Substrate- and species-specific processing enzymes for chloroplast precursor proteins

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Using different precursors of chloroplast proteins and stromal extracts from both *Chlamydomonas reinhardtii* and pea chloroplasts, we analysed the specificity of stroma-localized processing peptidases. By gel filtration of a stromal extract from isolated *Chlamydomonas* chloroplasts, fractions could be separated containing enzymic activities for processing the precursors of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) and of the protein OEE1 from the photosynthetic water-splitting complex (pOEE1). The enzymes differed not only in molecular size, but also in their sensitivity to inhibitors and in their pH optima. Obviously, in the stroma of *Chlamydomonas* chloroplasts different peptidases exist for processing of pSS and pOEE1, the latter being converted into an intermediate-sized form, iOEE1, which was found to be further processed to mature OEE1 by a

thylakoid-associated protease. To study the species-specificity of the stromal peptidases, stromal extracts from *Chlamydomonas* and pea chloroplasts were incubated with pSS from either of these organisms. In the heterologous combinations, the precursors were partly hydrolysed, but not to the correct size. In importation assays, pSS from pea (but also the precursor of the ribosomal protein L12 from spinach) could not enter into chloroplasts from *Chlamydomonas*. In contrast, the algal pSS was imported into chloroplasts from pea, although it was not processed to mature SS. Our results indicate that the importation machinery and the pSS-processing enzymes in higher plants and green algae have different specificities and that in *Chlamydomonas* several stromal peptidases for different precursor proteins exist.

INTRODUCTION

Many chloroplast proteins are synthesized in the cytosol as higher-molecular-mass precursor proteins containing an N-terminal transit sequence (Keegstra, 1989; de Boer and Weisbeek, 1991). Protein importation into chloroplasts is a post-translational process, starting with the specific association of the precursor protein with a protein receptor at the chloroplast surface (Friedman and Keegstra, 1989; Su et al., 1992). Then the bound precursor protein is translocated across the chloroplast envelope and the transit sequence removed (Pilon et al., 1992; Schnell and Blobel, 1993). This maturation of precursor proteins has received considerable attention in recent years (Hageman et al., 1986; Musgrove et al., 1989; Bassham et al., 1991; Berry-Lowe and Schmidt, 1991; Clark and Lamppa, 1992; Cline et al., 1992). Some precursors are transformed by hydrolytic cleavage directly to the mature protein which is located either in the stromal compartment or inserted as an integral membrane protein into the thylakoids (della-Cioppa et al., 1986; Cline, 1988). Other precursors are first cleaved to intermediate-sized forms which are further processed by a second hydrolytic step. In particular, precursors of proteins residing in thylakoids, e.g. proteins of the oxygen-evolving complex (Cline et al., 1993) or plastocyanin (Kirwin et al., 1987; Weisbeek et al., 1989), contain two cleavage sites. Their transit sequence can be divided into an envelope-transfer and a thylakoid-transfer domain, the former segment being comparable in importation and processing properties with transit peptides of proteins in the stroma. The latter, however, is structurally and functionally similar to signal sequences of bacterial export proteins (von Heijne et al., 1989). The specificity of protein targeting into chloroplasts is determined by signals

residing in the envelope-transit peptide sequence (Anderson and Smith, 1986; Cheung et al., 1988). However, comparison of transit peptides of different precursor proteins from the same organism, or even of precursor sequences of functionally identical proteins from higher plants with those from algae, reveals little similarity in primary or secondary structure (Franzén et al., 1990). The only common feature of all transit sequences is the high number of positively charged and hydroxylated amino acids (Keegstra et al., 1989). Taking into account the endosymbiotic origin of chloroplasts, processing peptidases might have evolved late and eventually in parallel in different branches of the evolutionary tree. Therefore processing enzymes might not be very well conserved, and it is possible that there exist different enzymes for the removal of transit peptides from different precursor proteins. However, until now only a stromal peptidase from pea has been partially purified and it was found to act on different precursors in higher plants (Robinson and Ellis, 1984; Abad et al., 1989; Oblong and Lamppa, 1992). Little is known also about the relationship between processing enzymes in chloroplasts of different species.

