Plant Physiol. (1994) 105: 1301–1311

Import, Targeting, and Processing of a Plant Polyphenol Oxidase[1]

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A tomato (*Lycopersicon esculentum* L.) gene encoding a precursor of polyphenol oxidase (PPO) was transcribed and translated in vitro. The import, targeting, and processing of the [^{35}S]methionine-labeled precursor protein (pPPO) were studied in isolated chloroplasts. The protein was routed to the thylakoid lumen in two steps. The 67-kD precursor was first imported into the stroma in an ATP-dependent step. It was processed to a 62-kD intermediate by a stromal peptidase. Translocation into the lumen was light dependent and involved processing of the 62-kD to the 59-kD mature form. The mature polypeptide was soluble in the lumen and not bound to thylakoids. This two-step targeting pattern was observed in plastids from a variety of plants including pea (*Pisum sativum* L.), tomato, and maize (*Zea mays* L.). The ratio between the intermediate and mature forms observed depended on the plant species, leaf age, growth conditions, and illumination regime to which the plants had been subjected. Cu^{2+} was not required for pPPO import or processing. Furthermore, low concentrations of Cu^{2+} (1–5 μM) markedly inhibited the first import step. Tentoxin specifically inhibited pPPO import, leaving the precursor bound to the envelope membrane. The two-step routing of pPPO into chloroplasts, typical of thylakoid lumen proteins, is consistent with the two-domain structure of the transit peptide and appears to be a feature of all plant PPO genes isolated so far. No evidence was found for unorthodox routing mechanisms, which have been suggested to be involved in the import of plant PPOs. The two-step routing may account for some of the multiplicity of PPO observed in vivo.

PPOs (diphenol:O_2 oxidoreductase, EC 1.10.3.2) are Cu enzymes of an almost ubiquitous distribution in plants. The physiological function of the enzyme in plants is still obscure after a century of intensive research and in spite of the economical importance of PPO (Mayer and Harel, 1979, 1991; Mayer, 1987; Vaughn et al., 1988). Plant PPOs are nuclear-coded proteins (Lax et al., 1984; Kowalski et al., 1990). They are located in plastids, although reports of their occurrence in other cell compartments are quite abundant (Mayer and Harel, 1979; Mayer, 1987). PPO is often described as tightly bound to thylakoids but has also been observed in plastid envelopes or the thylakoid lumen (Mayer, 1987; Vaughn et al., 1988; Kowalski et al., 1992).

There is uncertainty and confusion regarding the size of the native enzyme and the mechanism of its import into plastids. The active form of PPO was frequently reported to be about 45 kD (Vaughan et al., 1975; Lanker et al., 1987), but other forms, e.g. 59 and 65 to 68 kD were also reported (Steffens et al., 1990; Ganesa et al., 1992; Shahar et al., 1992). PPOs appear to be encoded by gene families (Newman et al., 1993). The apparent variability in location as well as heterogeneity in size may result from differences in targeting, processing, and possibly modifications of the products of various genes. Although some of the multiplicity of PPO may have a genetic basis, it is affected by additional factors. Secondary reactions with the products of PPO action can produce denatured, modified, and cross-linked enzyme protein, which could be subjected in turn to proteolytic degradation. Nonspecific degradation has been shown to produce artifacts in the size of isolated PPO (Harel et al., 1973; Robinson and Dry, 1992). In addition, PPOs may exist in latent forms that are activated by proteolytic cleavage (Tolbert, 1973; Mayer and Harel, 1979; Mayer, 1987). Differences in the apparent size and location of PPO could thus result from reaction with the oxidation products of phenolic substrates or from proteolytic cleavage of the enzyme.

Immunoprecipitation of poly(A^+) RNA translation products with antibodies against *Vicia faba* PPO suggested that the protein synthesized in the cytoplasm is identical in size with that observed in chloroplasts (Flurkey, 1985, 1986). However, the absence of a cleavable transit peptide in a plastid protein is known only for a 6.7-kD polypeptide of the plastid envelope whose import does not require ATP hydrolysis (Salomon et al., 1990). Tentoxin induced loss of plastidic PPO (Vaughn and Duke, 1981). Observations using immunoelectron microscopy suggested that the toxin prevented processing of PPO into an active enzyme, leaving it bound to the plastid envelope (Vaughn and Duke, 1984). The apparent lack of a transit peptide and the observations of the effect of tentoxin prompted suggestions that the translocation pathway for PPO differs from that of other plastid proteins and involves

[1] This research was supported by grant 292–89 from the Basic Research Foundation administered by the Israel Academy of Sciences and Humanities to E.H. and grant US 1870–90 from The United States-Israel Binational Agricultural Research and Development Fund to J.C.S., E.H., and A.M.M.

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Abbreviations: DCPIP, 2,6-dichlorophenol indophenol; LDC, light/dark cycle of 24 h; LHCP, light-harvesting Chl *a/b* protein II; PMS, phenazine methosulfate; pPC (PC), precursor (mature) form of plastocyanin; pPPO (PPO), precursor (mature) form of polyphenol oxidase; RB, resuspension buffer containing 0.33 M sorbitol, 0.1 M Hepes-KOH (pH 7.9), and 5 mM $MgCl_2$.

vesicles that originate in the inner envelope membrane (Vaughn et al., 1988). However, all plant PPO genes isolated so far encode about 67-kD polypeptides. Furthermore, all of these polypeptides possess amino-terminal extensions with properties typical of nuclear-coded plastid proteins (Steffens et al., 1990; Cary et al., 1992; Shahar et al., 1992; Hunt et al., 1993; Newman et al., 1993). In vitro translation of potato trichome poly(A^+) RNA produces a 67-kD polypeptide, whereas the native enzyme is 59 kD (Steffens et al., 1990; Kowalski et al., 1992; Hunt et al., 1993).

The recent isolation of the entire gene family of tomato PPO (Newman et al., 1993) presents a good opportunity for studying the import, targeting, and processing of PPO in higher plants. The apparent contradictions among reports of the mechanism of transport, location, and properties of plant PPOs could thus be experimentally resolved. Such studies may also provide insight into the function(s) of the enzyme in higher plant tissues.

We report on the import, targeting, and processing of a tomato pPPO by isolated plastids. We show that the 67-kD in vitro transcribed and translated precursor is directed to the thylakoid lumen in two steps, giving rise to a 59-kD mature form. The energy requirement and the effect of Cu^{2+} and tentoxin on the import of PPO are described.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Pea (*Pisum sativum* L. cv Alaska) plants were grown in vermiculite for 6 d under LDCs of 16/8 h and 24/18°C, 85% RH. The light source (100 μmol m^{-2} s^{-1}) was a combination of fluorescent (F96T 12-cw-HO; Sylvania, Danvers, MA) and incandescent lamps. Plants were transferred to darkness for 40 h at the middle of the light period on d 7 and illuminated again for 1 h before harvesting. Etiolated maize seedlings (*Zea mays* L. cv Neve Ya'ar 170) were grown in darkness at 26°C for 11 d and greened by illuminating at 30 μmol m^{-2} s^{-1} with white fluorescent light for 8 h. Tomato (*Lycopersicon esculentum* L., cv VFNT Cherry) seedlings were grown in vermiculite for 20 d as described for pea. Plants were watered with half-strength Hoagland No. 2 basal salt mixture (Sigma) on d 7 (pea, maize) or d 14 (tomato).

