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Translocation of the precursor of 5-*enol*pyruvylshikimate-3-phosphate synthase into chloroplasts of higher plants *in vitro*

(shikimate pathway/transit peptide/glyphosate)

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ABSTRACT **5-*enol*Pyruvylshikimate-3-phosphate synthase (EPS*P* synthase; 3-phosphoshikimate 1-carboxyvinyltransferase; EC 2.5.1.19) is a chloroplast-localized enzyme of the shikimate pathway in plants. This enzyme is the target for the nonselective herbicide glyphosate (*N*-phosphonomethylglycine). We have previously isolated a full-length cDNA clone of EPS*P* synthase from *Petunia hybrida*. DNA sequence analysis suggested that the enzyme is synthesized as a cytosolic precursor (pre-EPS*P* synthase) with an amino-terminal transit peptide. Based on the known amino terminus of the mature enzyme, and the 5′ open reading frame of the cDNA, the transit peptide of pre-EPS*P* synthase would be maximally 72 amino acids long. To confirm this prediction and to assay directly for translocation of pre-EPS*P* synthase into chloroplasts *in vitro*, we cloned the full-length cDNA into an SP6 transcription system to produce large amounts of mRNA for *in vitro* translation. The translation products, when analyzed by $NaDodSO_4$/PAGE autoradiography, indicate a relative molecular mass for pre-EPS*P* synthase of ≈55 kDa. Uptake studies with intact chloroplasts, *in vitro*, indicate that pre-EPS*P* synthase was rapidly taken up into chloroplasts and proteolytically cleaved to the mature ≈48-kDa enzyme. The transit peptide was shown to be essential for import of the precursor enzyme into the chloroplast. To our knowledge, post-translational import into chloroplasts of a precursor enzyme involved in amino acid biosynthesis has not been reported previously. Furthermore, enzymatic analysis of translation products indicates that pre-EPS*P* synthase is catalytically active and has a similar sensitivity to the herbicide glyphosate as the mature enzyme. To our knowledge, pre-EPS*P* synthase represents the only example of a catalytically competent chloroplast-precursor enzyme.**

In addition to the reactions of photosynthesis, chloroplasts are known to play a central role in many important aspects of plant cellular metabolism. The biochemical pathways for biosynthesis of fatty acids (1), terpenoids (2), porphyrins (3), and several amino acids (4) are compartmentalized principally within the chloroplast. In recent years it has become clear that the majority of chloroplast-localized proteins are encoded by nuclear genes and are synthesized as precursor proteins on free cytoplasmic ribosomes (5). The import of these precursors into chloroplasts has been shown to be mediated by amino-terminal sequences (termed transit peptides) which post-translationally direct the uptake process (6). After uptake into the chloroplast, the transit peptide sequence is removed by a sequence-specific metalloprotease (7).

The enzyme 5-*enol*pyruvylshikimate-3-phosphate synthase (EPS*P* synthase; 3-phosphoshikimate 1-carboxyvinyltransferase; EC 2.5.1.19) catalyzes the transfer of the carboxyvinyl moiety of phospho*enol*pyruvate (*P*-*e*Prv) to shikimate-3-phosphate yielding EPS*P* and inorganic phosphate. EPS*P* synthase is an intermediate in the shikimate pathway that gives rise to the aromatic amino acids L-phenylalanine, L-tyrosine, and L-tryptophan (8). In addition to the biosynthesis of these amino acids, the shikimate pathway is utilized for the production of *p*-amino- and *p*-hydroxybenzoic acids, and a variety of other natural plant products (8). The biosynthesis of aromatic amino acids has been shown to occur in isolated chloroplasts *in vitro* (9), and shikimate-pathway enzymes (including EPS*P* synthase) have been identified in the chloroplast compartment (10). EPS*P* synthase is of considerable agronomic importance because it is known to be the target of the broad-spectrum, nonselective herbicide glyphosate (*N*-phosphonomethylglycine) (11). The primary mode of action of glyphosate is to competitively inhibit the binding of the substrate phospho*enol*pyruvate to the active site of the enzyme. Glyphosate is thus thought to inhibit plant growth by blocking the synthesis of aromatic amino acids (12).

We have described the isolation of a full-length cDNA clone for EPS*P* synthase from a *Petunia hybrida* cell line that overproduces the enzyme (13). Based on the known amino terminus of the mature enzyme, the DNA sequence analysis suggested that the enzyme is synthesized as a precursor polypeptide with an amino-terminal extension that would be maximally 72 amino acids long. For EPS*P* synthase, we were interested in characterizing the precursor protein and determining whether it could be translocated into chloroplasts *in vitro* and subsequently processed. In this report, we show that EPS*P* synthase is synthesized as a precursor (designated pre-EPS*P* synthase, ≈55 kDa) that is rapidly translocated into chloroplasts *in vitro* and processed to its mature form (≈48 kDa). The amino-terminal transit peptide was found to be essential for uptake of the precursor enzyme into the chloroplast. Furthermore, we report that pre-EPS*P* synthase is a catalytically active enzyme that is inhibited *in vitro* by the herbicide glyphosate.

