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In vitro synthesis and processing of a putative precursor for the small subunit of ribulose-1,5-bisphosphate carboxylase of *Chlamydomonas reinhardtii*

(polyadenylylated mRNA/wheat germ system/immunoprecipitation/specific endoprotease/free polysomes)

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ABSTRACT **Translation of polyadenylated mRNA of *Chlamydomonas reinhardtii* in a cell-free wheat germ system resulted in the synthesis of numerous discrete polypeptides. Among them was a species with molecular weight 20,000 that was immunoprecipitated specifically by antibodies raised against the authentic small subunit (16,500 daltons) of ribulose-1,5-bisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase(dimerizing), EC 4.1.1.39]. Since the immunoprecipitated polypeptide has a larger molecular weight by approximately 3500 than the small subunit (S) it was identified as a putative biosynthetic precursor (pS). Post-translational conversion of pS by a specific endoprotease yielded two detectable products: one apparently identical in size to S and the other, a small peptide, presumably representing the remainder of pS. The endoprotease requires sulfhydryl groups for its activity and is present in a *C. reinhardtii* postribosomal supernatant as well as in a free polysome fraction. The latter could account for the observation that completion of nascent chains in free polysomes yielded S but not pS. We propose that pS is an extrachloroplastic form of S and that the small peptide portion plays a role in the transfer of S into the chloroplast.**

Ribulose-1,5-bisphosphate (Rbu-1,5-P_2) carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] is a predominant protein of the chloroplast stroma (cf. 1). It has a molecular weight of 550,000 and is composed of several copies each of two nonidentical subunits (1–3). It is generally agreed that the large subunit is made within the chloroplast (4–6) whereas the small subunit (S) is synthesized on cytoplasmic ribosomes (7, 8). The "ectopic" synthesis of S raises the question of how this molecule is transferred across the two chloroplast envelope membranes in order to assemble with the large subunit to form the holoenzyme.

In this paper we report the *in vitro* synthesis of a putative precursor of S when polyadenylated mRNA from *Chlamydomonas reinhardtii* was translated in a cell-free wheat germ system. This precursor could be processed by a specific endoprotease, present in the algal postribosomal supernatant, to a polypeptide with molecular weight identical to that of authentic S. We propose that the additional sequence information of the putative precursor is required for the transfer of S across chloroplast envelope membranes.

MATERIALS AND METHODS

The wild-type strain (137c) of *C. reinhardtii* and CW 15, a mutant devoid of cell wall, were grown in Tris/acetate/phosphate medium (9). All experiments, except for the isolation of free ribosomes, were performed with the wild-type strain.

Abbreviations: Rbu-1,5-P_2, ribulose-1,5-bisphosphate; S, small subunit of Rbu-1,5-P_2 carboxylase; pS, putative precursor of S; $NaDodSO_4$, sodium dodecyl sulfate; F, small peptide representing difference between pS and S.

Purification of S of Rbu-1,5-P_2 Carboxylase and Preparation of Monospecific Antibodies to S. Rbu-1,5-P_2 carboxylase was isolated according to Iwanij *et al.* (2, 10) and S was purified by sodium dodecyl sulfate ($NaDodSO_4$)/gradient gel electrophoresis (11). Gels were stained with Coomassie brilliant blue, gel bands containing S were pooled, and protein was eluted electrophoretically. The eluate was extracted twice with 90% acetone and the precipitate was dissolved in 0.15 M NaCl/1% $NaDodSO_4$. Antibodies to S were raised in rabbits by injecting 1 ml of the protein (400 μg in 0.15 M NaCl/0.5% $NaDodSO_4$) mixed with an equal volume of complete Freund's adjuvant into the subscapular space. The same amount of antigen was given on the second and fourth weeks; 1 week after the last injection the animals were bled. Control serum samples were taken from the animals before immunization. Sheep antiserum to rabbit $F(ab')_2$ was a kind gift of S. Silverstein. IgG fractions from the sheep and rabbit sera were prepared by 40% $(NH_4)_2SO_4$ precipitation (12).

RNA Extraction and mRNA Isolation. A pellet of intact cells was resuspended in 50 mM Tris·HCl (pH 7.5)/0.15 M NaCl/5 mM EDTA/proteinase K, 40 μg/ml/2% $NaDodSO_4$ to a density of 2×10^8 cells per ml, and the mixture was stirred at room temperature for 15 min. RNA was extracted twice with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, and subsequently chromatographed on oligo(dT)-cellulose (13). Bound RNA was eluted from the column with 10 mM Tris·HCl (pH 7.5)/0.1% $NaDodSO_4$ at 50° and this fraction was designated as polyadenylated mRNA.

