NH$_2$-TERMINAL AMINO ACID SEQUENCES OF PRECURSOR AND MATURE FORMS OF THE RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT FROM CHLAMYDOMONAS REINHARDTII

GREGORY W. SCHMIDT, ANNE DEVILLERS-THIERY, HELENE DESRUISSEAUX, GUNTER BLOBEL, and NAM-HAI CHUA

From The Rockefeller University, New York 10021. Dr. Devillers-Thiery's present address is the Institut Pasteur, 75015 Paris, France.

ABSTRACT

A precursor (pS) to the small subunit (S) of ribulose-1,5-bisphosphate carboxylase is the major product of cell-free protein synthesis directed by poly(A)-containing RNA from Chlamydomonas reinhardtii. We present sequence data for in vitro-synthesized pS, for in vitro-synthesized S that is generated from pS by posttranslational incubation with a Chlamydomonas cell extract, and for in vivo-synthesized, mature S. We show that pS contains an NH$_2$-terminal extension of 44 amino acid residues that is removed by cleavage at the correct site when pS is converted to S by an endoprotease present in the Chlamydomonas cell extract.

KEY WORDS posttranslational protein import  chloroplasts  transient amino terminal extension  cleavage of transit sequence

A large number of proteins that are located within the chloroplast are synthesized on cytoplasmic ribosomes (9, 11). These proteins, therefore, must be transferred from the cytosol across the two chloroplast envelope membranes before reaching their final destination.

Among the major chloroplast proteins synthesized outside the organelle is the small subunit (S) of ribulose-1,5-bisphosphate carboxylase (RuBPCase), which accounts for up to 10% of the total proteins in plants (15). The in vitro synthesis of S from the unicellular green alga Chlamydomonas reinhardtii provided the first clues to the mechanisms by which the import of proteins into the chloroplast stroma proceeds (10). Thus, it was demonstrated (10) that S (16,500 mol wt) was synthesized as a larger precursor (pS) (~21,000 mol wt) when poly(A)-containing RNA from Chlamydomonas was translated in a wheat germ cell-free system. Furthermore, pS could be processed to a molecule, identical in size to mature S, by a soluble endoprotease that was present in a postribosomal supernate of Chlamydomonas cell homogenates. Because pS was synthesized on free polysomes, it was proposed that pS is an extrachloroplast precursor form of S, and that the extra sequence in pS plays a role in the posttranslational transport of the protein into the chloroplast stroma (10).

Considerable evidence in support of these pro-
proposals has since been accumulated. Precursors for S from spinach (8) and pea (5, 8, 13) have been synthesized in the wheat germ cell-free system. Moreover, posttranslational transport into chloroplasts (8, 13), accompanied by conversion of pS to S (8, 13) and assembly of the newly transported S with its large subunit partner in the stroma to form the holoenzyme (8) have been demonstrated in vitro.

As a step toward elucidating the posttranslational transport mechanism, it is desirable to characterize the molecular nature of pS. Determination of the disposition of the peptide extension in the precursor molecule and resolution of its complete amino acid sequence may provide some clues to how it functions in transport. Furthermore, because the Chlamydomonas pS can be converted completely to S by a soluble endoprotease, it is important to ascertain whether the processing in vitro occurs with fidelity and to characterize the nature of the proteolytic cleavage site in the precursor molecule. For these reasons, we undertook to determine (a) the complete amino acid sequence of the extra sequence in Chlamydomonas pS, (b) the partial NH2-terminal amino acid sequence of processed pS, and (c) the partial NH2-terminal amino acid sequence of S. The results of these experiments are presented here.

MATERIALS AND METHODS

[14]Asparagine was obtained from Schwarz/Man. Div., Becton, Dickinson & Co. (Orangeburg, N.Y.). The other tritiated amino acids and [14]methionine were purchased from Amersham Corp. (Arlington Heights, Ill.) at the highest specific activities available. [14]Cysteine was purchased from New England Nuclear (Boston, Mass.) and reduced to cysteine by the procedure of Caskay et al. (6) before use. ATP, GTP, creatine phosphokinase, spermine-HCI, oxaloacetate, citrate synthase, and unlabeled amino acids were obtained from Sigma Chemical Co. (St. Louis, Mo.), and Trasylol was purchased from FBA Pharmaceuticals, Inc. (New York).