METHODS

Materials. Analytical grade glyphosate and shikimate-3-phosphate (a gift from R. D. Sammons) were provided by Monsanto Agricultural Products. The following products were obtained from Promega Biotec (Madison, WI): cloning vectors pGEM-1 and pGEM-2, SP6 polymerase, T7 polymerase, RNasin, and rabbit reticulocyte lysate *in vitro* translation systems. Restriction enzymes were obtained from New England Biolabs and used according to the manufacturer's directions. [^{35}S]Methionine (1000–1100 Ci/mmol; 1 Ci

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Abbreviations: EPS*P*, 5-*enol*pyruvylshikimate-3-phosphate; glyphosate, *N*-phosphonomethylglycine; kb, kilobase(s); *P*-*e*Prv, phospho*enol*pyruvate; 2D, two dimensional.

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= 37 GBq) and phospho*enol*[^{14}C]pyruvate ([^{14}C]*P-e*Prv) (11 mCi/mmol) were obtained from Amersham. A SynChropak AX-100 HPLC column was from P. J. Cobert Associates (St. Louis, MO). A radioactive flow detector for HPLC (Radiomatic FLO-ONE/Beta IC) was from Radioanalytic (Tampa, FL). High-temperature silicone oil was from Aldrich. All other chemicals were of the highest grade available.

EPS*P* Synthase cDNA Clones. A full-length cDNA clone of EPS*P* synthase from *P. hybrida* was obtained as described (13). This cDNA clone contains 27 nucleotides of the 5′-untranslated leader, 1.5 kilobases (kb) that codes for the 72 amino acid transit peptide and 444 amino acids of the mature enzyme, and 0.4 kb of the entire 3′-flanking sequence. The full-length EPS*P* synthase cDNA was cloned as a 2.1-kb *Bgl* II/*Sal* I fragment into the *Bam*HI/*Sal* I sites of the plasmid pGEM. The plasmids pMON6140 and pMON6145 containing the EPS*P* synthase coding region are transcribed 5′ to 3′ from the T7- and SP6-promoters, respectively.

***In Vitro* Transcription/Translation of Plasmid DNA with SP6 and T7 RNA Polymerases.** Plasmid DNA (pMON6140 and pMON6145) containing the full-length EPS*P* synthase cDNA was linearized at the only *Pvu* I site located in the 3′-untranslated region. The linearized plasmid DNA was transcribed *in vitro* (uncapped) with SP6 or T7 polymerase essentially as described (14). The standard reaction buffer contained 40 mM Tris·HCl (pH 7.9), 6 mM $MgCl_2$, 10 mM dithiothreitol, 2 mM spermidine, 80 units of RNasin ribonuclease inhibitor, 0.5 mM each of ATP, GTP, CTP, and UTP, in a final reaction volume of 100 μl. The final RNA pellet was resuspended in 20 μl of sterile water and stored at −80°C. A standard translation reaction contained 200 μl of nuclease-treated rabbit reticulocyte lysate, 5.7 μl of a 19-amino acid mixture (minus methionine) at 1 mM each, 5.7 μl of RNA (total RNA transcripts derived from 0.63 μg of plasmid DNA), 16 μl of RNasin (20 units/μl) ribonuclease inhibitor, and 58.3 μl of [^{35}S]methionine (14–15 mCi/ml). The *in vitro* translation reaction was incubated at 30°C for 90 min. The translation products were stored frozen at −80°C.

