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Synthesis and Uptake of Cytoplasmically Synthesized Pyruvate, Pi Dikinase Polypeptide by Chloroplasts[1]

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ABSTRACT

Polyadenylated RNA was isolated from maize leaves and translated *in vitro*. In agreement with a previous report by others, we found among the translation products a 110-kilodalton pyruvate orthophosphate dikinase (PPDK) precursor that is about 16 kilodaltons larger than the polypeptide isolated from cells. This maize PPDK precursor polypeptide was taken up from the translation product mixture by intact spinach chloroplasts and yielded a mature PPDK polypeptide (94 kilodaltons). The uptake and processing support the proposal that the extra 16-kilodalton size of the polypeptide from *in vitro* translation of maize leaf mRNA represents a transit sequence which is cleaved after its entry into chloroplasts. Moreover, these results provide additional evidence that *in vivo* in maize leaf cells PPDK polypeptide is synthesized in the cytoplasm and is transported into the chloroplasts.

Location of PPDK in C_3 plant leaves was investigated by immunochemical analysis. Intact chloroplasts were isolated from leaves of spinach, wheat, and maize. A protein blot of stromal protein in each case gave rise to bands corresponding to authentic PPDK polypeptide. This result indicates that PPDK is present in chloroplasts of C_3 plant leaves as it is in the case of C_4 plants.

Synthesis and transport of some chloroplast proteins, such as the small subunit of RuBPC-SS[2] (EC 4.1.1.39) and light-harvesting Chl *a/b* protein complex, have been studied extensively (5, 11, 12, 14, 17, 19, 33). Until recently, however, little was known about the synthesis and transport of PPDK (EC 2.7.9.1), an essential enzyme in the C_4 photosynthetic pathway (23). In C_4 plant leaves, PPDK is found in chloroplast stroma (35). Hague *et al.* (22) translated the polyadenylated mRNA isolated from maize leaf *in vitro* and found that the polypeptide had an apparent mol wt of 110 kD which is larger than that of the authentic PPDK. They proposed that the extra 16 kD sequence represents a transit sequence which permits recognition and entry into the chloroplast of the polypeptide synthesized *in vivo* in maize leaf cell cytoplasm. Gee *et al.* (18) used immunoprecipitation to show the presence of PPDK polypeptide in *iojap*, a maize mutant devoid of functional chloroplasts, thus supporting the contention that PPDK polypeptide is nuclear encoded and is synthesized in the cytoplasm. Aoyagi and Bassham (3) found that in both maize and wheat, the product of *in vitro* translation of RNA extracted from leaves is a 110 kD polypeptide, whereas the product from RNA extracted from seeds is a 94 kD polypeptide; thus, chain length of the polypeptide as synthesized in the cytoplasm is presumably organ specific in both a C_4 and a C_3 plant.

In this paper, we demonstrate that maize PPDK precursor can be recognized by and enter into isolated spinach chloroplasts and can be processed into mature protein. We show that PPDK of a C_3 plant leaf is present in the chloroplast stroma as is the case for the C_4 plant leaf PPDK.

MATERIALS AND METHODS

Plant Material. Wheat (*Triticum aestivum* L. var Anza) was grown in a growth chamber at 15°C with a quantum density of 250 μE m^{-2} s^{-1} and a photoperiod of 8 h. Corn (*Zea mays* L. Golden Bantam) was grown in a growth chamber at 27°C with a quantum flux density of 600 μE m^{-2} s^{-1} with a photoperiod of 16 h. Spinach (*Spinacia oleracea* L. cv Highpack) was grown in a green house. In each case, the plants were fertilized with half-strength Hoagland solution.

Preparation of Maize Leaf mRNA. Total RNA was extracted according to the method of Nelson *et al.* (29). In a coffee grinder, 15 g of maize leaves (3 weeks old) which had been frozen in liquid N_2 were ground into powder in dry ice and then thawed to room temperature in 4 mM guanidinium thiocyanate (1 ml/g tissue). Polyadenylated RNA was prepared by fractionation of total RNA on a column of oligo-dT cellulose (Collaborative Research, Lexington, MA), followed by ethanol precipitation (9).