In Vitro Transcription Plasmids

Hy-19, a full-length PPO cDNA from tomato stem chlorenchyma (Yu, 1992), which is identical with the open reading frame of the *ppo*B gene described by Newman et al. (1993), was subcloned in pGEM-7Z f(+) after ligation at the *Bam*HI/*Apa*I sites of the vector. The *cab*30 gene from *Lemna gibba* L. encoding LHCPII was cloned in pSP65 as previously described (Kohorn et al., 1986). The gene encoding the precursor of PC was cloned in the pSP64-derived transcription plasmid pSPPC74 (Smeekens et al., 1986).

In Vitro Transcription and Translation

Cesium chloride-purified plasmid DNAs were linearized 3′ to the transcription template with *Apa*I, *Hin*dIII, or *Eco*RI for *ppo*B, *cab*30, and PC, respectively. Linearized DNA was purified by phenol extraction and ethanol precipitation and transcribed with SP6 RNA polymerase using the RiboMax RNA production system (Promega) according to the instructions of the manufacturer. The 1-mL reaction mixtures containing 30 to 100 μg of linearized DNA were incubated for 4 h at room temperature. The DNA template was removed by digestion with RQI RNase-free DNase (Promega) in the presence of RNAsin (48 units), and the RNA was extracted with phenol and precipitated overnight with an equal volume of 7.5 M NH_4 acetate and 2 volumes of ethanol at −20°C. The precipitate was washed with 70% (v/v) ethanol, resuspended in 80 mM Hepes-KOH (pH 7.5), and precipitated again with ethanol. The RNA was dissolved in dimethyl pyrocarbonate-treated H_2O and stored at −70°C. Although capping with diguanosine triphosphate [G(5′)ppp(5′)G] was practiced at first, it proved unnecessary when using the RiboMax system.

The LHCPII and PC RNAs were translated in a wheat germ system as previously described (Chitnis et al., 1986; Kohorn et al., 1986) except that 30 mM K acetate was added to the translation mixture. PPO B was translated using a rabbit reticulocyte lysate kit (Stratagene) according to the instructions of the manufacturer. Usually 100,000 to 200,000 cpm of ^{35}S-labeled protein precursors were obtained per μL of translation mixture containing 20 to 40 ng of RNA in the wheat germ system and 180,000 to 400,000 cpm with the reticulocyte lysate.

Isolation of Chloroplasts and Suborganellar Fractions

Harvested leaves were cooled on ice and ground in a razor blade blender (Chitnis et al., 1986) as previously described (Yalovsky et al., 1992). Chloroplasts were isolated as described by Yalovsky et al. (1992) and suspended in a small volume of resuspension buffer. The plastid suspension (5–7 mg of Chl) was layered over a three-step gradient of Percoll (32, 50, and 70% [w/v], 6 mL each) in 0.3% (w/v) Ficoll 400,000, 0.9% (w/v) PEG 4000, 0.3% (w/v) BSA, 0.3 M sorbitol, 1 mM $MgCl_2$, 5 mM EDTA, and 0.1 M Tricine-KOH (pH 7.6) in 30-mL glass centrifuge tubes. The gradients were centrifuged at 15,000*g* in a HB-4 rotor (Sorvall) for 10 min. The lower green band containing intact plastids (25–35% of the total amount of Chl) was collected and diluted in a large volume of RB. The plastids were pelleted by accelerating the SS-34 rotor (Sorvall) to 1900*g* and immediately applying the brake and resuspended in fresh RB. The pea plastids used in most experiments, except when the effect of growth conditions and developmental stage on PPO import were studied, contained 0.55 to 0.60 pg Chl and 7.5 to 8.5 pg protein per plastid. Gradients were modified when etioplasts were isolated (maize) or younger leaves were analyzed (pea, maize). Gradients of 25, 40, and 60% (w/v) Percoll or 15 to 65% continuous Percoll gradients were used in these cases. Envelopes were isolated as described by Yalovsky et al. (1992). The stromal fraction was obtained by passing a suspension of intact plastids in RB four times through an 18-gauge hypodermic needle, followed by centrifuging at 10,000*g* for 2 min. The supernatant obtained by an additional centrifugation at 250,000*g* for 15 min (TLA rotor, Beckman TL100 microfuge) was regarded as the stroma fraction. Thylakoids were isolated following import by intact plastids as previously

described (Chitnis et al., 1986) or collected from the band of broken plastids in Percoll gradients, washed, and resuspended in RB when used in organelle-free assays. Lumen proteins were obtained by breaking RB-washed thylakoids (2 mg Chl mL^{-1}) in a Yeda press (100 kg cm^{-2}) and keeping the supernatant of a 15-min, 250,000*g* centrifugation.

Import Assays

Import assays were performed essentially as described previously (Chitnis et al., 1986). All of the experiments were repeated at least two to three times, and the results shown are representative of the observations obtained. The suspensions of intact plastids were divided equally into 15-mL glass centrifuge tubes using 30 to 60 μg Chl per tube, depending on the experiment. When plastids with different Chl contents were compared, the reactions contained an equal amount of protein or number of plastids. The reaction mixture contained 0.1 M Hepes-KOH (pH 7.6), 0.33 M sorbitol, 2.5 mM MgATP, 1 mM EDTA, 8 mM L-Met and 200,000 to 400,000 cpm of L-[^{35}S]Met-labeled polypeptide in a total volume of 300 μL. Import assays involving pLHCPII contained, in addition, 1 mM *S*-adenosyl Met and 1 mM δ-aminolevulinic acid. Plastids were incubated for 30 min (unless otherwise stated) at 25°C and 30 μmol m^{-2} s^{-1} incandescent light while the tubes were gently shaken in a close-to-horizontal position. The reaction was terminated, plastids were treated with thermolysin, intact organelles were reisolated, and subchloroplast fractions were obtained as previously described (Chitnis et al., 1986). The intact plastids recovered after thermolysin treatment were suspended in 50 mM Tricine-KOH (pH 7.9), 5 mM EDTA in an Eppendorf tube, and the suspension was centrifuged for 10 min at 14,000 rpm. The precipitate, consisting mainly of thylakoids, was washed again with Tricine-EDTA, suspended in denaturing buffer, heated for 30 min at 55°C (Chitnis et al., 1986), and centrifuged for 1 min before loading on gels. The stromal proteins were precipitated by 10% TCA, and the precipitate was washed with 80% acetone and solubilized for SDS-PAGE.

Immunoblotting

Electrophoretic transfer of proteins to nitrocellulose membranes following SDS-PAGE and immunodetection were performed according to the method of Ausubel et al. (1987) and the modifications described by Hunt et al. (1993). The primary antibody was polyclonal rabbit anti-*Solanum bertaultii* 59-kD trichome PPO (Kowalski et al., 1992). [^{125}I]Protein A (Amersham, 30 mCi ng^{-1} at 1:1000 dilution) and autoradiography were used for visualization.

Other Techniques

Membranes were sonicated twice for 30 s with an ultrasonic sonicator W-385 (Heat Systems Ultrasonics, Inc., Farmingdale, NY) at 50% output. Thylakoid membranes and other subchloroplast fractions were treated with trypsin as described before (Yalovsky et al., 1992). The reactions were performed in 200 μL of 0.1 M Tricine-KOH (pH 8.0). A freshly prepared trypsin (L-1-tosyl-amido-2-phenyl-ethyl chloromethyl ketone-treated, Sigma) solution was added to a final concentration of 40 μg/mL, and the mixtures were incubated at 37°C for 10 min. The reaction was stopped by adding PMSF and soybean trypsin inhibitor (type I-s, Sigma) to final concentrations of 2.5 mM and 0.25 mg/mL, respectively. Thylakoids were pelleted by centrifugation and resuspended in the same buffer containing inhibitors. The treatment was repeated twice before solubilizing the membranes for SDS-PAGE analysis.

