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ADP-Glucose Transport by the Chloroplast Adenylate Translocator Is Linked to Starch Biosynthesis[1]

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ABSTRACT

In organello **starch biosynthesis was studied using intact chloroplasts isolated from spinach leaves (*Spinacia oleracea*). Immunoblot analysis using a specific antiserum against the mitochondrial adenylate (ADP/ATP) translocator of *Neurospora crassa* shows the presence of an adenylate translocator protein in the chloroplast envelope membranes, similar to that existing in mitochondria and amyloplasts from cultured cells of sycamore (*Acer pseudoplatanus*). The double silicone oil layer-filtering centrifugation technique was employed to study the kinetic properties of adenylate transport in the purified chloroplasts; ATP, ADP, AMP, and most importantly ADP-Glc were shown to be recognized by the adenylate translocator. Similar to the situation with sycamore amyloplasts, only ATP and ADP-Glc uptake was inhibited by carboxyatractyloside, an inhibitor of the mitochondrial adenylate translocator. Evidence is presented to show that the ADP-Glc transported into the chloroplast stroma is utilized for starch synthesis catalyzed by starch synthase (ADP-Glc:1,4-α-D-glucan 4-α-D-glucosyltransferase). The high activity of sucrose synthase producing ADP-Glc observed in the extrachloroplastic fractions suggests that starch biosynthesis in chloroplasts may be coupled with the direct import of ADP-Glc from the cytosol.**

Among various types of plastids in plant cells, the chloroplast is the best characterized in that it produces both ATP and NADPH for photosynthetic CO_2 fixation (Benson-Calvin cycle). Although it is well known that some photosynthate (*e.g.* 3-phosphoglycerate and/or triose-P) is exported to the cytosol across the envelope membranes and eventually transformed to sucrose, a portion of the recently fixed carbon is converted to assimilation starch in a transient form (2, 26). It is commonly accepted that ADP-Glc[3] pyrophosphorylase (Glc-1-P adenyltransferase) utilizes Glc-1-P and photochemically generated ATP for the synthesis of ADP-Glc, which serves as the immediate glucosyl donor for starch synthase in the chloroplast (2, 26). Although an adenylate translocating system exists in the chloroplast envelope membranes (14), little is known about its detailed molecular structure or the functional properties related to its putative role in photosynthetic C metabolism (16). Recently, we have reported the existence of a putative adenylate translocator in the envelope membranes of amyloplasts isolated from cultured cells of sycamore (*Acer pseudoplatanus*) (22, 25) in which ADP-Glc transport was shown to be tightly linked to starch biosynthesis (24).

It is generally believed that amyloplasts and chloroplasts are ontogenically and evolutionarily related to each other (9). Thus, it is at least conceivable that an analogous adenylate transport system may be operative in chloroplasts, although we have failed to detect it previously in the sycamore chloroplast envelopes (22). In the work communicated in this paper, we have reexamined the adenylate translocator in chloroplasts from spinach (*Spinacia oleracea*) leaves and analyzed a direct transport of ADP-Glc across the envelope membranes that is possibly linked to starch biosynthesis in the stroma.

MATERIALS AND METHODS

Chloroplast Isolation

Chloroplasts were isolated using a continuous Percoll gradient as described previously (32), but with the following modifications. Fresh spinach (*Spinacia oleracea* L.) leaves (80 g), illuminated prior to harvest, were homogenized in a blender for 4 s in 400 mL of 0.33 M sorbitol, 2 mM Na_2-EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM Na-PPi, 100 μM reduced glutathione, and 50 mM Hepes-NaOH buffer (pH 6.8). The crude homogenate was filtered through one layer of Miracloth, and the filtrate was centrifuged for 90 s at 2500*g*. Additionally, Percoll (1.129 g/mL) was replaced by a denser Percoll solution (1.270 g/mL) obtained by evaporation of the commercially available product (24). By this method, a better

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[3] Abbreviations: ADP-Glc, ADP-glucose; CAT, carboxyatractyloside; FBP aldolase, fructose-1,6-bisphosphate aldolase; Fru-6-P, fructose-6-P; Fru 2,6-P_2, fructose 2,6-bisphosphate; Glc-1-P, glucose-1-P; Glc-6-P, glucose-6-P; Glc-6PDH, glucose-6-P dehydrogenase; HK, hexokinase; OPP, oxidative pentose-P; 6PGDH, 6P-gluconate dehydrogenase; SS, sucrose synthase.

separation of intact chloroplasts free of starch granules liberated from the broken plastids was achieved. Purified intact chloroplasts were resuspended in 5 mL of sampling buffer containing 25 mM Hepes-NaOH (pH 7.5), 20 mM KCl, 1 mM EDTA, and 0.35 M mannitol (1.029 g/mL) (final Chl content of 0.27 ± 0.043 mg/mL [n = 5]). The intactness of the chloroplasts was determined employing the protection method as previously reported (24); because the cytosolic contamination of ADH was very small, plastidic FBP aldolase was used as the chloroplast marker enzyme.

Immunoblot Analysis

Total envelope membranes were obtained after mechanical disruption of the intact chloroplasts in a tightly fitted glass homogenizer and subsequent centrifugation of the homogenate at 400g for 10 min. Starch granules were pelleted and the resulting supernatant fluid was centrifuged at 100,000g for 1 h. Envelope membranes and thylakoid membranes in the sedimented precipitates were readily recognizable and the former was subjected to electrophoresis and immunoblot analysis using antisera (immunoglobulin G) against the mitochondrial ADP/ATP-translocator of *Neurospora crassa* (22, 25).