PPDK Polypeptide Standard and Mol Wt Standards. PPDK was isolated from mature maize leaves and purified to homogeneity as described previously (1, 34). Polypeptide mol wt standards were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Isolation of Intact Spinach Chloroplasts. Thirty-five g of young spinach leaves were cut into small pieces, immersed in 100 ml of ice cold extraction buffer (0.33 M sorbitol, 0.2 mM $MgCl_2$, and 20 mM Tricine-NaOH, pH 7.8), and homogenized 3 times for 3 s each with a Waring Blendor. The brei was filtered through 6 layers of cheesecloth and centrifuged at 2,200*g* for 30 s. The pellet was resuspended in 40 ml of the above buffer and centrifuged again at the same speed for 30 s.

The next step in preparation of intact chloroplasts required the preparation of a Percoll gradient as described by Mourioux and Douce (28). Thirty ml of medium containing 50% Percoll (Sigma Chemical Co.), 330 mM sorbitol, 50 mM Tricine-NaOH (pH 7.8), 2 mM EDTA, and 0.15% BSA were pipetted into a centrifuge tube. The tube was placed in a precooled Sorvall SS 90 vertical rotor and centrifuged at 3°C at 10,000*g* for 100 min. The crude chloroplast pellet was resuspended in a small volume (1-2 ml) of the sorbitol/Tricine-NaOH buffer used in the first step of the chloroplast isolation and layered on the 30-ml pre-

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[2] Abbreviations: RuBPC-SS, ribulose 1,5-bisphosphate carboxylase small subunit; PPDK, pyruvate orthophosphate dikinase.

formed Percoll gradient. After centrifugation for 10 min at 5,000*g* at 4°C in the Sorvall SS 90 rotor, an intact chloroplast layer was obtained as a broad band near the bottom, whereas the stripped chloroplasts and extrachloroplastic membrane system formed a band at the sample-gradient interphase.

Precursor Uptake by Spinach Chloroplasts. Precursor uptake was carried out according to the method of Grossman *et al.* (21) and Bartlett *et al.* (10) with some modifications. Maize poly(A)RNA was translated in a rabbit reticulocyte lysate cell-free system (Amersham Corp.), using 200 μCi of [^{35}S]methionine (1126 mCi mmol^{-1}) (New England Nuclear) at 27°C for 1.5 h.

For polypeptide uptake, 200 μl of the translation product was incubated with intact spinach chloroplasts which had been prepared by Percoll gradient centrifugation as described above. The incubation mixture (600 μl) contained 400 μg Chl, 50 mM Tricine-NaOH (pH 8.0), 8.3 mM methionine, 0.33 M sorbitol, and 10 mM ATP. The chloroplasts were incubated in 5-ml test tubes at 27°C for 1 h with illumination (8,000 lux) and gentle shaking. For dark controls, test tubes were wrapped with aluminum foil.

After incubation, the chloroplast suspension was diluted with 5 ml of buffer containing 50 mM Tricine-NaOH (pH 8.0) and 0.33 M sorbitol and centrifuged at 4,000*g* for 3 min. The pellet was resuspended in 0.5 ml of the dilution buffer and treated with 300 μg ml^{-1} of trypsin for 30 min at 0°C, in order to digest any translation products that might be absorbed to the outer chloroplast membrane. After this treatment, the chloroplast suspension was diluted with 3 ml of the same buffer containing 1 mM phenylmethylsulfonylfluoride, 1 mM benzamidine-HCl, and 5 mM ϵ-amino-*n*-caproic acid, and pelleted by centrifugation. The pellet was resuspended in 1 ml of dilution buffer by gentle mixing. The intact chloroplasts were again isolated by centrifugation at 5,000*g* for 5 min through a layer of 5 ml of 50% Percoll gradient prepared by the method described above.

