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Localization of Carbamoylphosphate Synthetase and Aspartate Carbamoyltransferase in Chloroplasts

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ABSTRACT

The localization of carbamoylphosphate synthetase (CPSase) and aspartate carbamoyltransferase (ACTase), the first two enzymes of the pyrimidine biosynthetic pathway, in chloroplasts was investigated. In dark-grown radish (*Raphanus sativus*) seedlings, light induced a prominent increase in CPSase activity, but had little effect on ACTase activity. Both enzymes were found in chloroplasts isolated from radish cotyledons and leaves of spinach (*Spinacia oleracea*), soybean (*Glycine max*), and corn (*Zea mays*). The higher activity of ACTase relative to CPSase is discussed in relation to the instability of carbamoylphosphate, the product of the CPSase, and to the control of pyrimidine synthesis. Based on these results, the function of CPSase and ACTase in chloroplasts is discussed.

The two enzymes, Gln-dependent CPSase[1] (EC 6.3.5.5) and ACTase (EC 2.1.3.2) are involved in the *de novo* biosynthesis of pyrimidine compounds in all organisms (see reviews 7, 17). In the first step of the pyrimidine pathway, CPSase catalyzes the synthesis of CarP from CO_2 with Gln as the nitrogen donor and ATP as an energy source. The carbamoyl moiety of CarP is then transferred by ACTase to form CarAsp. Using ^{14}C-labeled compounds, CO_2, CarP, and CarAsp are incorporated into UMP by intact cells of excised roots of *Cucurbita pepo* (10). Four enzymes, including CPSase and ACTase, of the *de novo* pathway were found in the cytosol fraction of *Vinca rosea* cells (9). These two results indicate the presence of the *de novo* pathway in cytosol of plant cells. Alternatively, plants and other organisms can convert the nucleosides and bases to nucleotides by the so-called salvage pathway. The UMP formed through either pathway is then phosphorylated to UTP, a part of which is used for the synthesis of CTP.

Chloroplasts possess all the machinery required for protein synthesis, so, they are genetically autonomous in some ways. Genes in chloroplast DNA are transcribed by chloroplast RNA polymerases and the messages are translated on chloroplast ribosomes. Thus, intact isolated spinach chloroplasts are able to incorporate [^3H]uridine into the RNA (6) and *Acetobularia* chloroplasts incorporate [^{14}C]uracil into RNA (18). These results indicate that uridine and uracil can be (a) transported across the envelope into chloroplasts, (b) converted to UMP *via* the salvage pathway, (c) phosphorylated to UTP, and (d) subsequently incorporated into RNA by RNA polymerase. On the other hand, it was reported (8) that isolated chloroplasts of *Bryophyllum daigremontianum* Berger incorporate the label from [^{14}C]orotic acid, an intermediate of the *de novo* pathway, into nucleotides and RNA in the presence of 5-phosphoribosyl-1-pyrophosphate and ATP. These results indicate that (e) orotate can be transported across the envelope and (f) chloroplasts possess some enzymes of the *de novo* pathway, those which convert orotate via orotidine monophosphate to UMP.

It is not known whether chloroplasts contain the entire components required for pyrimidine *de novo* biosynthesis, or whether chloroplasts depend upon the cytosol for the synthesis of pyrimidine precursors (orotate, uracil, or uridine). In this paper, we provide direct evidence for the presence of the first two enzymes of pyrimidine biosynthetic pathway, CPSase and ACTase, in chloroplasts purified from four plant species. The higher activity of ACTase relative to CPSase found in chloroplasts and reported in extracts of many organisms, is discussed in relation to the instability of CarP *in situ* and to the control of pyrimidine synthesis.

MATERIALS AND METHODS

Plant Materials. Radish seeds (*Raphanus sativus* var. Wase-Yonjunichi) were germinated and grown on moistened absorbent cotton in the dark at 22 to 25°C (19, 20). The dark-grown seedlings were exposed to continuous illumination 3 d after germination in a growth chamber (NK Biotron, LH-200 RD, 7.6 m W/cm^2, 25°C). Spinach (*Spinacia oleracea*), soybean (*Glycine max*), and corn (*Zea mays*) were grown in the field, and the leaves of 3- to 5-week-old plants were used for the isolation of chloroplasts.