Adenylate Transport and Starch Synthesis

Unless otherwise indicated, transport experiments were performed as described previously (24) using 0.4-mL Bio-Rik plastic tubes (Bio-Plastics Co., Ltd.). A conventional single-silicone oil layer centrifugation technique (23) was employed using a Beckman Microfuge B to assay the time-course uptake of adenylates in chloroplasts. Seventy-five microliters of silicone oil (1.066 g/mL at 4°C) was layered onto a 100 μL pelleting layer (0.55 M sucrose in 10% $HClO_4$ [1.075 g/mL, 4°C]). Intact chloroplasts were preincubated for 30 min with 1 mM ATP and subsequently incubated with a variety of labeled adenylates in Eppendorf tubes at either 4 or 25°C; 40-μL aliquots were then layered onto the silicone layer in each transport assay. Incubation stopped soon after the chloroplasts traverse to the silicone layer during centrifugation. Because of the presence of $HClO_4$ in the pelleting layer, the stromal fraction containing radiolabeled adenylates of sedimented chloroplasts is uniformly released into the layer, whereas the starch-containing fraction is pelleted. Radioactivity in each fraction was measured separately in experiments assaying ADP-[^{14}C]Glc uptake, whereas only the soluble fraction was analyzed for the uptake of [^{3}H]ATP, -ADP, or -AMP. To examine the inhibitory effect of CAT on adenylate transport, chloroplasts were preincubated with the reagent at 4°C for 5 min. The inhibitory effect of CAT and ATP on ADP-Glc uptake was followed after preincubation of the intact chloroplasts with one of these reagents. In experiments measuring ADP-[^{14}C]Glc transport coupled with starch synthesis, totally broken chloroplasts lysed by addition of 0.05% Triton X-100 were used as a negative control.

For the kinetic studies of adenylate uptake, the double silicone oil layer centrifugation technique (23–25) was employed throughout this investigation using a refrigerated TOMY MRX-151 centrifuge (Tokyo) and a TOMY TMH-3 rotor (80 tubes were centrifuged at the same time at 724g, temperature set at either 4 or 25°C and incubation time for 26 s). In this technique, the incubation medium containing labeled metabolites is sandwiched between two layers of silicone oil (light and heavy, 1.003 and 1.066 g/mL, respectively). Transport starts during centrifugation, when the chloroplasts traverse the incubation medium (1.059 g/mL) for a short period of time (incubation time), depending on the centrifugal force employed.

For a precise analysis of adenylate transport mediated by the translocator located in the inner membranes, determination of the intermembrane space by measuring the permeation of [^{14}C]sucrose is necessary (16, 23, 24). The stromal space can then be estimated by subtracting this value from the [^{3}H]H_2O permeable total space.

Analytical Methods

Unless otherwise indicated, methods used for assaying enzyme activities, starch, and protein content were carried out as previously described (8).

SS and invertase activities were measured according to the assay method reported previously after some modifications (17). SS activities were determined in the direction of ADP-Glc formation and, because it was shown that the addition of 1 mM ATP in the assay mixture strongly inhibits the ADP-Glc synthesis by SS, 1 mM ATP was replaced by 2 mM UTP. Details of this new assay system will be reported elsewhere. SS activities were determined using three different preparations derived from the spinach leaves (see Table I): (a) crude leaf filtrates, (b) desalted crude leaf filtrates, and (c) preparation of SS partially purified according to the method of Rorem *et al.* (27). Chl was quantified according to the method of Wintermans and De Mots (34).

Reagents

ATP, ADP, AMP, ADP-Glc, and CAT, as well as HK (H4502), Glc-6PDH (G5885), and P-glucoisomerase (P5381) were purchased from Sigma. Radiochemicals ([^{3}H]ATP, [^{3}H]ADP, [^{3}H]AMP, ADP-[^{14}C]Glc, [^{3}H]H_2O, and [^{14}C]sucrose) were purchased from Amersham UK. Wackersilicone AR 20 Pharmas from Wacker-Chemie (Munich, Germany) and SH 704 from Toray Silicone (Osaka, Japan) were used in the transport experiments.

RESULTS

Immunoanalysis of Adenylate Translocator in Chloroplasts

Before proceeding to the step of immunoblot analysis, we had to be absolutely sure that the envelope membranes were totally free from contamination with mitochondrial membranes. The activity of the mitochondrial marker enzyme Cyt c oxidase was found to be very small in the purified chloroplast preparations (Table I); in addition, Cyt c oxidase activity was undetectable in the isolated envelope membranes. In contrast to our previous observations indicating the absence of the ADP/ATP translocator in sycamore chloroplast membranes (22), the immunoblot presented in Figure 1 clearly demon-

Table I. *Some Enzyme Activities of Spinach Chloroplasts*

The crude filtrate of spinach leaves (see "Chloroplast Isolation") was centrifuged at 2500*g* for 90 s. The resulting pellet was separated on a continuous Percoll gradient. To facilitate the comparison between chloroplastic gluconeogenic enzymes and SS in their contributions to the synthesis of ADP-Glc in the whole plant cells, enzyme activities in the crude filtrate and purified chloroplasts are expressed as the total activity of the extract/g fresh weight of original spinach leaves. Data from three independent experiments were obtained. Values ranging from the smallest to the largest determined are given to indicate experimental variation. Enzyme samples used for invertase and SS were (a) crude leaf filtrate, (b) desalted leaf filtrates, and (c) partially purified enzyme (26). Data concerning the recovery of invertase and SS during the purification step in sample (c) are not included.

