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Direct transport of ADPglucose by an adenylate translocator is linked to starch biosynthesis in amyloplasts

(carboxyatractyloside/double silicone oil layer centrifugation/starch synthase/sucrose synthase/sycamore)

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ABSTRACT **Starch biosynthesis has been studied by using amyloplasts isolated from cultured cells of sycamore trees (*Acer pseudoplatanus* L.). Highly purified intact amyloplasts, free from mitochondria and starch granules derived from broken amyloplasts, were isolated from a Percoll step gradient. Subsequently, the double silicone oil layer centrifugation technique was used to study adenylate transport in the amyloplasts. An adenylate-specific carrier was found to be active in the uptake of ATP, ADP, AMP, and, most importantly, ADPglucose (ADP-Glc). Kinetic analyses showed that the uptake of these adenylates was mutually competitive with each other. In contrast to the mitochondrial adenylate carrier, in amyloplasts only ATP and ADP-Glc uptake were inhibited by carboxyatractyloside. Evidence is presented that the ADP-Glc transported into the amyloplast stroma can be used in starch synthesis catalyzed by starch synthase (ADP-Glc:1,4-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.21). We propose that starch biosynthesis in amyloplasts is tightly coupled with the direct transport of ADP-Glc synthesized in the cytosol by sucrose synthase (ADP-Glc:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13).**

The amyloplast is a specifically differentiated plastid that synthesizes and accumulates reserve starch in the stroma. Although it is frequently hypothesized that chloroplasts and amyloplasts are ontogenically and evolutionarily related to each other, they differ in their carbon and adenylate flow (1). Chloroplasts produce ATP and NADPH necessary for photosynthetic CO_2 fixation (Benson–Calvin cycle). Although it is established that fixed products are exported to the cytosol by means of the phosphate translocator (2), some of them are utilized for the formation of starch in the chloroplast. ADPglucose pyrophosphorylase (glucose-1-phosphate adenylyltransferase, EC 2.7.7.27) utilizes the photochemically generated ATP and glucose 1-phosphate (Glc-1-P) for the synthesis of ADPglucose (ADP-Glc), which serves as the glucosyl donor in starch formation. This enzyme is believed to play a key role in the overall gluconeogenic mechanism in the chloroplast (3). On the other hand, amyloplasts possess neither ATP-generating nor CO_2-fixing capabilities. Provided an analogous mechanism of starch biosynthesis operates in both organelles (3), one must postulate the presence of adenylate and sugar-phosphate shuttles in the amyloplast envelope membranes. It can be envisioned that imported ATP and carbon compounds from the cytosol would be utilized eventually for the synthesis of ADP-Glc by ADP-Glc pyrophosphorylase. However, the extreme physical fragility of this starch-filled organelle has made it difficult to isolate structurally and functionally competent amyloplasts and to examine their transport capabilities linked to the mechanism of gluconeogenesis (4, 5).

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Previously, we reported the detection, by immunoanalysis, of a putative adenylate (ATP/ADP) translocator in the amyloplast envelopes isolated from cultured sycamore cells (6). We have now examined the kinetic properties of this transporter by using intact amyloplast preparations and have found evidence for the direct transport of ADP-Glc across the amyloplast envelope, which is tightly linked to the formation of starch.

MATERIALS AND METHODS

Cell Culture and Amyloplast Isolation. Amyloplasts from nonstarved suspension culture cells of sycamore trees (*Acer pseudoplatanus* L.) were isolated according to the Percoll step-gradient method (7, 8) with some modifications; the final gradient consisted of 2 ml each of Percoll layers of ρ = 1.057, 1.084 (including protoplast lysate), 1.098, and 1.210 g/ml. To eliminate contamination by starch granules derived from broken amyloplasts, an extra dense layer of Percoll (ρ = 1.210 g/ml), obtained after evaporation of the commercial product, was placed in the bottom of the gradient. After centrifugation at 2000 × *g* in a swinging-bucket type rotor, amyloplasts were recovered from the interface between the Percoll layers with ρ = 1.098 and 1.210 g/ml, whereas free starch granules pelleted down to the bottom (see Fig. 1*a*). Collected amyloplasts were sedimented at 1000 × *g* after dilution in MEM buffer (10 mM Mops, pH 7.5/1 mM EDTA/0.5 M mannitol) and resuspended in sampling buffer (25 mM Hepes, pH 7.5/20 mM KCl/1 mM EDTA/0.45 M mannitol) [final 6-phosphogluconate dehydrogenase (6PGDH) specific activity = 460 ± 90 units/liter]. The intactness of the amyloplasts was determined as reported (1).