SDS-PAGE was performed as described by Yalovsky et al. (1992) using 8% (w/v) acrylamide gels for PPO and 12% for PC and LHCP. Samples were loaded so as to include equivalent amounts of plastid material, according to the Chl content of the intact plastid fraction recovered after the thermolysin treatment. Protein content or plastid number was used when plastids of different Chl concentrations were compared. The large subunit of Rubisco (55 kD) and GroEL (60 kD) were used as molecular mass standards in addition to ones obtained commercially.

Radioactivity in gels was estimated by laser scanning the exposed x-ray films using a Soft Laser Scanning densitometer (Biomed Instruments, Fullerton, CA) or by extracting the excised gel strips with hyamine hydroxide as previously described (Yalovsky et al., 1992). Plastid concentration was determined by counting in a hemocytometer. Chl and protein determinations were carried out as described by Chitnis et al. (1986).

Chemicals

[^{35}S]L-Met and [^{125}I]protein A were purchased from Amersham International, RiboMax and pGEM-7Z f(+) were obtained from Promega, and diguanosine triphosphate was from Pharmacia. Restriction enzymes were purchased from Promega and New England Biolabs. Other chemicals were purchased from Sigma.

RESULTS

Hy-19 PPO Gives Rise to a Single, 67-kD Translation Product

A tomato leaf PPO cDNA (designated Hy-19) (Yu, 1992; J.C. Steffens and H. Yu, unpublished data) was subcloned in pGEM-7Z f(+). This cDNA was identical with the open reading frame of the *ppo*B gene described by Newman et al. (1993). Hy-19 was transcribed in vitro using SP6 RNA polymerase, and the mRNA obtained was translated using reticulocyte lysate and [^{35}S]Met. One major translation product of about 67 kD was observed along with several contaminants of lower molecular mass. The latter resulted from endogenous reticulocyte mRNAs (Fig. 1, cf. + and − PPO RNA lanes) and could be eliminated by treatment with micrococcal nuclease (Kohorn et al., 1986; results not shown). These contaminants were neither imported nor processed by plastids and were, therefore, ignored in further work. Translation of PPO B mRNA with wheat germ S30 extracts produced mainly low molecular mass products.

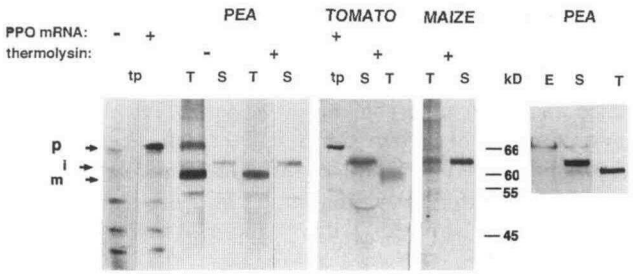

Figure 1. Import and processing of a PPO precursor by isolated chloroplasts. Intact pea and tomato chloroplasts and maize etiochloroplasts (approximately 10^8 plastids in each case) were incubated with ^{35}S-labeled precursor of PPO B (250,000 cpm) for 30 min in light. Plastids were treated with thermolysin to remove envelope-bound precursor at the end of the incubation period (+) or left untreated on ice for 30 min (−). Intact plastids were reisolated, disrupted, then fractionated into thylakoids (T) and stroma (S), and analyzed by SDS-PAGE and fluorography. Precursor (p), intermediate (i), and mature (m) forms of PPO are indicated as well as the position of molecular mass standards. tp, Translation products obtained using reticulocyte lysate in the presence (+) or absence (−) of PPO mRNA. The envelope (E), stromal, and thylakoid fractions shown in the right panel were obtained after fractionation of burst plastids on Suc gradients following import of ^{35}S-pPPO without treatment with thermolysin.

The Precursor of PPO B Is Processed into Two Forms by Isolated Plastids

The ^{35}S-labeled 67-kD polypeptide coded by Hy-19 was imported and processed by intact plastids from pea, maize, and tomato leaves (Fig. 1). The 67-kD polypeptide could thus be regarded as the precursor form of tomato PPO B. It was observed in envelope membranes and occasionally also in thylakoid membranes when plastids had not been treated with thermolysin at the end of the import period. Further analysis showed that the presence of precursor molecules in thylakoid fractions was apparently due to contamination by envelope membranes (Fig. 1, right).

Two processed forms of PPO B were observed inside pea and tomato plastids following import and digestion of envelope-bound species by thermolysin. A 62-kD form was found in the stroma, and a 59-kD species was observed in thylakoids (Fig. 1). Only the 62-kD stromal form was observed in etiochloroplasts from dark-grown maize leaves that had been illuminated for 8 h. Further work was carried out mainly with pea plastids, which were superior to plastids from other sources in importing and processing pPPO. This was undertaken after we determined that observations with pea plastids presented a faithful picture of pPPO targeting and processing (see "Discussion").

The 62-kD Stromal Form Is an Intermediate in the Conversion of pPPO to the 59-kD Thylakoid Form

Time-course experiments using subfractionation of plastids following import, with and without thermolysin treatment, showed that the 62-kD polypeptide was an intermediate in the conversion of the precursor to the mature form found in thylakoids (Fig. 2). A time-dependent decrease in the amount of envelope-bound precursor was followed by a transient build up of the 62-kD form in the stroma and accumulation of the 59-kD form in thylakoids (Fig. 2). The latter coincided with a decrease in the stromal 62-kD polypeptide. The small amounts of precursor molecules observed in the thylakoid fractions were removed by treating the plastids with thermolysin prior to subfractionation. This suggested that the presence of precursor molecules in thylakoid fractions was due to contamination by envelope membranes (Fig. 2A). The

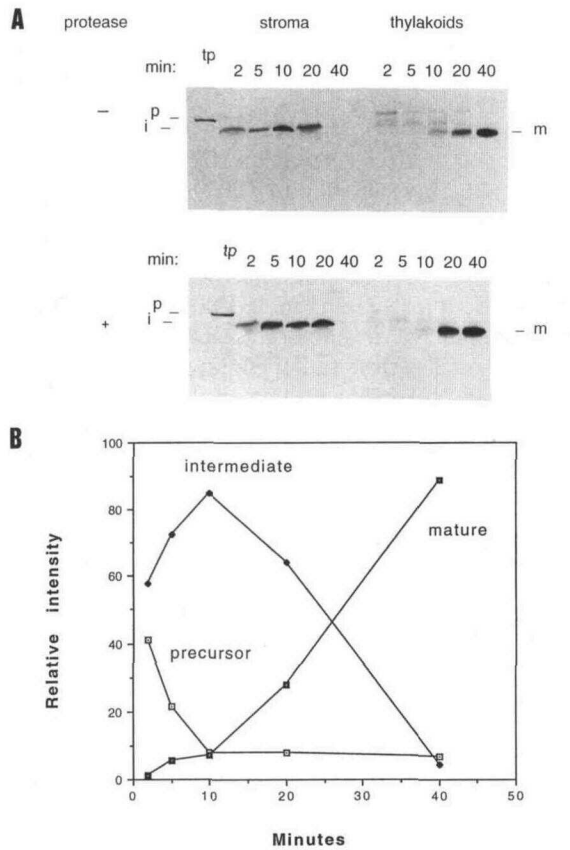

Figure 2. Time course of pPPO import into plastids. A suspension of intact pea plastids was divided into samples (40 μg of Chl, 7×10^7 plastids) that were incubated with [^{35}S]pPPO (250,000 cpm) in 300 μL of import mixture at 25°C in light for the indicated time. At the end of the incubation period, the plastids were treated with thermolysin (+) or left untreated on ice for 30 min (−). Intact plastids were reisolated and fractionated into stroma and thylakoids. Since differences in Chl content among the reisolated plastid samples were negligible, the entire fraction was loaded onto the gel in each case and analyzed by SDS-PAGE and fluorography. A, Fluorography of gels; 40-h exposure of x-ray films. The lanes marked "tp" contained 10,000 cpm of the translation products given to plastids. The precursor (p), intermediate (i), and mature (m) forms of PPO are indicated. B, Radioactivity in precursor, intermediate, and mature forms as percentages of total radioactivity at each time in plastids that were not treated with thermolysin. Radioactivity was estimated by laser scanning of the exposed x-ray films. Estimation of radioactivity by extracting excised gel strips and counting by liquid scintillation gave a practically identical picture.