Parameter		Activity	
		Crude filtrate	Purified chloroplasts
		m units/g fresh wt	
Contamination markers			
Cyt *c* oxidase (mitochondria)		270–410	0.22–0.78
Alcohol dehydrogenase (cytosol)		160–210	n.d.[a]
Gluconeogenic enzymes			
FBP aldolase		345–375	78–98
FBPase		62–80	2.2–3.1
P-glucoisomerase		250–408	4.1–8.7
P-glucomutase		398–678	6.8–12.3
Others			
Invertase	(a)	40–221	n.d.
	(b)	25–32	n.d.
	(c)	7–15	—[b]
SS (ADP-Glc synthesis)	(a)	150–320	n.d.
	(b)	n.d.	n.d.
	(c)	21.4–25.6	—
(UDP-Glc synthesis) (a,b,c)		n.d.	n.d.
Protein (mg/g fresh wt)		1.10–1.40	0.15–0.21
Starch (mg/g fresh wt)		0.23–0.43	0.04–0.06
Chl (mg/g fresh wt)		0.10–0.16	0.02–0.03

[a] Not detectable. [b] Not measured.

strates the existence of an adenylate translocator protein in spinach chloroplast envelopes. Although the molecular size of the antigenically positive polypeptide was found to be similar to the one existing in sycamore amyloplast and mitochondria membranes (22–25), the relative amount of translocator in the chloroplast membranes is markedly higher compared with amyloplasts.

ATP/ADP/AMP/ADP-Glc Transport in Intact Chloroplasts

To assess critically the coupling of ADP-Glc uptake with starch synthesis, any interference caused by insoluble starch synthase bound to liberated starch granules must be eliminated. Inspection by EM as well as the protection method (see "Materials and Methods" [24]) showed that, in agreement with our previous report using the same isolation procedure (32), more than 90% (91.5 ± 2.7, $n = 3$) of the chloroplasts were intact after a 30-min incubation at 25°C.

As a preliminary step in the transport experiments, a time course of ATP/ADP/AMP uptake by chloroplasts with and without preincubation in ATP (1 mM) was performed. A more rapid uptake was observed with the samples preincubated with ATP (data not shown), indicating that chloroplasts are depleted of adenylates during isolation and that an adenylate "counter exchange" reaction is operating (14) (our unpublished data). Consequently, throughout the present investigation, chloroplasts were always preincubated with 1 mM ATP.

Results of the time course analysis of ATP, ADP, AMP, and ADP-Glc (50 μM) uptake, measuring radioactivities in the soluble fraction at 4°C as well as the inhibitory effect of CAT (100 μM), a noncompetitive inhibitor of the mitochondrial ADP/ATP translocator (25, 33), are summarized in Fig. 2A. The concentrations of the adenylates in the stroma determined at steady-state were: ATP, 283 ± 35 μM ($n = 4$); ADP, 101 ± 19 μM ($n = 4$); AMP, 92.6 ± 9 μM ($n = 3$); and ADP-Glc, 36 ± 9 μM ($n = 3$). The low steady-state level for ADP-Glc at 4°C is likely due to its slow uptake and efficient coupling with the starch synthase reaction in the stroma (see below). In contrast with the situation with mitochondria (25, 33), [^{3}H]ADP and [^{3}H]AMP transport was not significantly inhibited by CAT, although a strong inhibitory effect was observed against the uptake of [^{3}H]ATP (Fig. 2A). Similar results were observed previously in amyloplasts (24). It is shown in Figure 2B that the steady-state concentrations determined at 25°C were 234 ± 37 μM ($n = 3$), 224 ± 25 μM ($n = 3$), and 121 ± 13 μM ($n = 3$) for ATP, ADP, and AMP, respectively. It should be noted, however, that transport is slow compared with that observed in amyloplasts (24), reaching the steady-state concentrations at approximately 10 min at 4°C (Fig. 2A) and 3 min at 25°C (Fig. 2B). Importantly, it was found that when the incubation was carried out at 25°C, ADP-Glc (200 μM) inhibited the uptake of ATP, ADP, and AMP (Fig. 2B). Conversely, transport of ADP-[^{14}C]Glc (200 μM) was inhibited by ATP (140 μM) and CAT (140 μM) (Fig. 2C).

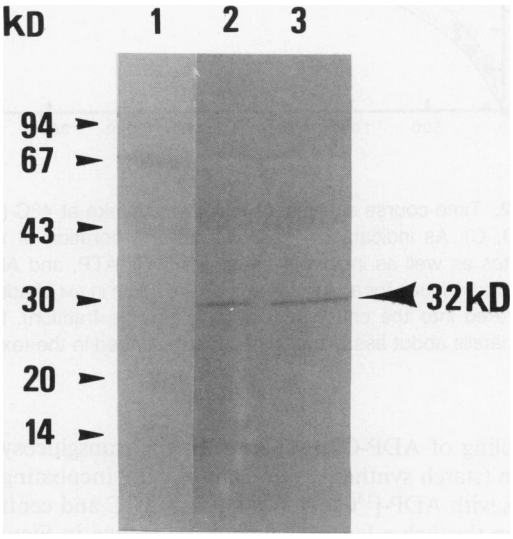

Figure 1. Immunoblot analysis of chloroplast membrane fractions. Molecular mass markers (lane 1), 20 μg (lane 2), and 40 μg (lane 3) protein samples from chloroplast envelope membranes were subjected to SDS-PAGE, followed by immunoblotting using alkaline phosphatase-conjugated antisera against the mitochondrial ADP/ATP-translocator of *N. crassa*.