In Vitro Synthesis and Immunoprecipitation of pS

Poly(A)-containing RNA from C. reinhardtii was purified and translated in a wheat germ cell-free system as described previously (8), except for the following changes: incubation mixtures for in vitro protein synthesis contained 18 amino acids at 24 μM, 15 μCi/ml [14]methionine and 300 μCi/ml of a tritiated amino acid, and when [14]cysteine was employed, [14]lysine replaced [14]methionine as the internal standard for sequence analysis. To prevent amino terminal acetylation of the in vitro products, citrate synthase (50 U/ml) and oxaloacetate (250 μM) were present in the incubation mixtures to consume endogenous acetyl coenzyme A (CoA-SH) (20).

High concentrations of citrate synthase were found to severely inhibit protein synthesis when the ammonium sulfate-precipitated enzyme was dissolved in 1 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) (pH 7.5) and added directly to the translation mixtures. Consequently, immediately before its addition to the translation mixtures, citrate synthase (250 U in 0.5 ml 1 mM HEPES, pH 7.5) was desalted on 5-ml columns of Sephadex G-25 medium equilibrated with 20 mM HEPES (pH 7.5) by the centrifugation methods of Neal and Fiorini (17).

Immunoprecipitation of the in vitro translation products was performed with purified IgG from rabbits injected with purified S (15). Translation mixtures were centrifuged at 140,000 g for 1 h and the postribosomal supernates were precipitated by adjusting to 10% trichloroacetic acid and incubated at 0°C for 30 min. The precipitate was solubilized by sonication in 100 mM Tris-base, 0.2% SDS, followed by addition of diethiothreitol (DTT) to 10 mM. The solubilized proteins were further denatured and reduced by boiling for 1 min. The solutions were then adjusted to 100 mM Tris-HCl (pH 8.6), 1% Triton X-100 (Rohm and Haas, Co., Philadelphia, Pa.), 0.15 M NaCl before addition of Trasylol to 100 U/ml and anti-small subunit IgG to 1 mg/ml. After incubation at 26°C overnight, sufficient amounts of formalin-fixed Staphylococcus aureus (Cowan's strain) were added to absorb completely the IgG-antigen complexes. After incubation at 37°C for 1 h the bacteria were washed three times by centrifugation and sonication in 0.1% SDS, 10 mM Tris-HCl (pH 8.6), and 0.5 M NaCl. Finally, the IgG-antigen conjugates were eluted from the bacteria by resuspending the latter in 10 mM Tris-HCl (pH 8.6), 5% SDS, 0.1 M DTT, and incubating for 5 min at 22°C. After centrifugation at 7,000 g for 5 min, the supernates were heated at 100°C for 1 min, adjusted to pH 5 with acetic acid and then to 90% acetone. The precipitates obtained after incubation at 0°C for 30 min were then solubilized in 50% heptfluorobutyric acid and loaded into the sequencer cup.

Purification of S

S was purified from RuBPCase holoenzyme obtained by centrifugation of Chlamydomonas cell supernatants on 10-30% sucrose gradients as described previously (8). The 18S peak was collected and associated with SDS and fractionated on Sephadex G-100 column as described by Rutner and Lane (22).

Automated Sequential Edman Degradation

S purified from RuBPCase holoenzyme was subjected to Edman degradation in a Beckman 90C sequencer (Beckman Instruments, Inc., Fullerton, Calif.) using a DMAA program (102974). After conversion with 1 N HCl, dithiothreitol derivatives were analyzed by gas chromatography on Chromasorb 400 (Beckman Instruments, Inc.) as described by Pisano and Bronzert (21) and by high-pressure liquid chromatography on a μBondapak C 18 column (300 × 3.9 mm i.d., Waters Associates Inc., Milford, Mass.) using an isocratic solvent of CH3CN:0.01 N sodium acetate, 42:58, vol/vol (pH 4.0), adapted from Zimmerman et al. (23).2

In vitro translation products labeled with a ‘H- and 35S-amino acid were immunoprecipitated with anti-S IgG as described above. The immunoprecipitates were subjected to Edman degradation as above, except that the amino acid derivative obtained at each cycle was dried under an air stream and dissolved in Liquifluor (New England Nuclear) for scintillation counting.

