Deletion of the Carboxyl-terminal Portion of the Transplant Peptide Affects Processing but Not Import or Assembly of the Small Subunit of Ribulose-1,5-bisphosphate Carboxylase*

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Import of the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase into the chloroplast has been proposed to involve two proteolytic cleavages which convert the 20-kDa precursor (pSSU) into the mature 14-kDa subunit (SSU) via an 18-kDa intermediate. A deletion mutant (PSd48/57) of pSSU which lacks 10 amino acids in a conserved region in the carboxyl-terminal portion of the transit peptide is converted into a series of 16-18-kDa polypeptides in addition to the mature 14-kDa SSU when imported into isolated pea chloroplasts. We examined import and processing of this mutant pSSU to determine whether the 16-18-kDa SSUs undergo further maturation in the chloroplast stroma to yield 14-kDa SSU. The ratio of incorrectly processed to 14-kDa SSU is stable up to 60 min following import. This indicates that processing of PSd48/57 involves a single proteolytic cleavage which occurs during or immediately following transit across the chloroplast envelope. The carboxyl-terminal portion of the transit peptide confers either sequence specificity for the processing protease or provides a three-dimensional structure necessary for consistent cleavage at the mature amino terminus of SSU. Incorrectly processed SSUs were incorporated into the holoenzyme demonstrating that removal of the entire transit sequence is not necessary for assembly of the holoenzyme.

Import of the small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco, EC 4.1.1.39) into the chloroplast is mediated by the transit peptide, a sequence at the amino-terminal end of the precursor protein (1). We recently reported (2) that deletions in each of three regions thought to constitute a common framework in chloroplast transit peptides (1, 3) have varying effects on both transport and processing of small subunit precursor (pSSU) imported into isolated pea chloroplasts. Of particular interest is a series of mutants in which portions of a conserved region in the carboxyl-terminal portion of the transit peptide have been removed. One deletion mutant (PSd48/57) lacks 10 amino acids in the transit peptide immediately adjacent to the amino terminus of the mature protein but is imported as efficiently as wild-type pSSU (4). Approximately 25% of imported PSd48/57 is processed into a polypeptide identical in size (14 kDa) to wild-type SSU. The remainder is recovered as a series of incorrectly processed SSUs, most of which have a molecular mass of between 16 and 18 kDa (4).

We have now examined import of this mutant pSSU in more detail to determine if the incorrectly processed SSUs are intermediates in a multistep or processive reaction which normally produces mature length SSU. We have also assayed subsequent assembly of imported SSUs into the holoenzyme. We find no evidence for further processing of the 16-18 kDa SSUs following import and discuss this result in the context of current hypotheses about proteolytic maturation of pSSU during import into the chloroplast.

MATERIALS AND METHODS

Construction of the deletion mutant PSd48/57 (4), transcription of plasmid DNA with SP6 polymerase (2), translation of synthetic mRNAs in rabbit reticulocyte lysates, and determination of radioactivity present in gel strips have been described (4). Each import reaction contained an approximately equal molar quantity of precursor as determined from the number of methionine residues in each precursor and the radioactivity incorporated into wild-type and PSd48/57 pSSUs synthesized in individual translation reactions (4). Transport experiments with isolated pea chloroplasts were performed as described by Wasmann et al. (5). Aliquots of the uptake reaction were taken at 0, 1, 3, and 5 min, or at 0, 15, 30, and 60 min, and added to 2 ml of sorbitol/Hepes (4°C) containing 400 nM nigericin to inhibit further transport (6). Samples were kept in the dark and at 4°C during subsequent steps. Intact chloroplasts were immediately reisolated without protease treatment (except where indicated, Fig. 3) by pelleting through 40% Percoll. Soluble protein extracts were prepared from lyed chloroplasts by centrifugation as described (5). Samples of soluble protein from a volume equivalent to 4 μg of chlorophyll in each lystate were prepared for SDS-PAGE as described by Laemmli (7) or nondenaturing PAGE (Laemmli buffers without SDS).

Two-dimensional (nondenaturing/SDS) PAGE for analysis of SSU assembly into the holoenzyme was performed as follows. Gels containing soluble proteins separated by nondenaturing PAGE (6% polyacrylamide) were stained for 1 min (0.1% (w/v) Coomassie blue, ethanol/acetic acid/H2O (45:5:49, v/v/v)), destained for 3 to 5 min in ethanol/acetic acid/H2O (20:4:74, v/v/v), and rinsed for 15 min in distilled H2O. The stained bands containing rubisco holoenzyme were excised and incubated in 50 μl of SDS sample buffer (7) for 15 min at room temperature. The sample buffer was removed, and the gel strips were incubated at 60°C for 1 h. Labeled proteins in the gel strips were separated on SDS-polyacrylamide (15%) gels and visualized by autoradiography. Quantitative analysis of the fluorograms was performed using a GS 300 densitometer (Hoefer Scientific Instruments). RESULTS