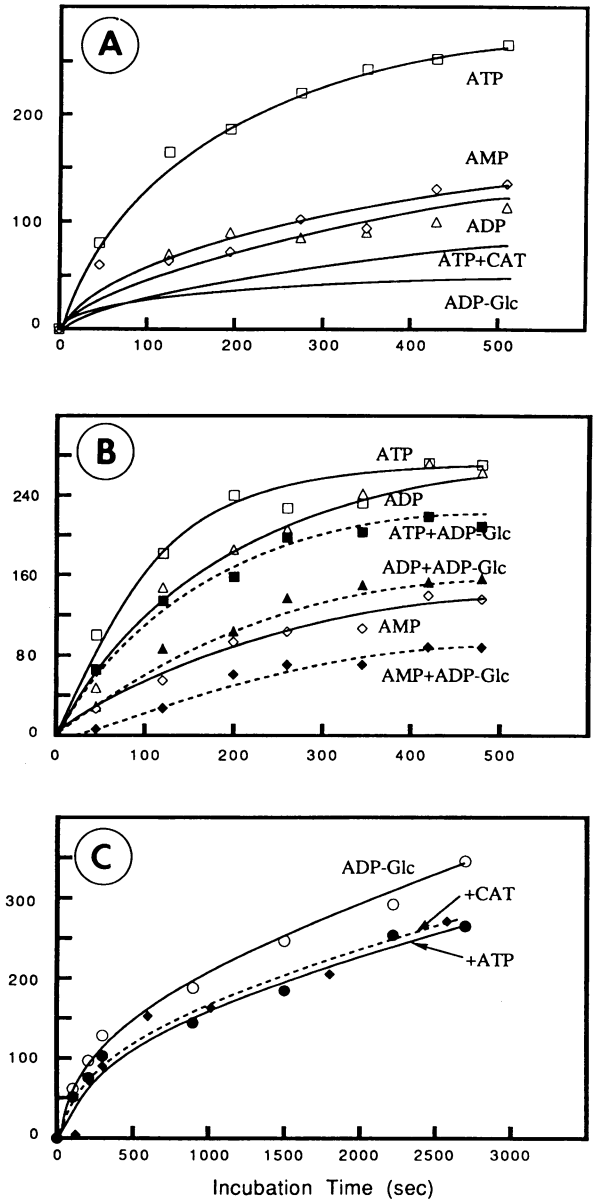

Figure 2. Time-course analysis of adenylate uptake at 4°C (A) and 25°C (B, C). As indicated in each frame, incorporation of various adenylates as well as inhibitory effects of CAT, ATP, and ADP-Glc were tested. Values for adenylate uptake are given in μM of adenylate incorporated into the chloroplast stroma (soluble fraction). Experimental details about assay conditions are described in the text.

Coupling of ADP-Glc uptake with the transglucosylation reaction (starch synthesis) was examined by incubating chloroplasts with ADP-[^{14}C]Glc (200 μM) at 25°C and centrifugal filtration through a layer of silicone. As shown in Figure 3A, [^{14}C]Glc incorporation into starch was inhibited by CAT and ATP (Fig. 3B). These observations are basically identical with those obtained with sycamore amyloplasts (see Fig. 4 in ref. 24). Thus, the results indicate the coupling of a specific transport mechanism for ADP-Glc across the chloroplast envelope membranes with starch biosynthesis. Using chloroplasts disrupted by treatment with 0.05% Triton and incubated under exactly the same conditions as those employed with the intact chloroplasts, it was found that the transglucosylation activity was not inhibited by CAT (Fig. 3B). This is essentially identical with the case of the disrupted amyloplasts (24).

Results of subsequent kinetic analyses of adenylate uptake at 4 and 25°C employing the double silicone, oil layer centrifugation technique are presented in Figures 4 and 5, respectively. The uptake of ADP at 4°C was inhibited by ATP (75 μM), AMP (200 μM), and most importantly, ADP-Glc (200 μM) (Fig. 4B). It has been reported that in rat liver mitochondria, the inhibitory effect of CAT on the adenylate transport is much stronger when mitochondria are preincubated with the reagent (6). Similarly, ATP transport was only partially inhibited by CAT as shown in Figure 4C when chloroplasts used were not preincubated with CAT. The inhibitory effect of ADP-Glc on ATP, ADP, and AMP transport measured at 25°C (Fig. 5B–D) was found to be more pronounced than at 4°C. K_m and V_{max} values for adenylate uptake measured at 4 and 25°C are summarized in Table II. A high affinity for the three adenylates can be envisioned from the low values of K_m observed. The V_{max} for the three adenylates tested were one order higher compared with the maximum rate of transglucosylation determined (see Fig. 3B).

