SYNTHESIS OF THE LARGE SUBUNIT OF SPINACH RIBULOSE BISPHOSPHATE CARBOXYLASE MAY INVOLVE A PRECURSOR POLYPEPTIDE

Peter LANGRIDGE

Division of Plant Industry, CSIRO, PO Box 1600, Canberra City, ACT 2601 and *Department of Botany, Australian National University, Canberra, ACT, Australia*

Received 20 November 1980

1. Introduction

The major soluble protein found in plant leaves, ribulose bisphosphate carboxylase (RuBP) carboxylase), is composed of 8 large subunits (M_r 52 000–56 000) and 8 small subunits (M_r 12 000–15 000) [1]. The small subunit (SSU) is coded by the nuclear genome [2] and synthesised on cytoplasmic ribosomes [3]. The initial product of translation of SSU mRNA is a polypeptide of M_r 20 000 which is transported into the chloroplast before being processed down to the 14 000 M_r, mature form [4–6]. The large subunit (LSU) is coded by the chloroplast DNA [7–10] and is the major product of protein synthesis by isolated chloroplasts of peas [11], spinach [12] and *Euglena* [13]. Translation of chloroplast RNA preparations in an *Escherichia coli* cell-free system has been reported to yield a polypeptide indistinguishable from mature LSU isolated from purified RuBP carboxylase [14,15].

In this paper I present results which suggest that the LSU of spinach RuBP carboxylase may be synthesized via a precursor polypeptide of app. M_r of 1000–2000 larger than the LSU of the native enzyme. The electrophoretic mobility and the charge/mass ratio of the LSU polypeptide produced by translation of spinach chloroplast RNA in an *E. coli* system are different from those of the LSU polypeptide synthesised by isolated spinach chloroplasts. Furthermore, the slightly larger in vitro synthesized polypeptide can be converted to a polypeptide of the same mobility as mature LSU by incubation with a soluble chloroplast extract but not by incubation with chloroplast thylakoid membranes.

Present address: Institut für Biologie III der Universität Freiburg, 78 Freiburg i.Br., FRG

2. Materials and methods

Chloroplasts were isolated from leaves (2–4 cm long) of young spinach (*Spinacia oleracea*) plants grown in liquid culture and the RNA was extracted by a phenol–sarkosyl procedure [16]. Light-dependent incorporation of [^{35}S]methionine into protein by intact, isolated chloroplasts was carried out for 30 min at 20°C as in [12]. Spinach chloroplast RNA was translated in vitro using a cell-free system from *E. coli* [10].

The products of the protein-synthesizing systems were precipitated at −20°C by the addition of 5 vol. acetone. Samples were prepared and electrophoresed on gradient (12.5–20%) polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) [17]. To obtain optimal resolution of the 55 000 M_r region, electrophoresis was for 15 h at 33 mA (twice normal current). The separation of proteins on the basis of charge involved carboxymethylation and citraconylation [18] of the samples and electrophoresis on polyacrylamide slab gels containing 6 M urea in the buffer 1 system of [19]. For the electroelution [20] and partial proteolysis of LSU protein [21], a method based on the modifications in [10] was followed.

In the preparation of soluble and membrane fractions for the assay of processing activity, chloroplasts were lysed in a hypotonic buffer (50 mM Tricine–KOH (pH 8.4) 10 mM $MgCl_2$, 2 mM EDTA, 4 mM β-mercaptoethanol) and centrifuged at 5000 × *g* for 5 min to give a soluble chloroplast extract. The pellet of membranes was washed twice and resuspended in the above buffer to give the chloroplast membrane fraction.

3. Results

The products of translation of spinach chloroplast RNA in an *E. coli* cell-free system were separated by SDS–polyacrylamide gel electrophoresis and the fluorograph was compared with that of the labelled proteins synthesized in a light-driven, isolated chloroplast system. The in vitro translation products, (fig. 1, track 1) contain a polypeptide in the 55 000 M_r region that migrates slightly more slowly than the product synthesized in isolated chloroplasts (fig. 1, track 3), which is known to be LSU protein [11]. The difference in the mobilities was not an artifact of electrophoresis due to the presence of unlabelled SU protein in the product from isolated chloroplasts since it could be shown (fig. 1, track 2) that the addition of carrier LSU did not alter the mobility of the 55 000 M_r in vitro product.

