Imported large subunits of ribulose biphosphate carboxylase/oxygenase, but not imported β -ATP synthase subunits, are assembled into holoenzyme in isolated chloroplasts

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The large subunit of ribulose biphosphate carboxylase from *Anacystis nidulans* 6301, and the β subunit of chloroplast ATP synthase from maize, were fused to the transit peptide of the small subunit of ribulose biphosphate carboxylase from soybean. These proteins were assayed for post-translational import into isolated pea chloroplasts. Both proteins were imported into chloroplasts. Imported large subunits were associated with two distinct macromolecular structures. The smaller of these structures was a hybrid ribulose biphosphate carboxylase holoenzyme, and the larger was the binding protein oligomer. Time-course experiments following import of the large subunit revealed that the amount of large subunit associated with the binding protein oligomer decreased over time, and that the amount of large subunit present in the assembled holoenzyme increased. We also observed that imported small subunits of ribulose biphosphate carboxylase, although predominantly present in the holoenzyme, were also found associated with the binding protein oligomer. In contrast, the imported β subunit of chloroplast ATP synthase did not assemble into a thylakoid-bound coupling factor complex.

Key words: chimeric proteins/coupling factor/import/plastid genes/RuBP carboxylase

Introduction

Chloroplasts are semi-autonomous organelles which contain sufficient DNA to encode only a small number of their proteins. Instead, most chloroplast proteins are encoded in the nucleus, synthesized on cytoplasmic ribosomes and transported into chloroplasts by a post-translational process (reviewed by Cashmore *et al.*, 1985; Schmidt and Mishkind, 1986), which depends upon an amino-terminal transit peptide (Schreier *et al.*, 1985; van den Broeck *et al.*, 1985; Lubben and Keegstra, 1986). The mechanism of action of the transit peptide is not clear, but it is usually removed following import to yield a protein of lower mol. wt than the cytoplasmically synthesized precursor.

Two of the proteins encoded on the plastid genome are the large (L) subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (rubisco) and the β subunit of ATP synthase (reviewed by Rochaix, 1985). The rubisco L subunit is the

focus of some discussion on the possibility of altering its structure to improve the efficiency of carbon dioxide fixation by increasing the affinity of the enzyme for carbon dioxide, or to improve the ratios of carboxylation to oxygenation (Ellis and Gatenby, 1984; Gutteridge and Gatenby, 1987). In studies designed to introduce modifications into the primary structure of the rubisco L-subunit an essential feature is the ability to reconstitute the active or modified rubisco holoenzyme. Expression plasmids have been developed that direct the synthesis of *Escherichia coli* of active dimeric (L₂) rubisco from *Rhodospirillum rubrum* (Somerville and Somerville, 1984), and also a higher-plant type L₈S₈ holoenzyme containing small (S) subunits using genes from cyanobacteria (Christeller *et al.*, 1985; Gatenby *et al.*, 1985; Gurevitz *et al.*, 1985; Tabita and Small, 1985). Although an active cyanobacterial/higher-plant hybrid rubisco can be synthesized in *E. coli* (Bradley *et al.*, 1986; van der Vies *et al.*, 1986), the synthesis and assembly of a higher plant enzyme outside of the chloroplast has been elusive, even when the genes for the two subunits have been expressed together in *E. coli* (Gatenby *et al.*, 1987). One suggestion to account for these difficulties in rubisco assembly is that the plant L subunit adopts an inactive, insoluble conformation after, or during synthesis (Gatenby, 1984). A chloroplast protein has been identified that will bind to newly synthesized L subunits within the organelle and may be involved in rubisco holoenzyme assembly (reviewed by Musgrove and Ellis, 1986). The possible requirement for this binding protein (BP) oligomer in assembly might explain the failure to assemble higher-plant subunits in experimental systems other than isolated chloroplasts. We are, therefore, presented with two related problems: if an L subunit gene can be satisfactorily modified, how can its product be used in higher-plant chloroplasts for incorporation into rubisco holoenzyme? Secondly, can an experimental system be devised in which to study the assembly and properties of mutated, foreign or hybrid rubisco L subunits in the context of the higher-plant photosynthetic pathway?

In this paper we have attempted to resolve these problems by fusing the transit peptide of the S subunit of rubisco from soybean, to the L subunit of rubisco from the cyanobacterium *Anacystis nidulans* 6301 and examining the import and assembly of a foreign L subunit in isolated pea chloroplasts. It was found that the fusion protein was imported. Furthermore, the imported L subunit assembled into a hybrid cyanobacterial/pea holoenzyme, which appeared to occur via an initial association with the BP oligomer. In separate experiments imported S subunits were also found associated with the BP oligomer.