Pre-EPS*P* Synthase Enzyme Assays. *In vitro* translations of SP6/T7 transcripts were done as described above with 20 amino acids (at 1 mM each) in the absence of radiolabeled methionine. The translation products (100 μl) were passed through a Sephadex G-50 column (1 ml) prior to enzyme assay. The enzyme assay contained 5 μl of translation products in a 50 μl reaction volume with 0.25 μCi [^{14}C]*P-e*Prv (11 mCi/mmol)/1 mM *P-e*Prv/1 mM shikimate-3-phosphate/protease inhibitors (1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/5 mM ε-aminocaproic acid)/phosphatase inhibitor (5 mM KF)/50 mM Hepes·KOH, pH 7.5. The reaction mix was incubated at 25°C for 10 min. Glyphosate was added to the reaction mixture as the sodium salt at pH 7.0. The reactions were terminated by incubating the tubes in a boiling water bath for 2 min. The denatured proteins were removed by centrifugation at 11,000 × *g* for 5 min, and the supernatant (20 μl) was taken for HPLC analysis. Analytical separations of [^{14}C]*P-e*Prv and [^{14}C]EPS*P* were done on a SynChropak AX-100 (250 × 4.1 mm I.D.) anion-exchange column with 0.5 M KH_2PO_4 [adjusted to pH 5.5 with 50% (wt/wt) NaOH] as the mobile phase at 1 ml/min at room temperature. The HPLC column effluent was directed to a flow-through radioactivity detector that counted [^{14}C] at 75% efficiency. Quantitative determination of the enzyme activity was based on the percent conversion of [^{14}C]*P-e*Prv to [^{14}C]EPS*P* in each assay.

Translocation of Pre-EPS*P* Synthase into Chloroplasts *in Vitro*. Intact chloroplasts were isolated from lettuce (*Latuca sativa*, var. longifolia) by centrifugation in Percoll/Ficoll gradients as modified (15). The final pellet of intact chloroplasts was suspended in sorbitol/Hepes [0.5 ml of sterile 330 mM sorbitol in 50 mM Hepes·KOH (pH 7.7)], assayed for chlorophyll (16), and adjusted to a final chlorophyll concentration of 4 mg/ml (using sorbitol/Hepes). The yield of intact chloroplasts from a single head of lettuce was 3–6 mg of chlorophyll. These chloroplasts were deemed homogeneous based on phase contrast and transmission electron microscopy.

A typical 300-μl uptake experiment (modified from ref. 15) contained 5 mM ATP, 8.3 mM unlabeled methionine, 322 mM sorbitol, 58.3 mM Hepes·KOH (pH 8.0), 50 μl of reticulocyte lysate translation products, and intact chloroplasts from *L. sativa* (200 μg of chlorophyll). The uptake mixture was gently rocked at room temperature (in 10 × 75 mm glass tubes) directly in front of a fiber optic illuminator set at maximum light intensity (150-W bulb). Aliquots of the uptake mix (50–125 μl) were removed at various times and fractionated over 100-μl silicone-oil gradients (in 250-μl polyethylene tubes) by centrifugation at 11,000 × *g* for 30 sec. Under these conditions, the intact chloroplasts form a pellet under the silicone-oil layer and the incubation medium (containing the reticulocyte lysate) floats on the surface. After centrifugation, the silicone-oil gradients were immediately frozen in dry ice. The chloroplast pellet was then resuspended in 50–100 μl of lysis buffer (10 mM Hepes·KOH, pH 7.5/1 mM phenylmethylsulfonyl fluoride/1 mM benzamidine/5 mM ε-aminocaproic acid/aprotinin at 30 μg/ml) and centrifuged at 15,000 × *g* for 20 min to pellet the thylakoid membranes. The clear supernatant (stromal proteins) and an aliquot of the reticulocyte-lysate incubation medium from each uptake experiment were mixed with an equal volume of 2× $NaDodSO_4$/PAGE sample buffer for electrophoresis (see below).

$NaDodSO_4$/PAGE was carried out according to Laemmli (17) in 12% (wt/vol) acrylamide slab gels (60 mm × 1.5 mm) with 3% (wt/vol) acrylamide stacking gels (5 mm × 1.5 mm). The gels were fixed in 30% (vol/vol) methanol and 10% (vol/vol) acetic acid, dried under vacuum, and taken for direct autoradiography with Kodak XAR-5 x-ray film. Quantitation of bands on the x-ray film was done with a Hoefer GS-300 scanning densitometer interfaced to a Spectra-Physics SP4100 recording/computing integrator.

RESULTS

Plasmid Constructions Containing the EPS*P* Synthase cDNA. The cloned cDNA of *P. hybrida* EPS*P* synthase was obtained as a *Bgl* II/*Sal* I fragment of 2.1 kb. This DNA fragment was inserted in opposite orientations into the plasmid pGEM, giving rise to pMON6145 (SP6 promoter) and pMON6140 (T7 promoter) (Fig. 1). Both cDNAs were cloned into the *Bam*HI site of the pGEM polylinker at position −9 of the 5′-untranslated leader. After linearizing the template cDNA at the *Pvu* I site located 0.4 kb past the stop codon, *in vitro* transcription reactions with SP6 polymerase gave rise to a major RNA species of ≈2.0 kb (data not shown). This 2.0-kb RNA transcript was used for *in vitro* translation (see below).