2 Gates, F. T. Personal communication.
RESULTS

In Vitro Synthesis of pS

As demonstrated previously (10), pS can be purified by immunoprecipitation (Fig. 1, lane 2) from the numerous products (Fig. 1, lane 1) that are synthesized from total poly(A) RNA of Chlamydomonas in a wheat germ cell-free system. Automated Edman degradation of the radiolabeled, immunoprecipitated pS resulted consistently in low yields (<5%), suggesting that the NH₂-termini were blocked. A precedent for blocked NH₂-termini was reported by Palmiter (20), who established that ovalbumin is acetylated when synthesized in a reticulocyte lysate system, and that blockage can be prevented if the endogenous CoASAc of the cell-free extract is depleted by concomitant incubation with oxaloacetate and citrate synthase. We found that the procedure of Palmiter (20) can also be applied to the wheat germ cell-free system. In the presence of the CoASAc scavenging system, radiolabeled pS was obtained that gave consistently high yields when subjected to automated Edman degradation. These results suggest that without the scavenging system pS could be acetylated at its NH₂-terminus in the wheat germ cell-free system. Fig. 1 compares the electrophoretic mobilities of pS synthesized in the absence (lanes 2 and 3) or presence (lane 4) of oxaloacetate and citrate synthase. It can be seen that in SDS polyacrylamide gel electrophoresis the presumably acetylated form of pS (lane 3) moves more slowly than the presumably nonacetylated pS (lane 4). Both precursor forms can be converted to S by posttranslational incubation with a postribosomal supernate derived from a Chlamydomonas cell homogenate. Because the mobilities of both species of "processed" S are indistinguishable, the acetylation is most likely confined to the peptide segment that is unique to pS.

Amino Terminal Location of pS Extension

As a first step toward determining the primary structure of the peptide extension of pS, it was necessary to establish its disposition in the pS molecule. To this end we determined the sequence of the first 12 NH₂-terminal residues of mature S purified from the RuBPCase holoenzyme. A striking feature of this sequence is the occurrence of methionine at positions 1, 2, and 11 (Fig. 2). With this information at hand, we proceeded to synthesize nonacetylated pS (see above) labeled only in [³⁵S]methionine and subjected it to 60 consecutive cycles of automated Edman degradation. Judging from the peaks of radioactivity (Fig. 3), it was possible to assign Met to positions 1, 24, 45, 46, 123456789101112131415 16 1 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55

FIGURE 1 Differences in the electrophoretic mobility of S forms synthesized in the presence and absence of citrate synthase/oxaloacetate. Analysis was by electrophoresis in SDS gels containing a 7.5–15% acrylamide gradient followed by autoradiography (8). Lane I: total translation products synthesized in the wheat germ cell-free system in the presence of poly-acetylated RNA from Chlamydomonas; lane 2: immunoprecipitate of total translation products with anti-S IgG fraction; lane 3: the same as lane 2, except that total translation products were adjusted to 25 mM EDTA, 100 U Trasylol/ml, and then treated for 1 h with 1.5 mg protein/ml of a postribosomal supernate from Chlamydomonas cell extracts (9) before immunoprecipitation; lane 4: the same as lane 3, except that in vitro protein synthesis was carried out in the presence of citrate synthase/oxaloacetate.

FIGURE 2 Summary of NH₂-terminal sequence data for the S forms of C. reinhardtii.

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FIGURE 3  NH₂-terminal sequence analysis of pS. Each panel is representative of automated Edman degradation of immunoprecipitated pS synthesized in the presence of the indicated 'H-labeled amino acid as described in Materials and Methods. Smooth curves reflect the expected repetitive yield of each sequence run as calculated from that obtained for internal standards of either [³⁵S]methionine or, in the case of [³⁵S]cysteine, [³H]lysine. Arrows indicate the reproducible occurrence of the amino acid within the NH₂-terminal sequence of pS.

and 55 of pS. The consecutive Met at positions 45 and 46 and of another Met at position 55 is in perfect register with the NH₂-terminal doublet of Met and the Met at position 11 of mature S. This demonstrates that the peptide extension of 44 residues in pS is located at the amino terminus. Because of its postulated role in posttranslational transport, we have proposed (9) that this peptide extension be designated the “transit sequence.”

Primary Structure of pS Transit Sequence

The NH₂-terminal location of the pS transit sequence indicated that its entire amino acid sequence could be determined simply by consecutive Edman degradations of double-labeled pS. To this end we synthesized nonacylated pS with both [³⁵S]methionine and a single tritiated amino acid present. Thus, each batch of pS to be sequenced contained [³⁵S]methionine as a control and as an internal marker for calculating the repetitive yield. When [³⁵S]cysteine was employed, [³H]lysine or [³H]alanine served as internal markers. The repetitive yield exceeded 98% in all cases.