The import and fate of the maize chloroplast β -ATPase subunit fused to the rubisco S subunit transit peptide was also examined. This protein was also imported into pea chloroplasts but the β subunit failed to assemble into a thylakoid-bound ATPase. These results indicate that chloroplast, or chloroplast related, proteins can be imported effi-

ciently by fusion to a transit peptide. This offers the potential of directing modified versions of such proteins back into the chloroplast following plant transformation.

Results

Comparison of the efficiency of import of TPSS, TPCF- β and TPLS into isolated chloroplasts

The structures of the chimeric fusion proteins TPSS, TPCF- β and TPLS are shown in Figure 1. All three possess the N-terminal transit peptide of the S subunit of soybean rubisco that has been fused to either the S subunit of pea rubisco (TPSS), the β subunit of maize ATPase (TPCF- β) or the L subunit of *A. nidulans* rubisco (TPLS). In the latter two proteins, as a result of the cloning strategy used, cleavage of the transit peptide should result in a β subunit deficient

Fig. 1. Chimeric precursor proteins. Proteins were synthesized by *in vitro* transcription and translation of cloned DNA. The resulting proteins are diagrammed to show their composition. Bars are drawn to scale; the numbers on the horizontal axis refer to the number of amino acids. The different types of shading show the origin of that section of peptide. Rubisco S subunit precursor transit peptide (TP) [hatched], pea rubisco S subunit mature peptide (S) [white], rubisco L subunit (L) [white]; β -ATPase [dotted]; 25-amino-acid upstream region (see text) [diagonal lines].

Fig. 2. Import and processing of TPLS and TPCF- β . Precursor proteins were incubated with chloroplasts as described in Materials and methods, treated with protease to remove external molecules and analyzed by SDS-PAGE and fluorography. Fluorographs of the starting translation and the imported, protease-protected molecules are shown. Different exposures were used for each of the proteins, hence the relative intensities of the bands is not representative of import efficiencies. Odd-numbered lanes show translations; even-numbered lanes show imported proteins. Lanes 1 and 2, TPCF- β ; lanes 3 and 4, TPLS. The migration of mol. wt standards is shown: bovine serum albumin, 68 kD; ovalbumin, 43 kD; α -chymotrypsinogen, 26 kD; β -lactoglobulin, 18 kD.

in 37 N-terminal amino acids but with four added at the fusion junction, and an L subunit with an additional 26 amino acids present at its N terminus.

Plasmids encoding the chimeric proteins were linearized, transcribed by SP6 RNA polymerase and translated either in a wheatgerm or a rabbit reticulocyte translation system. In an initial experiment, the radiolabelled precursors TPLS or TPCF- β were incubated with isolated pea chloroplasts, the chloroplasts treated with the protease thermolysin and the protease-protected proteins compared to the original translation products. Figure 2 shows a fluorograph of an SDS-polyacrylamide gel, which demonstrates that the imported β subunit (lane 2) is smaller than the precursor molecule in the translation reaction (lane 1), and that the imported L subunit (lane 4) is smaller than its precursor molecule (lane 3). Both TPCF- β and TPLS were processed to the size anticipated if the soybean transit peptide had been removed.

To examine the rates of import of TPCF- β and TPLS, compared with an S subunit precursor TPSS, a time-course experiment was carried out (Figure 3). The time-course for the import of TPLS and TPCF- β was examined over 20 min. The relative rates of import of TPLS and TPCF- β varied from experiment to experiment, such that the rate of import of TPCF- β varied from 10 to 50% of that of TPSS, and the rate of import of TPLS varied from 10 to 25% of that found for TPSS. These values were determined using an equal number of precursor molecules per chloroplast at the beginning of the import reaction. The import efficiency of the TPSS soybean/pea hybrid was almost the same as the import efficiency of a native pea TPSS into pea chloroplasts (Lubben and Keegstra, 1986) and so the chimeric S subunit protein was used as a suitable control in these studies. A factor that contributed to the variability in these rates is that there was some partial degradation of the L and β subunits following import. These degradation products can be seen in Figure 2 (lanes 2 and 4) and were not included in the calculations of import rate. In one experiment with TPLS, degradation products as well as intact L subunits were counted to determine rates. In this case import was 75% of that found for

Fig. 3. Time course of import for TPSS, TPCF- β and TPLS. Chloroplasts were incubated with the translation products for the indicated times. Aliquots were removed and quantitated for import proteins as described in Materials and methods. Results are shown as the percentage of molecules found inside chloroplasts relative to that present at the start of the incubation. Imported S subunit ○; imported β subunit △; imported intact L subunit □; imported L subunit included in the size range 55–25 kD ■.