relatively small amounts of the 62-kD form observed in the thylakoid fractions were protected from thermolysin. Quantification of the 67-, 62-, and 59-kD bands supported the precursor-intermediate-end product relationship among the three forms (Fig. 2B). The radioactivity in the intermediate and mature forms after 40 min of import amounted to 32.6% of that given to the plastids as [^{35}S]pPPO.

Imported 59-kD Polypeptide Accumulates in the Thylakoid Lumen

The 59-kD mature form observed in thylakoids following import of PPO B was not an intrinsic membrane protein. It appeared to be loosely bound on the luminal side of the thylakoid membrane or soluble in the lumen. When washed thylakoids were broken in a Yeda press and the suspension was centrifuged at 250,000*g*, the imported 59-kD form was found in the supernatant fraction (Fig. 3A). Limited digestion of washed thylakoids with trypsin or thermolysin following import of pPPO into intact plastids had no effect on the 59-kD polypeptide (Fig. 3A). However, the latter was completely digested if thylakoids were broken first by a Yeda press, sonicated, or treated with a mixture of 0.1% (w/v) octyl and nonyl β-D-glucopyranoside (Fig. 3A). The imported 59-kD polypeptide was also released as a soluble species by washing the thylakoids with 0.8 M Tris (pH 8.3), 2 M Na bromide and other treatments that solubilize lumen proteins (results not shown).

It could be argued that targeting pPPO in pea might be affected because pea thylakoids lack PPO activity and do not react with antibodies prepared against tomato trichome PPO (Fig. 4B). The seed coats of wild pea, *Pisum elatius*, possess PPO activity during seed ripening, contrary to cultivated pea (Marbach and Mayer, 1975). However, thylakoids from *P. elatius* leaves did not react with the tomato antibodies (Fig. 3B), and its plastids could not, therefore, be used to examine the possibility mentioned above. Tomato thylakoids were highly reactive to the antibodies, the prominent band having an apparent molecular mass of 59 kD (Fig. 3B). The native PPO in washed thylakoids was protected from limited digestion by trypsin or thermolysin (Fig. 3B). The stromal fraction was devoid of PPO immunogenicity. Sonication released all of the thylakoid PPO in a soluble form, whereas breaking in the Yeda press left most of the native tomato PPO still attached to the membrane (Fig. 3B).

The Ratio between the 62- and 59-kD Forms Depends on the Developmental Stage of Plastids and Other Factors

Isolated plastids exhibited a marked variability in both overall amount of precursor imported and the predominant processed form observed at the end of incubation. This appeared to be dependent on the plant species studied (Fig. 1), plant age, and growth conditions, e.g. illumination regime, mineral nutrition. Thus, the ratio between the 62-kD stromal and 59-kD luminal forms differed with the leaf age in pea, with the 59-kD form predominating in older leaves and the stromal species being more prominent in the uppermost, youngest leaf (Fig. 4A). A similar situation was observed in maize leaves; only the 62-kD polypeptide was observed at

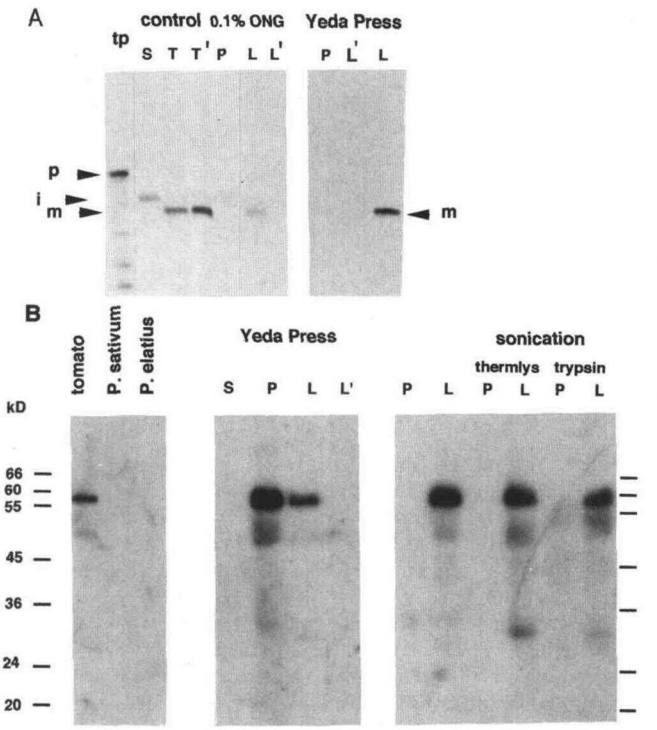

Figure 3. Localization of the mature form of imported and native PPO. A, Intact pea plastids (150 μg of Chl) were incubated in light with [^{35}S]pPPO (600,000 cpm) for 30 min. Intact organelles were reisolated after thermolysin treatment and subfractionated into stromal (S) and thylakoid (T) fractions. Aliquots (equivalent to 10 μg of Chl) were kept without further treatment ("control"), and the remaining thylakoids were suspended in 200 μL of 0.1 M Tricine-KOH (pH 8.0) and digested with trypsin (8 μg for 10 min at 37°C). The thylakoids were washed with buffer containing PMSF and trypsin inhibitor (see "Materials and Methods"). The trypsin-treated membranes were extracted with a mixture of 0.1% octyl β-D-glucopyranoside (ONG) and nonyl β-D-glucopyranoside, broken using a Yeda press, or left untreated (T'), using the equivalent of 20, 80, and 40 μg of Chl, respectively. The detergents and Yeda press-treated membranes were then fractionated by centrifuging at 250,000*g* for 15 min into a pellet (P) and a lumen (L) fraction. Half of the latter fraction was again treated with trypsin (L'). The various fractions were analyzed by SDS-PAGE and fluorography. An aliquot (10,000 cpm) of the translation products (tp) was run alongside the fractions. Precursor (p), intermediate (i), and mature (m) forms are indicated. B, Immunoblots of pea and tomato plastid suborganellar fractions that have been challenged with antibodies prepared against purified *S. bertaultii* PPO and visualized using [^{125}I]protein A. Left, Washed thylakoids from tomato, *P. sativum*, and *P. elatius*. Center, Blots of stroma and fractions obtained from tomato thylakoids (20 μg of Chl) after breaking in a Yeda press. Membrane (P) and the lumen fractions, before (L) and after treatment with trypsin (L'), are as described for A. Right, Isolated tomato thylakoids were treated with 8 μg of trypsin or 40 μg of thermolysin for 10 or 30 min, respectively, washed in the presence of inhibitors, sonicated, and fractionated into membrane and lumen fractions. The positions of molecular mass standards are indicated.