Our previous work (Su and Boschetti, 1993) identified two stromal peptidases (SPP-1 and SPP-2) in *Chlamydomonas reinhardtii* chloroplasts which cleaved the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) at specific sites. The two enzymes had different molecular masses, pH optima and inhibitor sensitivities. SPP-1 transformed pSS only to an intermediate-sized form (iSS). SPP-2, however, converted pSS (and also iSS) directly into the mature protein (SS) (Su and Boschetti, 1993). The physiological role of SPP-1 is not clear, but SPP-2 is considered to be the pSS-processing enzyme active *in vivo* in the chloroplast stroma. Because both enzymes were

Abbreviations used: LHClI, light-harvesting complex II; SS, small subunit of ribulose-1,5-bisphosphate carboxylase; pSS, precursor of SS; iSS, intermediate-sized form of SS; SPP-1, SPP-2, stromal precursor peptidases from *Chlamydomonas reinhardtii* described in the text; OEE1, oxygen-evolving enhancer protein 1; pOEE1, precursor of OEE1; iOEE1, intermediate-sized form of OEE1; rL12, ribosomal protein L12; poly(A)⁺, polyadenylated; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK'); Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK').

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characterized using pSS from *Chlamydomonas* as the only substrate, the question of enzyme specificity arose. Are other precursor proteins either from the same organism or even from other species also hydrolysed by these pSS-directed proteases? In the present report we provide evidence that, in *Chlamydomonas*, several stromal peptidases for different precursor proteins exist and that the pSS-processing enzyme of *Chlamydomonas* is very substrate- and species-specific.

EXPERIMENTAL

Chemicals

Most chemicals were purchased from Fluka (Buchs, Switzerland). Tosyl-lysylchloromethane ('TLCK') (Tos-Lys-CH₂Cl) and tosylphenylalanylchloromethane ('TPCK') (Tos-Phe-CH₂Cl) were from Sigma (St. Louis, MO, U.S.A.). [³⁵S]Methionine (specific radioactivity > 1000 Ci/mmol) was obtained from Amersham International (Rahn AG., Zürich, Switzerland).

Organisms, growth conditions, plasmids

The cell-wall-deficient mutant *Chl. reinhardtii* cw-15 was obtained as strain CC-277 from the *Chlamydomonas* Center, Duke University, Durham, NC, U.S.A. Cells were grown synchronously in a 14 h light/10 h dark cycle at 25 °C in the high-salt buffer of Sueoka (1960). After two days, they were harvested in the middle of the third light period.

Pea plants (*Pisum sativum* var.) were grown in soil for 12 days under natural light conditions.

Plasmid pSP64 containing cDNA coding for pSS-2 of *Chl. reinhardtii* was obtained from Dr. M. L. Mishkind (Rutgers University, New Brunswick, NJ, U.S.A.); plasmid pGMZ-3Z with the complete sequence coding for pOEE1 of *Chl. reinhardtii* was supplied by Dr. J. D. Rochaix and Dr. P. Künstner (University of Geneva, Geneva, Switzerland); plasmid pT7-pS with the gene for pSS from pea was given to us by Dr. D. Schnell (Rockefeller University, New York, NY, U.S.A.); RNA transcribed from plasmid pGWS-1 (SolL12-T7/18-A) containing the cDNA of the precursor of the ribosomal protein L12 (prL12) from spinach was supplied by Dr. A. R. Subramanian (Max Planck-Institut für Molekulare Genetik, Berlin, Germany).