TPSS. Therefore, the import rates which were determined by counting only intact L or β subunits should probably be regarded as underestimates. To test whether the L or β subunits could be imported into chloroplasts in the absence of a transit peptide, appropriate plasmids were constructed that lacked the soybean transit peptide coding sequence. Radiolabelled L or β subunits synthesized from these plasmids *in vitro* failed to import into isolated chloroplasts (data not shown). We conclude that both TPLS and TPCF- β are

imported efficiently into chloroplasts, but that import is dependent on the presence of the rubisco S subunit transit peptide.

Assembly of imported L subunit into rubisco holoenzyme

It is known that cyanobacterial rubisco L subunits can assemble with higher-plant S subunits to form an active enzyme when the genes for the two subunits are expressed in *E. coli* (Bradley *et al.*, 1986; van der Vies *et al.*, 1986), or if plant S subunits are added to cyanobacterial L₈ *in vitro* (Andrews and Lorimer, 1985). Since imported cyanobacterial L subunits might also combine with plant S subunits within the chloroplast, we examined chloroplast lysates following TPLS import to see if the L subunit had been assembled into a hybrid rubisco molecule.

In preliminary experiments, the radiolabelled TPLS protein was imported into chloroplasts, which were lysed and the stromal fraction analyzed by sucrose gradient centrifugation. It can be seen in Figure 4 (panel B) that a rapidly sedimenting peak of radioactivity is present in the lower part of the gradient. This peak was located in the same fractions as rubisco holoenzyme, as determined by non-denaturing PAGE and protein staining of the fractions (data not shown). In a control experiment imported S subunit was also found to sediment with the rubisco holoenzyme (Figure 4, panel A). The conversion of imported L subunit to a rapidly sedimenting form occurred within the chloroplasts. The TPLS present in the *in vitro* translation reactions examined prior to import remained near the top of sucrose gradients in a position suggesting that they were monomeric or dimeric molecules. Co-sedimentation of imported L subunit with rubisco holoenzyme on sucrose gradients might indicate that the cyanobacterial L subunit had assembled into a hybrid enzyme, but the resolution obtained with these gradients would make it difficult to distinguish between alternative possibilities, such as cyanobacterial L₈ formation or an aggregation state. Further analysis of the fate of imported L subunit made use of non-denaturing PAGE on gels of various polyacrylamide concentrations.

Fig. 4. Analysis of imported rubisco L or S subunits by sucrose gradient centrifugation. Chloroplasts were incubated with either TPLS or TPSS, treated with protease, lysed and the stromal contents analyzed by ultracentrifugation through gradients of 0.20–0.70 M sucrose as described in Materials and methods. Separate gradients were used for TPSS (panel A) and TPLS (panel B). Fractions were analyzed by SDS-PAGE and fluorography. Labelled bands containing intact L subunit or S subunit were excised, solubilized and quantitated by liquid scintillation counting. Results are shown as millions of molecules recovered per fraction for imported S subunit (○) and imported L subunit (□).

Fig. 5. Import time-course of TPLS (panel A) or TPSS (panel B) labelled with [³⁵S]methionine into pea chloroplasts. Aliquots of chloroplasts were removed from the import reactions at various times, washed, treated with protease and centrifuged through Percoll step gradients. The pellet obtained from chloroplasts containing 100 μ g chlorophyll was resuspended in 30 μ l 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, frozen and thawed, and a stromal fraction obtained by centrifugation. The protein concentration was measured and an identical amount of protein from each time point was electrophoresed on a non-denaturing polyacrylamide gel (6%) with a 3% stacking gel. Gels were fluorographed. **Panel A:** TPLS import at 0 (lane 1); 5 (lane 2); 10 (lane 3); 20 (lane 4); 30 (lane 5); 60 (lane 6) and 90 min (lane 7). **Panel B:** TPSS import at 0 (lane 1); 0.5 (lane 2); 1 (lane 3); 2 (lane 4); 5 (lane 5) and 10 min (lane 6). B marks the position of the binding protein oligomer and R marks the position of rubisco holoenzyme.

Fig. 6. Assembly of imported S and identification of the binding protein oligomer (B) and rubisco holoenzyme (R). In lanes 1 and 2 pea chloroplasts were incubated with [35 S]methionine-labelled TPSS for 10 min and a stromal fraction prepared. Aliquots were electrophoresed as in Figure 5, stained with Coomassie blue and fluorographed. Lane 1 shows the stained protein and lane 2 is the fluorographs of the same sample. In lanes 3–5 unlabelled stroma was electrophoresed on a non-denaturing gel (5% acrylamide) and either stained with Coomassie blue (lane 3), or immunoblotted using antiserum against binding protein (lane 4) or rubisco (lane 5) and using [125 I]protein A detection.