Extraction of Radish Cotyledons. For developmental studies, 50 to 100 cotyledon pairs were excised at selected time intervals, and homogenized in the presence of 10% (w/w) insoluble-PVP, with a mortar and pestle in 10 ml of chilled grinding medium containing 0.1 M Tricine-KOH (pH 7.8), 10 mM KCl, 3 mM Gln, 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000*g* for 20 min. A portion of the supernatant was passed through a column of Sephadex G-25, equilibrated with the grinding medium, and the eluate was used for enzyme assays (see below). Chl (19) and RNA (20) were estimated as described previously.

Preparation and Purification of Chloroplasts. Chloroplasts were purified from greened radish cotyledons (3 d dark then 40 h light) or from spinach, soybean and corn leaves. Fifty g of cotyledons or leaves were homogenized with a blender in a solution containing 0.33 M sorbitol, 50 mM Tricine-KOH (pH 7.8), 2 mM EDTA, 1 mM $MgCl_2$, 20 mM NaCl, 20 mM *iso*-ascorbic acid, 3 mM Gln, 4 mM DTT, and 0.2% BSA. After filtration through two layers of Miracloth, each filtrate was centrifuged at 170*g* for 3 min to remove cell debris. The crude chloroplast fraction obtained by centrifugation at 1,000*g* for 10 min was resuspended in the homogenizing medium. For sucrose

[1] Abbreviations: CPSase, carbamoylphosphate synthetase; ACTase, aspartate carbamoyltransferase: CarP, carbamoylphosphate; CarAsp, carbamoylaspartate; OCTase, ornithine carbamoyltransferase (EC 2.1.3.3); ALAD, 5-aminolevulinic acid dehydratase (EC 4.2.1.24); PEPCase, phosphoenolpyruvate carboxylase.

density gradient centrifugation, the 1,000*g* fraction was applied onto the semicontinuous sucrose density gradient prepared according to Miflin and Beevers (12). All the sucrose solutions contained 50 mM Tricine-KOH (pH 7.8), 3 mM Gln, 2 mM DTT, 1% Ficoll, 1% Dextran T-40, and 0.2% BSA. The centrifugation was carried out as in (12) using a RPS-25 rotor in a Hitachi 55-P2 ultracentrifuge. The gradient was collected in about 1.4-ml fractions using a Hitachi tube slicer TS-U. For the rapid purification of chloroplasts, crude fraction was purified by a Percoll density gradient centrifugation (22). The 1,000*g* fraction was loaded onto a linear Percoll gradient which contained 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 20 mM NaCl, 2 mM *iso*-ascorbate, 4 mM DTT, 0.2% BSA, and 3 mM Gln, and centrifuged in a Kubota RS-I swinging bucket rotor at 10,000*g* for 10 min. After centrifugation the heavy chloroplast fraction was recovered as described in (22).

Enzyme Assays: CPSase. Two different assay methods modified from the procedures of O'Neal and Naylor (13) were used. In method I, coupled assay using Orn and OCTase, in which the formed CarP is converted to acid-stable citrulline, the assay mixture in a small scintillation vial contained 90 mM Hepes-KOH (pH 8.0), 4 mM Gln, 4 mM ATP, 10 mM $MgCl_2$, 5 mM $NaH^{14}CO_3$ (280–350 dpm/nmol), 1 mM DTT, 4 mM Orn, 2 units of OCTase (1 unit synthesized 1 μmol of citrulline/min from Car and Orn at 37°C) and 0.2 ml of enzyme preparation in a total volume of 0.5 ml. The reaction was started by addition of $NaH^{14}CO_3$ and allowed to proceed for 10 min at 30°C. The reaction was stopped by adding 0.2 ml of 2 N $HClO_4$. The vials were heated at 80°C for 1 h. Five ml of Bray's scintillation mixture (Scintisol) were then added to each vial; after thorough mixing the ^{14}C incorporated into citrulline was counted with an Aloka LSC-700 scintillation counter. In method II, the CarP formed in the absence of Orn and OCTase was converted to hydroxyurea. The reaction was stopped by adding 0.1 ml of 0.6 M hydroxylamine (pH 6.5). The vials were then transferred to an 80°C water bath for 10 min, after which they were acidified with 0.2 ml of 2 N $HClO_4$ and then kept at 80°C for 1 h. The ^{14}C in acid-stable hydroxyurea was counted as indicated above. The assay method for ACTase was based on methods of Lovatt *et al.* (10). The reaction mixture in the vial contained 0.1 M Tris-HCl (pH 8.0), 10 mM Asp, 4.8 mM [^{14}C]CarP (13 dpm/nmol), and 0.25 ml of enzyme preparation in a total volume of 0.5 ml. The reaction was begun by the addition of [^{14}C]CarP. After incubation at 30°C for 20 min, the reaction was terminated by adding 0.2 ml of 33% HCOOH. The samples were heated and then ^{14}C in CarAsp was counted as above. Blanks of CPSase and ACTase with either heated enzyme or with acid in each assay were subtracted from the observed activity. The following assay methods as described in the relevant literature were employed: ALAD (19), PEPCase (22), and Cyt *c* oxidase (4). The data presented are the results of typical experiments and each experiment has been repeated at least 3 times.