***In Vitro* Translation of the EPS*P* Synthase mRNA.** To make large amounts of pre-EPS*P* synthase by *in vitro* translation, we used mRNA derived by transcription of linearized plas-

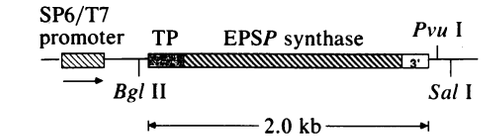

FIG. 1. Plasmid constructions containing the full-length EPS*P* synthase cDNA. A 2.1-kb *Bgl* II/*Sal* I fragment was inserted into the plasmid pGEM to give pMON6140 (T7 promoter) or pMON6145 (SP6 promoter). TP, transit peptide sequence.

mid templates containing the full-length cDNA. As indicated in Fig. 1, these cDNA templates are expressed under the control of SP6 or T7 promoters. Fig. 2*A* shows an NaDodSO$_4$/PAGE autoradiograph of total *in vitro* translation products made with the EPS*P* synthase mRNA. A major band of pre-EPS*P* synthase appeared at ≈55 kDa for constructs cloned in either the SP6- (pMON6145) or T7-promoter orientations (pMON6140). Based on the cDNA sequence, the predicted molecular weight for pre-EPS*P* synthase is 55,682. Several lower molecular weight proteins were also present in the *in vitro* translations; these polypeptides may result from premature termination at either the transcriptional or translational levels. The low molecular weight polypeptides were not translocated into chloroplasts (see below).

Two-dimensional (2D) gel electrophoresis of the translation products shows that pre-EPS*P* synthase has a significantly higher pI value than the mature enzyme (Fig. 2*B*). This difference in pI values between the precursor and mature form of the enzyme is not surprising since DNA sequence analysis indicates that the transit peptide is enriched in basic amino acids and has an overall net charge of +8. In addition, the 2D-gel autoradiograph shown in Fig. 2*A* indicates that only the precursor form of EPS*P* synthase was made by *in vitro* translation of SP6-derived mRNA.

Pre-EPS*P* Synthase Is Catalytically Active and Inhibited by Glyphosate. To establish whether the EPS*P* synthase cDNA encoded for an authentic pre-enzyme, we measured the enzymatic activity present in reticulocyte lysates programmed with SP6 mRNA. Our enzyme assay for EPS*P* synthase is based on the conversion of [^{14}C]*P-e*Prv and unlabeled shikimate-3-phosphate to [^{14}C]EPS*P*. The radiochromatogram shown in Fig. 3*A* indicates that reticulocyte lysates programmed with SP6 transcripts contained significant EPS*P* synthase activity, as indicated by the peak of radioactivity that coeluted from the HPLC column at the position of the [^{14}C]EPS*P* standard. Based on the amount of pre-EPS*P* synthase enzymatic activity measured in SP6-programmed reticulocyte lysates, each *in vitro* translation reaction made approximately 170 ng of pre-enzyme from the RNA equivalent of 0.63 μg of plasmid DNA. These calculations are based on a specific activity of purified EPS*P* synthase of ≈40 units/mg. Fig. 3*B* shows that a reticulocyte lysate message blank contained no EPS*P* synthase activity.

Glyphosate is an inhibitor of the mature EPS*P* synthase in plant chloroplasts (10). In light of the observation that pre-EPS*P* synthase is catalytically active, we were interested in determining its glyphosate sensitivity. Enzyme assays for

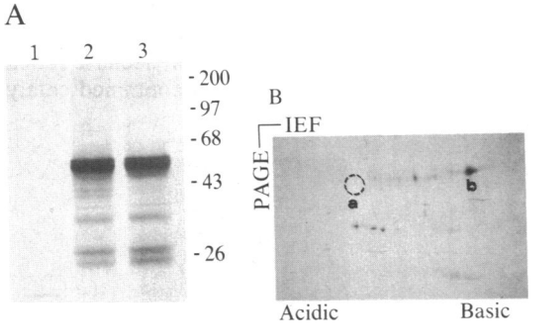

FIG. 2. NaDodSO$_4$/PAGE autoradiography of pre-EPS*P* synthase made by *in vitro* translation with rabbit reticulocyte lysates. (*A*) Total *in vitro* translation products of pre-EPS*P* synthase mRNA derived by transcription with SP6 (lane 2) and T7 (lane 3) RNA polymerases. Lane 1, no mRNA control. Molecular size markers (kDa) are shown to the right. (*B*) 2D-gel electrophoresis of SP6 translation products; isoelectric focusing (IEF) followed by NaDodSO$_4$/PAGE. The positions of the mature (a) and precursor (b) enzymes are indicated. The pH range was from 3.5 to 9.0.