Vesicles from amyloplast envelopes were prepared according to the method described by Liedvogel and Kleinig (9) for obtaining vesicles from daffodil chromoplasts. Vesicles were resuspended in sampling buffer but without mannitol (hypotonic buffer; 1 mg of protein/ml).

Adenylate Transport. Unless indicated otherwise, transport experiments were performed with Bio-Rik 0.4-ml plastic tubes from Bio Plastics (Osaka) at 4°C in a refrigerated TOMY (Tokyo) MRX-151 centrifuge. A conventional single silicone oil layer centrifugation technique (10) was used for assaying the time course of uptake of adenylates in intact amyloplasts. In experiments examining the inhibitory effect of carboxyatractyloside (CAT) on the adenylate transport, amyloplast preparations were preincubated at 4°C with the reagent (100 μM) for 30 min. Subsequent kinetic studies of adenylate

Abbreviations: ADP-Glc, ADPglucose; CAT, carboxyatractyloside; Glc-1-P (or Glc-6-P), glucose 1-phosphate (or glucose 6-phosphate); 6PGDH, 6-phosphogluconate dehydrogenase; UDP-Glc, UDPglucose.

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uptake were carried out by using the double silicone oil layer centrifugation technique. The precise details of this experimental technique will be described elsewhere (J.P.-R., M.F., and T.A., unpublished results). A summary of the procedure is as follows: amyloplasts resuspended in sampling buffer (40 μl, ρ = 1.031 g/ml) are separated by a layer of light silicone oil (75 μl, ρ = 1.003 g/ml) from the labeled metabolite-containing incubation layer (30 mM Hepes, pH 7.5/1 mM EDTA/25 mM KCl/0.4 M sucrose; 60 μl, ρ = 1.059 g/ml). The incubation of amyloplasts with metabolite in the incubation layer begins immediately after inversion of the light silicone layer during centrifugation. The incubation time varies as a function of the centrifugal force (g); we determined empirically that centrifugation at 750 × g corresponds to a 37-sec incubation period, which was employed throughout the present kinetic analyses. The reaction stops when amyloplasts traverse the incubation layer to the heavy silicone oil layer (75 μl, ρ = 1.066 g/ml), which is located below the incubation medium. Finally, the amyloplasts sediment to the pelleting layer (100 μl of 0.55 M sucrose containing 10% $HClO_4$, ρ = 1.075 g/ml) located under the lower silicone layer.

Since the sorbitol-permeable space (intermembrane space) is accessible to every metabolite tested, real values of metabolite transport mediated by carrier proteins located in the amyloplast inner membranes are obtained after determining the sorbitol-impermeable space (^3H_2O-permeable space minus [^{14}C]sorbitol-permeable space) (2).

Coupling of ADP-Glc Transport and Starch Synthesis. Amyloplasts incubated at 4°C with 100 μM ADP-[^{14}C]Glc in the sampling buffer were filtered by using the single silicone oil layer centrifugation technique. Parallel experiments using either starch granules or mechanically disrupted amyloplasts were carried out as negative controls. After centrifugation, the radioactivity from each fraction was measured to estimate the amount of ADP-Glc transported into the stroma or the rate of the transglucosylation reaction.