Following import of L subunit into chloroplasts, stromal fractions were prepared and examined on non-denaturing gels containing 5, 6 or 7% polyacrylamide. In Figure 5A it can be seen that imported radiolabelled L subunit is found associated with two protein complexes resolved in the gel. The smaller of these protein complexes co-migrated with rubisco holoenzyme as determined by Coomassie blue staining. The larger protein co-migrated with a protein of similar mobility to the BP oligomer. To confirm the identity of these proteins they were transferred to nitrocellulose and incubated with antisera raised against either the BP, or rubisco holoenzyme. The bound antibody was located by incubation with [125 I]protein A followed by autoradiography, and the results showed that the larger protein complex was the BP oligomer and the smaller one was rubisco holoenzyme (Figure 6, lanes 3–5).

If chloroplasts which contained imported L subunit were maintained in the light and samples removed at various times, the distribution of L subunit between the BP oligomer and the rubisco holoenzyme changes (Figures 5A). Imported L subunit was initially found associated with the BP, but during a 90-min incubation the amount of L subunit associated with the BP decreased, and that found in the holoenzyme increased. This change in the distribution of L subunit between the two proteins is shown graphically in Figure 7. The radioactivity present in each of the BP and rubisco bands is presented as a fraction of their combined total at each time point. The radioactivity observed in Figures 5A and 7 was due entirely to imported L subunit since the addition of chloramphenicol to inhibit chloroplast protein synthesis did not alter the result obtained, and the levels of endogenous L subunit synthesis obtained by adding appropriate amounts of isotope and cold methionine in mock import reactions were negligible. In addition to radiolabelled L subunit associated with the binding protein and rubisco, there was frequently L subunit present as an aggregate that failed to enter the resolving gel at the junction of the stacking gel.

Fig. 7. Distribution of imported L in either binding protein or rubisco during a time-course experiment. The binding protein (B) or rubisco (R) bands shown in Figure 5A were excised, treated with Fluorosol (National Diagnostics) and counted. The counts in the two bands at each time point were combined and are expressed graphically as a function of that total for either binding protein (□) or rubisco (■).

Although we were always able to observe a decrease in the label present in the binding protein and an increase in that present in rubisco, the amount of label present at the gel interface appeared to be variable.

If imported L subunit assembled into rubisco holoenzyme, then it would be part of a hybrid enzyme containing pea S and possible pea L subunits. The cyanobacterial L might, however, assemble into a homologous L_8 structure such as that found when the L subunit is synthesized in *E. coli* (van der Vies *et al.*, 1986). To determine if the non-denaturing gels used in this study could distinguish between L_8 and L_8S_8 structures, the experiment shown in Figure 8 was carried out. Cyanobacterial L_8 from an *E. coli* expression strain and pea L_8S_8 were isolated by sucrose gradient centrifugation and analyzed on non-denaturing gels of various polyacrylamide concentration, followed by immunoblotting. It can be seen in Figure 8 that L_8 (lanes 3 and 6) migrates more rapidly than L_8S_8 (lanes 1 and 4) and it can be resolved in a mixture of the two forms (lanes 2 and 5). Therefore we conclude that the imported cyanobacterial L is assembled into a hybrid holoenzyme. Since isolated chloroplasts are used, presumably the pea S is contributed from a free pool or by recycling of S from pre-existing holoenzyme.

Association of imported S subunit with the BP oligomer

It has been demonstrated previously that imported S subunit will assemble into rubisco (Chua and Schmidt, 1978; Smith and Ellis, 1979), and this is shown in Figures 4A and 6. We have found that imported S subunit is also associated with the BP oligomer. In Figure 6, S subunit has been imported into isolated chloroplasts and the stromal fraction analyzed by non-denaturing gel electrophoresis. Lane 1 shows a stained gel and lane 2 shows an autoradiograph. It can be seen that, in addition to the majority of imported S subunit in the holoenzyme, there is also ~5–15% present in the BP band. In a time-course experiment of S subunit import (Figure 5B), S is found associated with both protein complexes, but we do not observe the gradual change in the distribution of imported subunit between BP and rubisco, as was the case for L subunit (Figure 5A). Furthermore, although for imported L subunit it takes ~30 min for an equal distribution of label between BP and rubisco to occur (Figure 7), for imported S subunit there is more label

Fig. 8. Rubisco holoenzyme isolated from pea chloroplasts and cyanobacterial L_8 synthesized in *E. coli* were analyzed by electrophoresis on non-denaturing gels. Proteins were transferred to nitrocellulose, incubated with antisera against pea rubisco and detected using alkaline phosphatase conjugated antibody. Lanes 1–3 show results from a 7% gel; lanes 4–6 show the results from a 5% gel. Lanes 1 and 4, rubisco holoenzyme (R); lanes 2 and 5, rubisco holoenzyme and L_8 mixed; lanes 3 and 6, L_8 (L).