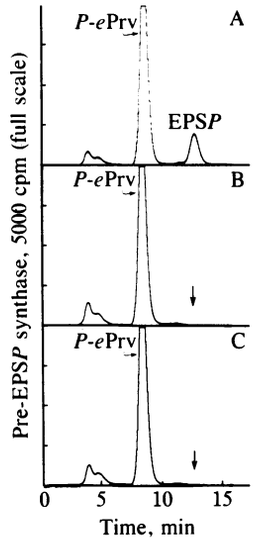

FIG. 3. HPLC radioassay of pre-EPS*P* synthase activity. (*A*) Reticulocyte lysate with SP6 mRNA. (*B*) Reticulocyte lysate without SP6 mRNA (message blank). (*C*) Reticulocyte lysate with SP6 mRNA plus 1 mM glyphosate. The arrow indicates the position at which [^{14}C]EPS*P* elutes.

pre-EPS*P* synthase activity in SP6-programmed reticulocyte lysates were, therefore, conducted in the presence of increasing amounts of the herbicide glyphosate. The radiochromatogram in Fig. 3*C* shows that 1 mM glyphosate completely inhibited the incorporation of radioactive substrate into EPS*P*. A titration of EPS*P* synthase activity (Fig. 4) indicates that the sensitivity to glyphosate for the precursor was essentially identical to that observed for the mature chloroplast enzyme (IC_{50} ≈ 10 μM in each case).

Translocation of Pre-EPS*P* Synthase into Chloroplasts *in Vitro*. To see if pre-EPS*P* synthase is taken up and processed by chloroplasts, the total translation products containing [^{35}S]methionine-labeled pre-EPS*P* synthase (shown in Fig. 2*A*) were incubated with freshly isolated, intact chloroplasts from *L. sativa*. Fig. 5*A* shows that pre-EPS*P* synthase was rapidly translocated into chloroplasts and cleaved to a mature ≈48-kDa product. The NaDodSO$_4$/PAGE autoradiograph shows the disappearance of the precursor enzyme from the incubation medium, and the subsequent appearance of a lower molecular weight, processed form in the chloroplast

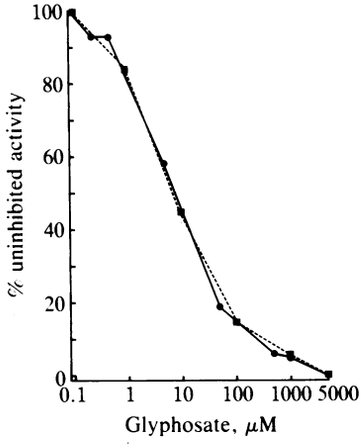

FIG. 4. Glyphosate inhibition of pre- and mature-EPS*P* synthase activities. Pre-EPS*P* synthase (●) from reticulocyte lysates was assayed as in Fig. 3. Mature EPS*P* synthase (■) was from a Sephadex G-50 filtrate of *P. hybrida* (MP4-G) plastid proteins.