Chloroplast isolation

Intact chloroplasts from *Chlamydomonas* were prepared by a modification of the procedure of Mendiola-Morgenthaler et al. (1985). After harvesting by centrifugation, the cell pellet was resuspended in isolation medium containing 250 mM sorbitol, 35 mM Hepes/KOH, pH 7.8, 1 mM MnCl₂, 5 mM MgCl₂ and 2 mM EDTA/KOH, pH 7.8, and broken down in a Yeda press at 486.4 kPa. The final concentration of EDTA in the homogenate was then adjusted to 8 mM by the addition of a concentrated solution of EDTA/KOH, pH 7.8. After incubation for 20 min at 0 °C with gentle shaking, the homogenate was centrifuged at 3000 g for 5 s. The pellet was carefully resuspended in isolation medium and fractionated on step gradients of Percoll, as described previously (Mendiola-Morgenthaler et al., 1985). The intact chloroplasts banding between 40 and 60% (v/v) Percoll were collected and washed three times with isolation medium. Depending on the experiment to be performed, the final chloroplast pellet was suspended in either importation buffer or lysis buffer as described below.

Intact chloroplasts from pea were isolated as described by Nivison et al. (1988) with the following modifications: 12-day-old pea plants were homogenized in a grinding medium containing 350 mM sorbitol, 50 mM Hepes/KOH, pH 8.3, 5 mM

MgCl₂, 2 mM EDTA and 2 mM EGTA by passing them through a pharmaceutical roller mill for ointment preparation (Asra SWD2; Greve and Behrens, Hamburg, Germany). Chloroplasts were isolated by centrifugation through a step gradient of 70, 50 and 30% (v/v) Percoll.

Preparation of crude stromal extract and thylakoid lumen extract

Intact chloroplasts were suspended and held in lysis buffer (50 mM Tris/HCl, pH 8.0) for 30 min on ice and then centrifuged at 5000 g for 15 min at 4 °C. The supernatant was centrifuged again at 30000 g for 60 min at 4 °C. The main processing activity tested with pSS remained in the supernatant, designated as 'crude stroma'.

The pellet obtained after the first low-speed centrifugation of lysed *Chlamydomonas* chloroplasts was suspended again in lysis buffer and homogenized by passing through a French press at 6900 kPa at 4 °C. After centrifugation at 30000 g for 30 min at 4 °C the clear supernatant was recovered as 'thylakoid lumen extract'.

Partial purification of stromal peptidases from *Chlamydomonas*

The crude stromal extract from *Chlamydomonas* was loaded on to a Superose 6 gel-filtration column (HR10/30, f.p.l.c. system; Pharmacia) which had been equilibrated with Tris buffer (50 mM Tris/HCl, pH 8.0). Fractions were collected and tested for processing activity (see below). The fractions containing SPP-1 activity were stored at -70 °C. SPP-2-active fractions were adsorbed on a Mono Q anion-exchange column (HR5/5, f.p.l.c. system; Pharmacia) pre-equilibrated with the same Tris buffer. The column was eluted with a linear salt gradient (0–1 M NaCl) in Tris buffer. SPP-2-active fractions were concentrated in an Amicon C-10 ultrafiltration unit (Amicon Corp., Danvers, MA, U.S.A.), and the small volume of the enzyme was rechromatographed on Superose 6. The fractions containing SPP-2 activity were stored at -70 °C and used for up to 4 weeks with no observed loss of processing activity.

Transcription of cloned genes, isolation of poly(A)⁺ mRNA from *Chlamydomonas* and translation *in vitro*

Plasmids pSP64 and pGMZ-3Z containing cDNA of genes encoding pSS and the precursor of the oxygen-evolving enhancer protein I (pOEE1) of *Chlamydomonas* respectively were transcribed using SP6 RNA polymerase as described (Su et al., 1992). The encoded precursor proteins, pSS and pOEE1, were synthesized from the capped transcriptions in a standard reticulocyte lysate translation system in the presence of [³⁵S]methionine.

For the synthesis of pea pSS, plasmid pT7-pS was transcribed by the method of Schnell et al. (1990). Translation was carried out as described above.