Fig. 9. Localization of imported β subunit. Chloroplasts containing imported β or imported S subunits were fractionated as described in Materials and methods. The amount of imported molecules found associated with each fraction was quantitated by SDS-PAGE, fluorography and liquid scintillation counting. Results are expressed as the percentage found associated with a fraction compared to the total amount recovered, and are shown for S subunit ($\square$) and β subunit ($\blacksquare$) for each fraction: str, stroma; env, envelopes; thyl, thylakoids; w thyl, washed thylakoids.

associated with rubisco in the first 30 s of the experiment than with BP, and this does not alter during the course of the experiment (Figure 5B).

Imported β subunit fails to assemble into thylakoid ATP synthase

The β subunit of ATPase is part of a multi-subunit structure located on the stromal surface of the thylakoid membrane. Since imported rubisco L subunit will assemble into holoenzyme, we were interested to see if the imported β subunit would also assemble into a membrane-bound ATPase.

To examine the location of the β subunit, chloroplasts containing the imported subunit were lysed and subjected to fractionation (Figure 9). As a control, the location of imported rubisco S subunit in a parallel experiment was also examined. The fractions obtained from chloroplasts correspond to the stroma, envelopes, thylakoids and washed thylakoids. The presence of the β or S subunits in each fraction was

identified by SDS-PAGE, followed by scintillation counting of the excised bands. About 50% of the β subunit was found in the stromal fraction, with a smaller amount (35%) associated with the thylakoid pellet. In the control experiment using rubisco S, 75% was found in the stroma and 17% in the thylakoid fraction (Figure 9). Extensive washing of the thylakoids removed about two-thirds of both the imported β and S subunit. The remaining imported β subunit which was associated with the thylakoids did not co-purify with CF_1 when it was isolated using chloroform extraction, followed by velocity sedimentation on sucrose gradients. These data support the conclusion that the imported β subunit was not assembled into thylakoid-bound ATPase.

Discussion

Both the maize β ATPase subunit and the *A. nidulans* rubisco L subunit were imported into pea chloroplasts when fused to the transit peptide of the S subunit of rubisco from soybean. Import was dependent on the presence of the transit peptide: in its absence, import of the L or β subunits was not observed. Following import, the proteins were processed to a size that suggests that the N-terminal transit peptide had been removed. In addition to the full-length processed β and L subunits, it could also be observed that there were a number of smaller polypeptides, presumably arising by proteolysis. We were unable to detect any precursor forms of the β and L subunits within the organelle.

Neither the TPCF- β , nor the TPLS proteins contain sequences present in the mature S polypeptide, and yet their import efficiency was reasonable. This is an important point since although it is well established that the transit peptide alone is sufficient for import of some proteins into chloroplasts (reviewed by Cashmore *et al.*, 1985; Schmidt and Mishkind, 1986), the requirement for parts of the mature sequence following the cleavage site is not so clear. Wasmann *et al.* (1986) demonstrated that the efficiency of import of neomycin phosphotransferase II is improved by the presence of part of the mature S subunit, and that import is very inefficient if only the transit peptide is present. This is in contrast to the efficiencies of import of TPLS and TPCF- β that we report here, which contain only the transit peptide of TPSS. Clearly, at this stage, it is important not to establish models of what is required for efficient import until a wider range of proteins have been examined. Possibly the requirements may vary between particular proteins.