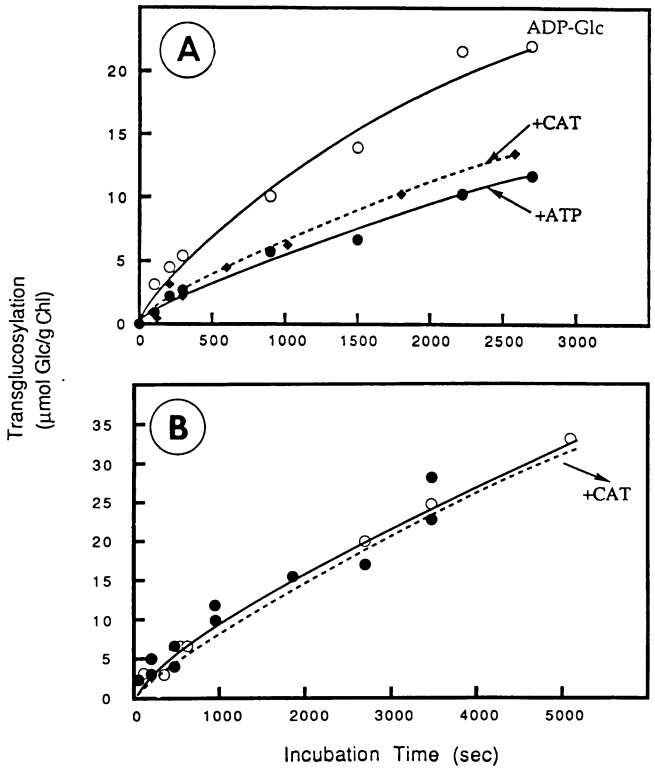

Figure 3. Time-course analysis of ADP-[^{14}C]Glc uptake coupled with the transglucosylation reaction (starch biosynthesis) and effect of CAT using intact (A) and broken chloroplasts (B) at 25°C. Intact (A) and broken (B) chloroplasts were incubated with 200 μM ADP-[^{14}C] Glc ± CAT (140 μM) or ATP (140 μM) under the same conditions used for Figure 2. Values of transglucosylation are given in μmol of [^{14}C] Glc transferred/g Chl.

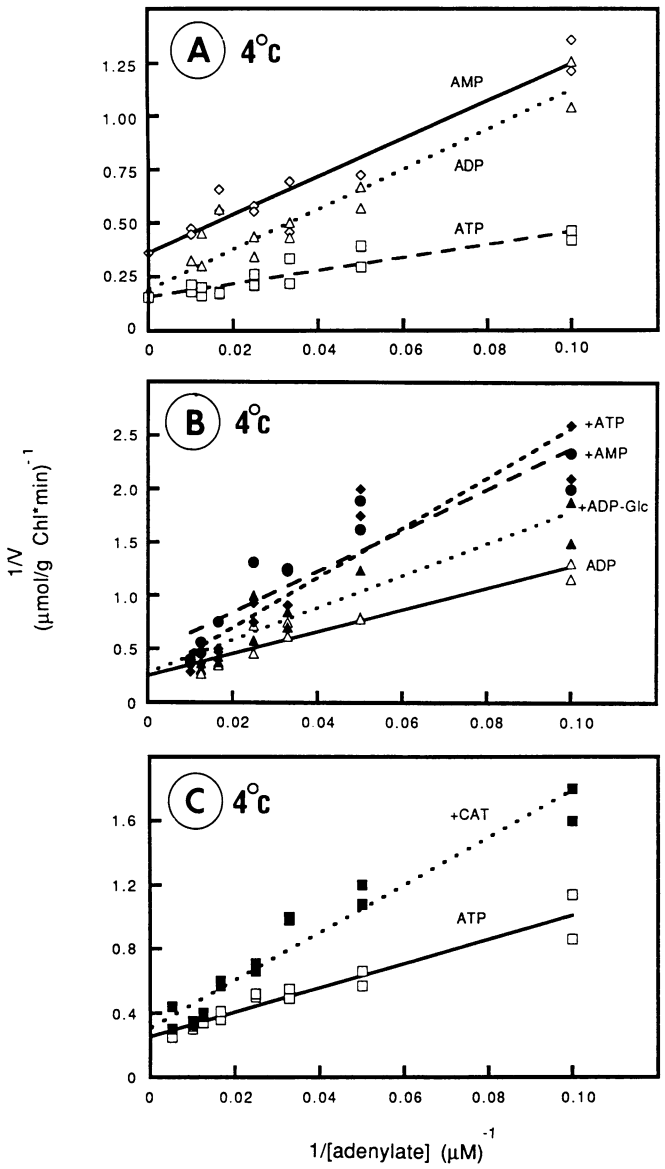

Figure 4. Kinetic analysis of adenylate uptake at 4°C by intact chloroplasts. Experimental details of the double silicone oil layer centrifugation method (23, 24) are described in the text. Data obtained are presented in double-reciprocal plots. A, Comparative analysis of ATP, ADP, and AMP transport; B, effect of ATP (75 μM), AMP (200 μM), and ADP-Glc (200 μM) on ADP transport; C, effect of CAT (100 μM) on ATP uptake.

Analyses of [^{3}H]H_2O and [^{14}C]sucrose permeable spaces yielded the values of 0.026 ± 0.007 and 0.010 ± 0.004 mL/mg Chl, respectively (n = 5).