Poly(A)⁺ mRNA from *Chlamydomonas* was isolated from 2.5 × 10⁸ cells suspended in 4 ml of lysis buffer (0.4 M KCl, 0.1 M Tris/HCl, pH 7.4, 1% sodium laurylsarcosinate). RNA was prepared by three extractions with warm phenol/chloroform/3-methylbutan-1-ol (50:50:1, by vol.) and precipitation overnight at -20 °C with 2 vol. of cold ethanol. After pelleting at 10000 g and 4 °C for 10 min, the RNA was dried under vacuum, resuspended in 100 µl of ETS buffer (10 mM Tris/HCl, pH 7.5, 10 mM EDTA, pH 7.5, 0.2% sodium laurylsarcosinate) and 300 µl of dimethyl sulphoxide, and incubated for 5 min at 65 °C, followed by the addition of 2.6 ml of cold ETS buffer and 230 µl of 4 M KCl. Poly(A)⁺ RNA was adsorbed by affinity chromatography on poly(U)-Sephacrose 4B (Pharmacia) previously equilibrated with ETS buffer. The affinity column was washed first

with 10 bed vol. of ETS buffer and then with 10 bed vol. of ETS buffer containing 0.1 % formamide. The poly(A)⁺ RNA was eluted by 2.5 ml of 70 % formamide and 2.5 ml of ETS buffer. The eluted RNA was precipitated by the addition of 200 μ l of 8 M LiCl and 10 ml of cold ethanol. The pellet was resuspended in 0.2 ml of sterile double-distilled water and stored at -70°C as poly(A)⁺ mRNA. Both poly(A)⁺ mRNA and prL12 mRNA were translated as described above.

Organelle-free processing assay

The processing activity in a preparation of chloroplast stromal enzymes was assayed by measuring the conversion of radiolabelled precursor proteins into smaller-sized products. Incubation mixtures were composed of 1 vol. of buffer (50 mM Tris/HCl, pH 8.0) containing radiolabelled precursor proteins equivalent to 10^6 d.p.m. (about 0.5 μ l of reticulocyte translation mixture) and 1 vol. of chloroplast stromal extract. After incubation for 90 min at 25°C , the reaction was stopped by the addition of 1 vol. of sample buffer (6 % SDS, 4 % 2-mercaptoethanol, 6 % sucrose, 30 mM Tris/HCl, pH 7.6) followed by heating in boiling water for 5 min. Samples were analysed by SDS/PAGE. For a rough estimate of the molecular mass of the proteins, a set of marker proteins was run on each SDS/polyacrylamide gel. However, identity of two proteins or small differences in size were demonstrated exclusively by direct comparison of the migration distances on the same gel. After fluorography, the radioactive bands were excised from the dried gel and the radioactivity was measured by liquid-scintillation counting. Alternatively, to determine the ratio of precursor to processed protein in Figure 4, the radioactivity on the dried gel was quantified using a PhosphorImager (Molecular Dynamics).

For the studies of *in vitro* processing of light-harvesting complex II (LHCII) apoprotein precursors, the genes of which are not yet cloned, the translation products of poly(A)⁺ mRNA were incubated with the enzyme to be tested. By immunoprecipitation from the reaction mixture (Schmidt et al., 1984), radioactive products cross-reacting with LHCII antiserum (Michel et al., 1983) were isolated and analysed by SDS/PAGE as above.

Chloroplast importation assay

Importation of precursor proteins into chloroplasts of *Chlamydomonas* and pea was assayed as follows: 50 μ l of chloroplasts (1 mg/ml chlorophyll) and 50 μ l of translation mixture containing one of the radiolabelled precursor proteins were diluted to a final volume of 300 μ l in importation buffer (250 mM sorbitol, 50 mM Hepes buffer, pH 7.8) supplemented with 10 mM ATP and incubated for 30 min at 25°C in white light (Osram R 125, 300 W, distance 80 cm). The chloroplasts were reisolated by a first washing in importation buffer, treatment on ice for 30 min with 50 μ g/ml thermolysin in the presence of 1 mM CaCl_2 , then centrifugation through a cushion of 40 % Percoll in importation buffer for 5 min at 5000 g and 4°C , and three washings with importation buffer.

