A processing intermediate of a stromal chloroplast import protein in *Chlamydomonas*

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Proteins synthesized in the cytoplasm and destined for importation into the chloroplast across the double envelope membrane contain an N-terminal transit sequence which upon import is cleaved off by a stromal-processing peptidase. Since for stromal-residing proteins no intermediates have ever been found *in vivo*, it is assumed that precursor proteins are cleaved to the mature size by one proteolytic event which occurs immediately after translocation across both envelope membranes. During import of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) into isolated chloroplasts of *Chlamydomonas* we identified an intermediate-sized product, called iSS. It might be identical to a previously described iSS obtained *in vitro* by a partially purified soluble chloroplast protease [Su and Boschetti (1993) Eur. J. Biochem. 217,

1039–1047]. The kinetics of the formation of iSS in chloroplasts suggest that pSS is processed to the mature small subunit (SS) not by one, but by two steps via this intermediate product. Since, after an induction period, the ratio of iSS/SS was constant under various experimental conditions of import, the formation of iSS was considered not to be a side-reaction. The location of iSS in the intermembrane space of the envelope, as suggested by protease treatment of chloroplasts, questions the one-step translocation mechanism of precursor import into chloroplasts.

Key words: *Chlamydomonas reinhardtii*, isolated chloroplasts, protein import, small subunit ribulose-1,5-bisphosphate carboxylase, two-step processing.

INTRODUCTION

A great number of chloroplast proteins are synthesized as precursor proteins containing an N-terminal transit sequence which is necessary and sufficient for their post-translational import into the chloroplast stroma. Import experiments with isolated chloroplasts demonstrated that in higher plants the import of a particular precursor protein into the stroma was competitively inhibited by different precursor proteins or precursor analogues [1–3]. This implied that the examined proteins enter the chloroplast by a common recognition and translocation pathway. At first the precursor proteins associate with a proteinaceous receptor on the surface of the outer envelope membrane [4,5]. This binding step requires the hydrolysis of low concentrations of both ATP and GTP in the cytoplasm or in the intermembrane space [6–8]. The import step across the protein translocation complex requires the hydrolysis of ATP in the stromal compartment [9,10]. Whether the translocation, which most probably occurs at contact sites of the inner and outer membranes, is a single-step process or can be dissected in two steps, is still discussed [11–13]. During or immediately after import the transit sequence is cleaved off by a stromal processing peptidase (SPP). Thereby stromal-residing proteins are processed to mature-sized proteins, while luminal proteins are cut to an intermediate-sized stromal form which is further processed by a second protease only upon transfer into the thylakoid lumen [14–16]. A cDNA encoding a metalloproteinase assumed to be the SPP was isolated from pea [17]. Over-expression in *Escherichia coli* led to an active proteinase. *In vitro* processing of 11 precursor proteins of various origins and destined for different chloroplast compartments suggested that in higher plants a general stromal enzyme was responsible for the cleavage of the precursor proteins in an endoproteolytic one-step process [18].

Recent experiments, in contrast, revealed the existence of several different SPPs in the unicellular green alga *Chlamydomonas reinhardtii*. Each type of such SPP specifically cleaved one single precursor protein or a set of several precursor proteins [19,20]. For the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS), two specific SPPs could be identified and separated: one of them, SPP-2, was present in the stroma with relatively high activity and processed pSS *in vitro* to the mature form SS. The second, SPP-1, produced the intermediate-sized form iSS by cleaving pSS at the N-terminal side of methionine-25 situated close to the middle of the transit sequence containing 46 amino acids [21]. As SPP-2 cleaved both pSS and iSS *in vitro* very efficiently to the mature SS, this enzyme was considered to be responsible for the *in vivo* processing, while the physiological role of SPP-1 remained obscure.

In the present paper we present results on the formation of iSS under more physiological conditions, i.e. during import of pSS into isolated, intact chloroplasts. It seems that in *Chlamydomonas*, the stromal protein SS is processed in two steps, whereby the detectable intermediate-sized iSS might be located in the intermembrane space of the chloroplast envelope. This two-step processing is distinctly different from the processing of luminal proteins where the second cleavage step occurs at the thylakoid membranes.

