverse sources, all of which were synthesized in the wheat germ system. Fig. 6 shows that none of these proteins are degraded by the thylakoidal peptidase, further emphasizing the specificity of the reaction catalyzed by this enzyme. Since precursors of stromal proteins were among the substrates tested, we conclude that the reaction mechanism of the thylakoid peptidase is also radically different to that of the stromal processing peptidase.

Several observations suggest that the thylakoidal peptidase encounters only the plastocyanin processing intermediate, and not pre-plastocyanin, in the chloroplast. During in vitro import experiments, the processing intermediate, but not pre-plastocyanin, is transiently apparent in the stromal phase (10). Furthermore, the isolated stromal processing enzyme efficiently processes pre-plastocyanin to the intermediate size but no further (11). Clearly, pre-plastocyanin is very rapidly processed to the intermediate form upon entry into the stroma. The thylakoidal peptidase is, however, capable of processing pre-plastocyanin to the mature size (Fig. 7) indicating that removal of the envelope transfer domain by the stromal peptidase is not a prerequisite for cleavage by the thylakoidal enzyme. Thus, we cannot formally rule out the possibility that some pre-plastocyanin is transported into the thylakoids without prior cleavage by the stromal peptidase.

Although the thylakoidal enzyme recognizes pre-plastocyanin, processing takes place 5-10-fold more slowly than cleavage of PCI. It is probably for this reason that processing of pre-plastocyanin was not observed in an earlier study using crude thylakoid extracts containing rather low levels of processing activity (11).

The thylakoidal enzyme appears to process pre-plastocyanin to the mature size in a single step and does not produce the intermediate form which is generated by the stromal peptidase. This further emphasizes the differing reaction specificities of the two enzymes. It is apparent from Fig. 7 that incubation of pre-plastocyanin with the thylakoidal peptidase leads to the production of a low molecular weight

FIG. 5. Effects of protease inhibitors on the thylakoidal peptidase. Lane 1, PCI translation; lane 2, processing incubation with no additions and in the absence of EDTA; lane 3, +100 µg/ml leupeptin; lane 4, +2 mM phenylmethylsulfonyl fluoride; lane 5, +10 mM EDTA; lane 6, +10 mM EGTA; lane 7, the peptidase was incubated with 1 mM iodoacetate for 60 min at 4 °C before mixing with PCI; lane 8, as lane 7 but with 10 mM iodoacetate; lane 9, as lane 2. Lanes M, import marker as in Fig. 2. Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

FIG. 6. Reaction specificity of the thylakoidal peptidase. The thylakoidal peptidase was tested for activity against PCI (incubation 1), Brassica napus acyl carrier protein (incubation 2), precursor of pea ribulose bisphosphate carboxylase small subunit, an imported stromal protein (incubation 3), yeast prepro-α-factor (incubation 4), and prepropratin (incubation 5). In each case, equal aliquots of in vitro translation mixture were incubated with buffer A (lanes B) or peptidase (lanes P). Samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by fluorography.

FIG. 7. Processing of pre-plastocyanin by the thylakoidal peptidase. A, pre-plastocyanin (pre-PC) was synthesized as described for PCI and incubated with buffer A (lane 1) or thylakoidal peptidase (lane 2). PCI was incubated for the same period with buffer A (lane 3) or thylakoidal peptidase (lane 4). B, 10-20% gradient SDS-polyacrylamide gel showing pre-plastocyanin (lane 1) and pre-plastocyanin after incubation with stromal (lane 2) or thylakoidal (lane 3) processing peptidase. Lanes M, as in Fig. 2. Pep. denotes small polypeptide, possibly the pre-sequence of pre-plastocyanin, generated by the thylakoidal peptidase. PC, plastocyanin.
polypeptide; this may well be the cleaved pre-sequence (molecular weight 6,500).

**DISCUSSION**

The primary objective of the experiments described in this report was to analyze the mechanism by which the plastocyanin import intermediate is processed to the mature size in the thylakoid vesicles. In particular, we have sought to determine whether cleavage is carried out by a processing peptidase specific for imported precursors or processing intermediates. Thylakoidal protease activities involved in protein degradation have been reported by several groups (18-21) and our initial aim was therefore to purify the peptidase to a high degree in order to minimize contamination by these activities. The procedure employed results in a 36-fold purification and is successful in removing the vast majority of thylakoid proteins. The partially purified peptidase cleaves PCI to yield mature-size plastocyanin but no further proteolytic products and displays very little activity against a variety of non-chloroplast proteins. We conclude that the enzyme is a processing peptidase with restricted reaction specificity. The basis for this reaction specificity is currently unknown, primarily because sequence data is available for only two thylakoid polypeptides; this may well be the cleaved pre-sequence (molecular weight 6,500).

The thylakoidal peptidase appears to have little in common with the stromal processing peptidase, other than the specific nature of the reactions carried out by both enzymes. The pH optimum of the thylakoidal enzyme (6.5-7) is considerably lower than that of the stromal enzyme (8.5-9) (6). EDTA is a good inhibitor of the stromal peptidase but markedly stimulates processing by the thylakoidal enzyme. Furthermore, the detergent Triton X-100 also inhibits the stromal enzyme but is essential for the activity of the isolated thylakoidal peptidase.

The exact location of the thylakoidal peptidase remains to be determined. Recent evidence\(^5\) indicates that the enzyme is tightly bound to the thylakoid membranes rather than soluble in the thylakoid lumen. Intact thylakoid vesicles do not process PCI (11), suggesting that the active site of the peptidase is not located on the stromal face of the thylakoid membrane. These findings suggest that processing of plastocyanin to the mature size occurs during or after transport into the thylakoid lumen, rather than upon binding to the thylakoids. Further investigation is clearly required to resolve the sequence of events taking place during protein transport into the thylakoid lumen.

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