RESULTS

pSS and pOEE1 synthesized *in vitro* are imported into chloroplasts, processed to their mature form and correctly located in the chloroplasts

In order to study the question of whether there exist several enzymes to remove transit peptides from different precursor proteins, different highly labelled, but radiochemically pure precursor proteins from the same organism were required. In a

first experiment, pSS and pOEE1, both from *Chl. reinhardtii*, were used. To be sure that the precursor proteins synthesized *in vitro* indeed corresponded to their native proteins, their importation into isolated intact chloroplasts from *Chlamydomonas* was tested. After importation, the reisolated chloroplasts were treated with thermolysin, which degraded proteins adhering to the outer chloroplast membrane without affecting inner-membrane proteins (Joyard et al., 1987). Figure 1 shows that both radiolabelled substrates, pSS (21 kDa) and pOEE1 (30 kDa), were imported into isolated chloroplasts and processed to products that migrated with the same molecular masses as the mature native proteins SS and OEE1 (16 kDa and 26 kDa respectively). When these chloroplasts were separated into a soluble and a membrane fraction, the mature radioactive SS was found exclusively in the stromal fraction and OEE1 was located only in the membrane fraction, where the native proteins are expected to be found.

pSS and pOEE1 synthesized *in vitro* can be processed to their mature forms *in vitro*

A crude stromal extract was prepared from isolated *Chlamydomonas* chloroplasts. With this stromal extract, pSS was processed to mature SS. pOEE1, however, was only shortened to an intermediate-sized form, iOEE1, (Figure 2) by this stromal preparation. As OEE1 is an extrinsic protein located on the inner side of the thylakoid membrane, its maturation is expected to occur in a two-step process as found in pea (James et al., 1989; Mould et al., 1991). Indeed, a processing peptidase, which converted the plastocyanin importation intermediate into the mature form, has been solubilized from thylakoids of pea by a mild detergent (Kirwin et al., 1987). The enzyme could also be released from thylakoids by sonication (Hageman et al., 1986). In *Chlamydomonas*, for the second step of maturation of pOEE1

Figure 1 Importation of pSS and pOEE1 into isolated intact chloroplasts

pSS (a) and pOEE1 (b) were synthesized from pSP64 and pGMZ-3Z respectively (lane 1), and incubated with isolated intact chloroplasts as described in the Experimental section (lane 2). The chloroplasts were separated into membrane fraction (lane 3) and soluble fraction (lane 4). All samples were analysed by SDS/PAGE and autoradiography. Note that the intensity of the pOEE1 band in lane 3 is less than in lane 2 because of the loss of membrane material during disruption of the thylakoids by ultrasonication and washing of the membrane fraction.

Figure 2 Proteolytic processing of pSS and pOEE1 by stromal and lumen extracts

In vitro synthesized pSS (lane 1) and pOEE1 (lane 3) were incubated with stromal extract (lanes 2 and 4). When pOEE1 was incubated with thylakoid lumen extract which contained some stromal enzymes, mature OEE1 was formed (lane 5). The samples were analysed by SDS/PAGE and autoradiography.

Figure 3 Effect of pH on pOEE1 processing by crude stromal extract (○) and thylakoid lumen extract (□)

After incubation of pOEE1 with crude stromal extract at different pH values, samples were analysed by SDS/PAGE and autoradiography. The graphs show the relative ratio of radioactivity found in iOEE1 (for stromal extract) or OEE1 (for lumen extract) to that present in unprocessed pOEE1. In all determinations, the sum of radioactivity recovered from pOEE1 plus OEE1 or iOEE1 was 2×10^4 d.p.m. $\pm 5\%$.

Figure 4 Chromatographic separation of pOEE1- and pSS-processing enzymes from stromal extract

A crude stromal extract from *Chlamydomonas* was chromatographed on Superose 6 gel as described in the Experimental section. The eluted fractions were assayed with radiolabelled precursor pSS (a) or pOEE1 (b) as the substrate. The products were separated by SDS/PAGE. The autoradiogram is shown.

a protease located in the thylakoids is also necessary. In fact, when iOEE1 was incubated *in vitro* with 'crude stroma' and a membrane-free 'thylakoid lumen extract' (see the Experimental section), a new radioactive band corresponding in molecular mass to the mature native OEE1 appeared (Figure 2). Therefore, in *Chlamydomonas* also, proteins of the thylakoid lumen, such as pOEE1, which have to pass through not only the envelope membranes but also the thylakoid membrane, are processed in two steps: first by a stromal enzyme and then by an enzyme found in the chlorophyll-free supernatant obtained after homogenization of thylakoids in the French press and in the absence of detergents. Therefore this thylakoid protease seems to be either a soluble luminal protein or is loosely attached to the inner side of the thylakoid membrane.