In experiments measuring the time-dependent uptake of ADP-Glc in starch-depleted vesicles, the method employed was basically that described by Pfaff and Klingenberg (11) using mitochondria. After incubation with ADP-[^{14}C]Glc (200 μM), 40 μl of the vesicular suspension was layered on top of 200 μl of the hypotonic buffer as described above containing 0.1 M sucrose and centrifuged at 10,000 × g for 2 min; the pelleted vesicles were washed with hypotonic buffer (200 μl).

Analytical Methods. Plastidial 6PGDH and cytochrome c oxidase have been used as marker enzymes of amyloplasts and mitochondria, respectively (7). The sucrose synthase (ADP-Glc:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13) activity in the protoplast lysates (7) was assayed as described (12), replacing ATP and UDP with UTP and ADP, respectively. Starch content was estimated as reported (7).

To examine the covalent $\alpha(1 \rightarrow 4)$ linkage formed between [^{14}C]glucose and the terminal end of the amylose molecules, TLC separation of the β-amylolytic products of the labeled starch granules isolated from the amyloplasts incubated with ADP-[^{14}C]Glc was performed. The identification of radiolabeled adenylates transported in amyloplasts was carried out by using ascending cellulose TLC [isobutyric acid/water/ammonia/EDTA (37 g/liter) (500:280:21:8, (vol/vol)] followed by radioautography.

Reagents. The radiochemicals {[^{3}H]ATP, [^{3}H]ADP, [^{3}H]-AMP, ADP-[^{14}C]Glc, [^{3}H]UTP, UDP[^{14}C]glucose (UDP-[^{14}C]Glc), [^{14}C]Glc, ^3H_2O, and [^{14}C]sorbitol} were purchased from Amersham. β-Amylase, hexokinase (no. H4502), glucose-6-phosphate dehydrogenase (no. G5885), and phosphoglucoisomerase (no. P5381) were purchased from Sigma. Wackersilicone AR 20 Pharmas from Wacker-Chemie (Munich) and SH 704 from Toray Silicone (Osaka) were used in the transport experiments.

RESULTS

Isolation of Intact Amyloplasts. Previous attempts to isolate intact amyloplasts have not been successful, principally because of the extreme physical fragility of the amyloplasts (4, 5). As a prerequisite to study the characteristics of adenylate transport across the amyloplast envelopes coupled with starch biosynthesis, it was essential to isolate pure organelles having structural integrity. Interference caused by the adenylate translocator associated with the mitochondrial inner membranes and the insoluble starch synthase (ADP-Glc:1,4-α-D-glucan 4-α-D-glucosyltransferase, EC 2.4.1.21) bound to free starch granules must be completely eliminated.

By employing a discontinuous Percoll gradient centrifugation technique, intact amyloplasts free from contamination of other organelles were obtained at the interface between the Percoll layers with ρ = 1.098 and 1.210 g/ml, and the denser free starch granules sedimented to the bottom of the tube (Fig. 1 a and b). The absence of free starch granules is necessary to prevent breakage of amyloplasts during transport experiments. In our studies, ≈89% (89% ± 4.6%; mean ± SD, n = 9) of the loaded amyloplast sample was recovered after transport.