The radioactive 55 000 M_r band from the in vitro translation system was eluted from the gel, mixed with unlabelled LSU protein, subjected to partial proteolysis by *Staphylococcus aureus* protease, papain or chymotrypsin, and electrophoresed on a SDS–polyacrylamide gel. Comparison of the Coomassie blue-stained pattern of the peptides from the unlabelled LSU protein with the fluorograph of the labelled peptides from the radioactive product (fig. 2) showed the two proteins to be almost identical. Partial proteolysis of the 55 000 M_r product synthesised in isolated chloroplasts gave an equivalent pattern (not shown). From this data it was concluded that translation of spinach chloroplast RNA in an *E. coli* system yields LSU protein in an apparently larger form than that synthesized in isolated chloroplasts. The observed difference in electrophoretic mobility between the two products was reproducibly found to be equivalent to ~1000–2000 M_r.

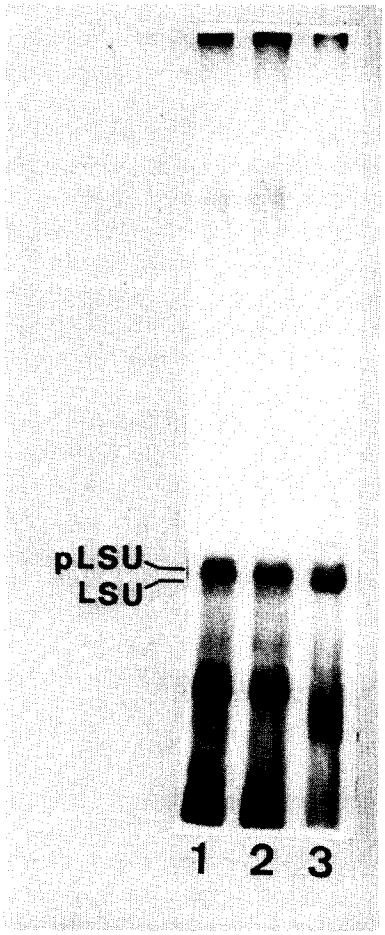

Fig.1. Fluorograph of SDS–polyacrylamide gradient gel showing [^{35}S]methionine-labelled proteins synthesized by the translation of spinach chloroplast RNA in an *E. coli* cell-free system (tracks 1,2), and by isolated spinach chloroplasts (track 3). Tracks 1 and 2 are the same except that 5 μg RuBP carboxylase were included in the sample run in track 2. The LSU and proposed LSU precursor polypeptide (pLSU) are indicated on the fluorograph. Most of the lower M_r products have been run off the bottom of the gel.

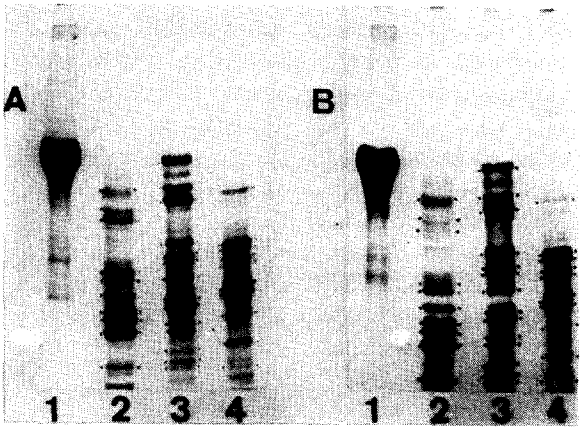

Fig.2. Comparison of the partial proteolytic digests of the LSU of spinach RuBP carboxylase and the 55 000 M_r product of the cell-free translation of spinach chloroplast RNA. The radioactive 55 000 M_r band was located by autoradiography of the SDS-polyacrylamide gel. It was cut out, eluted, mixed with unlabelled carrier LSU, digested with proteases for 30 min at 37°C and electrophoresed on SDS–polyacrylamide gradient gels. Treatments were (1) undigested, (2) *S. aureus* protease (120 μg/ml), (3) chymotrypsin (40 μg/ml), (4) papain (10 μg/ml). (A) Coomassie blue stained pattern; (B) fluorograph. The black dots correspond to the major bands staining with Coomassie blue.