MATERIALS AND METHODS

Organism and cultivation

All experiments were performed with the cell-wall deficient mutant *cw-15* of *Chlamydomonas reinhardtii* which was obtained as strain CC-277 from Dr. E. Harris (Chlamydomonas Genetics Centre, Duke University, Durham, NC, U.S.A.). Highly syn-

Abbreviations used: SPP, stromal processing peptidase; SS, small subunit of ribulose-1,5 bisphosphate carboxylase/oxygenase (EC 4.1.1.39); pSS, precursor of SS; iSS, intermediate form of SS; L1, L6, L11, hour 1, 6, 11 during the light period of synchronously cultured cells.

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chronous cultures in high salt medium of Sueoka [22] were obtained in cylindrical 1-litre vessels by applying a light/dark cycle of 12/12 h under constant temperature of 25 °C and gentle aeration with about 4% CO₂ in air as described previously [23].

Protein import into isolated chloroplasts

For chloroplast isolation the method of Mendiola-Morgenthaler and colleagues [24] with the modifications described by Su and Boschetti [19] was used. Synthesis and radioactive labelling of pSS with [³⁵S]methionine in a wheat germ translation system was described by Rothen and colleagues [25]. Chloroplast protein import, and the enzymatic digestion of pSS *in vitro* by stromal extracts were performed according to Su and Boschetti [19]. Electrophoretic analysis, autoradiography and evaluation of methionine incorporation by PhosphorImager were done as mentioned in Su and Boschetti [21].

N-terminal amino acid sequence analysis

After SDS polyacrylamide gel electrophoresis the proteins were transferred by the semi-dry method to PVDF membranes (ProBlott Membranes, Applied Biosystems, Paul Bucher, Basel). Transfer buffer: 50 mM boric acid/NaOH, pH 9.0, and 0.05 % SDS in water/methanol (4:1, v/v). The membranes were stained with Coomassie Brilliant Blue. Radioactive spots were cut out and N-terminal amino acid sequence analysis was carried out as described by Su and Boschetti [21].

Trypsin and thermolysin treatment of chloroplasts

After 20 min of incubation the protein import assays were centrifuged (5 s at 2500 g) and the pellet resuspended in 300 µl import buffer [19]. Maximally 15 µl of a protease solution (thermolysin or trypsin; Sigma), giving the desired final concentrations, were added. After 30 min on ice with gentle agitation the samples were centrifuged again and the pellet resuspended in 40 % Percoll in import buffer. After centrifugation (3 min, 2500 g) the pellet was transferred in a new Eppendorf tube, resuspended again in 300 µl import buffer, centrifuged and the pellet solubilized for electrophoresis on a 15 % SDS polyacrylamide gel.

RESULTS

Formation, identification and characterization of iSS in isolated chloroplasts

Starting from a cloned *rbcS*-2 cDNA, a transcript was produced *in vitro* and subsequently translated by a wheat germ extract containing [³⁵S]methionine into the highly labelled precursor protein pSS (Figure 1a, lane 1). By light-driven protein import into isolated chloroplasts this pSS became processed not only to its mature form SS, but also to low amounts of an intermediate-sized form iSS which was detectable only under high labelling conditions. Until now, in intact cells such an intermediate form has escaped detection even after protein labelling with [³⁵S]methionine and immunoprecipitation, probably because of a too low detection limit. In previous work, *in vitro* treatment of pSS with a stromal fraction enriched in the enzyme SPP-1 resulted also in the production of an intermediate-sized iSS with methionine-25 as the new N-terminal amino acid [19]. Electrophoresis on SDS polyacrylamide gel demonstrated that the molecular masses of the *in vitro* produced iSS and the iSS from protein import (*in organello*) were identical within the resolution limits of less than six amino acids (Figure 1a, lanes 2 and 3). Determination by

Figure 1 Characterization and localization of the processing intermediate iSS formed during import of pSS into isolated *Chlamydomonas* chloroplasts

(a) Characterization: autoradiogram after electrophoresis on SDS polyacrylamide gel. Lane 1: [³⁵S]-labelled pSS synthesized in wheat germ extract, starting material for protein import; lane 2: solubilized, washed chloroplasts after import of pSS; lane 3: pSS processed *in vitro* with a stroma fraction enriched in SPP-1; lane 4: pSS processed *in vitro* with an enriched SPP-2 preparation. (b) Localization of iSS in the chloroplast. After import of pSS the chloroplasts were disrupted and separated in supernatant (lane 5) and washed membranes (lane 7). Lane 6: solubilized chloroplasts.