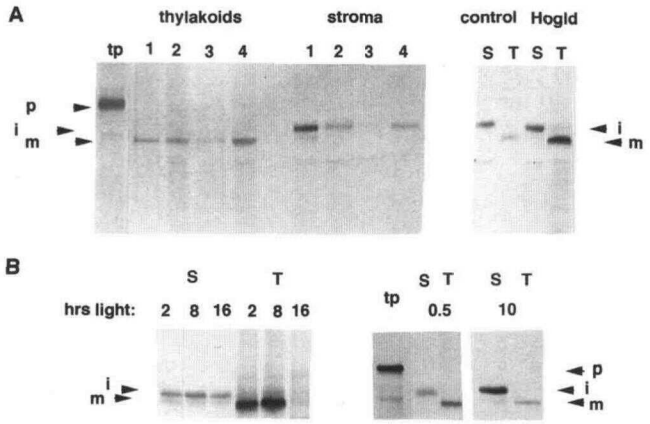

Figure 4. The relative amount of stromal and thylakoid forms of PPO following import into pea plastids depends on developmental stage and growth conditions. A, Import and processing of pPPO by plastids from the first four leaves (including stipules) of 10-d-old pea seedlings. 1, Uppermost leaf and apex; 4, first, oldest leaf. Approximately 4×10^7 plastids (11, 18, 32, and 57 μg of Chl in youngest through oldest leaf sample, respectively) were incubated with [^{35}S]pPPO (200,000 cpm) for 30 min in light. Intact plastids were reisolated after thermolysin treatment and subfractionated and analyzed as described for Figure 2. Right, Plastids (40 μg of Chl) from control plants (8 d old, 16-/8-h LDCs for 6 d, followed by 2 d in darkness and 1 h in light before harvest) and seedlings supplemented with half-strength Hoagland solution on d 7. B, Left, Plastids (7×10^7) from plants grown in 16-/8-h LDCs for 8 d, transferred to darkness for 40 h at the middle of the light period, and reilluminated for the times indicated before isolation of plastids. Right, Plastids (7×10^7) from 8-d-old plants illuminated for 30 min and 10-d-old plants illuminated for 10 h following the 40-h destarching period. Intact plastids were incubated with [^{35}S]pPPO (200,000 cpm) for 30 min in light and treated with thermolysin, then subfractionated into stroma (S) and thylakoids (T), and analyzed as described for Figure 2.

early stages of greening of etiolated leaves (Fig. 1). In green maize leaves pPPO was processed primarily to the 59-kD form by the fully differentiated top one-third of the leaf, whereas in plastids from the developing, bottom part, mainly the 62-kD form was observed (results not shown). Chloroplasts from young pea leaves (8–9 d old) were more efficient in importing and processing pPPO than older ones (10–12 d old) but converted it primarily to the 62-kD stromal form (results not shown). Mineral nutrition also affected both the overall amount of precursor imported and the ratio between the stromal and luminal forms. When pea seedlings were supplemented with half-strength Hoagland solution on d 7, their plastids imported significantly more pPPO, which was converted mainly to the luminal 59-kD species (Fig. 4A, right).

Our plant-growing protocol involved a 40-h destarching period in the dark to enable isolation of intact plastids fit for import and processing of proteins. Therefore, we examined the effect of the illumination regime on the import and processing of pPPO. Dark-grown pea or maize plants were unable to import pPPO. The same was true for light-grown plants that were transferred to darkness for 96 h or longer. Eight-day-old pea seedlings that were grown in a 16-h light/8-h dark regime were transferred to darkness for 40 h in the middle of the light period. The plants were then illuminated for 2, 8, or 16 h before isolation of plastids for the determination of pPPO import. Figure 4B shows the results of a representative experiment that was repeated three times. Plastids from plants that were illuminated for 2 or 8 h contained markedly more mature 59-kD PPO than those that received 16 h of light (Fig. 4B). No significant differences were observed in the amounts of the stromal form in the three treatments (Fig. 4B, left). Similar results were obtained with 8-d-old plants.

Thus, pea plants can be manipulated to produce predominantly one of the two processed forms of PPO. For example, plastids from 10-d-old plants that have been illuminated for 30 min following the destarching period produced mainly the thylakoid form, whereas those from 8-d-old plants illuminated for 10 h made mainly the stromal form (Fig. 5, bottom right).

Energy Requirements for Import and Processing of pPPO

The import of pPPO was strongly dependent on the availability of MgATP (Fig. 5). GTP was less effective. MgATP supported PPO import also in the dark, but the precursor was processed only to the 62-kD stromal form (Fig. 5, top). Nigericin, an electroneutral H^+/K^+ antiporter, abolished import in the absence of added ATP, and the addition of MgATP restored import and formation of the 62-kD intermediate form (Fig. 5, bottom). Light was required for conversion of the 62- to the 59-kD polypeptide or for traversing the thylakoid membrane. The addition of apyrase or hexokinase and Glc to remove exogenous ATP did not significantly affect import in illuminated plastids, suggesting that ATP was act-

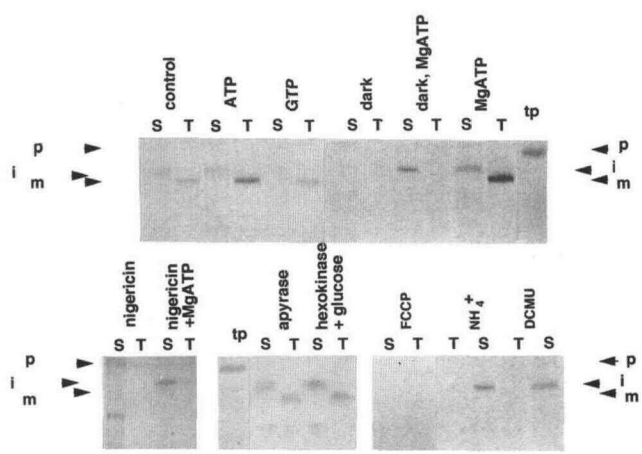

Figure 5. Energy requirements of import and processing of pPPO by pea plastids. Plastids (30 μg of Chl) were preincubated for 15 min in darkness with inhibitors or enzymes before the addition of [^{35}S]pPPO (200,000 cpm) and MgATP or GTP and incubation for additional 30 min in light. ATP, GTP, and MgATP, 2.5 mM; nigericin, 0.2 μM; carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone, 20 μM; NH_4 acetate, 3 mM; DCMU, 1 μM. Treatment with thermolysin at the end of the import period, subfractionation, analysis, and other details are as described for Figure 2.

ing from within the plastid. Carbonyl cyanide *p*-trifluoromethoxy phenylhydrazone (2 μM), a potent inhibitor of photophosphorylation, abolished import in light even in the presence of added ATP, whereas NH_4^+ (3 mM), an uncoupler, caused partial inhibition (Fig. 5). DCMU (1 μM), an inhibitor of photosynthetic electron transport, markedly inhibited import even in the presence of light and added MgATP, allowing the formation of only some 62-kD polypeptides (Fig. 5).

Tentoxin Specifically Inhibits the Import of pPPO into Plastids

Tentoxin practically abolished the import of pPPO into isolated plastids at 10 μM (Fig. 6A). The import of pLHCPII and pPC was similarly affected. MgATP restored tentoxin-inhibited import of pLHCPII and pPC (Fig. 6B; see also Cline et al., 1992) but had little effect on that of pPPO (Fig. 6A). Tentoxin-blocked pPPO import was restored by 0.1 mM DCPIP or PMS and MgATP; however, mainly the stromal form of PPO was observed under these conditions (Fig. 6A). Reduced DCPIP or PMS had no such effect. Neither reduced nor oxidized DCPIP or PMS affected pPPO import or processing in the absence of tentoxin (not shown). Incubation of pPPO with 10 μM tentoxin at 4°C for 10 min had no effect on the subsequent import and processing of the precursor in which the toxin was diluted to a final concentration of 0.1 μM. Therefore, it is unlikely that tentoxin interacts directly with the precursor.