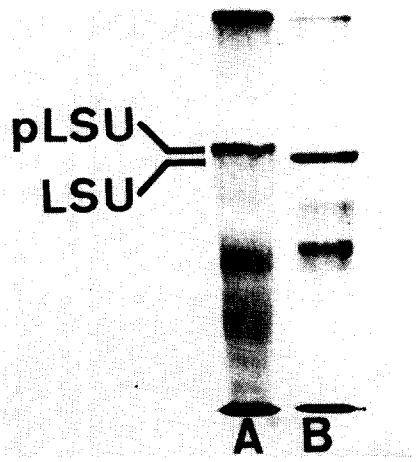

Fig.3. Fractionation on a net-charge basis of the [^{35}S]methionine-labelled products synthesized (A) by the translation of spinach chloroplast RNA in an *E. coli* cell-free system, and (B) by isolated spinach chloroplasts. The protein samples were carboxymethylated and citraconylated prior to electrophoresis on a 6 M urea–acrylamide gel and fluorography. LSU and pLSU as in fig.1.

The two protein products could also be distinguished when fractionated on a charge rather than a M_r basis (fig. 3).

The possibility that chloroplasts may be capable of processing the LSU synthesized by the in vitro translation of chloroplast RNA was examined since the product synthesized in the isolated chloroplast system was the same size as authentic LSU prepared from purified RuBP carboxylase. Chloroplast RNA was incubated for 20 min in the *E. coli* translation system, at which time further protein synthesis was inhibited by the addition of chloramphenicol and unlabelled methionine. Different amounts of membrane or soluble chloroplast fractions were added and incubation was continued for a further 30 min. In fig. 4 it can be seen that 3 μl soluble chloroplast extract were sufficient to completely convert the in vitro 55 000 M_r product into a smaller polypeptide equivalent to the LSU which is synthesized in isolated chloroplasts, whereas chloroplast membranes had no such effect. The shift in electrophoretic mobility of the LSU band resulting from the addition of soluble chloroplast extracts was not due to distortions in that region of the gel caused by large amounts of unlabelled LSU since it was found that, if the in vitro product was mixed with chloroplast extract, immediately heated in SDS and loaded onto the gel, there was no shift in the position of the pLSU band (fig. 4C). These results show that there is a processing activity in the soluble fraction from chloroplasts which can convert the apparently larger precursor form of LSU into the smaller mature form. In some gel patterns a faint band can be seen immediately below the main in vitro 55 000 M_r product (e.g. fig. 4A, track a). It has almost the same mobility as LSU itself and it is possible that some processing of the pLSU takes place in the *E. coli* cell-free system.

4. Discussion

The experiments described here show that the in vitro translation of spinach chloroplast RNA yields a form of LSU of RuBP carboxylase which appears to be 1000–2000 M_r larger than the LSU synthesized in isolated chloroplasts. Although incorrect initiation or termination by the *E. coli* translation system could account for the larger form, it seems unlikely since incubation with a soluble chloroplast fraction converts it to the same apparent size as the mature form. Also, the differences between the two polypeptide forms are unlikely to be attributable to conformational differences because they persist through the strongly denaturing conditions used both in the SDS–polyacrylamide gel electrophoresis and in the urea–gel analysis of the charge/mass ratio. Post-translational modification of the protein, such as glycosylation, could also explain the difference, but I favour the view that the in vitro synthesized polypeptide is simply longer than the mature LSU and that synthesis of LSU in chloroplasts normally proceeds via such a precursor which is immediately processed into the mature protein by a soluble component of the chloroplasts.