To the reisolated chloroplasts, sterile water containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine-HCl, and 5 mM ϵ-amino-*n*-caproic acid was added to lyse the chloroplasts. NaCl was added to give a final concentration of 100 mM and the mixture was centrifuged at 12,000*g* for 10 min to separate supernatant and membrane fractions. To each fraction, 10 μl of PPDK antiserum (2) was added, and any PPDK polypeptide was immunoprecipitated overnight at 4°C. The method of Kessler (26), employing *Staphylococci* A. cells (Bethesda Research Laboratories), was used to bind the PPDK-immunoglobulin-G conjugate. This conjugate was released from the complex by eluting in the buffer containing 50 mM Tris-HCl (pH 7.5), 3% β-mercaptoethanol, 3% SDS, and 40% glycerol for 1 h followed by heating at 85°C for 2 min and centrifugation at 12,000*g* for 5 min.

The samples were run on 15-cm-long 6.4 to 12.8% gradient polyacrylamide SDS slab gels. After SDS-PAGE, the gel was stained in 0.1% (w/v) Coomassie brilliant blue R in 45% methanol, 10% acetic acid, and destained in 45% methanol, 10% acetic acid. The gel was then immersed in Enhance (New England Nuclear) for 1 h and in H_2O for 40 min. After vacuum drying, the gel was exposed to an x-ray film (Kodak X-AR) with an intensifying screen at −70°C for 10 d.

Preparation of Chloroplast Stroma for Protein Analysis. The intact spinach chloroplast layer was pipetted out after centrifugation in a 50% Percoll gradient and buffer was added to give a Chl concentration of 2 mg ml^{-1}. The Chl content was measured by the method of Arnon (6). Sterile water was added to the chloroplast solution and the solution was frozen in liquid N_2, thawed to room temperature, vortexed vigorously to lyse chloroplasts, and centrifuged at 14,000*g* for 20 min. The supernatant was brought to 2% SDS, 12% glycerol, 20 mM DTT, and 2% bromophenol blue before electrophoresis.

Maize and wheat chloroplast stroma protein preparations were made by the method of Sugiyama and Hatch (35) with minor modifications. About 8 g of each leaf was harvested from young seedlings (7 d), sliced into 1 to 2-mm sections with a razor blade, and blended for 10 s in 50 ml of extraction buffer consisting of 20 mM Tris-Hcl (pH 8.3), 0.4 M sorbitol, 5 mM $MgSO_4$, 2 mM DTT, and 0.2 mM EDTA using a Polytron (Brinkmann Instruments, Inc., New York, NY) equipped with a PT 20ST probe generator set at 6. The homogenate was filtered through Miracloth and centrifuged at 1,000*g* for 3 min. The pellet was resuspended in 30 ml of the same buffer and centrifuged again at the same speed for 2 min. The pellet was resuspended in 2 ml of sterile distilled H_2O and was first frozen in liquid N_2 and then thawed. After vigorous vortexing to lyse the chloroplasts, the suspension was centrifuged at 14,000*g* for 2 min. The supernatant was collected and concentrated using a microconcentrator (Centricon; Amicon, Danvers, MA), until the volume was reduced to one-fortieth of the starting volume, since the protein content assay of the original supernatant of the chloroplast stroma showed the protein concentration to be too low (between 0.3 and 0.7 mg ml^{-1}) to load on the gel directly. The concentrated extract was prepared in the same manner as spinach chloroplast protein before electrophoresis. Protein content was assayed by the method of Bradford (3).

Protein Blot. The samples were loaded onto 6.4 to 12.8% SDS polyacrylamide gradient gel. Following SDS-PAGE, the protein was electrophoretically transferred to cyanogen bromide paper (15) and probed with PPDK antibody, and then with ^{125}I-labeled protein A (30 mCi mg^{-1}, Amersham Corp.) according to the method described previously (2). The paper was blotted dry and exposed overnight at −70°C to an x-ray film with an intensifying screen.

RESULTS

After uptake of *in vitro* synthesized protein, spinach chloroplast stromal extract was reacted with PPDK antibody prepared against maize leaf PPDK (2). The darkest polypeptide band observed has an apparent mol wt of 94 kD (Fig. 1, lane 3). The observed faint band of mol wt 110 kD corresponds in size with the 110 kD polypeptide obtained by both Hague *et al.* (22) and our previous studies on immunoprecipitation of *in vitro* translation product of poly(A)RNA isolated from maize leaf (3) (Fig. 1, lane 2). The results suggest the processing of a 110 kD precursor polypeptide into a mature-sized one in the chloroplasts of both maize and spinach *in vivo*.