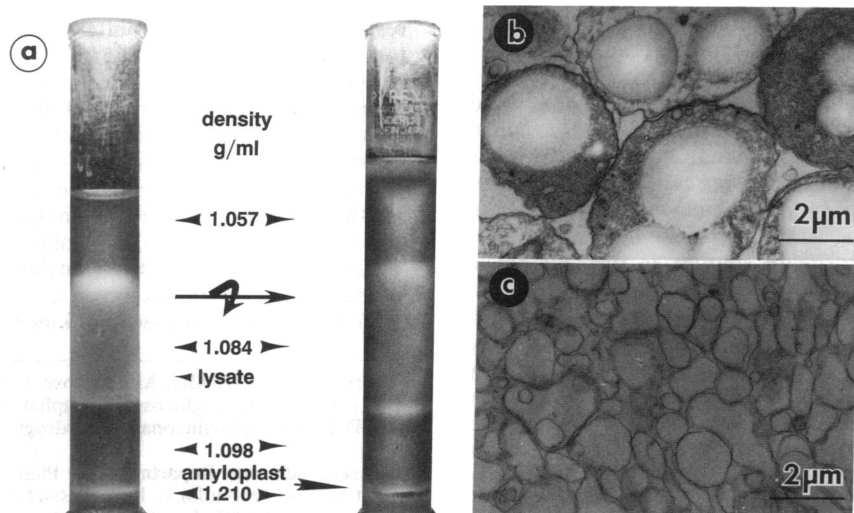


FIG. 1. Isolation of amyloplasts. (a) Discontinuous Percoll gradient centrifugation. Electron micrographs of intact amyloplasts (b) and vesicles (c) are shown.

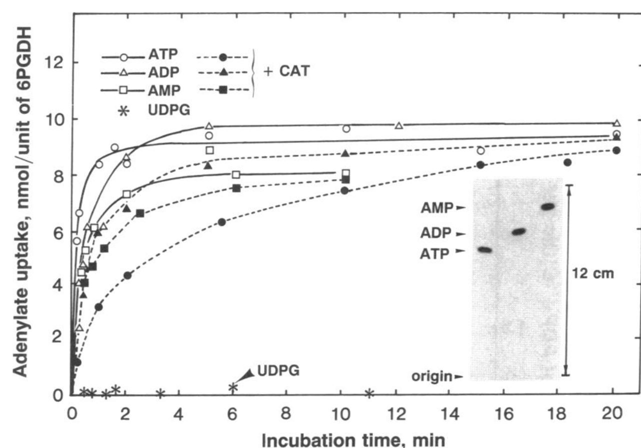


FIG. 2. Time course of uptake of [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]AMP (55 μM) by amyloplasts with and without preincubation with CAT (100 μM). (*Inset*) Radioautogram showing the adenylates transported to amyloplasts after a 2-min incubation with the radiolabeled adenylate (55 μM). UDPG, UDP-Glc.

By assaying marker enzymes of amyloplasts and mitochondria, the ratio of cytochrome *c* oxidase to 6PGDH activities (0.014 ± 0.006, *n* = 13) was several times lower than that obtained by using the original method (8) (0.066 ± 0.025, *n* = 7). Thus, contamination of amyloplast preparations by mitochondrial membranes is principally due to their adsorption to starch granules, and we are convinced that the influence of mitochondrial contamination on the adenylate transport by amyloplasts is negligible.

ATP/ADP/AMP Transport in Amyloplasts. To calculate the concentration of metabolite transported in the stromal phase, the volume of the sorbitol-impermeable space was estimated (see *Materials and Methods*). The ^3H_2O- and [^{14}C]sorbitol-permeable spaces yielded values of 0.122 ± 0.02 and 0.055 ± 0.009 ml/unit of 6PGDH, respectively (*n* = 6).

As presented in Fig. 2, it was found that the ATP/ADP/AMP transport into amyloplasts is rapid and required only 2 min to reach the steady-state level of 498 ± 196 μM (*n* = 6) for the three compounds tested. No appreciable uptake of either UDP-[^{14}C]Glc or [^{3}H]UTP (data not shown) was observed. As shown in the *Inset* of Fig. 2, [^{3}H]ATP, [^{3}H]ADP, and [^{3}H]AMP remained structurally unchanged after transport. We tested the effect of CAT, a noncompetitive inhibitor of the mitochondrial ADP/ATP translocator (13). In contrast to the case of mitochondria, uptake of ATP but not ADP and AMP was significantly inhibited by CAT (Fig. 2).

As a consequence of the rapid adenylate transport into amyloplasts, we performed a series of experiments to examine the kinetic properties of the adenylate translocator by employing the double silicone oil layer centrifugation technique. The results presented in Fig. 3*a* give K_m values of 24.5 ± 7 μM (*n* = 5) for ATP, 32 ± 6 μM (*n* = 4) for ADP, and 47 ± 7.7 μM (*n* = 4) for AMP.