Other Materials. Plant seeds were obtained from Takii Seed Co, Kyoto. The $NaH^{14}CO_3$ and $K^{14}CNO$ were purchased from New England Nuclear and Amersham-Japan, respectively. The [^{14}C]CarP was synthesized from $K^{14}CNO$ and KH_2PO_4 according to the method of Spector *et al.* (21). OCTase was purchased from Sigma. Scintisol was the product of Wako Pure Chemical Co., Tokyo.

RESULTS AND DISCUSSION

Changes in Developmental Pattern of CPSase and ACTase Activities in Radish Cotyledons during Growth and Chloroplast Development. Figure 1 shows the fresh weight, content of Chl, content of RNA, and the activities of CPSase and ACTase in radish cotyledons during the course of germination and growth in the dark, or upon exposure of the dark-grown seedlings to continuous illumination to induce chloroplast development. After seed germination (1–2 d), RNA started to accumulate in the cotyledons of dark-grown seedlings, reaching a maximum level at 4 d, whereas fresh weights of the cotyledons remained almost constant. Fresh weight, Chl accumulation, and RNA content increased significantly after light exposure. It is known that illumination of dark-grown plants induces a rapid increase in RNA polymerase activity and the RNA content of chloroplasts (2). Accordingly, nucleoside triphosphates would be required for the synthesis of chloroplast RNA during chloroplast development.

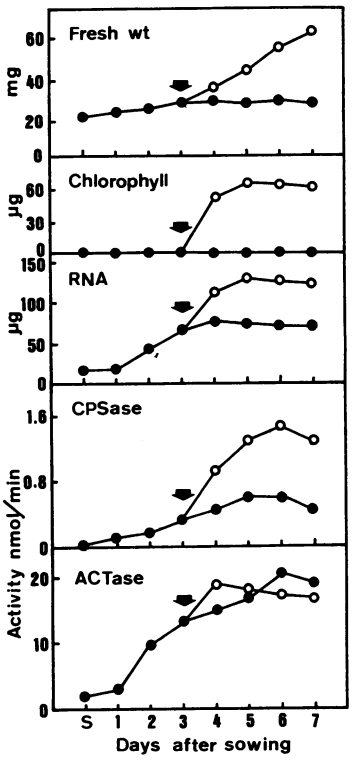

FIG. 1. Developmental changes of enzyme activities in cotyledons of dark- and light-grown radish seedlings. (●), seedlings grown in darkness; (○), seedlings exposed to continuous illumination at 3-d stage as indicated by arrow. (S), seeds soaked for 3 h. CPSase was assayed by the method I. Fresh weight, contents of Chl and RNA, and enzyme activities were expressed on a per pair cotyledon basis.

In cotyledons of dark-grown seedlings, CPSase and ACTase levels started to increase after germination. Illumination of seedlings induced a significant increase in CPSase activity, and at the 5 d stage the activity in cotyledons of seedlings exposed continuously to light from the 3-d stage was 2.2 times as high as that in the tissues maintained in the dark. Light exposure had little effect on ACTase activity.

Localization of CPSase and ACTase in Chloroplasts. To demonstrate the occurrence of CPSase and ACTase in intact chloroplasts, crude chloroplasts (the 1000*g* fraction) were applied onto the semicontinuous sucrose density gradient (Fig. 2). Markers used for the cell organelles were as follows; Chl (chloroplasts), ALAD (chloroplast stroma), Cyt *c* oxidase (mitochondria), PEPCase (cytosol). There were two Chl-containing fractions. ALAD was found in the higher density Chl-containing fraction, indicating that it contains intact chloroplasts (3, 19), whereas the lighter fraction represents broken chloroplasts. PEPCase present in the crude chloroplast fraction was essentially absent from the two chloroplast fractions in the gradient, indicating no contamination of cytosol in these fractions. Although a little activity of Cyt *c* oxidase was still present in the intact chloroplast fraction, peaks