A precursor form of LSU had not been reported, possibly due to the small difference in electrophoretic mobility between the presumptive precursor and the mature form. Only by extending the electrophoresis run does the difference become apparent. In preliminary experiments, pea chloroplast RNA translated in vitro in an *E. coli* system produced a major product in the 55 000 M_r region that migrated slightly more slowly than the LSU protein synthesized by isolated pea chloroplasts (not shown). A comparable situation may also occur in maize. Using a linked system comprising *E. coli* RNA polymerase and a rabbit reticulocyte lysate in [8], two closely migrating 55 000 M_r polypeptides were produced if a cloned fragment of

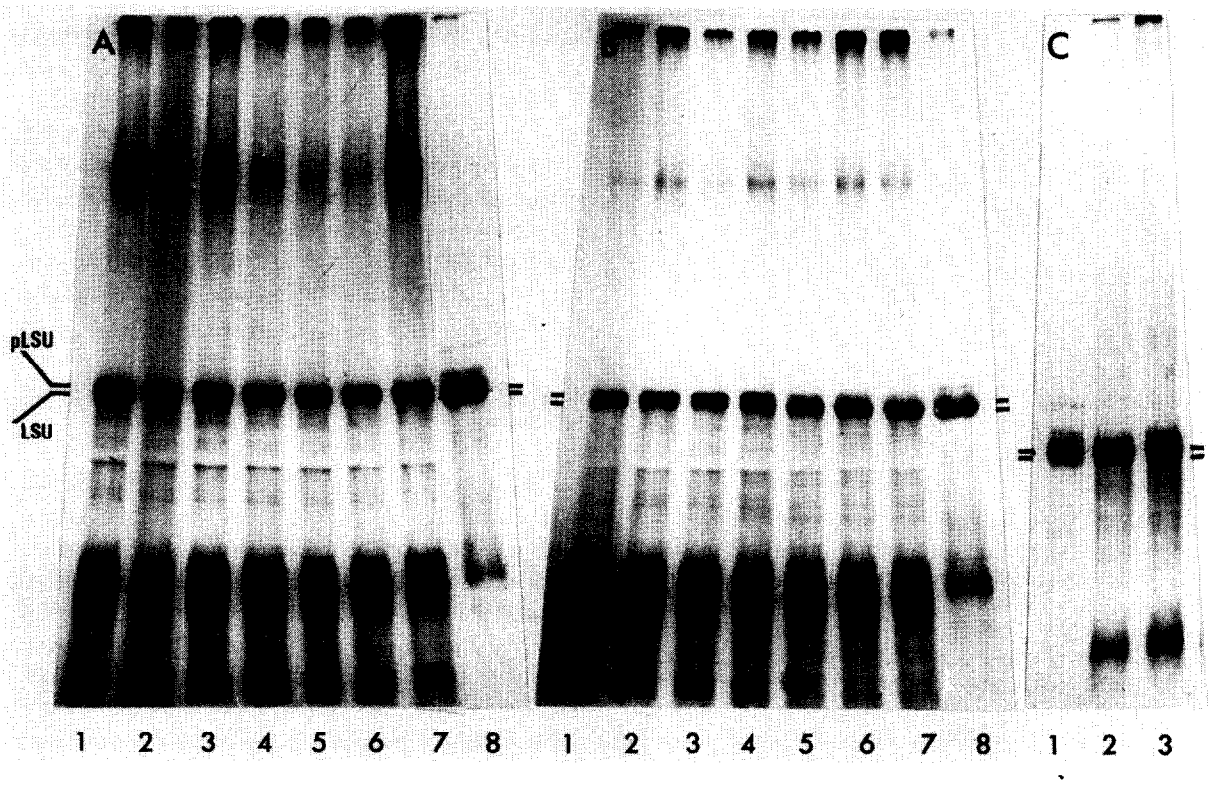

Fig.4. Processing of the [^{35}S]methionine-labelled 55 000 M_r product synthesized in the *E. coli* cell-free system programmed with spinach chloroplast RNA. Various concentrations of chloroplast membrane (A) and soluble (B) extracts were added to the in vitro product after translation had been terminated by the addition of chloramphenicol to 50 μg/ml and unlabelled methionine to 1 mM final conc. The volumes of extract added to each 50 μl assay were: (1) 0, (2) 0.1 μl, (3) 0.3 μl, (4) 1.0 μl, (5) 3 μl, (6) 10 μl and (7) 30 μl. Incubation was for 30 min at 25°C and the samples were then electrophoresed and fluorographed. Track 8 shows the proteins synthesized by isolated spinach chloroplasts. (C) 3 μl and 10 μl soluble chloroplast extract were added to the in vitro translation product and loaded onto a gel without further incubation (tracks 2,3). The products synthesized by isolated spinach chloroplasts are shown in track 1. LSU and pLSU as in fig.1.

maize chloroplast DNA carrying the LSU gene was used as template. The smaller band corresponded to mature LSU protein and the second, slightly larger band was related to LSU and may be analogous to the precursor form described here. The rabbit reticulocyte lysate may, therefore, be capable of processing some of the precursor to the mature form.