There could be a number of reasons why genes have been retained in the plastid genome. One possibility is that the regulation of expression or activity of multisubunit proteins is best accomplished when the transcription or translation of some of their subunits remain in the plastid. Another possibility is that organelle-synthesized proteins contain structural features that prevent or interfere with their post-translational import (von Heijne, 1986). The successful import of the maize chloroplast encoded β subunit indicates that this particular protein does not contain features that prevent its post-translational transfer into chloroplasts. In addition, the chloroplast β subunit shares extensive homology with the mitochondrial ATPase β subunit, but the latter is nuclear-encoded and is imported post-translationally using an N-terminal transit peptide (Takeda *et al.*, 1985). Since structural impediments do not prevent chloroplast β subunit import, the retention of its gene in the organelle may well

be due to regulatory or assembly requirements. It is interesting in this regard that the transcriptional units for the β and ϵ subunits are linked (Gray *et al.*, 1986), and their reading frames overlap (Krebbers *et al.*, 1982), suggesting translational coupling. If the co-ordinated expression of these two proteins is an integral part of the assembly process for chloroplast ATPase this could account for their coding sites; however, this then raises the question of why a different arrangement exists for the mitochondria. The post-translational import of cyanobacterial L also demonstrates that this protein does not possess features that prevent its passage into the chloroplast. Although the particular *A.nidulans* gene we used is not chloroplast encoded, it does possess good primary sequence homology with the L subunit from higher plants (Shinozaki *et al.*, 1983), and can form a functional hybrid enzyme with higher plant subunits (Andrews and Lorimer, 1985; van der Vies *et al.*, 1986). Hurt *et al.* (1986) have reported the import of *Chlamydomonas* L subunit into yeast mitochondria, which supports our view that there are unlikely to be difficulties in the import of a range of L subunits from various organisms into chloroplasts.

When the cyanobacterial L subunit was imported into chloroplasts it was initially found associated with a large macromolecular structure (Figure 5A), which we have identified as the BP oligomer. During a time-course experiment the amount of L subunit associated with BP decreased and that present in the smaller rubisco holoenzyme increased (Figure 4A). This result is consistent with the view that imported L subunit associates with the BP oligomer as a first step in rubisco assembly, and then becomes assembled into holoenzyme. Imported L subunit was also present at the junction of the stacking and resolving gel, and throughout the length of the lane. It is not clear what role, if any, this material has in the assembly process but its presence should be noted in any model involving rubisco assembly. It is important to note that this result does not necessarily demonstrate a precursor-product relationship, since L subunits that assembled into holoenzyme could come from sources other than the BP complex. The reduction in L subunit associated with the BP complex could then arise by changes in the equilibrium between the complex and these other sources of L subunit. If imported L subunit does assemble into rubisco by prior association with the BP oligomer, this assembly process does not require chloroplast protein synthesis since we obtain identical results when chloramphenicol is present. The involvement of the BP oligomer in rubisco assembly has been suggested by Barraclough and Ellis (1980) and Roy *et al.* (1982). The data in Figure 5A is reminiscent of data presented by Barraclough and Ellis (1980), except that in their results the presence of chloroplast-synthesized L subunit in BP or rubisco was examined. Although there are some slight differences in the time of appearance or loss of L subunit associated with BP or rubisco, the similarity of data indicates that the L subunit is assembled into holoenzyme using a similar pathway whether it is a homologous pea L subunit synthesized within the chloroplast, or a heterologous cyanobacterial L subunit imported into the organelle.

The association of cyanobacterial L subunit with BP could reflect a simple recognition of its conserved structure to allow it to be directed through the postulated assembly pathway. It does, however, raise the question of whether cyanobacteria require a BP for assembly. The ease with which cyano-

bacterial rubisco will assemble in *E.coli* has led to the suggestion that a protein to assist in assembly is not required, whereas in plants such a requirement may exist (reviewed by Gutteridge and Gatenby, 1987). Perhaps cyanobacteria do use such a protein and its function is supplied in *E.coli* by a related protein to allow assembly.

In our initial experiments we were interested in comparing the efficiency of import of TPCF- β and TPLS with that of TPSS as a control. Upon analysis of our import reactions on non-denaturing gels, it was observed that not only was imported S subunit assembled into rubisco, as anticipated from previous reports (Chua and Schmidt, 1978; Smith and Ellis, 1979), but it was also found associated with BP (Figure 6). During a time-course experiment it can be seen that there is a rapid accumulation of S subunit into holoenzyme (Figure 5B). Although there is also an increase in S subunit associated with BP, we do not see a temporal alteration in the amounts of S present in the two structures, as observed for imported L subunit (Figure 5A). The presence of S associated with BP is intriguing and might indicate that this complex is the site for assembly of L and S subunits. The rates of assembly of imported L and S subunits into rubisco are very different, with the rate for L being much slower. We do not believe this is due to any significant non-homology of the cyanobacterial L subunit with the pea assembly process, since similar rates are obtained for pea L synthesized in chloroplasts (Barraclough and Ellis, 1987). Because isolated chloroplasts are deprived of a source of imported S subunit, any holoenzyme assembly must use either a pool of pre-existing S or must use S that can be recycled from existing holoenzyme via subunit exchange. If the pool is small, or unstable (Schmidt and Mishkind, 1983), or recycling does not occur, then assembly might become stalled, leading to a slow rate of holoenzyme formation. If this interpretation is correct, then the rapid assembly of S subunit would indicate that the assembly mechanism is poised ready to sequester any imported S. This process might be so rapid as to make it difficult to see any possible precursor-product relationship for S subunit import with the methods used here, whereas a slower rate of assembly of imported L subunit may facilitate detection of such a relationship with the BP.