Edman-degradation of the positions of [³⁵S]methionine in the amino acid sequence of iSS, which was recovered from one- or two-dimensional SDS gels by blotting on nylon membranes, revealed that the N-terminus of the *in organello*-iSS coincided with position 26, i.e. with the amino acid just following methionine-25 (Figure 2). The main radioactivity peak at sequencing cycles 21 and 22 must coincide with the two methionines –46 and –47 which mark the N-terminus of mature SS. The tailing of such peaks is a common phenomenon and due to sequence lag. Taking into account that at each sequencing cycle repetitive yield is about 94 % and radioactivity after 21 cycles is 230 cpm, an eventual N-terminal methionine should result in an initial peak of about 840 cpm. Obviously, *in organello*-iSS contains no N-terminal methionine. The slightly enhanced radioactivity in cycles 1 and 2 is most probably due to residual radioactive

Figure 2 N-terminal sequence analysis of iSS recovered from isolated chloroplasts after import of [³⁵S]methionine-labelled pSS

After import, the chloroplasts were solubilized, the proteins separated by SDS gel electrophoresis and transferred to PVDF-membranes. Pieces of membranes containing iSS were subjected to Edman degradation for 27 cycles. (a) The radioactivity released at each cycle was determined by liquid scintillation counting. (b) The amino acid sequence of pSS [34]. Vertical bar: start of mature SS with N-terminal methionine-46; small arrow: *in vitro* cleavage site for SPP-1 [21]; heavy arrow: N-terminus of *in organello*-iSS. (c) SPP-1 cleavage site divides the transit sequence of pSS from *Chlamydomonas* in two parts with similar amino acid sequences. Double bars: *in vitro* cleavage sites of SPP-1 and SPP-2; single bars: separation into segments to facilitate sequence comparison; + : amino acids repeated in the two sequence parts; ● : conservative amino acids; ↔, — : any amino acid.

contamination washed off from the nylon membrane during the first sequencing steps. The results show that the *in organello*-iSS differs from the iSS produced *in vitro* by SPP-1 only by the absence of the N-terminal methionine. Therefore, both iSS might originate from pSS by the action of the same processing enzyme SPP-1, but within the chloroplast the N-terminal methionine might be sensitive to exoproteolysis. The occurrence of such type of aminopeptidases in pro- and eukaryotic cells is well known [26–28]. However, it cannot be excluded that *in organello*-iSS is the direct product of a still unknown processing enzyme.

When after import chloroplasts were broken and the homogenate separated into a supernatant fraction and washed membranes, iSS together with SS were found exclusively in the soluble fraction, while the pelleted fraction contained pSS only (Figure 1b). Obviously, after having passed the chloroplast envelope, iSS and SS became detached from membranes.

Time course of formation of iSS in chloroplasts

The formation of iSS in intact chloroplasts raised again the question about its physiological significance. To decide whether iSS could be an intermediate product, or represented merely a by-product of the processing of pSS, we determined the kinetics of the appearance of iSS during import of pSS into chloroplasts. These chloroplasts were isolated from highly synchronized *Chlamydomonas* cells harvested in the middle of the light period, i.e. 6 h after onset of light (L6). Import was started by the addition of radiolabelled pSS and stopped by chilling on ice, followed by reisolation of intact chloroplasts by centrifugation. Figure 3a shows that about 1 min after starting, SS was easily detectable and iSS became discernible. Later in the course of the import experiment, the amount of labelled SS markedly increased while an increase of iSS was less obvious, especially due to variable yields upon reisolation of chloroplasts.

To determine the kinetics of the appearance of the two processing products, their relative amounts were quantified at different time points during import by measuring the radioactivity in the protein bands of an SDS/PAGE with a PhosphorImager. Figure 3b represents the quantitative evaluation of a similar experiment as shown in Figure 3a, but with the incubation time extended to 80 min, when chloroplasts began to disintegrate. In order to compare the data from different assays and to compensate for the different degree of loss of chloroplast material in individual assays due to chloroplast disintegration, the radioactivity in SS and in iSS was calculated relative to that in pSS still adsorbed to the envelope of the reisolated, intact and washed chloroplasts. The shapes of the curves in Figure 3b demonstrated that the formation of iSS and SS as well as their relative amounts followed roughly the same kinetics, except for the very beginning of protein import. After an induction period of about 10 min a steady state was attained when the ratio of iSS/SS became low and constant. The early peak in the iSS/SS ratio may be explained by fast formation of iSS and subsequent reduction of its accumulation rate relative to SS which is formed at a slower initial rate, but continuously. The lowering of iSS-accumulation to a steady state might be expected when some of the iSS is subjected to further reactions, eventually by processing to SS with SPP-2 as soon as iSS has reached a substantial substrate level. Therefore, the kinetics shown in Figure 3b are compatible with iSS being the product of an independent side-reaction or with iSS representing a facultative or even obligatory intermediate in the maturation of pSS to SS. In fact, some authors previously hypothesized on the possibility that maturation of SS could occur in two steps [31,33]. Here we provide the first demonstration