Since in light, as a result of the proton-pumping activity of the photosynthetic electron-transport chain, the thylakoid lumen becomes acidic relative to the stroma, it was of interest to test the pH optima of the stromal and lumen processing enzymes. Figure 3 shows that the pH optima for pOEE1 processing by the crude stromal extract and by the thylakoid lumen extract are different.

Whereas the stromal protease, which is responsible for transforming pOEE1 to iOEE1, has its main activity at a pH about 7.5, the thylakoid lumen enzyme, producing the mature OEE1, has a pH optimum of about 5. If the protease for the second maturation step is indeed located in the interior of the thylakoids, it would be active mainly during illumination. The proton-pumping activity of the photosynthetic electron-transport chain would then ultimately not only be responsible for ATP production, but also for processing and hence for synthesis of an active form of a component of the electron-transport chain itself, namely OEE1, which is part of the oxygen-evolving complex of photosystem II.

The stromal peptidases for pOEE1 and pSS are not the same

As both precursor proteins, pOEE1 and pSS, are processed by the stromal extract, the question may be studied of whether they are cleaved by the same stromal peptidase. Therefore the crude stromal extract from *Chlamydomonas* was chromatographed on a Superose 6 column. By this gel filtration, soluble proteins could be separated according to their molecular mass and collected in a series of fractions. On the one hand, Figure 4a shows that two pSS-processing activities (SPP-1 and SPP-2) were separated as described previously (Su and Boschetti, 1993). SPP-1 processed pSS to an intermediate form iSS of about 19 kDa and its active peak was located in fraction 8 at a molecular mass of 340 kDa, whereas SPP-2 cleaved pSS to the mature form, SS, and its maximal activity was eluted in fraction 20 with a molecular mass of 90 kDa. On the other hand, when the same series of fractions was tested with pOEE1 as substrate, the main activity for production of the intermediate-sized iOEE1 did not coincide with the fractions containing SPP-1 or SPP-2, but was eluted with a molecular mass between those of SPP-1 and SPP-2 (Figure 4b). Quantification of the radioactivity with a PhosphorImager revealed fraction 14 as the most active, giving the highest ratio of iOEE1 to pOEE1.

A further indication that the enzymes SPP-1 and SPP-2 are different from the stromal protease that cleaves pOEE1 to iOEE1 comes from inhibitor studies. Tos-Phe-CH₂Cl and Tos-Lys-CH₂Cl, the most potent inhibitors of SPP-2 and SPP-1 (Su and Boschetti, 1993), had no effect on the activity of the pOEE1-processing enzyme (Figure 5). Obviously, stromal enzymes of different size and different sensitivity to inhibitors are responsible for removing the transit sequences from pSS and pOEE1 to form SS and iOEE1, the stromal forms of these proteins. It seems that several processing enzymes with different specificities are involved in protein importation into the chloroplast stroma.

However, when we incubated the *in vitro* translation products of poly(A)⁺ mRNA from *Chlamydomonas* in the same way as for pSS or pOEE1, and then immunoprecipitated the processed products with antiserum against an LHCII apoprotein (Michel et al., 1983), a processing activity for one of the pLHCII proteins was eluted in about the same fractions as SPP-2 (results not shown). Unfortunately, because of low amounts of pLHCII, further distinction of the pLHCII-processing activity from SPP-2 could not be obtained.