The ability to transport proteins into chloroplasts which are chloroplast encoded, or related to chloroplast proteins, suggests an additional route for the genetic modification of plants in which chloroplast genes fused to transit peptide coding sequences can be transferred to the nucleus. This may be an alternative strategy to chloroplast transformation, and our results have shown that for two proteins at least, all that is required for successful import is fusion to only a transit peptide. The ability of a foreign imported L subunit to assemble into rubisco would suggest that suitably modified L subunits can be imported into the chloroplast with some confidence that they can assemble into holoenzyme. The failure of imported β subunits to assemble into thylakoid ATPase reveals the potential difficulties involved in perturbing the structure of multisubunit proteins. It has been suggested that ATPase assembles via a concerted mechanism requiring the presence of all subunits (Nelson, 1986). A previous report demonstrated the assembly of ATPase in isolated chloroplasts, but all the subunits required for assembly were present (Nelson *et al.*, 1980). It is possible that assembly of β subunits could be obtained if the other nuclear-encoded ATP subunits were also imported, such as would exist *in vivo*.

Materials and methods

Enzymes and reagents

Most biochemicals were from Sigma unless listed otherwise. Nucleotides were from Pharmacia, radioisotopes from New England Nuclear and SP6 RNA polymerase and restriction enzymes from Pharmacia, New England Biolabs or Bethesda Research Laboratories. Wheat-germ was a gift from Vitamins, Inc. (Chicago) or General Mills. Wheat germ and rabbit reticulocyte lysates were also purchased from Promega, together with RNasin, Protoblot, pSP64, pGEM3 and pGEM4. Sequenase was from United States Biochemical Corporation. Antisera raised against the binding protein oligomer or rubisco holoenzyme were generous gifts from John Ellis.

Plasmid construction

Plasmid pSP81/4 (Lubben and Keegstra, 1986) encodes a chimeric rubisco S subunit precursor (TPSS) (previously designated prSP) containing a soybean (*Glycine max*) transit peptide and a pea (*Pisum sativum*) mature peptide. Plasmid pGM22/3, which codes for the transit peptide region of soybean rubisco S subunit precursor, was constructed by subcloning the *HindIII/SphI* fragment of pSP81/4 into *HindIII/SphI*-cut pGEM3.

Plasmid pTPSL1, which codes for the TPLS protein, was constructed by subcloning a 2.1-kb *PstI* fragment of DNA encoding the L subunit of rubisco from the cyanobacterium *A. nidulans* 6301 (Shinozaki *et al.*, 1983) into the *PstI* site of pGM22/3. The resulting plasmid encodes a protein containing the transit peptide of soybean rubisco S subunit precursor, a 1-amino-acid cloning artefact, 25 amino acids from the region upstream of the initiation codon of the native L subunit (in frame with the initiation methionine), and the entire L subunit protein (Figure 1).

Plasmid pTP β 3A, which codes for TPCF- β (Figure 1), was constructed using a series of vectors. It consists of a fragment of the maize (*Zea mays*) β ATPase gene extending from a *SmaI* site at codon 37 at the 5' end, to a *PvuII* site located 123 nucleotides downstream from the termination codon (Krebbers *et al.*, 1982). This fragment was fused to a partially Klenow repaired *SalI* site in the polylinker from pUC18, such that the *SphI* site was at the 5' end. A partial repair was necessary to maintain the correct reading frame and this was confirmed by DNA sequencing. This fragment was subcloned into pGM22/3 using the *SphI* and *EcoRI* sites. The resulting plasmid codes for a protein containing the entire transit peptide of soybean rubisco S subunit precursor, a 4-amino-acid cloning artefact, and all but the first 37 N-terminal amino acids of the β subunit.

In vitro transcription/translation

Transcription of linearized DNA templates to produce mRNA was performed as described previously (Lubben and Keegstra, 1986). The mRNA was precipitated with 2.5 vol of ethanol, collected by centrifugation, and dissolved in 10 mM dithiothreitol, 0.2 U/ml RNasin at 40°C for 30 min.

