ADP-glucose drives starch synthesis in isolated maize endosperm amyloplasts: characterization of starch synthesis and transport properties across the amyloplast envelope

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We recently developed a method of purifying amyloplasts from developing maize (*Zea mays* L.) endosperm tissue [Neuhaus, Thom, Batz and Scheibe (1993) Biochem. J. **296**, 395–401]. In the present paper we analyse how glucose 6-phosphate (Glc6P) and other phosphorylated compounds enter the plastid compartment. Using a proteoliposome system in which the plastid envelope membrane proteins are functionally reconstituted, we demonstrate that this type of plastid is able to transport [^{14}C]Glc6P or [^{32}P]P_i in counter exchange with P_i, Glc6P, dihydroxyacetone phosphate and phosphoenolpyruvate. Glucose 1-phosphate, fructose 6-phosphate and ribose 5-phosphate do not act as substrates for counter exchange. Besides hexose phosphates, ADP-glucose (ADPGlc) also acts as a substrate for starch synthesis in isolated maize endosperm amyloplasts. This process exhibits saturation kinetics with increasing concentrations of exogenously supplied [^{14}C]ADPGlc, reaching a maximum at 2 mM. Ultrasonication of isolated amyloplasts greatly reduces the rate of ADPGlc-dependent starch synthesis, indicating that the process is dependent on the intactness of the organelles. The plastid ATP/ADP transporter is not responsible for ADPGlc uptake. Data are presented that indicate that ADPGlc is transported by another translocator in counter exchange with AMP. To analyse the physiology of starch synthesis in more detail, we examined how Glc6P- and ADPGlc-dependent starch synthesis in isolated maize endosperm amyloplasts interact. Glc6P-dependent starch synthesis is not inhibited by increasing concentrations of ADPGlc. In contrast, the rate of ADPGlc-dependent starch synthesis is reduced by increasing concentrations of ATP necessary for Glc6P-dependent starch synthesis. The possible modes of inhibition of ADPGlc-dependent starch synthesis by ATP are discussed with respect to the stromal generation of AMP required for ADPGlc uptake.

INTRODUCTION

Starch is one of the major storage compounds in plants [1]. In leaves, it accumulates during the day and is nocturnally degraded to supply the stroma and cytosol with the carbohydrates required for various anabolic reactions [2].

In contrast, starch synthesis in storage tissues proceeds continuously. As most heterotrophic plastids do not possess a fructose-1,6-bisphosphate phosphatase [3,4], the conversion of triose phosphates into hexose phosphates required for starch biosynthesis is not possible within the plastid. The absence of a plastid fructose bisphosphatase agrees with results obtained from randomization experiments using specifically labelled glucose molecules. Using such an experimental approach, it was possible to show that starch synthesis in intact endosperm tissues from wheat and maize, or in potato tubers, occurs via the uptake of C_6 units into the corresponding plastids [5,6].

By using isolated wheat endosperm amyloplasts, glucose 6-phosphate (Glc1P) was identified as the most efficient precursor of newly synthesized starch [7,8]. This result was later confirmed for amyloplasts purified from a soya-bean cell suspension culture [9]. In contrast, in plastids isolated from pea embryos, cauliflower buds, maize endosperm or green-pepper fruits the most efficient carbon precursor for starch synthesis is Glc6P, supplied at physiological concentrations [4,10–13].

ADP-glucose (ADPGlc) has also been identified as an exogenous precursor for starch biosynthesis. Amyloplasts isolated from an *Acer pseudoplatanus* (sycamore) cell suspension culture were shown to use externally supplied ADPGlc as a carbon source for starch biosynthesis [14]. This result was criticised for several reasons. (1) No enzyme reaction was known that allowed cytosolic synthesis of ADPGlc (for a review see [15]). (2) In some types of amyloplasts, exogenously supplied ADPGlc induced only low rates of starch biosynthesis [12]. Recently, a new ADPGlc pyrophosphorylase (ADPGlcPPase) isoenzyme has been discovered which is located in the cytosol of barley and maize endosperm tissue, representing between 85 and 95% of the total activity [16,17]. This observation led us to analyse the precursor-dependence of starch biosynthesis in isolated maize endosperm amyloplasts in more detail. Cytosolic ADPGlc has yet to be identified as an efficient precursor for starch biosynthesis in cereal storage tissue. We therefore needed to examine to what extent ADPGlc contributes to starch biosynthesis in a cereal endosperm amyloplast.