Protein importation into chloroplasts of *Chl. reinhardtii* is species-specific

Several groups have reported that a precursor protein from one organism can be processed *in vitro* by stromal extracts from different species, indicating closely related conserved processing enzymes. However, most of these observations have been limited to higher plants. In order to study whether the processing

Figure 5 pSS- and pOEE1-processing enzymes show different sensitivities to inhibitors

Precursor proteins pSS (a) and pOEE1 (b) were incubated under different conditions: lane 1, precursor protein only; lane 2, precursor protein incubated with crude stromal extract without inhibitors; lane 3, precursor proteins incubated with SPP-1 (corresponding to fractions 6–10 of Figure 4) and 1 mM Tos-Lys-CH₂Cl; lane 4, precursors incubated with SPP-2 (corresponding to fractions 20–24 of Figure 4) and 1 mM Tos-Phe-CH₂Cl. The samples were analysed by SDS/PAGE and autoradiography.

Figure 6 Importation of pSS from *Chlamydomonas* and pea into isolated intact chloroplasts from both organisms

pSS from *Chlamydomonas* and pea were synthesized *in vitro* and incubated with isolated chloroplasts from pea and *Chlamydomonas*. After importation, the reisolated chloroplasts were treated with thermolysin and analysed by SDS/PAGE and autoradiography. Lanes 1 and 4, pSS from *Chlamydomonas* and pea respectively, not incubated as controls; lanes 2 and 5, pSS from *Chlamydomonas* and pea respectively, imported into chloroplasts from *Chlamydomonas*; lanes 3 and 6, pSS from *Chlamydomonas* and pea respectively imported into pea chloroplasts.

Figure 7 *In vitro* processing of pSS from *Chlamydomonas* and pea by crude stromal extract from both species and by partially purified enzymes from *Chlamydomonas*

The radiolabelled precursor proteins (lanes 1 and 5) were incubated with crude stromal extract (lanes 2 and 6) or with partially purified enzymes SPP1 (Lanes 3 and 7) or SPP-2 (lanes 4 and 8). Autoradiograms after SDS/PAGE are shown.

enzymes of higher plants and green algae can recognize the same precursor as substrate, we synthesized pSS from *Chlamydomonas* and pea. In a first set of experiments, importation of these precursors into isolated chloroplasts was studied. Figure 6 shows that pSS from *Chlamydomonas* can be imported into pea chloroplasts, but cannot be processed to its mature form in pea chloroplasts (lane 3). The imported pSS can only be shortened to an intermediate form iSS* which, on the SDS/polyacrylamide gel, is slightly larger than iSS produced by SPP-1 of *Chlamy-*

domonas. These results confirm earlier findings (Mishkind et al., 1985). In contrast, pSS from pea can only be imported into chloroplasts from pea (Figure 6, lanes 5 and 6). In an importation assay with *Chlamydomonas* chloroplasts and pSS from pea, no radioactivity was found in thermolysin-treated reisolated chloroplasts. Obviously, pSS from pea is not recognized as a substrate by the chloroplast importation machinery of *Chlamydomonas*.

Also prL12 from spinach could not be imported into *Chlamydomonas* chloroplasts (results not shown).

Stromal peptidases for the precursor protein pSS are species-specific

In another set of experiments, *in vitro* synthesized pSS from *Chlamydomonas* and pea were tested for *in vitro* processing by stromal extracts from these organisms (Figure 7). pSS from *Chlamydomonas* can be cleaved to an intermediate product by a crude stromal extract from pea. It corresponds on SDS/PAGE to the intermediate product detected by the importation assay using isolated pea chloroplasts. Interestingly, pSS from pea was processed *in vitro* to an intermediate form iSS(pea) by the crude stromal extract from *Chlamydomonas* (Figure 7, lane 6), but it was not imported into intact chloroplasts from *Chlamydomonas*.

However, after separation of the processing enzymes, SPP-1 and SPP-2, by gel filtration of a crude stromal extract from *Chlamydomonas*, in the fractions containing the processing activity for *Chlamydomonas* pSS absolutely no processing of pea pSS could be detected (Figure 7, lanes 7 and 8). Therefore in *Chlamydomonas* and pea the stromal enzymes for processing pSS are specific for their endogenous substrates and do not cross-react with homologous precursors from the other species.

Also the heterologous prL12 from spinach was not processed *in vitro* by the stromal extract of *Chlamydomonas* (results not shown).