In vitro translations were either carried out as described previously using [³H]leucine or [³⁵S]methionine (Lubben and Keegstra, 1986), or used protocols supplied with wheat germ or rabbit reticulocyte lysates from Promega. Reactions were stopped by the addition of either 60 mM leucine or 100 mM methionine in 2 × uptake buffer (Cline *et al.*, 1985) and frozen at -70°C until used for import.

Import assays

The preparation of wheat germ S23 extract and pea chloroplasts and *in vitro* assays for import of proteins into chloroplasts have been described previously (Cline *et al.*, 1985). Quantitation of proteins and calculations of numbers of molecules per chloroplasts were determined from the number of leucine residues per protein molecule: TPSS, 13; TPCF- β , 56; and TPLS, 44. Processed molecules, without transit peptides, contained one less leucine per molecule than the precursor. The number of chloroplasts per sample was calculated from the protein content of the sample and the number of chloroplasts per μ g protein at the beginning of the experiment. Protein assays were performed according to Bradford (1976). Chloroplasts were counted by phase-contrast microscopy.

Analysis of rubisco assembly

Imported proteins were assayed for assembly into rubisco holoenzyme by sucrose gradient centrifugation (Kahana and Edelman, 1982). A control sample containing imported TPSS was treated identically. Chloroplasts were lysed by two freeze-thaw cycles in isotonic buffer (Cline *et al.*, 1981) and membranes removed by centrifugation at 48 000 g for 30 min. The supernatant, containing the stromal contents, was layered on a linear sucrose gradient (0.2–0.70 M) and centrifuged at 155 000 g for 10 h. Fractions were collected and quantitatively precipitated in 80% acetone with 5 μ g ovalbumin as carrier for several hours at -20°C and centrifuged at 12 000 g for 10 min. Protein pellets were washed twice with acetone and resuspended

in 50 mM Tris-HCl, pH 8, at 50°C. SDS-PAGE sample buffer was added and radiolabelled proteins analyzed by SDS-PAGE. Two gels were run for each gradient so that total protein could be visualized after staining with Coomassie blue and labelled proteins could be detected by fluorography. Labelled protein bands were excised, solubilized and quantitated by liquid scintillation methods (Cline *et al.*, 1985). Non-denaturing gel electrophoresis (PAGE) was performed using conditions as described (Laemmli, 1970) except that neither SDS nor β -mercaptoethanol were present. Resolving gels were 5, 6 or 7% acrylamide. The L₈ form of cyanobacterial rubisco was synthesized in *E. coli* using plasmid pDB53 that encodes the *rbcL* gene (Gatenby *et al.*, 1985). MC1061 cells containing pDB53 were grown to mid-log phase, collected by centrifugation and sonicated in a minimal volume of 100 mM Tris-HCl, pH 8.0, 10 mM MgSO₄, 10 mM β -mercaptoethanol. The extract was centrifuged at 48 000 g for 30 min and the supernatant used for isolation of the L₈ form on sucrose gradients. Analysis of proteins by Western blotting used standard techniques (Vaessen *et al.*, 1981).

Localization of imported β -ATPase subunit

Following import of TPCF- β , chloroplasts were treated with protease, repurified on Percoll cushions and lysed by either freeze-thaw cycles as described above for TPLS assembly, or by hypotonic lysis. Thylakoids were collected by centrifugation at 8000 g for 20 min, and then washed to remove extrinsic proteins. To accomplish this, thylakoids were resuspended in 1 ml of uptake buffer (Cline *et al.*, 1985), centrifuged briefly and washed once with the same volume of this buffer. They were then washed twice with Buffer A (10 mM Tricine-OH, pH 8.0), twice in Buffer A containing 0.15 M NaCl and once more in Buffer A. Following this wash procedure, an aliquot was removed to analyze by SDS-PAGE and fluorography for ³H-labelled imported CF₁- β subunit. The remainder was resuspended in 400 μ l of Buffer B (10 mM Tris-SO₄, pH 7.6, 1 mM EDTA, 2 mM ATP, 5 mM dithiothreitol) and extracted with chloroform (Lien and Racker, 1971). The aqueous supernatant, which contained the CF₁, was centrifuged at 12 000 g for 30 min at room temperature. An aliquot of the supernatant from this centrifugation was saved for analysis by SDS-PAGE and fluorography to detect imported β subunit. The remainder of the supernatant was centrifuged on 0.20–0.70 M sucrose gradients in buffer B (Lien and Racker, 1971). Fractions from the gradients were assayed for trypsin-activated ATPase activity (Lien and Racker, 1971). Aliquots of the fractions were also analyzed by SDS-PAGE for the presence of Coomassie blue-stained CF₁ subunit proteins and radiolabelled β as described above.

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