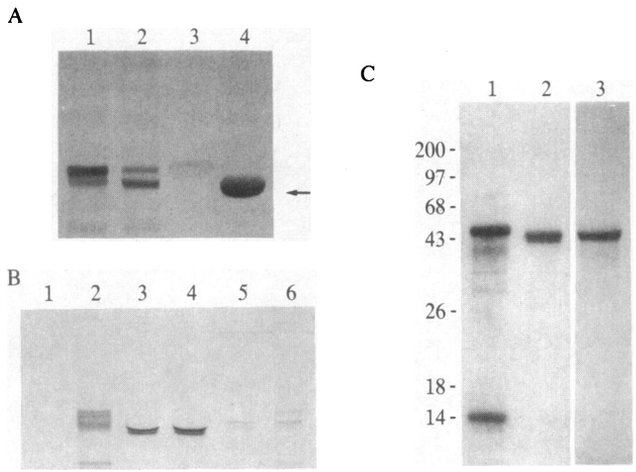

FIG. 5. Uptake of pre-EPS*P* synthase into chloroplasts *in vitro*. (*A*) NaDodSO$_4$/PAGE autoradiography of the incubation medium (lanes 1 and 2) and reisolated chloroplasts (lanes 3 and 4) (stromal proteins) at zero (lanes 1 and 3) and 15 min (lanes 2 and 4). Arrow, mature enzyme. (*B*) NaDodSO$_4$/PAGE autoradiography of incubation medium (lanes 1 and 2), chloroplast proteins (lanes 3 and 4) (stroma), and thylakoid membranes (lanes 5 and 6) with (lanes 1, 3, and 5) and without (lanes 2, 4, and 6) treatment after uptake with trypsin/chymotrypsin (200 μg/ml each, 30 min at 4°C). Equivalent volumes of the total chloroplast stromal and thylakoid fractions were loaded in each of the lanes. The uptake experiment was for 15 min. The processed form of EPS*P* synthase appears as a doublet in the gel; this is an artifact of electrophoresis (unpublished data). (*C*) Comigration during NaDodSO$_4$/PAGE of purified *P. hybrida* EPS*P* synthase with the processed, SP6-derived enzyme after uptake into *L. sativa* chloroplasts. Lane 1, Coomassie stain of chloroplast stromal proteins (the two major bands represent the large and small subunits of ribulose-1,5-bisphosphate carboxylase); lane 2, Coomassie stain of purified EPS*P* synthase (2.5 μg); lane 3, autoradiograph of lane 1. Molecular size markers (kDa) are shown to the left.

fraction. Some of the processed enzyme was also present in the incubation medium at 15 min due to chloroplast lysis. Treatment after uptake of the incubation mixture with trypsin and chymotrypsin showed that the pre-enzyme in the incubation medium was completely degraded, whereas the processed enzyme in the chloroplast fraction was fully protected (Fig. 5*B*). These results indicate that EPS*P* synthase was translocated across the chloroplast envelope into a protease inaccessible space. Furthermore, subfractionation of the reisolated chloroplasts indicated that the mature EPS*P* synthase was localized in the stromal, as opposed to thylakoid, fraction (Fig. 5*B*). Based on nucleotide sequence, the predicted molecular weight for the mature *P. hybrida* enzyme is 47,790. The ≈48-kDa polypeptide localized in the reisolated chloroplast fraction comigrated during NaDodSO$_4$/PAGE with the purified, mature enzyme of *P. hybrida* (Fig. 5*C*).

A time course of pre-EPS*P* synthase uptake and cleavage by *L. sativa* chloroplasts is shown in Fig. 6*A*. After a 2-min initial lag period, uptake and processing of the precursor increased linearly for about 10 min. The uptake of pre-EPS*P* synthase into the chloroplast was correlated with the disappearance of the precursor from the incubation medium. Under these *in vitro* uptake conditions, approximately 80% of the available pre-EPS*P* synthase was imported into the chloroplasts during the first 15 min of the incubation. The autoradiograph shows the processed enzyme after uptake into the chloroplast (Fig. 6*B*).

The Transit Peptide Is Required for Uptake of EPS*P* Synthase into Chloroplasts. To show that the transit peptide is required for uptake, we obtained the mature enzyme (lacking the transit peptide) from the chloroplast stroma after

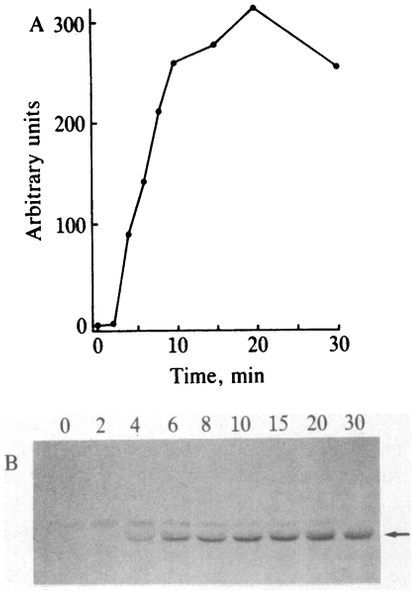

FIG. 6. Time course of pre-EPS*P* synthase uptake into chloroplasts. (*A*) Quantitation by densitometry of NaDodSO$_4$/PAGE autoradiograph of stromal proteins. (*B*) Autoradiograph. Incubation time in min is indicated above each lane. Arrow, mature enzyme.

an initial 15-min uptake experiment. A mixture of stromal proteins (containing the labeled mature enzyme) was diluted with unlabeled reticulocyte lysate and used in a second uptake experiment with intact chloroplasts. Fig. 7 shows that the mature EPS*P* synthase was not translocated into chloroplasts, or bound to the outer-envelope membrane, during a 15-min incubation. As a control experiment, we found that the rate of uptake of pre-EPS*P* synthase into chloroplasts was unaffected by the addition of stromal proteins to the incubation mixture (data not shown). From these data we conclude that the transit peptide of EPS*P* synthase is required for uptake of the enzyme into chloroplasts.