Preparation of Free Ribosomes. Cultures of CW 15 were pretreated with cycloheximide (10 μg/ml) for 10 min to prevent polysomal runoff before harvesting by centrifugation (14). Sedimented cells were resuspended in 25 mM Tris·HCl (pH 7.5)/25 mM KCl/25 mM $MgCl_2$/5 mM dithiothreitol and broken by passage through a chilled French pressure cell at 1500 lb/in^2. Under these conditions chloroplasts were fragmented. The homogenate was centrifuged at $50{,}000 \times g_{max}$ for 10 min, and then 7.5 ml of the resulting supernatant was layered onto a 2-ml cushion of 1.87 M sucrose in the same buffer mixture and centrifuged at $105{,}000 \times g_{av}$ for 5 hr. The resulting pellet consisted of free ribosomes from the cytoplasm as well as from the chloroplast. Electron microscopic examination of a section through the entire pellet revealed only ribosomes, with no contamination by membranes.

Preparation of a Postribosomal Supernatant. A $50{,}000 \times g_{max}$ supernatant (see above) derived from a homogenate of the wild-type strain was subsequently centrifuged for 6 hr at $105{,}000 \times g_{av}$ to yield a postribosomal supernatant. The latter was dialyzed against 20 mM triethanolamine (pH 7.5) and aliquots were kept frozen at −80° for up to 1 month without loss of endoproteolytic activity.

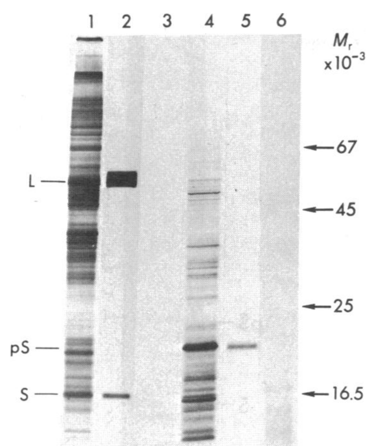

FIG. 1. Characterization of antibodies to S and of products obtained from the translation of *C. reinhardtii* polyadenylated mRNA in a cell-free wheat germ system. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, polypeptides of a *C. reinhardtii* supernatant (50,000 × g_{av} for 10 min) derived from cells labeled with [*1*-^{14}C]acetate (2 μC/ml) in the light for 2 hr; 2, direct immunoprecipitation of the ^{14}C-labeled 50,000 × g_{av} supernatant with anti-S IgG fraction; 3, polypeptides synthesized in the wheat-germ system without added mRNA; 4, polypeptides synthesized in the wheat germ system in the presence of polyadenylated mRNA; 5, indirect immunoprecipitation of *4* with anti-S IgG fraction; 6, indirect immunoprecipitation of *4* with preimmune IgG fraction. Molecular weight standards are: bovine serum albumin, 67,000; ovalbumin, 45,000; chymotrypsinogen, 25,000; and S, 16,500. L = large subunit of Rbu-1,5-P_2 carboxylase.

***In Vitro* Protein Synthesis.** The preparation of a wheat germ extract (15) and its subsequent use in a protein synthesizing system will be detailed elsewhere. Algal polyadenylated mRNA or free ribosomes were used in a concentration of 1.0 or 30.0 A_{260} units, respectively, per ml of incubation mixture. The latter also contained 200 μCi/ml of [^{35}S]methionine (300 Ci/mmol).

Indirect Immunoprecipitation of Products Synthesized *In Vitro*. The cell-free translation mixture was adjusted to 1% (wt/vol) Triton X-100 and centrifuged at 104,000 × g_{av} for 1 hr. The resulting postribosomal supernatant was used for immunoprecipitation in a mixture containing 0.1 M Tris·HCl (pH 8.6)/0.8 M NaCl/1% Triton X-100; 3 μl of the rabbit anti-S IgG fraction (57 mg of protein per ml) or the preimmune IgG fraction (60 mg of protein per ml) was added to 460 μl of the mixture. After incubation at 37° for 1 hr, 30 μl of the IgG fraction of sheep antiserum to rabbit F(ab′)$_2$ (61 mg of protein per ml) was added and the mixture was incubated further at 4° for 24–48 hr. The immunoprecipitate was washed twice in 1 ml of 10 mM Tris·HCl (pH 8.6)/0.5 M NaCl/1% Triton X-100 and once in 1 ml of 10 mM Tris·HCl (pH 8.6). The precipitate was resuspended in 2% $NaDodSO_4$/50 mM Tris·HCl (pH 7.5)/10 mM dithiothreitol and alkylated with 25 mM iodoacetamide. Analysis by gradient polyacrylamide gel electrophoresis in $NaDodSO_4$ and subsequent autoradiography of the dried slab gels was as previously described (16, 17).