Enzyme Activities

We have measured several enzyme activities involved in the chloroplast C metabolism and the results are summarized in Table I. The presence of contaminating subcellular compartments in the purified intact chloroplast preparations was monitored by the respective marker enzyme activities and it was found that, in agreement with our previous report (32), only trace amounts of mitochondrial and cytosolic fractions were present. Activities of chloroplast stromal enzymes involved in the gluconeogenic reaction sequence leading to the synthesis of ADP-Glc from triose-P by ADP-Glc pyrophosphorylase are high enough to achieve the maximum rate of transglucosylation presented in Figure 3.

Using different plant tissues obtained at various developmental stages, it is well established that SS is universally present in green tissues (5, 10) utilizing UDP and ADP as glucose acceptors during sucrose breakdown (1, 29). In the present work, however, no activity of SS synthesizing UDP-Glc was observed in three different preparations (see "Analytical Methods"). Instead, we found that SS activities synthesizing ADP-Glc in the crude leaf filtrates as well as in the partially purified SS are several times higher than those of the gluconeogenic enzymes in chloroplasts involved in the production of Glc-1P from triose-P. Thus, our results indicate the possible existence in the plant cell of isozymic forms of SS.

DISCUSSION

Adenylate transport across the chloroplast envelope membranes was first reported by Heldt (14), who noted that the counter-exchange activities were low. Heldt thus argued that the inward translocation of ADP does not participate in photophosphorylation and that the high specificity for ATP suggested that ATP produced by either glycolysis or respiration at night could be imported from the cytosol to sustain some metabolic requirements in the chloroplast (14). However, the present investigation has clearly demonstrated an adenylate translocating system in the spinach chloroplast envelope membranes showing high affinity for the uptake of various adenylates, including ADP-Glc. This system involves the counter-exchange transport of adenylates (our unpublished data). It should be noted that some basic properties of this chloroplast translocator are similar to those observed with sycamore amyloplasts (24) and mitochondrial adenylate translocators (25). The high affinity for external ADP suggests that cytosolic ADP may indeed play an important role as a Pi acceptor (ADP donor) during photophosphorylation, in agreement with the view of Heber and Santarius (13). Considering the relatively high content of adenylate translocator molecule in the chloroplast envelopes (see Fig. 1), the V_{max} values observed (see Table II) are unexpectedly low and may not be high enough to sustain the photophosphorylation activity *in vivo*. Such a discrepancy between *in vitro* and *in vivo* systems has been discussed by Heber and Santarius with chloroplasts (13) and by Chappell and Haarhoff with mitochondria (4).

It is often stated that, as a self-sustaining organelle, the chloroplast can readily produce ATP from the ADP generated in the Benson-Calvin cycle as the unique Pi acceptor for photophosphorylation. However, one must bear in mind that the chloroplast itself and the metabolic systems within it constitute an open system typified by a continuous dissipation of metabolite (21) as well as energy and its communication with the rest of the subcellular compartments, in particular the cytosol. In this context, it is not inconceivable that a continuous turnover and "flux" of both metabolites and