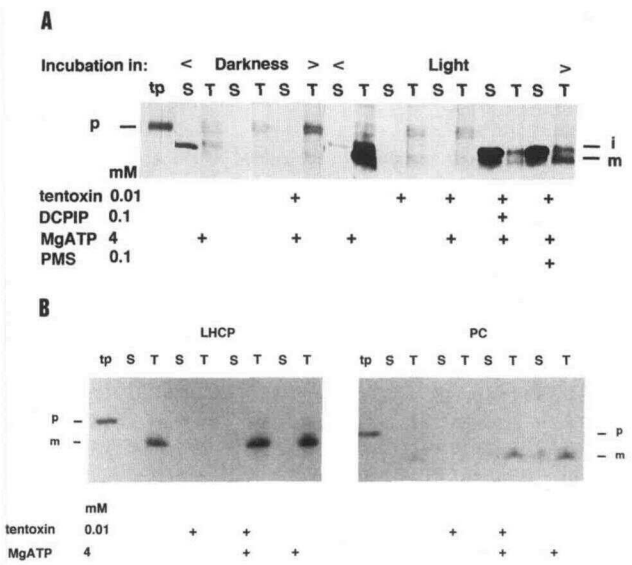

Figure 6. The effect of tentoxin on the import of pPPO and precursors of other plastid proteins by isolated pea plastids. A, Plastids (60 μg of Chl) were preincubated in darkness for 15 min with tentoxin before addition of [^{35}S]pPPO (400,000 cpm), followed by incubation in light or darkness in the presence of effectors for 30 min. Intact plastids were reisolated after thermolysin treatment, subfractionated into stromal (S) and thylakoid (T) fractions, and analyzed by SDS-PAGE and fluorography. tp, An aliquot (10,000 cpm) of the precursor used in the reactions. Positions of the precursor (p), intermediate (i), and mature (m) forms of PPO are indicated. B, Plastids (60 μg of Chl) were preincubated in darkness in the presence or absence of 10 μM tentoxin for 15 min. ^{35}S-labeled pPC or pLHCPII (400,00 cpm) and MgATP were added, and the plastids were further incubated in light for 30 min. The lanes marked "tp" contained 10,000 cpm of the respective precursors. The positions of the precursor (p) and mature (m) forms of the proteins are indicated in each case. Other details are as described for A.

Low Concentrations of Divalent Cu Ions Block the Import of pPPO into Plastids

Since PPO B has three putative Cu-binding domains (Newman et al., 1993), we examined the possibility that Cu^{2+} might be required for some step in the import, targeting, or processing of its precursor. Surprisingly, 1 μM $CuSO_4$ (and even 0.1 μM, not shown) strongly inhibited the import of pPPO (Fig. 7A). $CaSO_4$ had no effect (not shown). The effect appeared to be quite specific to Cu^{2+} ions, since 50 μM Zn^{2+} or Fe^{2+} had only a limited effect on pPPO import (Fig. 7B). The effect of Cu ions on PPO import was counteracted by EDTA if the latter was added before the addition of Cu^{2+}. The effect of EDTA depended on the ratio between its concentration and the concentration of Cu ion (Fig. 7C).

The effect of Cu^{2+} was specific for pPPO import; it had no effect on the import or processing of pLHCPII by the pea plastids used in the experiments (Fig. 7D). The import of pPC, also a Cu metalloprotein targeted to the lumen, was only moderately affected by 50 μM $CuSO_4$, 10 times the concentration that abolished the import of pPPO (Fig. 7D).

DISCUSSION

The Use of a Heterologous System for Studying pPPO Import

We used primarily pea plastids in the study of pPPO import, despite the fact that the precursor is coded by a tomato gene. Pea plastids are widely used in the study of protein import because of the relative ease of their isolation and their stability in vitro. Pea plastids will import and process precursors of nuclear-coded plastid proteins regardless of source, sometimes at higher rates than their respective native proteins. An additional advantage of pea is the absence of both active plastid PPO and proteins that cross-react with anti-tomato PPOs (Fig. 3). Import of relatively large amounts of pPPO into pea plastids could be used to study possible function(s) of the enzyme.

We have concluded from preliminary experiments that observations with pea plastids on pPPO import and processing are representative of the import in plastids from other sources, including tomato. PPO may be mistargeted in pea plastids, since the latter do not contain the protein, but this is unlikely in view of the correspondence between the structure of the transit peptide and the fate of the protein.

PPO Is Imported into Plastids and Targeted to the Thylakoid Lumen by the Conventional Mechanisms

The sequence of the Hy-19 cDNA from tomato indicated that the gene encodes a polypeptide of 67.2 kD (Yu, 1992). This was indeed the size of the in vitro translation product of its mRNA, which was imported and processed by plastids

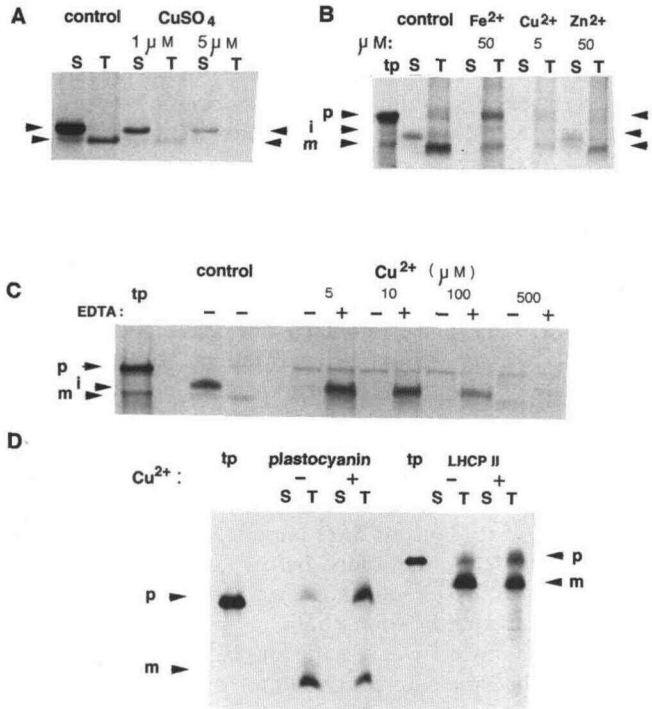

Figure 7. Low concentrations of Cu^{2+} block the import of pPPO into pea plastids. A and B, Plastids (30 μg of Chl) were incubated for 20 (A) or 30 min (B) in light with [^{35}S]pPPO (300,000 cpm) in the presence or absence of metal ions at the concentrations indicated. Intact plastids were reisolated after treatment with thermolysin and subfractionated into stroma (S) and thylakoids (T). C, Plastids (30 μg of Chl) were preincubated for 2 min in the presence (+) or absence (−) of 1 mM EDTA. [^{35}S]pPPO (400,000 cpm) and Cu^{2+} at the concentrations indicated were added, and the reaction mixtures were further incubated in light for 15 min. The control was incubated in the absence of EDTA and Cu^{2+} and subfractionated into a stromal (left) and thylakoid (right) lane. The Cu-treated lanes contain the entire plastid fraction of each import reaction. D, The effect of $CuSO_4$ on the import and processing of the precursors of PC and LHCPII. ^{35}S-labeled precursors of LHCP or PC (400,000 or 500,000 cpm, respectively) were added to intact plastids (20 μg of Chl). The plastids were incubated in light for 30 min in the presence (+) or absence (−) of 50 μg of $CuSO_4$. Intact plastids were reisolated after thermolysin treatment, subfractionated into stroma (S) and thylakoids (T), and analyzed by SDS-PAGE and fluorography. Aliquots of the precursors (20,000 cpm for PC, 10,000 cpm for LHCP) were loaded in the lanes marked "tp." The position of the precursor (p) and mature (m) forms are indicated for each protein.