The results of consecutive Edman degradation of the various double-labeled pS preparations comprising all 20 amino acid residues are shown in Figs. 2 and 3. Most sequence determinations were performed in duplicate with independent
preparations of pS. Reproducible ambiguities in the assignment are evident only in positions 3 (Val and Ser) and 37 (Ala and Val). It is not possible to discern whether these ambiguities are due to artifacts or major contaminants, or whether they reflect a heterogeneity resulting from the presence of several genes for S in C. reinhardtii.

Among the notable features in the composition of the pS transit sequence are the dominance of Ala (14 or 15 residues out of 44), an abundance of Val, Ser, and Pro, a predominance of basic over acidic residues (3 Lys and 3 Arg vs. 1 Asp and 1 Glu), and the complete absence of 7 amino acid residues (Cys, Phe, Gly, His, Gln, Trp, and Tyr). The sequence reveals a certain clustering of the six basic residues (positions 6-32), of the two acidic residues (positions 41-44) and of the five (or six) Ser residues (positions 3-20). There is a five-residue-long stretch at positions 5-9 (Ala-Lys-Ser-Ser-Val) that appears to be repeated at positions 17-21 (Ala-Arg-Ser-Ser-Val).

The predicted secondary structure of the NH2-terminal sequence of pS as calculated from the parameters elaborated by Chou and Fasman (7) is shown in Fig. 4. The NH2-terminal sequence up to Arg-15 is probably an α-helix. Arg-15 is likely to initiate a β-turn to reverse the direction of the chain, which then continues in the form of a random coil. At Glu-41 the random coil is interrupted by another β-turn. This is followed by a short random coil extending through the proteolytic cleavage site (see below) and another β-turn initiated at Val-51.

In Vitro Conversion of pS to S

The previously observed endoproteolytic conversion of pS to “processed” S by a Chlamydomonas postribosomal supernate (10) indicated that cleavage occurs at the correct site because the “processed” S had a mobility during SDS polyacrylamide gel electrophoresis identical to that of mature S. To provide definitive evidence on this point, we examined the NH2-terminal sequence of “processed” S that was generated from pS labeled with [35S]methionine and [3H]valine (Fig. 5). The occurrence in the “processed” S of Met in positions 1, 2, and 11, and of Val in positions 3 and 7 demonstrates that the NH2-terminal sequence of “processed” S is identical to its mature counterpart. This result, together with the previous observation (10) that cleavage generated a small fragment (which could represent the balance between pS and S), suggests that the proteolytic activity present in the Chlamydomonas postribosomal supernate is a specific endopeptidase. The presence of this activity in the postribosomal supernate of Chlamydomonas is most likely a result of its leakage from broken chloroplasts because a similar activity has been detected in association with intact chloroplasts from higher plants (8, 13).

The “processed” S was compared with mature S also by two-dimensional polyacrylamide gel electrophoresis (isoelectric focusing in the first dimension, SDS in the second dimension). It can be seen (Fig. 6) that, like mature S, “processed” S can be resolved into three major species that possess identical molecular weights, but differ in isoelectric points. This heterogeneity of mature and of “processed” S could be explained by the presence in Chlamydomonas of several genes coding for S, a conjecture consistent with the sequence ambiguities in pS at positions 3 and 37 (see above).
Removal of the transit sequence from pS occurs most likely by a single endoproteolytic cleavage between Asp-44 and Met-45. From the predicted secondary structure of the NH₂-terminus of pS (Fig. 4), it appears that the cleavage site adjoins a β-turn. It is thought that the processing enzyme is a sulfhydryl protease (10), and it is interesting to note that a sulfhydryl protease exhibiting specificity for glutamine and Asp and possibly glutamine and aspartic acid has been characterized in mung-bean cotyledons (2).

**DISCUSSION**

The results presented here demonstrate clearly that the difference in electrophoretic mobility between *Chlamydomonas* pS and S is due to the presence of a 44-amino-acid-residue-long extension at the NH₂-terminus of pS. In vitro processing of the precursor by a soluble endoprotease present in postribosomal supernates of *Chlamydomonas* is effected by the endoproteolytic removal (10) of this transit sequence from the NH₂-terminus of pS, thereby generating the NH₂-terminal sequence of S. The size difference between the precursor and the mature protein can be fully accounted for by the transit sequence (4,273 mol wt). However, the possibility of slight trimming at the carboxy terminus of the precursor cannot be rigorously ruled out because the amino acid sequence of this end of the molecule has not been determined.