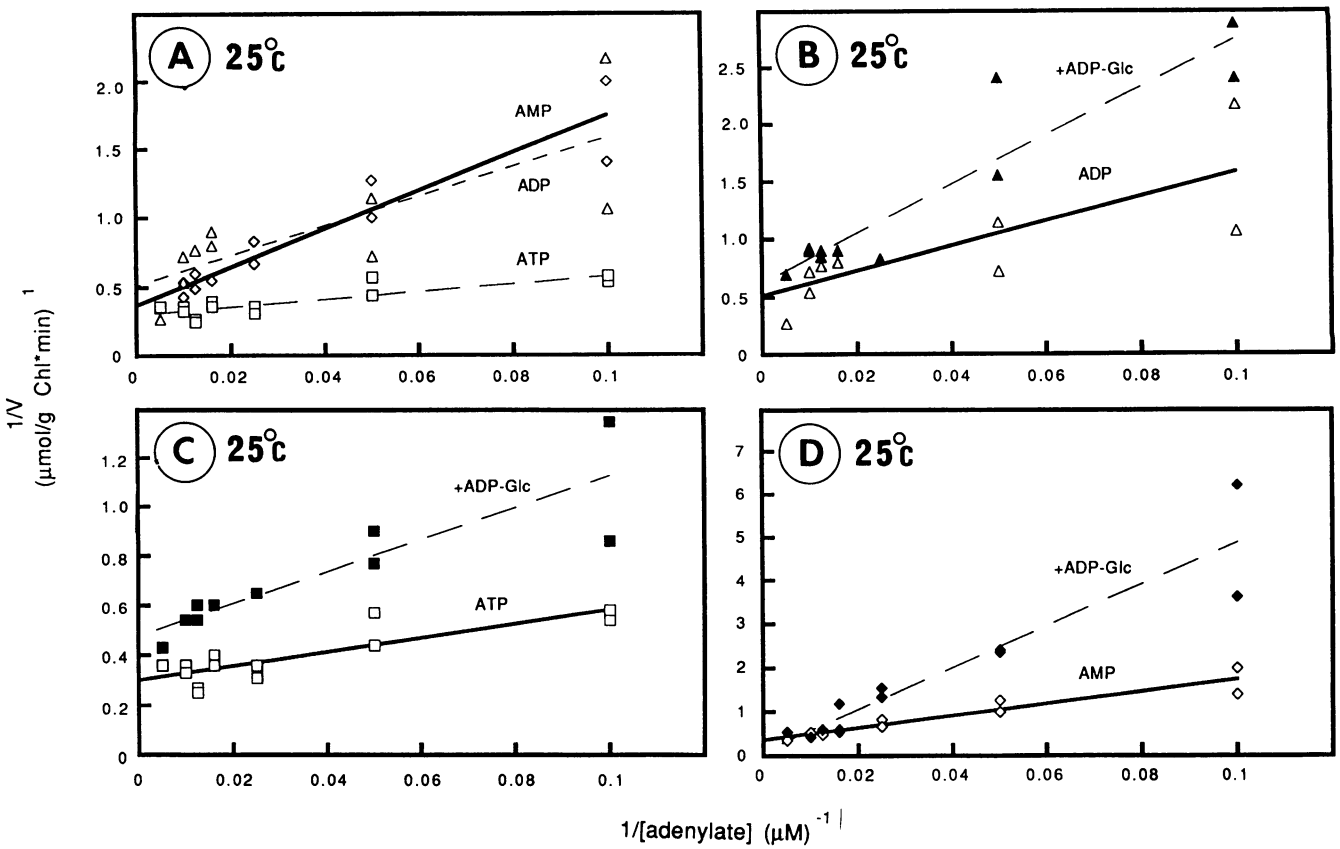

Figure 5. Kinetic analysis of adenylate uptake at 25°C by chloroplasts. Experimental conditions employed were essentially identical with those shown in Figure 4 except that incubation was performed at 25°C. A, Comparative study of ATP, ADP, and AMP transport; B–D, Effect of ADP-Glc (200 μM) on the transport of ADP, ATP, and AMP, as indicated.

energy exist across the envelope membranes and that direct entry of either ADP or ADP-Glc is coupled with photophosphorylation. To advance this conceptual idea, a hypothetical mechanistic scheme concerning the gluconeogenesis and C flux mediated by ADP-Glc and Pi translocator proteins is illustrated in Figure 6. Further analytical studies of the level of adenylates in the cytosol as well as their inward movement are evidently necessary to verify this proposed model, which clearly seems counter to the classic concept of the "chloroplast as a complete unit of photosynthesis" (3).

It is generally accepted that triose-P synthesized in the Benson-Calvin cycle is converted to Glc-1-P in the chloroplast stroma. This hexose-P and photochemically produced ATP are then utilized by the regulatory enzyme ADP-Glc pyrophosphorylase for the synthesis of ADP-Glc, which in turn serves as the glucosyl donor for starch synthase (26). According to this almost axiomatic concept and common scheme, one can predict that green leaves exposed to $^{14}CO_2$ for a short period of time should synthesize starch with ^{14}C symmetrically distributed in the Glc. However, experiments, now classic, have shown that ^{14}C in the Glc moiety of sucrose, starch, and phosphate esters (UDP-Glc, Glc-1-P, and Glc-6-P) is asymmetrically distributed (11, 12, 18). Furthermore, because chloroplasts are equipped with an array of enzymes involved in the conversion of triose-P to starch (gluconeogenesis), one can predict that leaves fed [1-^{14}C]Glc will show a redistribution of

Table II. *Kinetic Analysis of ATP, ADP, and AMP Uptake at 4° and 25°C*

Values given are the average of triplicate analyses [see Figs. 4 (4°C) and 5 (25°C)]

	K_m	
	4°C	25°C
	μM	
ATP	20.4 ± 3.4	9.3 ± 3.2
ADP	39.7 ± 6.5	19.7 ± 5.4
AMP	54.8 ± 13.5	36.8 ± 7.7
	V_{max}	
	μmol adenylate/min·g Chl	
ATP	4.3 ± 1.5	7.8 ± 1.8
ADP	3.6 ± 1.8	5.8 ± 2.0
AMP	3.9 ± 1.2	6.7 ± 2.4

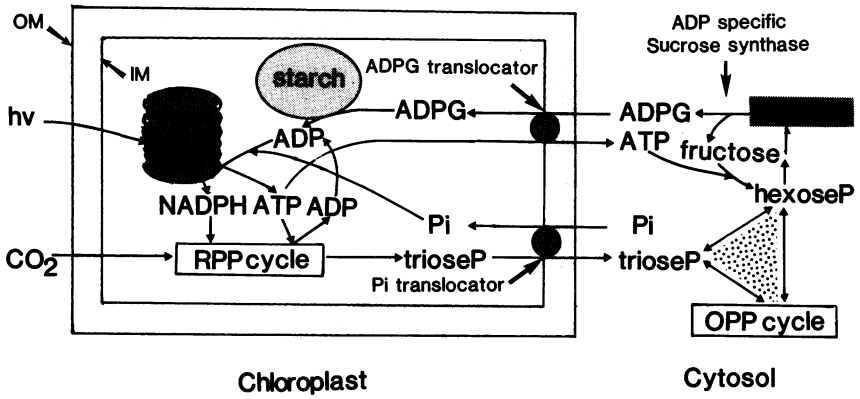

Figure 6. Proposed mechanism for starch biosynthesis in chloroplasts and C flux to cytosol. Products (triose-P) of photosynthetic assimilation of atmospheric CO_2 (RPP-Benson-Calvin cycle) are transported to the cytosol by counter-exchange with cytosolic Pi via the Pi-translocator and eventually converted to sucrose by sequential steps including oxidative pentose-P (OPP) cycle. ADP-Glc, synthesized by the reversal of the extrachloroplastic SS, is imported across the chloroplast membranes (IM and OM) via the adenylate (ADP-Glc) translocator localized in IM. ADP-Glc transported is utilized for starch synthesis by starch synthase and released ADP can serve as Pi acceptor in photophosphorylation in the thylakoid membrane system.