from a variety of plants (Fig. 1). The 67-kD precursor was processed to a 62-kD product located in the stroma and a 59-kD one observed in the thylakoid lumen (Figs. 1 and 3). No product corresponding to the widely reported 45-kD polypeptide (Vaughan et al., 1975; Vaughn and Duke, 1984; Flurkey, 1985, 1986; Lanker et al., 1987; Vaughn et al., 1988; Steffens et al., 1990) has been observed either as a translation product of Hy-19 or following import and processing by plastids. Our observations are in accordance with accumulating evidence on the structure of PPO genes in higher plants. In vitro translation products of poly(A$^+$) RNA from *S. berthaultii* glandular trichomes contained a single species reacting with anti-PPO, having a molecular mass of 67 kD (Steffens et al., 1990). This was suggested to be the precursor of the 59-kD purified trichomal PPO (Kowalski et al., 1992) and was further supported by the structure of cDNA clones isolated from potato (Hunt et al., 1993). Cloned tomato PPO genes appeared to code for 66.1- to 70.6-kD precursors of 57.0- to 61.6-kD mature products (Shahar et al., 1992; Yu, 1992; Newman et al., 1993). Cary et al. (1992) reported recently that a full-length *V. faba* PPO cDNA clone had a predicted mature molecular mass of 58 kD.

Most of the Reported Multiplicity of PPO Should Be Attributed to Postimport Events

The multiple forms reported for PPO apparently arise from a single type of protein precursor of about 67 kD, carrying a transit peptide of 88 to 110 amino acid residues. Some multiplicity may arise from partial processing, like that leading to the formation of the 62-kD stromal intermediate. Partial processing has been observed with other lumen proteins. Castor bean leukoplasts were able to import pPC but processed it only to its stromal intermediate (Halpin et al., 1989). The incomplete processing was ascribed to lack of a thylakoid protein transport and maturation system. Biosynthetic Thr deaminase from tomato has a transit peptide typical of lumen proteins. However, the enzyme isolated from floral tissues retained the C-terminal portion of its transit peptide, suggesting that only partial processing had taken place (Samach et al., 1991). The protein was soluble and apparently resided in the stroma.

Slight variations in molecular mass could also result from expression of different members of a PPO gene family as has been shown for tomato (Newman et al., 1993), e.g. the discrete doublet of PPO bands in the range of 57 to 60 kD observed in extracts of leaves or trichomes from several species (Kowalski et al., 1992; Robinson and Dry, 1992; Shahar et al., 1992; Hunt et al., 1993). However, most of the reported multiplicity appears to result from events that occur after processing and arrival of PPO at its final destination. Although glycosylation and aggregation may contribute to PPO multiplicity observed, proteolysis, quinone adduct formation, and cross-linking during isolation of the proteins are apparently a more common cause (Mayer, 1987; Flurkey, 1990; Mayer and Harel, 1991; Robinson and Dry, 1992).

The Targeting of PPO Corresponds to the Sequence of Its Transit Peptide

The sequence of Hy-19 and other plant PPOs corresponded well with our observations of the two processing steps involved in pPPO targeting in isolated plastids. The N-terminal 88 amino acid residues are typical of transit peptides of plastid lumen proteins, e.g. PC and the three polypeptides of the oxygen-evolving complex of PSII (De Boer and Weisbeek, 1991). The 48-residue N-terminal domain of pPPO B, rich in hydroxy amino acids, is characteristic of the stroma-targeting domain of such proteins. This is followed by a lumen-targeting domain carrying two Arg residues, N terminal to a highly hydrophobic domain (Fig. 8). The first processing site is assumed to be located immediately after a Lys residue by analogy with other lumen proteins (Bassham et al., 1991).

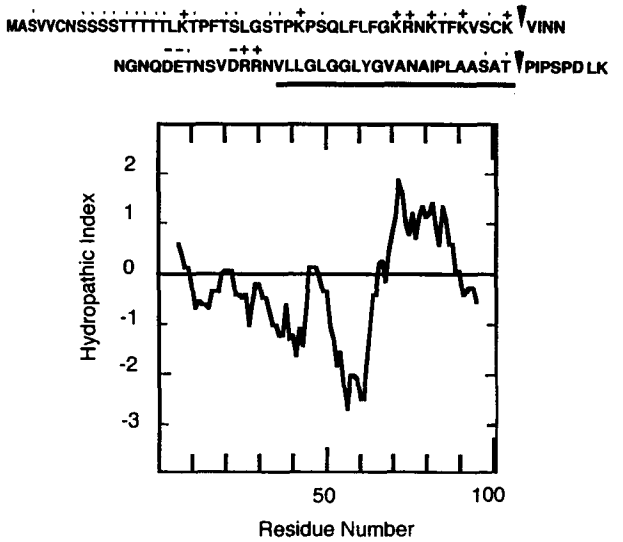

Figure 8. Amino acid sequence and hydrophobicity plot of PPO B transit peptide. Top, Amino acid sequence of the transit peptide. The arrows indicate the putative first processing site leading to the formation of the 62-kD intermediate, deduced by comparison to other lumen proteins (Bassham et al., 1991), and the final processing site, deduced from N-terminal microsequencing of the native protein (Yu 1992). Hydroxy (·) and charged amino acid residues and the hydrophobic domain in the lumen targeting part of the transit peptide (underlined) are indicated. Bottom, Kyte-Doolittle hydrophobicity plot of the first 96 amino acid residues of pPPO B.

The Two Processing Steps

The 62-kD intermediate could be formed in vitro by incubating the translation product of Hy-19 mRNA with a partially purified preparation of a stromal processing peptidase from pea chloroplasts (E. Harel, A. Sommer, and E. Ne'eman, unpublished data). The purified peptidase was able to process precursors of other nuclear-coded plastid proteins, e.g. pPC, the precursor of the small subunit of Rubisco, and that of subunit II of PSI. It was unable to process pLHCPI and pLHCPII. The peptidase might be related to the stromal processing peptidase described by Robinson and Ellis (1984).

The conversion of the 62-kD intermediate to the 59-kD form takes place apparently on the stromal face of thylakoids by a membrane-bound enzyme. The 62-kD form, prepared in vitro by the action of the purified stromal processing peptidase and unfolded using urea and DTT, was processed by isolated, washed thylakoids (E. Harel, A. Sommer, and E. Ne'eman, unpublished observations). However, the resulting 59-kD polypeptide was not inserted into the thylakoids nor was it translocated to the lumen. The transient appearance of a thylakoid-bound 62-kD polypeptide, which was resistant to thermolysin treatment of intact plastids (Fig. 2A) but digested by treating the isolated thylakoids (not shown), suggests that attachment of the 62-kD polypeptide to the membrane precedes its conversion to the 59-kD form.