The uptake of ATP was competitively inhibited by ADP and AMP (Fig. 3*b*), and as is the case for the mitochondrial ADP/ATP translocator (13), noncompetitive inhibition by CAT was observed. The lack of inhibition by UTP on the ATP transport indicates that the translocator is highly specific for adenylates. The results presented in Fig. 3*c* show the competitive inhibition of ADP uptake by ATP and AMP but no appreciable inhibition by CAT. Most importantly, ADP-Glc was a competitive inhibitor of ADP (Fig. 3*c*) and AMP transport (Fig. 3*d*).

Coupling of ADP-Glc Transport and Starch Biosynthesis. As a consequence of the overall results obtained, experiments were carried out to examine the direct uptake of ADP-[^{14}C]Glc and possible coupling with starch biosynthesis in amyloplasts. Radiolabeling of starch granules by using intact amyloplasts incubated with ADP-[^{14}C]Glc and subsequent filtration by silicone oil centrifugation showed an inhibitory effect exerted by CAT in the nonsaturable transglucosylation reaction, whereas the reaction occurring in the broken amyloplasts as well as with free starch granules was unaffected by CAT (Fig. 4*a*). In parallel experiments using UDP-[^{14}C]Glc and [^{14}C]Glc under identical incubation conditions, no transglucosylation was observed (data not shown). It must be noted that the broken amyloplasts exhibited a higher rate of transglucosylation compared to the starch granules alone, which was most likely due to the combination of both insoluble (starch-bound) and soluble forms of starch synthase (3).

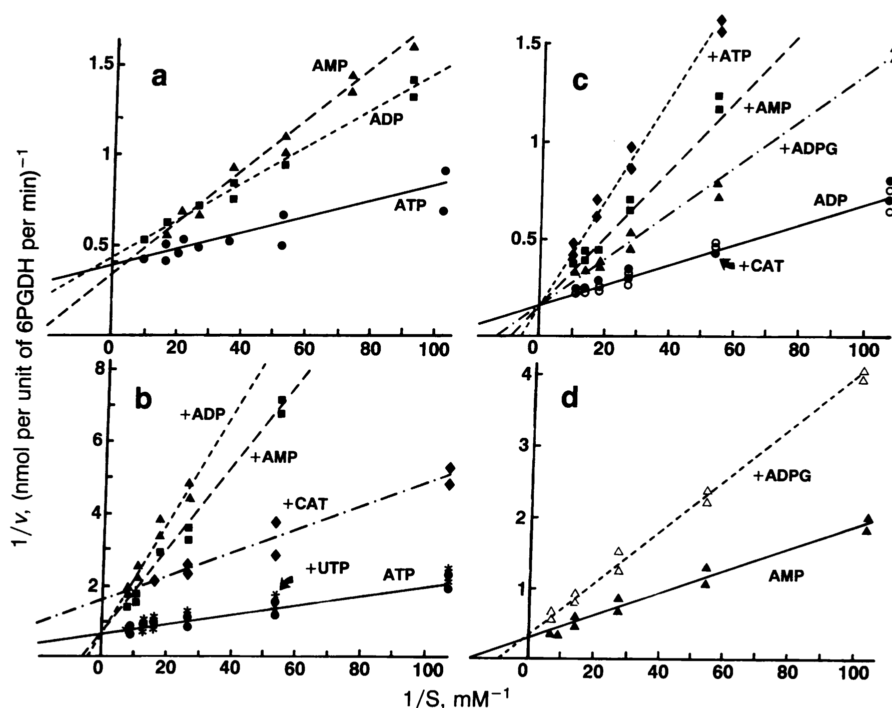


FIG. 3. Kinetic studies of adenylate uptake by amyloplasts. (*a*) Comparative study of ATP, ADP, and AMP transport. (*b*) Effect of ADP, AMP, and UTP (200 μM each) and CAT (100 μM) on the transport of ATP. (*c*) Effect of ATP, AMP, ADP-Glc (ADPG), and CAT (100 μM each) on the transport of ADP. (*d*) Effect of ADP-Glc (200 μM) on the transport of AMP.