Figure 3 Time course of iSS formation in chloroplasts

Import of pSS into isolated chloroplasts was stopped at the time indicated. Chloroplasts were washed, solubilized and the proteins separated by SDS/PAGE and analysed by fluorography. (a) Radioactivity pattern: T: pSS in wheat germ translation system. (b) Quantitative evaluation of the kinetics of iSS formation: In a similar experiment as in Figure 3a the radioactivity of the firmly bound pSS, or iSS and SS in washed chloroplasts was quantified on a SDS/PAGE by means of a PhosphorImager. The radioactivity was expressed relative either to the externally bound pSS or to the imported SS. ■, SS/pSS; ●, iSS/pSS; ▲, iSS/SS.

that in chloroplasts such an intermediate for a stromal protein is really formed. This interpretation would imply that during the steady state, the processing of pSS to iSS is the rate-limiting step of SS formation. During the induction period of such an import experiment, however, when iSS transiently accumulates relative to SS, the cleavage of iSS to SS would be rate-limiting. Such behaviour can be explained by a slow activation of the processing enzyme or of the transport of iSS to the site of its processing to SS.

Formation of iSS under different experimental conditions

If conditions could be found under which the formation of SS would become independent from iSS, the two-step mechanism would be wrong. Therefore we measured the formation of import products under different experimental conditions by varying different parameters, as, e.g., ATP-concentration and amount of pSS in the assay, the physiological state of chloroplasts and light intensity during import.

Concentration of ATP and amount of pSS

Figure 4a shows that import of pSS and formation of SS in chloroplasts of L6 was highly stimulated by addition of ATP to the assay, reaching a maximum at the non-physiological high ATP concentration of 10 mM. At higher concentrations, i.e. 20 mM, import became almost completely inhibited. Even at high import rates, however, iSS did not accumulate and the ratio iSS/SS remained constant over the whole concentration range tested. The inconsistency of the curves in Figure 4a at the low ATP concentrations of 0 and 1 mM is explained by difficulties in

Figure 4 ATP-dependence and light-dependence of import and processing of pSS in isolated chloroplasts

(a) After 20 min of import in the presence of different concentrations of ATP chloroplasts were washed, solubilized and subjected to SDS/PAGE. The radioactive proteins were quantified with a PhosphorImager. (b) Otherwise identical import assays were incubated at different light intensities, and the products were analysed as in Figure 4(a). On the y-axes the ratio of the import products relative to externally bound pSS or to SS is represented. ■, SS/pSS; ●, iSS/pSS; ▲, iSS/SS.

measuring the vanishing amounts of iSS and SS produced under these conditions. Also by increasing the amounts of pSS in the import assays the ratio iSS/SS did not alter (not shown). This indicates that formation of iSS is not due to a subordinate processing side-reaction that becomes the recourse if normal processing is limited.

Cell cycle dependence

Highly synchronous cell cultures of *Chlamydomonas* were obtained by cultivation under a light-dark regime [23]. At different time points of the cycle cells were harvested and chloroplasts, which hence were in different physiological conditions, were isolated and used for import experiments. Although the import capacity of chloroplasts from cells harvested at the beginning, in the middle and towards the end of the light period (L1, L6, L11) were different (Su, Q., Schild, C. and Boschetti, A., unpublished work), the ratio of iSS/SS remained about constant (not shown).

Light intensity during import

Import of pSS into isolated chloroplasts is light dependent. Figure 4b shows that import into chloroplasts from cells harvested at L6 could be stimulated by increasing light intensity

Figure 5 Sensitivity of iSS to proteases

pSS was imported into chloroplasts which then were treated with different concentrations of (a) thermolysin or (b) trypsin as indicated in the Materials and methods section. After reisolation of intact chloroplasts by centrifugation through Percoll the radioactivity in pSS (●), iSS (▲), and SS (■) was quantified as in Figure 3. The radioactivity of each individual protein band was first related to the sum of the radioactivities in all three bands of this sample. Then this ratio was expressed as percentage of the ratio calculated from the untreated sample.

until to about $5 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, when maximal import rate was achieved under our experimental conditions. It also shows that the ratio iSS/SS was almost independent from the level of energetization of the chloroplasts.