DISCUSSION

In this paper, we report that *in vitro* translation of SP6/T7 transcripts of the *P. hybrida* EPS*P* synthase cDNA yielded an ≈55-kDa precursor polypeptide. Based on cDNA sequence analysis, EPS*P* synthase contains an amino-terminal extension that would give rise to a precursor form of the enzyme of 55,682 (13). We have confirmed that *in vitro* transcription/translation of the EPS*P* synthase cDNA gives rise to an authentic form of the protein, because reticulocyte lysates programmed with SP6 RNA contained catalytically

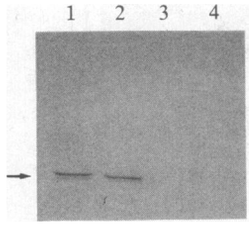

FIG. 7. Lack of uptake of mature EPS*P* synthase into chloroplasts. The conditions were the same as in Fig. 5*A* except that stromal proteins from a 15-min uptake experiment (containing [^{35}S]-methionine-labeled mature EPS*P* synthase) were mixed 1:1 (vol/vol) with unlabeled reticulocyte lysate and reincubated with intact chloroplasts. Medium, lanes 1 and 2. Reisolated chloroplasts, lanes 3 and 4. Zero min, lanes 1 and 3; 15 min, lanes 2 and 4. Arrow, mature enzyme.

active precursor enzyme. Assuming that the specific activity of the pre-enzyme is equivalent to that of the mature enzyme (40 units/mg), our SP6 system is capable of synthesizing up to 0.94 μg of pre-EPS*P* synthase per 1.0 μg of template DNA. Analysis of the translation products by 2D-gel electrophoresis confirmed that none of the mature enzyme was produced in the reticulocyte lysate system used. Thus, the enzyme activity observed after *in vitro* translation can be attributed entirely to the precursor form of the enzyme. To our knowledge, pre-EPS*P* synthase represents the first example of a nuclear-encoded chloroplast precursor-protein that is catalytically active. This observation suggests that prior to uptake into the chloroplast, the catalytic site of pre-EPS*P* synthase assumes a three-dimensional conformation similar to that of the mature enzyme.

In addition, we have shown that pre-EPS*P* synthase is able to bind the herbicide glyphosate *in vitro*. Glyphosate has been shown to be a potent inhibitor of EPS*P* synthase from a variety of sources (11), including the endogenous enzyme found in chloroplast lysates (10). Direct evidence that pre-EPS*P* synthase binds glyphosate with the same affinity as the mature enzyme stems from our observation that the IC_{50} for both forms of the enzyme are similar (≈10 μM) and the glyphosate-sensitivity curves are indistinguishable. This finding further suggests that glyphosate may interact with pre-EPS*P* synthase in the cytoplasm of plant cells, *in vivo*, and inhibit its uptake/catalysis, or exert some other as of yet unknown effect on the import and maturation process.

Uptake studies with intact chloroplasts from *L. sativa, in vitro*, indicate that pre-EPS*P* synthase is rapidly imported and processed to the mature form of the enzyme. We conclude that pre-EPS*P* synthase is taken up into the chloroplast stroma because (*i*) treatment after uptake of intact chloroplasts with nonpenetrant proteases showed complete protection of the processed enzyme, and (*ii*) chloroplast subfractionation indicated that essentially all of the processed enzyme was localized in the stromal, as opposed to the thylakoid, fraction. Furthermore, studies of the EPS*P* synthase distribution in isolated chloroplasts from *L. sativa* have shown that the endogenous enzyme is soluble and localized primarily within the stroma (G.d.-C., unpublished data). These observations are consistent with the report that EPS*P* synthase is a soluble enzyme located in the chloroplast fraction of *Pisum sativum* (10).

We have also carried out *in vitro* uptake studies using a homologous system (i.e., uptake into *P. hybrida* chloroplasts) and found that pre-EPS*P* synthase is translocated and processed in the same fashion as described here for *L. sativa*. Under our experimental conditions, however, chloroplasts from *P. hybrida* are easily lysed *in vitro* and thus unsuitable for translocation studies.

The requirement of the amino-terminal transit peptide for post-translational uptake of precursors into chloroplasts (5) and mitochondria (18) has been well documented. Of the chloroplast transit peptides for which sequence data is available, the 72-amino acid extension of EPS*P* synthase (M_r 7,937) represents the longest to date. A possible exception to this would be pyruvate orthophosphate dikinase; based on $NaDodSO_4$/PAGE its transit peptide is ≈16 kDa (19). Structurally, the EPS*P* synthase transit peptide is similar to other chloroplast transit peptides by virtue of its enrichment in basic amino acids, unusually high serine content, and lack of long hydrophobic stretches (D.M.S., unpublished data). We found that the transit peptide of EPS*P* synthase was essential for import of the precursor enzyme into chloroplasts *in vitro*. The transit peptide of the small-subunit precursor of ribulose-1,5-bisphosphate carboxylase is able to direct the uptake of a chimeric, foreign protein fused at its carboxyl terminus (20, 21). These experiments indicate that a transit peptide sequence is sufficient to target a protein to the chloroplast.