Sources of Materials. All reagents used were analytical grade whenever available. [^{35}S]Methionine (300 Ci/mmol) was obtained from Amersham/Searle; oligo(dT)-cellulose (T-2) was from Collaborative Research; and proteinase K was from Merck.

RESULTS

Polyadenylated mRNA from *C. reinhardtii* was found to be active in directing protein synthesis in a cell-free system derived from wheat germ. Addition of the algal mRNA increased the rate of incorporation 40- to 60-fold above the endogenous level (data not shown). Lane 4 in Fig. 1 shows that discrete polypeptides of widely different molecular weights were synthesized *in vitro* and prominent among these was a band with molecular weight about 20,000.* This band was immunoprecipitated specifically by anti-S IgG fraction (Fig. 1, lane 5) but not by a similar fraction from preimmune serum (Fig. 1, lane 6). By these immunological criteria and based on its larger molecular weight, we identified the 20,000 molecular weight band as a putative precursor to S (designated pS). An equivalent of pS could not be detected in the algal 50,000 × g_{av} supernatant (Fig. 1, lane 1), and immunoprecipitation of this supernatant with anti-S antibodies gave only two bands (Fig. 1, lane 2) corresponding to L and S (molecular weights 55,000 and 16,500, respectively) (2, 3) of *C. reinhardtii* Rbu-1,5-P_2 carboxylase. However, pS may have a short life *in vivo* and therefore could have escaped detection by this technique. It should be pointed out that in these experiments the assembled holoenzyme which contains both L and S was precipitated by anti-S antibodies. In separate experiments, not shown here, we found that anti-S antibodies gave a single precipitin line or arc with purified S but no reaction with purified L in double-diffusion and cross-immunoelectrophoresis assays.

Ultrastructural studies show that the majority of 80S ribosomes in *C. reinhardtii* are free in the cytoplasm (18). These ribosomes can be isolated readily free of any contamination by membranes (see *Materials and Methods*). Addition of isolated polysomes to the wheat germ system stimulated incorporation 30-fold above the endogenous level, and this incorporation was insensitive to chloramphenicol (100 μg/ml) but was inhibited more than 95% by cycloheximide (10 μg/ml) (data not shown). These results show that only cytoplasmic polysomes are active in the wheat germ system. The nascent chains, completed *in vitro*, yielded numerous polypeptides (Fig. 2, lane 2). Immunoprecipitation with anti-S IgG fraction gave only one major band having the same electrophoretic mobility as authentic S (Fig. 2, lane 4) whereas no major product was precipitated with the preimmune IgG fraction (Fig. 2, lane 5). Furthermore, similar immunoprecipitation experiments showed that synthesis of S by polysomes was totally inhibited by cycloheximide but unaffected by chloramphenicol (data not shown).

The synthesis of a putative precursor, resulting from the translation of mRNA in the cell-free wheat germ system, but not detectable among the polypeptides completed by free polysomes *in vitro* raised the possibility that precursor synthesis may have resulted from faulty initiation or termination. Alternatively, the free polysomes could contain a processing activity that converts the precursor into S. To examine this possibility, mRNA translation products were incubated subsequently with free polysomes in the presence of cycloheximide (100 μg/ml) to prevent completion of their nascent chains. Under these conditions, pS was converted to a product (Fig. 3, lane 6) with a mobility identical to that of S. Moreover, this processing activity was also present in a postribosomal supernatant derived from *C. reinhardtii* homogenate. The presence of increasing amounts of this supernatant during translation (Fig. 3, lanes 2–4) or added after chain completion (Fig. 3, lane 5) resulted in conversion of pS.

The observed processing of pS could occur either by

* The RNA fraction that was not retained by oligo(dT)-cellulose was also active in directing protein synthesis *in vitro*. The 20,000-dalton band was present among the products synthesized, but it represented only a minor fraction.