It appears that the uptake of the precursor polypeptide is light-dependent (Fig. 2); however, uptake dependence on added ATP could not be demonstrated unequivocally. Spinach chloroplasts are reportedly less favorable than pea chloroplasts for demonstrating ATP-dependent uptake of precursor polypeptide (at least in the case of RuBPC-SS) (20), possibly because of higher endogenous levels of chloroplast ATP even in the dark (25, 30). We chose spinach for these studies, however, because we have detected PPDK in spinach leaves but did not see PPDK in pea chloroplasts. Light-dependent RuBPC-SS precursor uptake into isolated chloroplasts has been previously considered to be evidence for an ATP requirement; thus, our failure to observe ATP-dependent PPDK precursor uptake in spinach chloroplasts may be due to inadequate uptake of ATP by these chloroplasts, perhaps because of suboptimal medium pH, P_i, or other factors (21).

Examination of chloroplast stromal enzymes from maize, spinach, and wheat leaves indicates that in both C_4 and C_3 leaves, PPDK is present mostly in the chloroplast (Fig. 3). Previously, we found that of total soluble protein in wheat leaves, PPDK polypeptide constitutes 0.1% (1), whereas we find it is 0.3% of the stroma protein. There is a similar 1/3 ratio in spinach (0.05%

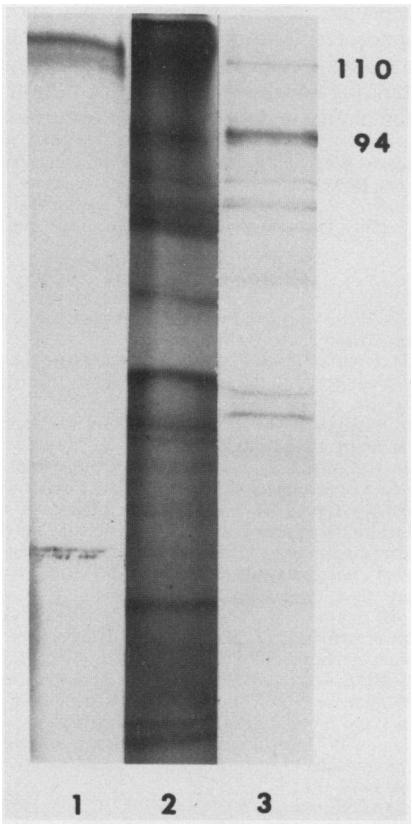

FIG. 1. Maize PPDK precursor uptake by spinach chloroplasts. The *in vitro* translation products formed in the presence of maize leaf poly(A) RNA were incubated with intact spinach chloroplasts. Subsequently, the chloroplasts were lysed and the stroma extract was reacted with maize PPDK antiserum (see "Materials and Methods" for details). Lane 1: PPDK precursor of 110 kD, 1/20 aliquot of total translation product precipitated by PPDK antiserum. Lane 2: total *in vitro* translation product; 1/20 aliquot. Lane 3: chloroplast-processed PPDK after uptake. The suspended chloroplasts were exposed to nine-tenths of the total translation product; after lysing, the entire stromal extract was loaded on this lane. The faint band above the 94 kD band is at about 110 kD and may be the unprocessed precursor.