Some precursor proteins, differing only slightly in size from the mature chain, are inserted into, or are transported across, a membrane. These precursors appear to function as a means of recognition for membrane transport mechanisms by providing a hydrophobic amino acid chain [4,22]. However, this is unlikely to be the role of the LSU precursor since it does not appear to be involved in membrane transport or insertion, and since the processing activity, like the RuBP carboxylase holoenzyme, seems to be soluble.

At least two alternative functions can be proposed for a LSU precursor. LSU prepared from RuBP carboxylase is essentially insoluble in aqueous solution below pH 9.3 whereas the products of in vitro translation are soluble at neutral pH. This apparent difference in solubility may be a property of the secondary or tertiary structure of the two polypeptide chains and the precursor may function as a soluble form of the LSU polypeptide preparatory to its assembly into the active enzyme.

A second possibility is that the LSU precursor form is required for the correct assembly of the RuBP carboxylase holoenzyme. The small subunit of the enzyme is synthesized as a precursor and subsequently processed to the mature form in the stroma of the chloroplast [5,6]. Although it is not known whether the processing of the small subunit occurs before or after its assembly into RuBP carboxylase, the precursor form may, in some unknown way, be necessary for correct assembly and the same requirement may apply to the precursor form of LSU.

Acknowledgements

I am greatly indebted to Drs P. R. Whitfeld and T. J. V. Higgins for their advice and encouragement.

References

[1] Baker, T. S., Eisenberg, D. and Eiserling, F. (1977) Science 196, 293–295.
[2] Kawashima, N. and Wildman, S. G. (1972) Biochim. Biophys. Acta 262, 42–49.
[3] Gray, J. C. and Kekwick, R. G. O. (1973) FEBS Lett. 38, 67–69.
[4] Dobberstein, B., Blobel, G. and Chua, N.-H. (1977) Proc. Natl. Acad. Sci. USA 74, 1082–1085.
[5] Chua, N.-H. and Schmidt, G. W. (1978) Proc. Natl. Acad. Sci. USA 75, 6110–6114.
[6] Highfield, P. E. and Ellis, R. J. (1978) Nature 271, 420–424.
[7] Chan, P.-H. and Wildman, S. G. (1972) Biochim. Biophys. Acta 277, 677–680.
[8] Coen, D., Bedbrook, J. R., Bogorad, L. and Rich, A. (1977) Proc. Natl. Acad. Sci. USA 74, 5487–5491.
[9] Malnoe, P., Rochaix, J. D., Chua, N. H. and Spahr, P.-E. (1979) J. Mol. Biol. 133, 417–434.
[10] Bottomley, W. and Whitfeld, P. R. (1979) Eur. J. Biochem. 93, 31–39.
[11] Blair, L. E. and Ellis, R. J. (1973) Biochim. Biophys. Acta 319, 223–234.
[12] Bottomley, W., Spencer, D. and Whitfeld, P. R. (1974) Arch. Biochem. Biophys. 164, 106–117.
[13] Vasconcelos, A. C. (1976) Plant Physiol. 58, 719–721.
[14] Hartley, M. R., Wheeler, A. and Ellis, R. J. (1975) J. Mol. Biol. 91, 67–77.
[15] Sagher, D., Grosfeld, H. and Edelman, M. (1976) Proc. Natl. Acad. Sci. USA 73, 722–726.
[16] Whitfeld, P. R., Herrmann, R. G. and Bottomley, W. (1978) Nucleic Acids Res. 5, 1741–1751.
[17] Spencer, D., Higgins, T. J. V., Button, S. C. and Davey, R. A. (1980) Plant Physiol. 66, 510–515.
[18] Wieland, F. and Engeser, H. (1979) FEBS Lett. 100, 90–94.
[19] Maurer, H. R. (1971) in: Disc Electrophoresis, Walter de Gruyter, Berlin.
[20] Stephens, R. E. (1975) Anal. Biochem. 65, 369–379.
[21] Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106.
[22] Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851.