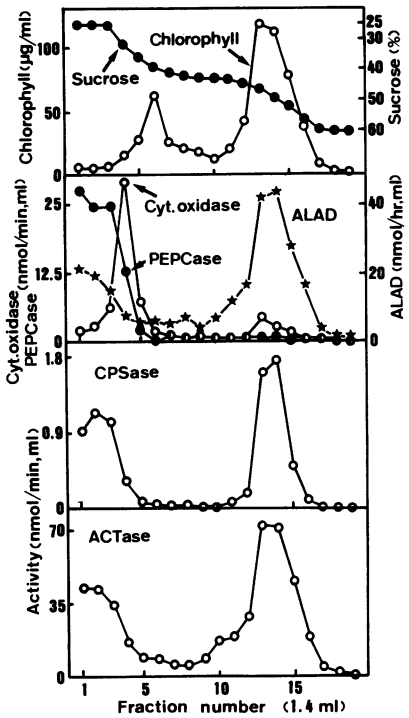

FIG. 2. Distribution of enzyme activities in separated fractions after sucrose density gradient centrifugation of a crude chloroplast fraction. The 1000*g* crude chloroplast fraction was subjected to sucrose density gradient centrifugation as described in the text. At the end of centrifugation, 1.4-ml fractions were collected and used for the following assays: Chl (broken and intact chloroplasts), Cyt *c* oxidase (mitochondrial enzyme), ALAD (chloroplast stroma), PEPCase (cytosolic enzyme), and CPSase and ACTase. CPSase was assayed by the method II.

of CPSase and ACTase activities in the gradient did not coincide with the main peak of Cyt *c* oxidase (fraction 4), indicating that mitochondria do not appear to possess CPSase and ACTase. From the results in Figure 2, it will be noted that CPSase and ACTase are localized in intact chloroplasts, whereas they were undetectable in broken chloroplasts indicating that they are stroma enzymes.

Occurrence of CPSase and ACTase in Chloroplasts of Other Plants. We attempted to learn whether CPSase and ACTase exist in chloroplasts of other plant species. In this experiment, chloroplasts were purified from radish cotyledons and from leaves of spinach, pea and corn by Percoll density gradient centrifugation (22). The chloroplasts thus purified from radish cotyledons showed the following characteristics: light-dependent CO_2 fixation and CO_2-dependent O_2 evolution, about 45 and 85 μmol/mg Chl·h, respectively; intactness 80 to 90% (maximum 92.8%); contamination of mitochondria (Cyt *c* oxidase activity) and peroxisomes (catalase activity) was less than 5 and 1.5%, respectively; free from activity of cytosolic marker enzyme (PEPCase). The reliability of assay methods for chloroplast CPSase and ACTase was demonstrated by the results shown in Table I. CPSase activity in isolated chloroplasts was dependent upon the inclusion of OCTase and Orn, ATP, and $MgCl_2$ in the reaction mixture. Dependency on Gln was not observed because the chloroplast isolation buffer contained 3 mM Gln. Activity of ACTase in chloroplasts was dependent on the added Asp. Table II shows that purified chloroplasts from radish cotyledons or leaves of spinach, bean and corn possess both activity of CPSase and ACTase. From the results in Figure 1 and Table II, we concluded that both CPSase and ACTase are localized in chloroplasts.

Table I. *Assays of CPSase and ACTase in Chloroplasts Purified from Radish Cotyledons*

The complete reaction mixtures and assay conditions were described in "Materials and Methods." The assay mixtures for CPSase and ACTase contained chloroplasts equivalent to 140 μg Chl and 63 μg Chl, respectively.

Conditions	CPSase	ACTase
	dpm/assay	
Complete system	9284	7128
Boiled chloroplasts	592	372
−Chloroplasts		182
−(OCTase + Orn)	2276	
−ATP	798	
$-MgCl_2$	3045	
−Gln	9470	
−Asp		932

Table II. *Localization of CPSase and ACTase Activity in Purified Chloroplasts of Some Plant Species*

Chloroplasts[a]	CPSase[b]	ACTase	ACTase/CPSase
	nmol/min mg Chl		
Radish cotyledons	24.3	512	21.9
Spinach leaves	10.5	75.4	7.2
Pea leaves	29.3	102	3.5
Corn leaves	3.7	31.0	8.4

[a] Purified by Percoll density gradient centrifugation. [b] According to the assay method I.