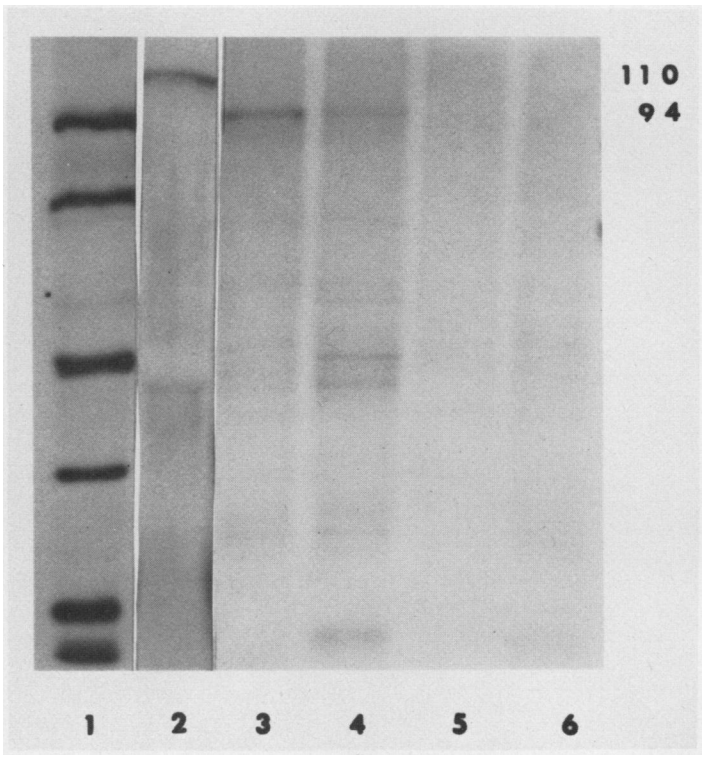

FIG. 2. Light-dependency of chloroplast uptake of PPDK precursor. The *in vitro* translation products formed in the presence of maize leaf poly(A)RNA were incubated with intact spinach chloroplasts as in Figure 1, but in light or dark and with or without ATP, as indicated below (lanes 3–6). Subsequently, the chloroplast stroma extract was reacted with PPDK antiserum to analyze for the PPDK polypeptide among the products transported into the chloroplasts. Lane 1: ^{14}C-labeled polypeptide mol wt standards, at 92, 69, 46, 30, and 14 kD, respectively from top to bottom. The lowest band is an artifact of degradation. Lane 2: total translation product was reacted with maize PPDK antiserum as in Figure 1, lane 1. Lane 3: incubation in light plus ATP. Lane 4: incubation in light minus ATP. Lane 5: incubation in dark plus ATP. Lane 6: incubation in dark minus ATP.

to 0.15%) and in maize (7% to 20%). In spinach, on the basis of Chl, there is from 2.5 to 3 times as much total soluble leaf protein as stroma protein. Most of the PPDK polypeptide thus must be in the stroma in spinach leaf cells.

DISCUSSION

Since trypsin treatment of the chloroplasts following uptake of the *in vitro* translation product would remove proteins bound to the outer membrane, the immunodetected bands seen in Figure 1, lane 3, are all from the chloroplast stroma. The faint band at 110 kD might be due to precursor PPDK polypeptide which was transported into the spinach chloroplasts but not processed. The major band at 94 kD is the mature PPDK polypeptide resulting from removal of the 16 kD leader sequence. Lower mol wt bands visible in lane 3 similar to bands commonly seen with PPDK preparations when conditions provide an opportunity for degradation (4, 8).

The light-dependent uptake of PPDK precursor polypeptide by spinach chloroplasts provides further evidence for the location of PPDK in the chloroplasts of a C_3 plant and a mechanism for synthesis, transport, and processing similar to that already known for RuBPC-SS and some other chloroplast proteins. The light-dependency of PPDK precursor uptake also may be related to reported light-dependences of PPDK mRNA level (22), polypeptide level (24), and enzyme activity (7, 32). If the rate of synthesis of PPDK mRNA were somehow affected by the level of PPDK precursor in the cytosol, light-dependent uptake of this precursor could be the key factor in each of these reported light effects.

The great majority of chloroplast proteins are synthesized on cytoplasmic ribosomes (17). Much remains to be learned about the size and specificity of transit sequences of the polypeptides of these proteins with respect to both species and protein. Recognition of the polypeptide precursor of RuBPC-SS seems not to be species-specific since the *Chlamydomonas* precursor polypeptide is taken up by spinach and pea chloroplasts (27). The entry of maize PPDK polypeptide into isolated spinach chloroplasts suggests an unspecific recognition with respect to species in this case also. It was noted earlier that in terms of immunochemistry, the PPDK polypeptides from maize and from several C_3 species appear similar (2).