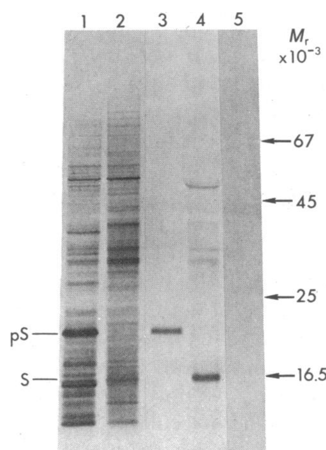

FIG. 2. Characterization of polypeptides synthesized by polysomes or polyadenylated mRNA of *C. reinhardtii* in a cell-free wheat germ system. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, polypeptides synthesized in the presence of polyadenylated mRNA; 2, products of polysome readout; 3, indirect immunoprecipitation of *1* with anti-S IgG fraction; 4, indirect immunoprecipitation of *2* with anti-S IgG fraction; 5, indirect immunoprecipitation of *2* with a preimmune IgG fraction. Molecular weight markers are the same as in Fig. 1.

exoproteolytic digestion or by endoproteolytic cleavage. If the processing were by an endoprotease it should be possible to recover two peptides: one with the size of authentic S and the other, a small fragment (F), representing the balance between pS and S. In previous experiments, this small peptide could have been lost during immunoprecipitation or eluted from the gel during staining and destaining. To avoid such a loss, the immunoprecipitate containing pS was incubated with *C. reinhardtii* postribosomal supernatant and the resulting mixture was analyzed directly by $NaDodSO_4$/polyacrylamide gel electrophoresis. After electrophoresis, the slab gel was dried without staining. It can be seen in Fig. 4 (lane 2) that the small peptide F, in addition to the presumptive S, was recovered, suggesting that processing had occurred endoproteolytically. In contrast, no proteolytic activity was detected in the postribosomal supernatant previously incubated at 95° for 2 min (Fig. 4, lane 1). However, it remains to be established by sequence analysis whether *in vitro* processing had occurred correctly and by a single endoproteolytic cleavage.

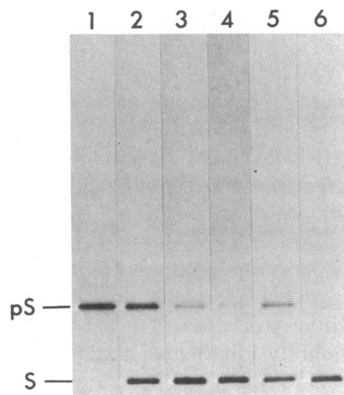

FIG. 3. Processing of pS *in vitro*. Fractions with processing activity were added either during or after the completion of translation of polyadenylated mRNA, and pS and S were immunoprecipitated by the indirect method. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, no addition; 2, 3, and 4, with increasing amounts (0.8, 1.6, and 2.4 A_{280} units/ml, respectively) of a *C. reinhardtii* postribosomal supernatant present during translation; 5 and 6, after completion of synthesis, cycloheximide was added to 100 μg/ml and the mixture was incubated with *C. reinhardtii* postribosomal supernatant (1.6 A_{280} units/ml, lane 5) or ribosomes (20 A_{260} units/ml, lane 6) at 25° for 90 min.

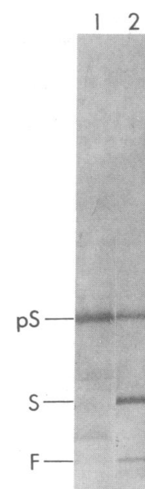

FIG. 4. Characterization of products resulting from the endoproteolytic cleavage of pS. Analysis was by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography. pS was immunoprecipitated with the indirect method. The resulting immunoprecipitate was resuspended in 50 mM Tris·HCl (pH 8.6)/5 mM EDTA/Trasylol, 100 units. Lane 1, heat-inactivated (95° for 2 min). Lane 2, untreated postribosomal supernatant was added to the suspension at a concentration of 2.4 A_{280} units/ml. After incubation at 25° for 90 min, the entire mixture was analyzed directly without prior precipitation. F = a small peptide fragment that presumably represents the difference between pS and S.

To investigate the nature of the endoprotease, aliquots of the postribosomal supernatant were treated with known protease inhibitors for 10 min at 25° before their processing activities were assayed. It was found (data not shown) that the proteolytic activity was unaffected by the following compounds: Trasylol (100 units/ml), phenylmethylsulfonylfluoride (4 mM), benzamidine (10 mM), L-1-tosylamide-2-phenylethylchloromethyl ketone (500 μg/ml), EDTA (50 mM), and soybean trypsin inhibitor (55 μg/ml). In contrast, the activity was completely inhibited by iodoacetamide (25 mM) and *N*-ethylmaleimide (25 mM). These results suggest that the enzyme is not a serine- or metalloprotease and that it is probably a sulfhydryl-protease.