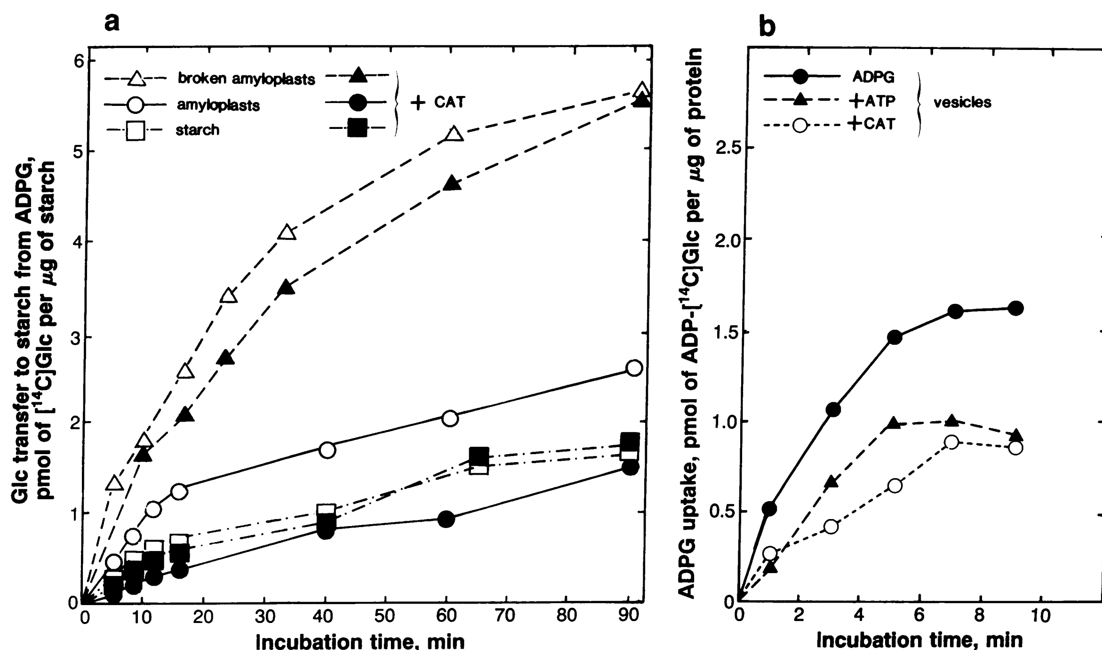


FIG. 4. (*a*) Time-course analysis of ADP-[^{14}C]Glc transport coupled with the transglucosylation reaction catalyzed by starch synthase in amyloplasts. Intact amyloplasts, mechanically disrupted amyloplasts, and starch granules were incubated with ADP-[^{14}C]Glc (85 μM) with and without preincubation with CAT (200 μM). (*b*) Time-course analysis of ADP-[^{14}C]Glc transport (200 μM) by amyloplast vesicles. ADPG, ADP-Glc.

It is extremely important for us to ascertain that the correct $\alpha(1 \rightarrow 4)$ linkage is produced under the incubation system. The [^{14}C]maltose derived from the digestion of starch by β-amylase (Sigma) was the only radiolabeled molecule detectable by TLC. The specific mode of action of β-amylase recognizing only $\alpha(1 \rightarrow 4)$-linked glucan, unable to hydrolyze $\alpha(1 \rightarrow 3)$-linked oligosaccharides, was recently reported by Lizzotte *et al.* (14) with the pea epicotyl enzyme.

A time-course analysis of ADP-Glc import into the stroma of amyloplasts with and without preincubation with CAT showed that steady-state levels of 25 and 45 μM ADP-Glc, respectively, were rapidly established. This concentration level is about 10 times lower than that observed for the transport of ATP, ADP, and AMP (see above). We conclude that it is likely due to the efficient coupling with the starch synthase reaction.