We have shown previously (4) that incubation of PSd48/57 pSSU with isolated chloroplasts for 30 min produces a
Fig. 1. Short term import of wild-type (wt) and Psd48/57 pSSUs by isolated pea chloroplasts. Soluble chloroplast protein samples were prepared from aliquots taken at 0-, 1-, 3-, and 5-min intervals during an import experiment. Labeled proteins were resolved by SDS-PAGE to show total SSU polypeptides imported (A) or by non-denaturing PAGE to assay assembly of imported SSU into the holoenzyme (holo) (B). A photograph of the fluorogram is shown. Timepoints (in minutes) are indicated in the lower margin. The amino acid sequences of the wild-type and Psd48/57 transit peptides are shown at the bottom of the figure.

### Table I

<table>
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<tr>
<th>Wild-type mature pSSU</th>
<th>Truncated pSSU</th>
<th>Mature pSSU</th>
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<tbody>
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<td>60</td>
<td>754</td>
<td>68</td>
<td>375 (24)</td>
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</table>

*ND, not determined.

Radioactivity (counts/min above background) incorporated into mature, truncated, and intermediate length (16-18 kDa) SSUs during short term (0-5 min) and long term (0-60 min) time-course experiments with wild-type and Psd48/57 pSSU.

Radiolabeled proteins were identified by fluorography following SDS-PAGE. Gel strips containing the labeled proteins were excised and prepared for liquid scintillation counting as described under "Materials and Methods." The percentage of the total radioactivity recovered in mature length SSU for Psd48/57 is indicated in parentheses.

of the radioactivity present in the mature and 16-18-kDa SSU polypeptides (Table I). Approximately 60% of the SSU recovered after import of the wild-type pSSU migrated with the holoenzyme. In samples of chloroplasts incubated with Psd48/57 pSSU approximately 45% of the total radioactivity recovered following import migrated with the pea holoenzyme. Assembly of imported Psd48/57 pSSU proceeded with kinetics similar to that of wild-type SSU (Fig. 1B).

To determine whether final maturation of incorrectly processed SSU can occur following import into the stroma, we examined the relative proportion of intermediate to mature length SSU polypeptides produced during longer incubations. Import of both wild-type and Psd48/57 pSSU was essentially complete after 15 min (Table I). We found no evidence that incorrectly processed SSU underwent further proteolysis during the course of the incubation (Fig. 2A). Densitometric analysis of the fluorograms showed that 16-18-kDa, mature, and truncated SSUs contributed approximately 77, 20, and 3%, respectively, to the total peak area at the 15-, 30-, and 60-min timepoints. These results agreed with those obtained by direct determination of the radioactivity present in the imported SSU polypeptides (Table I). Radioactivity recovered in gel strips containing the holoenzyme accounted for approximately 60 and 45%, respectively, of the total radioactivity in wild-type and Psd48/57 SSU (both mature and 16-18-kDa SSUs). An additional band at a position corresponding to that of unassembled SSU (5) was observed at the 0 timepoint in wild-type incubations and at later timepoints in the incubations with Psd48/57 pSSU (asterisk, Fig. 2B).

An additional experiment was conducted to determine whether incorrectly processed SSU generated during import of Psd48/57 pSSU could be assembled into the holoenzyme. Soluble protein samples from 30-min incubations with wild-type or Psd48/57 pSSU were separated first by non-denaturing PAGE followed by SDS-PAGE to examine the size distribution of polypeptides incorporated into the holoenzyme. When labeled proteins associated with the holoenzyme were resolved alongside samples of the chloroplast lysate, a single labeled species was found in samples from chloroplasts which had been incubated with wild-type pSSU (Fig. 3). In contrast, 16-18-kDa as well as mature 14-kDa SSUs were resolved in the holoenzyme sample from the transit peptide mutant (Fig. 3), indicating that incorrectly processed SSUs had assembled...
**Processing of Small Subunit Precursors**

**Fig. 2.** Long term import of wild-type (wt) and PSd48/57 pSSUs. Samples were prepared from aliquots taken at 0-, 15-, 30-, and 60-min intervals during an import experiment. **A and B show total SSU imported and SSU assembled into the holoenzyme (holo), respectively, as described in Fig. 1.** A photograph of the fluorogram is shown. Timepoints (in minutes) are indicated in the lower margin. An asterisk in the right margin indicates the position of unassembled SSU resolved by nondenaturing PAGE (5). The positions of the precursors are indicated in the left margin.