DISCUSSION

Our results show that mRNA for the (S of Rbu-1,5-P_2 carboxylase) of the green alga *C. reinhardtii* is translated in a cell-free wheat germ system into a precursor, pS, that is larger than S by about 3500 daltons. In contrast, completion of nascent chains on free polysomes ("readout") yielded S instead of pS. The precursor can be cleaved by a highly specific endoprotease present in *C. reinhardtii* postribosomal supernatant and also in association with polysomes to yield two products: a larger one with a molecular weight apparently identical to that of authentic S, and a smaller fragment, F that presumably represents the balance of pS. Thus, the failure to detect pS among the products of polysomal readout could be attributed to the presence of the endoprotease in polysome preparations. It is evident that the protease must be lacking from the wheat germ extract in order to permit the synthesis and accumulation of pS during the translation of mRNA.

We found that *in vitro* processing of pS is possible after

polypeptide chain completion and that conversion could be performed with pS complexed with antibodies in the form of an immunoprecipitate. Although the specific endoprotease has not yet been purified, our inhibitor studies show that it requires sulfhydryl groups for activity. The subcellular location of this protease is at present unknown. Because it is present in the postribosomal supernatant after cell breakage, it is likely to be a soluble enzyme normally located in the cytosol or the chloroplast stroma. Its association with polysomes therefore may not have physiological significance and is probably due to adventitious binding during cell fractionation.

The synthesis of pS raises the question as to its biological role. It has been shown previously (7, 8) that S is synthesized by cytoplasmic ribosomes. We have confirmed these results and further demonstrated that the synthesis occurs on free ribosomes. Because Rbu-1,5-P_2 carboxylase is found only inside the chloroplast it follows that S must be transported into the organelle for assembly. We propose therefore that pS is an extrachloroplastic form of S and that the additional sequence F in pS contains the information necessary for its specific binding to and subsequent transfer across the chloroplast envelope.

Although the precise localization of the F sequence in pS remains to be established, it is evident already that this sequence is not functionally equivalent to the amino-terminal chain extension ("signal sequence") found in presecretory proteins. The latter has been proposed (16, 17, 19) to function in the cotranslational attachment of the ribosomes to the microsomal membrane, thus providing the topological conditions for transfer of the remainder of the chain across the microsomal membrane. It has been shown (16, 17) that the signal sequence is removed by a membrane-associated enzyme—"signal peptidase"—before translation of the chain is completed; processing of the presecretory proteins (i.e., removal of the signal sequence) could not occur after polypeptide chain completion. In contrast to secretory proteins, pS is synthesized by free ribosomes (i.e., its F portion apparently does not trigger attachment of ribosomes either to the microsomal membrane or to the chloroplast envelope) and, furthermore, pS can be cleaved after chain completion by a soluble endoprotease. It should be pointed out that electron microscopic studies of *C. reinhardtii* reveal a relatively sparse rough endoplasmic reticulum (18) and, unlike the case with mitochondria in which some cytoplasmic ribosomes have been shown to be attached to the outer mitochondrial membrane (20), no equivalent attachment of the cytoplasmic ribosomes to the outer chloroplast envelope membrane has been detected so far.

As a clue to a possible mechanism of transport across the chloroplast envelope, it is tempting to draw analogies between pS and diphtheria toxin (cf. 21). The latter is synthesized as a single polypeptide chain that is subsequently cleaved to yield two chains, α and β, containing the amino and carboxy terminals, respectively, of the parent molecule. These two chains are linked by an -S-S- bridge. Only the α subunit traverses the plasma membrane of certain eukaryotic cells. Although the precise mechanism of this transfer is not yet known, it involves the prior binding of the β subunit to plasma membrane receptors (22). By analogy it is therefore conceivable that the F sequence in pS is functionally equivalent to the β subunit in that it binds to a receptor in the chloroplast envelope (possibly localized in regions where the inner and outer envelope membranes are in contact) to mediate transfer of the S portion from the cytosol into the chloroplast stroma.

It should be noted that Roy *et al.* (8) have examined the completion and release of polypeptides by wheat cytoplasmic polysomes and found that two labeled species (molecular weights, 12,000 and 20,000) were immunoprecipitated by anti-S serum. The 12,000 component was shown to contain tryptic peptides corresponding to those of the authentic S of wheat leaves whereas the identity of the 20,000 component was not established.

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