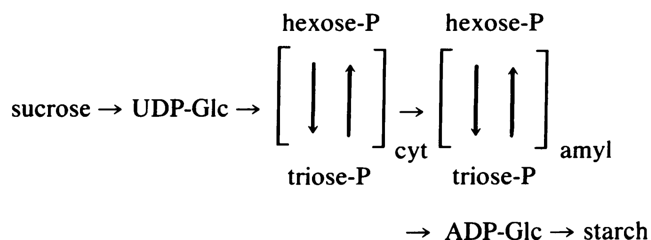
The presence of specific machinery transporting ADP-Glc across the amyloplast envelopes was further strengthened by experiments using starch-depleted vesicles prepared from the amyloplasts (Fig. 4*b*). Transport of ADP-Glc in the amyloplast vesicles was inhibited by ATP and CAT (100 μM). In contrast to the case of intact amyloplasts, a typical saturable reaction pattern was observed for the incorporation of ADP-[^{14}C]Glc into the vesicles (cf. Fig. 1*c*). This experiment is crucial for us to demonstrate the direct transport of ADP-Glc into amyloplasts, because only ADP-[^{14}C]Glc but not [^{14}C]ADP-Glc is commercially available, so that kinetic analysis of its direct entry into amyloplasts is unobtainable.

DISCUSSION

Since the initial discovery in 1963 showing that ADP-Glc serves as the glucosyl donor for starch synthase (15), the schematic pathway of starch biosynthesis in both chloroplasts and amyloplasts has remained essentially unchanged (3). Despite numerous investigations dealing with the enzymic mechanisms operating in amyloplasts, difficulties in isolating the intact organelle have prevented us to advance our knowledge. We have now been able to isolate amyloplasts that maintain their structural integrity (see Fig. 1) from cultured sycamore cells. This gives us the best possible chance to elucidate the intrinsic mechanisms underlying gluconeogenesis in amyloplasts.

It is generally accepted that the key regulatory enzyme for starch formation in chloroplasts is ADP-Glc pyrophosphorylase (3). Since this enzyme was also shown to be exclusively localized in amyloplasts (16), it is natural to postulate that a model of "ADP-Glc pyrophosphorylase controlled starch biosynthesis" equally operates in amyloplasts (3). According to this model, there must exist a supply of ATP and carbon compounds being transported from the cytosol to the amyloplast. In recent years, strong arguments have persisted as to what type of carbon compounds are imported into amyloplasts and utilized in starch biosynthesis. MacDonald and Rees (17), using soybean suspension cultures, and Echeverria *et al.* (18), using the maize kernels, suggested, on the basis of enzyme activity studies, that amyloplasts import triose phosphate mediated by a phosphate translocator similar to that present in chloroplasts (2). However, Keeling *et al.* (19), using wheat and maize grains, and Hatzfeld and Stitt (20), using potato tubers and maize endosperm, argued that the major gluconeogenic route involved is the direct import of hexose units, most probably Glc-1-P (21). Later, Borchert *et al.* (22), using pea roots, reported that Glc-6-P is the only hexose phosphate species transported.

Our own studies dealing with the enzymic capacities for gluconeogenesis in sycamore amyloplasts (7) have shown that activities of some key enzymes in the classical model of starch biosynthesis are marginally small, indicating that the following sequential step



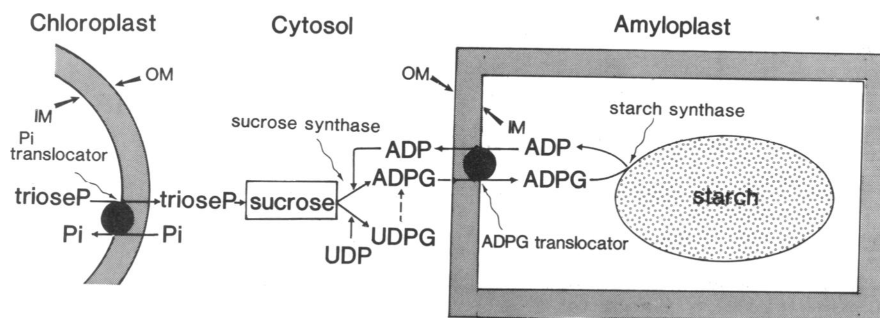


FIG. 5. Proposed model for starch biosynthesis in amyloplasts. ADPG, ADP-Glc; UDPG, UDP-Glc; trioseP, triose phosphate.