**Fig. 3.** A comparison of SSU present in the chloroplast lysates with SSU assembled into the holoenzyme. Soluble protein samples were prepared from protease-treated chloroplasts following a 30-min import experiment. Labeled proteins in the holoenzyme band were resolved by two-dimensional PAGE as described under “Materials and Methods” alongside samples of the total soluble protein recovered from the chloroplast lysates. A photograph of the fluorogram is shown. **wt, wild-type pSSU; PSd48/57, the carboxy-terminal transit peptide deletion mutant.** A longer exposure of the PSd48/57 lanes is shown. **i, imported SSU; a, SSU assembled into the holoenzyme. The position of the mature SSU is indicated in the left margin. The position of the truncated SSU is indicated by the open arrowhead in the right margin.**

pSSU inhibits processing at the mature amino terminus (Mmt, see Fig. 1) and produces an 18-kDa SSU when the chemically modified pSSU is incubated with processing protease in vitro (8). Mishkind et al. (9) showed that incorrect processing of Chlamydomonas pSSU by pea and spinach chloroplasts during import generates an 18-kDa SSU which is cleaved in a conserved region (Pro26-Lys31, Refs. 1 and 3) in the central portion of the transit peptide. Together these data have been interpreted as evidence that pSSU undergoes an initial cleavage in the central conserved region in the transit peptide followed by a second processing step at the mature amino terminus of SSU.

More recent evidence suggests that processing involves a single proteolytic cleavage whose specificity is determined by a conserved region at the carboxy-terminal end of the transit peptide. Reiss et al. (2) showed that deletion of 10 amino acid residues (Pro26-Gly25, see Fig. 1) which contain the putative first processing site in the transit peptide has little effect on import and generates only properly matured SSU. Deletions in the carboxy-terminal portion of the transit peptide clearly interfere with both binding of precursor to the chloroplast envelope (10) and with correct processing of pSSU during import into the chloroplast (2, 4, 10). The apparent low efficiency of import of PSd48/57 compared to wild type (10) is easily ascribed to the presence of incorrectly processed as well as mature length SSUs (4); taking them into account, import of this deletion mutant is equivalent to that of wild type. Similar results have been reported with the precursor to the mitochondrial enzyme ornithine carbamyltransferase (11). Deletion of 9 amino acids in the transit peptide adjacent to the amino-terminal end of the mature protein (analogous to PSd48/57) abolishes correct processing of ornithine carbamyltransferase without affecting transport.

**DISCUSSION**

Two conserved regions in the SSU transit peptide have been implicated in processing steps which convert the 20-kDa pSSU into the mature 14-kDa polypeptide inside the chloroplast. Robinson and Ellis (8) demonstrated that pSSU is converted to SSU via an 18-kDa intermediate when in vitro synthesized pSSU is incubated with a partially purified protease obtained from pea chloroplasts. Carboxymethylation of

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**TABLE I**

<table>
<thead>
<tr>
<th>Precursor</th>
<th>wt</th>
<th>PSd48/57</th>
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<tbody>
<tr>
<td>pSSU</td>
<td>i</td>
<td>a</td>
</tr>
<tr>
<td>SSU</td>
<td>i</td>
<td>a</td>
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**Fig. 1.** SSU positions resolved by nondenaturing PAGE (5). The positions of the precursors are indicated by the asterisk. The position of unassembled SSU resolved by nondenaturing PAGE (5). The positions of the precursors are indicated by the asterisk.
single proteolytic cleavage which occurs during or immediately following transit across the chloroplast envelope. Other bonds in the transit peptide or mature SSU sequence are hydrolyzed if this region has been removed (e.g. PSd48/57), altered by chemical modification (8), or if the amino acid sequence of this region is not closely related to the consensus sequence (e.g. Chlamydomonas pSSU, Ref. 9). The 18-kDa polypeptide which is thought to represent the product of the putative first processing step is apparently the result of cleavage at a preferred alternative site in the transit peptide sequence. Indeed, the first polypeptide species smaller than pSSU following import of PSd48/57 is approximately 18 kDa (Figs. 1A and 2A).

Both mature length and incorrectly processed SSU assembled rapidly into the holoenzyme. Assembly may have prevented further maturation of incorrectly processed SSUs. However, higher molecular weight SSUs which were apparently discriminated against in holoenzyme assembly were still present in the chloroplast lysates at the 30-min timepoint (Fig. 3). This argues against the possibility that the mature amino terminus of SSU is inaccessible to proteolytic processing due to association with the holoenzyme. Together these results are consistent with the hypothesis that pSSU processing involves a single proteolytic event. Site-directed mutagenesis of the carboxyl-terminal region of the transit peptide sequence should provide further information about the role of individual residues in specifying correct processing of pSSU by the chloroplast.

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REFERENCES