^{14}C in the Glc moiety of the starch molecules produced; triose-P derived from the cytosolic metabolism of [1-^{14}C]Glc can be imported into chloroplasts and subsequently transformed to hexose-P and starch-yielding Glc molecules labeled predominantly in the C3 and C4 positions. However, it was observed, in fact, that the Glc moiety of the starch is labeled mainly in the C1 position (19, 20). In attempting to explain this surprising observation, Schafer and Heber (28) reported the existence in intact chloroplasts of Glc uptake mediated by a Glc translocator (K_m = 23 mM) that also can transport Glc-6-P (7). Either direct uptake of [1-^{14}C]Glc coupled with the stromal HK producing [1-^{14}C]Glc-6-P or conversion of [1-^{14}C]Glc to [1-^{14}C]Glc-6-P by the HK existing in the intermembrane space of the chloroplast (30) and subsequent uptake will eventually produce starch molecules labeled in the C1 position of the Glc moiety. Nevertheless, it was later found that isolated chloroplasts incubated with [^{14}C]Glc do not produce radioactive starch (15).

Based on the present observations on adenylate uptake by the translocator and the activity of various stromal enzymes engaged in C metabolism, we tend to think that the proposed mechanism of starch synthesis illustrated in Figure 6 likely provides a possible clue for solving the above enigma. As shown in the figure, the key element in the overall scheme of starch biosynthesis in the chloroplast is a tight linkage with the cytosol. It can be seen that the product of CO_2 fixation, triose-P, is exported to the cytosol by the Pi translocator and eventually converted to sucrose. Under conditions of high sucrose levels, *e.g.* upon intense illumination for long periods, ADP-Glc produced by the extrachloroplastic SS (1, 29, our unpublished data) will be transported into the chloroplast and utilized as the glucosyl donor for starch synthase and possibly as ADP donor for photophosphorylation as well. Cytosolic triose-P can be channeled into the oxidative pentose-P pathway, thereby leading to a randomization of the carbons giving rise to the asymmetric ^{14}C distribution observed in sugar phosphates, sucrose, and starch (see above). Stitt *et al.* (31) have argued that the export of triose-P from the chloroplast is decreased during starch biosynthesis; therefore, triose-P may not be channeled into sucrose synthesis in the cytosol. Alternatively, we would like to propose that both sucrose and starch biosynthesis can proceed simultaneously without decreased metabolite flux between the cytosol and chloroplast (see Fig. 6). Because cytosolic Glc can be readily converted to sucrose and subsequently reconverted to ADP-Glc without randomization of the carbons, the pathway proposed here is thought to provide an answer for the conservative ^{14}C distribution in starch molecules synthesized in green leaves incubated with [1-^{14}C]Glc (19, 20). Additionally, fructose produced by extraplastidic SS during sucrose breakdown, coupled to the fructokinase reaction producing Fru-6-P, can possibly explain another unexplained finding, namely the increased levels of cytosolic Fru-6-P and Fru 2,6-P_2 during active periods of starch biosynthesis (31).

As has been described above, triose-P synthesis, conversion to Glc-1-P, and subsequent utilization for the ADP-Glc synthesis by ADP-Glc pyrophosphorylase, all occurring in the chloroplast, are postulated to be the principal mechanism of starch biosynthesis (2, 26). Although activities of plastidic gluconeogenic enzymes assumed to be involved in the conversion of triose-P to Glc-1-P (see Table I) appear not to be contradictory to this general model, we have deliberately eliminated it from Figure 6. Previously, it was reported that, although at a markedly lower rate of starch synthesis than in the whole plant, isolated chloroplasts incubated with $^{14}CO_2$ *in vitro* are capable of producing starch (15). However, these observations are not consistent with the above mentioned asymmetric ^{14}C distribution pattern (11, 12, 18) and the conservative ^{14}C distribution pattern (19, 20) obtained from experiments performed *in vivo*. Thus, the question arises as to what is the real mechanism of starch biosynthesis *in vivo*. Our recent experimental findings concerning the presence of extraplastidic SS that can specifically recognize ADP-synthesizing ADP-Glc likely support a mechanism of starch biosynthesis similar to that presented in Figure 6 (our unpublished data). It is noteworthy in this context that the diurnal rhythm of starch formation in plant leaves can be interpretable by the activity of SS, which will fluctuate by the level of intracellular sucrose (31). Indeed, we have found that ADP-specific SS behaves allosterically with respect to sucrose concentrations. Therefore, we want to emphasize that the complex mechanism(s) operating in SS, a decisive regulatory element of sucrose-starch transition, can also be envisioned from the properties of the enzyme as presented in Table I; upon a prolonged dialysis, there occurs loss of activities (sample b),

whereas partial purification ensures maintenance of its activities (sample c).

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