The mol wt of mature PPDK polypeptide subunits vary from 94 to 97 kD, while that of the precursor is about 110 kD, so that the transit sequence may be about 13 to 16 kD. As noted previously (22), this size is comparable to the 12 kD size reported for a subunit of another enzyme of C_4 carbon metabolism, NADP-dependent malic enzyme, found in bundle sheath chloroplasts. These are the largest transit sequences reported thus far for chloroplast stroma polypeptides, but sequences of similar size

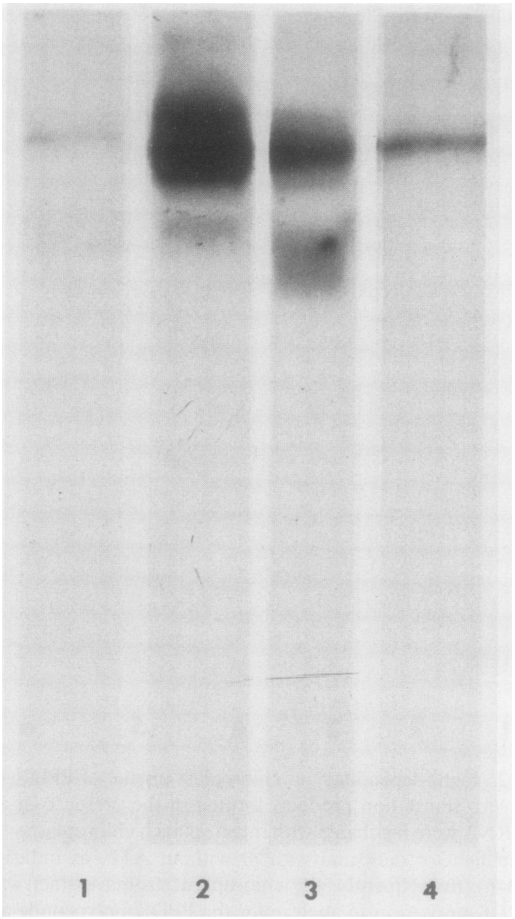

FIG. 3. Protein blot analysis of leaf chloroplast stroma. Lane 1: purified PPDK standard (0.375 μg). Lane 2: maize chloroplast stroma protein (30 μg). Lane 3: wheat chloroplast stroma protein (400 μg). Lane 4: spinach chloroplast stroma protein (300 μg). The level of PPDK polypeptide in wheat stroma is only about one-seventieth that in maize stroma. PPDK bands of equal intensity could have been obtained by using about 6 μg of maize stroma protein (instead of 30 μg) in lane 2 compared to the 400 μg of wheat stroma protein used in lane 3.

have been reported for proteins of chloroplast membranes synthesized in the cytoplasm (21). Whether or not there is any specificity of recognition sites with respect to leader size is unknown.

A role of PPDK in stomatal regulation has been proposed (16, 31), but the level of PPDK which we find in C_3 leaves, though only 1 to 2% of that of C_4 leaves, might indicate an additional role. In tissues undergoing glycolysis, there is no apparent function for PPDK, since phosphoenolpyruvate formation from carbohydrates occurs. In fact, since pyruvate kinase is active during glycolysis, PPDK activity would result in a futile cycle. The need for PPDK arises when there is a net conversion of pyruvate to phosphoenolpyruvate, for example, during gluconeogenesis or perhaps during conversion of alanine to glutamate, as has been suggested for the case of seeds (2).

In C_4 plant leaf mesophyll cells, the role of PPDK in the C_4 CO_2 fixation and transport pathway is well known (24). Such an intercellular C_4 transport does not apparently occur in C_3 leaves, but the possibility of intracellular transport remains. In view of the low level of activity of PPDK and other enzymes of the C_4 pathway relative to the rates of photosynthesis in C_3 plants so far examined, such a transport, if it exists would seem not be quantitatively important. Nevertheless, it might be of interest to examine levels of PPDK in those C_3 species that are capable of higher-than-expected rates of photosynthesis under conditions favorable to photorespiration. Also, the ability of C_3 plants to express a chloroplast-specific PPDK polypeptide may prove helpful in understanding how one step in the evolution of C_4 carbon transport in the plant kingdom may have occurred.

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