DISCUSSION

In chloroplasts of the green alga *Chl. reinhardtii* we have identified several stromal peptidases acting on the transit sequence of specific nuclear-encoded chloroplast precursor proteins, which *in vivo* are imported into the chloroplast. Two stromal peptidases, SPP-1 and SPP-2, cleaved pSS to an intermediate-sized form, iSS, and to the mature SS respectively. We have evidence that these enzymes work independently (Su and Boschetti, 1993). Now a pOEE1-processing enzyme which cleaved pOEE1 to its intermediate form, iOEE1, has also been found in the stromal extract of *Chlamydomonas*. These pSS- and pOEE1-specific enzymes were separated by gel filtration and therefore have different molecular masses. They differ also in their pH optima: SPP-1 and SPP-2 showed optimum activity at pH 8 and 9 respectively (Su and Boschetti, 1993), and the stromal peptidase for pOEE1 had its pH optimum at about 7.5. Furthermore, the enzymes can be distinguished by their different sensitivities to inhibitors. The activity of the stromal peptidase for pOEE1 was not affected by the serine protease inhibitors Tos-Lys-CH₂Cl and Tos-Phe-CH₂Cl which are the most potent inhibitors of SPP-1 and SPP-2 respectively. However, the same fractions containing SPP-2 might also contain processing activity for pLHCII.

Several groups have studied the importation of pOEE into chloroplasts (Kirwin et al., 1989; Ko and Cashmore, 1989; Cline et al., 1992). The results have shown that *in organello* both a stromal and a thylakoid peptidase are involved in the maturation of precursors of OEE1 and other proteins located in the thylakoid lumen. An exception might be the *in vivo* maturation of the thylakoid lumen protein plastocyanin, the precursor of which was found to be directly processed by a thylakoid peptidase

(Bauerle and Keegstra, 1991; Bauerle et al., 1991). All these data come from studies with several higher plants; targeting of thylakoid lumen proteins in green algae is less well studied. In the present report, processing of the precursor protein pOEE1 from *Chl. reinhardtii* was analysed by importation into isolated chloroplasts and *in vitro* by organelle-free processing. pOEE1 could be imported into chloroplasts from *Chlamydomonas*, processed to mature OEE1, and correctly located in the thylakoid membrane fraction. Whereas in importation assays an intermediate-sized protein, iOEE1, was barely detectable, *in vitro* pOEE1 was processed in two steps. When pOEE1 was incubated first with stromal extract, iOEE1 was formed and could be further processed to the mature OEE1 by a thylakoid lumen extract. Therefore our experiments with this alga support the facts obtained for higher plants about processing of thylakoid lumen proteins: pOEE1 is imported into the stroma, processed to iOEE, then translocated across the thylakoid membrane, and further processed to the mature OEE1 in the thylakoid lumen by a soluble protease.

It has been suggested that a stromal peptidase can process analogous precursor proteins from different species, such as pea, wheat and spinach (James et al., 1989; Abad et al., 1991; Oblong and Lamppa, 1992). This might be due to some homologies spread over the entire transit sequence of higher-plant importation proteins. However, comparison of transit sequences of pSS from pea and *Chlamydomonas* shows only low identity in the N-terminal part and no similarity in the 13 amino acids preceding the correct cleavage site of the algal protein (Mishkind et al., 1985). Indeed, here we demonstrate that, in *Chlamydomonas*, importation and processing of pSS are species-specific. Although pSS from *Chlamydomonas* can be imported into chloroplasts from pea, it is not correctly processed to SS, whereas pSS from pea cannot be imported at all into chloroplasts from *Chlamydomonas*. *In vitro*, pea pSS was not correctly cleaved by crude stromal extract from *Chlamydomonas*, but only to an intermediate-sized form. This incorrect protease activity was not due to an aberrant reaction of the endogenous pSS-processing enzymes, as the pSS-specific stromal peptidases, SPP-1 and SPP-2, from *Chlamydomonas* did not accept pea pSS as a substrate. All these results indicate that pSS-processing enzymes in *Chlamydomonas* are species-specific.

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