All these experiments taken together suggest that the formation of iSS and SS are not independent reactions.

Localization of iSS in the chloroplast

In Figure 1b we demonstrated that iSS is present in the chloroplast as a soluble component. Of the three chloroplast compartments containing water-soluble components only the stroma and the intermembrane space of the envelope are relevant in our context. The inter-envelope space is indeed on the way between the binding site of pSS on the outer envelope membrane and the final location of SS in the stroma. To discriminate between stromal and intermembranal localization of iSS a proteolytic approach was used.

Thermolysin is known to act on proteins associated with the outer surface of the organelle, but not to penetrate the outer envelope membrane. Trypsin, however, is able to enter the outer membrane and to develop its proteolytic activity also on proteins of the intermembrane space and on the surface of the inner envelope membrane [29,30]. We imported radiolabelled pSS into intact chloroplasts and treated them with increasing concentrations of thermolysin or trypsin. Figure 5 reveals different effects of these proteases on the import products. While thermo-

lysin degraded the externally bound pSS, but not iSS nor SS, trypsin completely digested pSS and iSS, leaving only SS intact which is known to be localized in the stroma. Even at the highest concentrations of thermolysin or trypsin we used, iSS plus SS or SS remained fully protected. Therefore, the differential degradation of iSS was not due to different specific activities of the proteases, but to a different accessibility of pSS, iSS and SS. These data suggested that in *Chlamydomonas* iSS, which is a processing intermediate of pSS, is located in the intermembrane space.

DISCUSSION

In the past, based on experiments with heterologous import systems or on proteolytic digestions of precursor proteins with stromal extracts, several authors hypothesized about a two-step processing of chloroplast import proteins. Indeed, a closer examination of the transit sequence of pSS from *Chlamydomonas* suggests that it might have originated from a duplicated DNA segment (Figure 2c). It is well known that pSS from *Chlamydomonas* can be imported into isolated pea chloroplasts, but is not correctly processed [19,31]. Experiments with such a heterologous import system led Mishkind and colleagues [31] to propose that processing of the algal pSS involves two proteolytic sites. Indeed, later two different enzymes were found and separated in chloroplast extracts from *Chlamydomonas* [21].

When immunoprecipitated pSS from *Chlamydomonas* was treated with a crude cell extract from *Chlamydomonas*, Marks and colleagues [32] observed in this homologous system the production of an intermediate-sized protein, but not of the mature SS. Robinson and Ellis [33] found that a 300-fold enriched processing enzyme from pea cleaved pSS(pea) *in vitro* to an intermediate as well as to the mature form. The fact that such membrane-free *in vitro* systems of *Chlamydomonas* and of pea had the capacity to generate intermediate-sized products suggested that the processing not only of thylakoid-residing proteins may proceed in two stages, but also of pSS leading to a stromal protein. Since, however, the formation of the mature SS by the enzyme SPP-2 was as efficient with pSS as with iSS as substrates, it could not be decided whether the production of iSS represented a normal step of the processing pathway or merely an artefact of the *in vitro* system. No physiological significance could be attributed to the corresponding enzyme. *In vivo*, i.e. in intact cells, no processing intermediates could ever be detected. The present demonstration of such intermediates and the kinetics of their formation in physiologically active isolated chloroplasts suggests that protein processing during import into chloroplasts includes several steps which depend on each other.

The localization of a detectable amount of iSS in the intermembrane space of the envelope argues against the hypothesis that protein translocation has to occur necessarily at contact sites where protein complexes of the outer and inner membrane form a physical entity. Recently, based on the work of Kessler and Blobel [12] a dynamic equilibrium has been postulated between the dissociated, individual inner and outer envelope import machineries and the translocation apparatus consisting of the

joined import complexes [11]. Furthermore, Scott and Theg [13] presented evidence that the outer envelope is able to import proteins independently from the inner membrane. Our results indicate that in *Chlamydomonas* the import of pSS might proceed discontinuously with transient accumulation of the intermediate iSS in the intermembrane space of the envelope. However, the localization of the enzyme SPP-1 which presumably is involved in the formation of iSS, remains to be determined.

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