The mechanism of post-translational import of proteins into mitochondria is similar to that for chloroplasts; mitochondrial precursors possess highly basic leader sequences that can range in length from 20 to 60 amino acids, the import process is energy dependent, and maturation occurs by sequence-specific proteolysis within the mitochondrion (22). For the mitochondrial precursor of cytochrome *c* oxidase subunit IV, the first 12 amino acids of the leader peptide have been shown to be sufficient for targeting of a foreign protein to the organelle (23). Data, however, suggest instead that an internal α-helical stretch within the leader is critical for binding and import of preornithine transcarbamylase into mitochondria (24). For chloroplast-precursor proteins (e.g., chlorophyll a/b protein, ribulose-1,5-bisphosphate carboxylase small subunit, ferredoxin, and plastocyanin), it has been proposed that transit peptide sequences share a structural framework homology (25), but there is no direct evidence indicating that a particular sequence region is important for recognition by the organelle.

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1. Roughan, G. & Slack, R. (1984) *Trends Biochem. Sci.* **9,** 383–386.
2. Camara, B. (1984) *Plant Physiol.* **74,** 112–116.
3. Castelfranco, P. A. & Beale, S. I. (1983) *Annu. Rev. Plant Physiol.* **34,** 241–278.
4. Miflin, B. J. & Lea, P. J. (1980) in *The Biochemistry of Plants, A Comprehensive Treatise*, ed. Miflin, B. J. (Academic, New York), Vol. 5, pp. 169–202.
5. Cashmore, A., Szabo, L., Timko, M., Kausch, A., Van den Broeck, G., Schreier, P., Bohnert, H., Herrera-Estrella, L., Van Montagu, M. & Schell, J. (1985) *Bio/Technology* **3,** 803–808.
6. Schmidt, G. W., Devillers-Thiery, A., Desruisseaux, H., Blobel, G. & Chua, N.-H. (1979) *J. Cell Biol.* **83,** 615–622.
7. Robinson, C. & Ellis, J. R. (1984) *Eur. J. Biochem.* **142,** 337–342.
8. Haslam, E. (1974) *The Shikimate Pathway* (Wiley, New York).
9. Bickel, H., Palme, L. & Schultz, G. (1978) *Phytochemistry* **17,** 119–124.
10. Mousdale, D. M. & Coggins, J. R. (1985) *Planta* **163,** 241–249.
11. Steinrücken, H. C. & Amrhein, N. (1980) *Biochem. Biophys. Res. Commun.* **94,** 1207–1212.
12. Jaworski, E. G. (1972) *J. Agric. Food Chem.* **20,** 1195–1198.
13. Shah, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegel, N. R., Rogers, S. R. & Fraley, R. T. (1986) *Science* **233,** 478–481.
14. Krieg, P. A. & Melton, D. A. (1984) *Nucleic Acids Res.* **12,** 7057–7070.
15. Bartlett, S. G., Grossman, A. R. & Chua, N.-H. (1982) in *Methods in Chloroplast Molecular Biology*, eds. Edelman, M., Hallick, R. B. & Chua, N.-H. (Elsevier, New York), pp. 1081–1091.
16. Arnon, D. I. (1949) *Plant Physiol.* **24,** 1–15.
17. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
18. Neupert, W. & Shatz, G. (1981) *Trends Biochem. Sci.* **6,** 1–4.
19. Aoyagi, W. & Bassham, J. A. (1984) *Plant Physiol.* **76,** 278–280.
20. Van den Broeck, G., Timko, M. P., Kausch, A. P., Cashmore, A. R., Van Montagu, M. & Herrera-Estrella, L. (1985) *Nature (London)* **313,** 358–363.
21. Schreier, P. H., Seftor, E. A., Schell, J. & Bohnert, H. J. (1985) *EMBO J.* **4,** 25–32.
22. Hay, R., Bohni, P. & Gasser, S. (1984) *Biochim. Biophys. Acta* **779,** 65–87.
23. Hurt, E. C., Pesold-Hurt, B., Suda, K., Oppliger, W. & Schatz, G. (1985) *EMBO J.* **4,** 2061–2068.
24. Horwich, A. L., Kalousek, F., Fenton, W. A., Pollock, R. A. & Rosenberg, L. E. (1986) *Cell* **44,** 451–459.
25. Karlin-Neumann, G. A. & Tobin, E. M. (1986) *EMBO J.* **5,** 9–13.