Comparison of ACTase/CPSase Activity Ratio. The activity of ACTase is more than 10 times as high as that of CPSase in the extracts (Fig. 1), and in the chloroplasts (Fig. 2, Table II) prepared from radish cotyledons. However the ACTase/CPSase ratio in the chloroplasts purified from mature leaves of different species was slightly lower, ranging from 3.5 to 8.3 (Table II). Extracts from wheat grain (11), *V. rosea* (9), *Bacillus subtilis* (16), rat liver (5), and kinetoplastida, *Trypanosoma brucei* (5) possess a higher activity of ACTase than CPSase, supporting the idea that the rate-limiting step of the pyrimidine *de novo* pathway is the formation of CarP by CPSase rather than the metabolism of CarP by ACTase (9, 17). The lower CPSase activity in cell-free extracts does not appear to result from the instability of CPSase *in vitro*. Although O'Neal and Naylor (13) reported that CPSase of pea shoots is highly unstable, and it is stabilized by Gln, Orn, and 2-mercaptoethanol, all of which are components of the isolation buffer of chloroplasts. Furthermore, we have found that chloroplast CPSase from radish cotyledons is stable at least for 6 h in the presence of Gln, DTT, EDTA, and BSA, whereas Orn had little effect (data not shown).

CarP, the product of CPSase and also the substrate of ACTase, is extremely unstable in a solution; its half-life is: 16 h at 0°C, 2 h at 30°C, and 30 to 50 min at 37°C (21), over the pH range of 5 to 9 (1). Under the conditions found in cytosol and in chloroplasts, especially when the day temperature may reach 40°C and the pH is 7.6 to 8.2, CarP would rapidly decompose. Since large amount of CarP would not accumulate (17), a large excess of ACTase activity would allow for the rapid conversion of almost all the CarP to CarAsp, which is a very stable compound (10). Plant ACTases have relatively low apparent K_m values for CarP, 0.1 to 0.13 mM (14, 17). We estimated that the K_m of the chloroplast ACTase of radish cotyledons was 0.14 mM, therefore CarP probably does not occur in free form in the systems. A similar explanation (15) has been reported for the high level of ribulose-1,5-bisphosphate carboxylase in chloroplasts where the molarity of active sites of the enzyme exceeds that of its substrate, ribulose-1,5-bisphosphate. In a solution, ribulose-1,5-bisphos-

phate decomposes nonenzymatically to two degradation products, both of which are inhibitors of ribulose-1,5-bisphosphate carboxylase (15).

CarAsp is converted by dihydroorotase to dihydroorotate. As the K_m of this enzyme for CarAsp in pea leaves is reported to be 6.2 mM, Ross (17) proposed that with such a low affinity for its substrate, the efficient operation of the pyrimidine pathway in plants depends upon a high concentration of CarAsp. Taken together, a large excess of ACTase rapidly converts CarP to the stable CarAsp and results in a relatively high CarAsp concentration required for efficient operation of the next enzyme, dihydroorotase, of the *de novo* pyrimidine pathway. In mammals these three enzymes, CPSase, ACTase and dihydrooratase, are present as a multienzymic protein (7).

Activities of CPSase, ACTase, orotatephosphoribosyl transferase, and orotidine-5′-monophosphate decarboxylase were found in the cytosol of *V. rosea* (9). Pyrimidine *de novo* pathway was demonstrated in nonchloroplastic tissue, roots of summer squash (10). These results may indicate the localization of pyrimidine *de novo* pathway in the cytosol of plant cells. In this study we demonstrated the occurrence of CPSase and ACTase in chloroplasts. By comparing the CPSase activity expressed on a mg Chl basis in the extract of greened cotyledons (40 h light) with that found in the intact isolated chloroplasts, chloroplastic CPSase was found to comprise about 60 to 65% of total CPSase present in radish cotyledon cells. The remainder would occur in the cytosol. However, it is still unclear if chloroplasts depend upon pyrimidine precursors derived from cytosol under any certain condition, though intact chloroplasts can incorporate orotate (8), uracil (18), and uridine (6) into RNA. As yet there is no evidence for the occurrence of the third and fourth enzymes of the pyrimidine *de novo* pathway, dihydroorotase and dihydroorotate dehydrogenase in chloroplasts.

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