Localization of the Processed PPO

The 62-kD intermediate was directed to the lumen and converted to the 59-kD fully processed species in isolated plastids (Fig. 2). The fact that a significant portion of endogenous tomato PPO was found soluble in the lumen (Fig. 3) suggests that a similar scenario occurs in vivo. The in vitro import results are in line with cytochemical and immunogold studies. Reaction products of PPO were observed in the lumen, and the enzyme was suggested to be located on the luminal face of the thylakoid membrane (Shomer et al., 1979; Sherman et al., 1991). The PPO-containing protein bodies in wild potato trichomes (K.C. Vaughn, S.P. Kowalski, and J.C. Steffens, unpublished data) and both trichomal and chlorenchyma PPO in tomato (D. Joel and J.C. Steffens, unpublished data) were located in the lumen by immunogold EM.

Tomato PPO is encoded by a family of seven genes, four of which contain targeting information resembling that of PPO B and lack potential membrane-spanning domains in the mature polypeptide (Newman et al., 1993). Some reports of PPO tightly bound to the thylakoid membrane could thus be due to postimport modification or, more likely, cross-linking reactions occurring during the isolation of the proteins. However, some of the genes coding for plant PPOs, e.g. those of *V. faba*, may be genuinely membrane bound. Three of the tomato genes, *ppo*A, A′, and C, contain a hydrophobic domain close to the C terminus, which could span the thylakoid, anchoring the protein in the membrane (Newman et al., 1993). Sonication of tomato thylakoids completely solubilized PPO, whereas breaking the membranes using a Yeda press released only part of the native protein (Fig. 3B). This might reflect the presence of products of several PPO genes in tomato leaves, differing in the strength of their attachment to the thylakoid membrane. We have recently subcloned *ppo*A and C in expression vectors and are studying the import and targeting of their precursors by isolated plastids.

The Dependence of pPPO Import and Processing on the Developmental Stage of the Plastid

Both the overall rate of pPPO import and the ratio between the 62- and 59-kD forms depended on the developmental stage of the plastid (Fig. 4A). It appears that translocation across the thylakoid membrane and formation of the 59-kD mature form may require photosynthetically active plastids possessing a developed lamellar system. The observations described in Figure 4A are not surprising, since the rate of import of several plastid proteins, particularly those related to photosynthesis, often depends on the developmental stage of the plastid. LHCPII, the apoprotein of the major light-harvesting complex of PSII, is efficiently imported and processed by plastids from developing and greening leaves. Import ability declines rapidly with leaf age and progress of greening (Chitnis et al., 1986; Dahlin and Cline, 1991).

The effect of the illumination regime on the relative amount of the 59-kD polypeptide (Fig. 4B) is more difficult to interpret. The transcription and translation of LHCPII genes as well as Chl accumulation are regulated by circadian rhythm (Bei-Paraskevopoulou and Argyroudi-Akoyunoglou, 1992). Circadian rhythm appears to affect also the potential of plastids to import and process pLHCPII (E. Harel, unpublished data). It is not clear whether this reflects a general import potential, which also affects pPPO. However, since

no 59-kD form was observed in thylakoids of plants that have received 16 h of light, but the 62-kD form was present (Fig. 4B, left), the effect of illumination on PPO import may be more specific, possibly on transfer across the thylakoid membrane. It appears that the import of pPPO was considerably reduced after 16 h of illumination. Alternatively, the 59-kD polypeptide might be unstable in thylakoids exposed to longer light duration.

Energy Requirements of pPPO Import

Import into the stroma is consistent with that of other nuclear-encoded plastid proteins (De Boer and Weisbeek, 1991). It is dependent on ATP but not membrane potential. ATP appears to be required inside the plastid, since light will support import in the absence of added ATP or when exogenous ATP is hydrolyzed by apyrase or hexokinase and Glc (Fig. 5).

Lumen proteins require proton motive force alone (the OE17 and OE23 polypeptides of the the oxygen-evolving complex of PSII) or in combination with ATP (OE33) for traversing the thylakoid membrane, whereas PC needs only ATP (Bassham et al., 1991; Cline et al., 1992). Only the 62-kD polypeptide was observed in plastids incubated with MgATP in the dark. Since ATP can readily cross the envelope of pea plastids (Robinson and Wiskich, 1977), it appears that light, and not merely ATP, is required for translocating PPO across the thylakoid membrane. Valinomycin (1 μM, results not shown) had no effect on formation of the 59-kD form, whereas nigericin (Fig. 5) and monensin (not shown), both at 1 μM, completely prevented it, even when MgATP was present. Translocation might, therefore, require ΔpH and/or photosynthetic electron transport as is suggested by the effect of DCMU (Fig. 5).

The Effect of Tentoxin

Our observations on the inhibition of PPO import by tentoxin support the claim that the effect of this mycotoxin on protein import by plastids is rather specific to PPO (Vaughn and Duke, 1981). Tentoxin inhibits chloroplast coupling factor 1 and would be expected to affect plastid protein import (Klötz, 1988). However, although inhibition of the import of pLHCP and pPC was relieved by the addition of MgATP (Cline et al., 1992; Fig. 6B), restoration of pPPO import required also the presence of an electron acceptor (Fig. 6A). In accordance with immunoelectron microscopic observations (Vaughn and Duke, 1981), a significant amount of pPPO remained bound to the plastid envelope in the presence of tentoxin. Its import was resumed upon removal of the inhibitor or addition of MgATP and an electron acceptor. However, mostly the 62-kD intermediate was observed under these conditions; translocation into the lumen remained blocked. The requirement of an electron acceptor is difficult to explain. Since DCPIP is apparently unable to cross the plastid envelope, its ability to restore PPO import implies the involvement of electron transfer reaction(s) at the envelope or perhaps at contact sites between thylakoids and the inner envelope membrane.

The Effect of Cu Ion

The import of pPPO into plastids was inhibited by relatively low concentrations of Cu^{2+} (Fig. 7). This differed from the import pattern of another Cu-containing lumen protein, PC. The import and processing of pPC by pea chloroplasts was not affected by low concentrations of Cu ions (Fig. 7D; Hibino et al., 1991; De Boer and Weisbeek, 1991). In Cu-deficient *Chlamydomonas*, Cyt *c*552 replaced PC in the photosynthetic electron transport chain. It was suggested that Cu^{2+} has a regulatory role in the import and processing of pPC by plastids (Merchant and Bogorad, 1986).

The effect of Cu^{2+} on pPPO import is quite specific. The effect of a 10-fold higher concentration of Cu^{2+} on the import of pPC was rather small, and pLHCPII import was unaffected (Fig. 7D). We suggest that Cu ions bind strongly to the precursor, rendering it less amenable to import, perhaps because binding of Cu prevents its unfolding. Unfolding has been demonstrated to be necessary for protein import into organelles, particularly into mitochondria (Pfanner and Neupert, 1990; De Boer and Weisbeek, 1991). We are currently examining this possibility using a series of Hy-19 mutants in which histidyl residues in one or more of the putative Cu-binding domains have been replaced (D.M. Hunt, A. Sommer, J.C. Steffens, and E. Harel, unpublished data).

It is not clear whether Cu^{2+} combines with the PPO precursor or with one of the processed forms in the course of the assembly of the enzyme. It would appear from our observations that association takes place only after the first processing step. It will be interesting to determine whether Cu^{2+} is required for PPO translocation across the thylakoid membrane or conversion of the 62- to the 59-kD form. An in vitro thylakoid import assay is obviously required for this purpose.

ACKNOWLEDGMENTS

We are grateful to Ms. Hamutal Mazrier for technical help and to Dr. Pierre Gulubinoff, The Hebrew University, for a gift of the Rubisco small subunit and GroEL.

Received January 24, 1994; accepted April 6, 1994.

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