Previous reconstitution experiments in vitro have provided compelling evidence that pS is transported across the chloroplast envelope by a posttranslational mechanism. Thus, isolated, intact chloroplasts from spinach (8) and pea (8, 13) import pS after its synthesis from poly(A)-containing RNA in a cell-free translation system. During, or immediately after, transport through the chloroplast envelope, a single endoproteolytic cleavage between Asp-44 and Met-45 occurs, thereby generating the NH₂-terminal sequence of S. This process is believed to occur on the stromal side of the chloroplast envelope, as indicated by the lack of such sequences in isolated thylakoids (13) and ATP-deficient chloroplasts (10).

**Figure 5** NH₂-terminal sequence analysis of pS processed to S in vitro. In vitro synthesis of pS was carried out in the presence of [³⁵S]methionine and [³H]valine. Postribosomal supernates of the translation mixtures were then incubated with cell extract from *Chlamydomonas* as described in Fig. 1 and in Materials and Methods. The immunoprecipitates obtained with an anti-S IgG fraction were then subjected to automated Edman degradation. Arrows indicate the coincidence of methionine and valine residues for both mature S and in vitro-synthesized and processed pS.

**Figure 6** Two-dimensional gel electrophoresis of RuBPCase holoenzyme and processed pS. 1. RuBPCase holoenzyme was purified from cell extracts of *Chlamydomonas*. 2. Processed pS was prepared as described in Fig. 5. Isoelectric focusing (IEF) in the first dimension was performed as described by O'Farrell (19). The second dimension was electrophoresis in SDS in a 7.5-15% polyacrylamide gel (8). Major isoelectric species of S possess pIs of 5.3, 5.65, and 6.15. The isoelectric species of processed pS with a pI of 6.95 is found as a very minor component of S.
roplast envelope, pS is converted to S. Moreover, most of the in vitro-synthesized and transported S assembles with the chloroplast-synthesized RuBPCase large subunit to form the holoenzyme (8). In vitro transport of the protein, therefore, occurs through both envelope membranes and thus reconstructs with fidelity the sequence of events which follows the synthesis of S by free ribosomes in vivo.

The presumptive function of the precursor form of S for transport across chloroplast envelopes is unlike that of the NH₂-terminal signal sequences for secretory (3, 4) and certain integral membrane proteins (16). Signal sequences trigger binding of translating ribosomes to the endoplasmic reticulum membranes and facilitate transport of proteins through the latter by a translation-coupled mechanism (3). In contrast, the peptide extension of pS does not lead to binding of translating ribosomes to the chloroplast envelope membranes. Furthermore, it facilitates transport into the chloroplast by a posttranslational rather than a cotranslational mechanism (8, 13). Because of these functional differences, we have called the peptide extension of pS the “transit sequence.” Transit sequences are considered to be extensions of those organelle proteins that are synthesized by free cytoplasmic ribosomes. It was proposed that transit sequences mediate posttranslational recognition by envelope receptors and subsequent transport across the envelope membrane (9).

Despite the occurrence of the transit sequence at the NH₂-terminus of pS, transport of S into chloroplasts occurs in a posttranslational fashion. It follows that the function of the transit sequence is latent until pS is completely synthesized. Thus, a conformational change of pS may be required before the transit sequence is able to interact with chloroplast envelope receptors. Alternatively, posttranslational modifications of the completely synthesized pS may be needed before transport through the chloroplast envelopes can proceed.

The sequence data presented here show that the primary structure of the transit sequence of Chlamydomonas pS differs significantly from the signal sequence shared by numerous secretory proteins (cf. reference 12). Thus, the chloroplast transit sequence does not contain clusters of the hydrophobic residues characteristic of the signal sequence core structure. Nevertheless, the transit sequence is apolar, for the most part because of the preponderance of alanine. It is also longer than the signal sequence and significantly more basic. The latter feature might facilitate interaction of the transit sequence with the chloroplast envelope membranes, which are highly negatively charged (18).

In addition to S (4-7), two other chloroplast proteins, ferredoxin (14) and the apoprotein (1) of chlorophyll-protein complex II, are also synthesized as larger precursors in a wheat germ cell-free system. In both cases, the weight difference between the precursor and the mature protein is 4,000-5,000 daltons. It would be of interest to compare the transit sequence of these precursors with that of the Chlamydomonas pS to see whether they are homologous.

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