(where hexose-P = hexose phosphate, triose-P = triose phosphate, cyt = cytosol, and amyl = amyloplast) is unlikely to operate.

Although we cannot totally exclude the role of ADP-Glc pyrophosphorylase in the ADP-Glc synthesis in amyloplasts, it will be pointed out that our independent experiments have shown that the import of Glc-6-P coupled with starch biosynthesis in the presence of ATP plus Mg^{2+} is marginally small, indicating that there may exist an alternative role of ADP-Glc pyrophosphorylase other than for ADP-Glc synthesis (J.P.-R. and T.A., unpublished results).

Naturally there occurs a fundamental question as to the source of ADP-Glc in the cytosolic phase. From the results of radiolabeling of starch in rice seeds, Lee and Su (23) found that Glc-1-P is not in equilibrium with ADP-Glc, eliminating the predominant role of ADP-Glc pyrophosphorylase in sucrose–starch transformation. It is noteworthy that the content of ADP-Glc in the cell-free extracts of starch-bearing plant tissues such as rice seeds was found to be exceedingly high (24). Using developing maize seeds, Liu and Shannon (4) reported that most of the ADP-Glc is unexpectedly confined to the cytosol and questioned whether ADP-Glc synthesis occurs exclusively in the stromal phase of amyloplasts. They also discussed that the P_i-to-phosphoglyceric acid ratio estimated in amyloplast stroma would not allow the active state of the ADP-Glc pyrophosphorylase, which regulates starch biosynthesis (cf. ref. 3). Thus a likely mechanism proposed for the production of such a high level of ADP-Glc is the transformation of the cytosolic sucrose primarily derived from the chloroplastic CO_2 fixation to ADP-Glc mediated by sucrose synthase as schematically illustrated in Fig. 5. Although it is generally believed that sucrose synthase preferentially utilizes UDP-producing UDP-Glc, the enzyme can equally recognize ADP as a glucose acceptor (25–27). By measuring the ADP-dependent activities in the sycamore protoplast lysates by the coupling enzyme assays, 1.9 ± 0.2 ($n = 3$) units/g of fresh weight was obtained. Results reported by previous investigators concerning the lower level of sucrose synthase and subsequent elevation of sucrose content in the cytosol in developing endosperm of starch-deficient mutants of maize also support the important role of sucrose synthase in sucrose–starch transformation (28, 29).

Our work on the adenylate translocator was originally initiated assuming that the import of ATP is needed for ADP-Glc synthesis. As described above, it was found, however, that the translocator actively imports a wide range of adenylates, most importantly ADP-Glc, which can be subsequently utilized for the transglucosylation reaction. According to this overall mechanism, hexose sugars available for sucrose synthesis are in equilibrium with those eventually utilized for starch formation (see Fig. 5). This conceptual idea is compatible with previous observations of C1–C6 redistributions in sucrose and starch molecules derived from various plant species incubated with [1-^{14}C]Glc and [6-^{14}C]Glc (19, 20).

Electron micrographs were kindly taken by Dr. K. Kojima (Nagoya University, School of Medicine). J.P.-R. acknowledges a predoctoral student fellowship from the Japanese Ministry of Education, Science and Culture (Monbusho). M.F. and A.M.V. are indebted to the Japan Society for the Promotion of Science (JSPS) for a postdoctoral fellowship (1989–1990) and for a fellowship (1990) from the Inoue Foundation (Tokyo), respectively. This is paper no. 91 in the series "Structure and Function of Chloroplast Proteins." Paper no. 90 is ref. 7.

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