In particular, we wished to address the following questions. (1) How is Glc6P, previously described as a precursor for starch synthesis in isolated maize endosperm amyloplasts [4], imported into the plastid? (2) Can isolated amyloplasts from maize endosperm use exogenously supplied [^{14}C]ADPGlc as a carbon precursor for starch biosynthesis? (3). Is the plastid ATP/ADP

Abbreviations used: PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid; Glc6P, glucose 6-phosphate; ADP-Glc, ADP-glucose; ADPGlcPPase, ADPGlc pyrophosphorylase; DHAP, dihydroxyacetone phosphate; Glc1P, glucose 1-phosphate; Fru6P, fructose 6-phosphate; Rib5P, ribose 5-phosphate.

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transporter responsible for transport of ADPGlc? (4) Is there metabolic interaction between Gcl6P- and ADPGlc-dependent starch synthesis in isolated amyloplasts from maize endosperm?

MATERIALS AND METHODS

Isolation of intact amyloplasts from maize endosperm

Maize (*Zea mays* L., var. Apache) was grown under field conditions in the botanical garden of the University of Osnabrück. Intact amyloplasts from maize endosperm were isolated exactly as described previously [4].

Isolation of envelope membranes

Envelope membranes from maize endosperm amyloplasts were purified according to a protocol developed for the enrichment of envelopes from various heterotrophic plastids [18,19]. The protein content of the enriched envelopes was determined using a detergent-insensitive assay containing a bicinchoninic acid/cupric sulphate solution (Sigma, Deisenhofen, Germany) according to the instructions of the supplier.

Starch synthesis in isolated intact amyloplasts from maize endosperm

Starch synthesis in isolated amyloplasts from maize endosperm was measured as described previously [4]. [^{14}C]Glc6P (specific radioactivity 1–2 MBq/mmol) was supplied by NEN (Dreieich, Germany). ADP[^{14}C]Glc (specific radioactivity 0.1–0.5 MBq/mmol) was supplied by Amersham (Hannover, Germany). Starch synthesis was measured for 30 min at 25 °C and terminated by incubation of the samples for 3 min at 95 °C. For details of extraction of radioactively labelled starch and quantification of starch synthesis see [4].

Reconstitution of maize envelope membrane proteins in proteoliposomes and uptake experiments

Proteoliposomes with incorporated proteins from maize endosperm amyloplasts were prepared essentially as previously described [20]. Vesicles were prepared using 100 mg/ml acetone-washed phosphatidylcholine (type IV-S from soya bean; Sigma) in 100 mM Tricine/NaOH (pH 7.5)/30 mM potassium gluconate. For counter-exchange experiments, the metabolites were added at the indicated concentrations. The suspension was homogenized by sonication on ice (2 × 5 min; 50 % line voltage; 20 % duty cycle; Vibra-Cell, Bioblock-Scientific Instruments, Freiburg, Germany) under a permanent stream of nitrogen. Envelope membrane protein was added at a concentration of 50 μg/ml of the liposome suspension. Membrane proteins were solubilized by adding Triton X-100 to a final concentration of 0.2 % (v/v); the sample was then rapidly (less than 10 s) combined with the liposome suspension (at a resulting lipid/detergent ratio of 50:1) and subsequently transferred to liquid nitrogen to allow incorporation into proteoliposomes after thawing of the sample (freeze–thawing). After thawing at room temperature the proteoliposomes were immediately sonicated for 30 s under a permanent stream of nitrogen gas (50 % line voltage; 20 % duty cycle). Unincorporated solution was removed from the liposomes by passing the preparation over a NAP-5 gel-filtration column (Pharmacia, Freiburg, Germany) equilibrated with 10 mM Tricine/NaOH, pH 7.5, containing 100 mM potassium gluconate and 50 mM sodium gluconate. Gel filtration was carried out in the cold-room at 4 °C. The eluted proteoliposomes (1 ml) were stored on ice and used for transport measurements within the subsequent 60 min.

Uptake experiments were performed in 1.5 ml reaction vessels. The incubation was initiated by adding 100 μl of proteoliposome suspension to 100 μl of extraction medium containing the indicated labelled substrates. The latter medium was kept at 30 °C by incubation in an Eppendorf incubator. Uptake was allowed to proceed for 2 min and stopped by the addition of 100 μl of an inhibitor mix (3 mM 4,4′-di-isothiocyanostilbene-2,2′-disulphonate, 5 mM pyridoxal phosphate) and transfer to a 1 ml column of Dowex AG-1 (X8; Cl^- form; 100–200 mesh) equilibrated with 200 mM Tricine/NaOH, pH 7.5. Proteoliposomes were eluted from the column by washing with 3 × 500 μl of the equilibration medium, and the radioactivity in the eluate was quantified in a Tricarb 2500 scintillation counter (Canberra-Packard, Frankfurt, Germany). [^{3}H]ATP, [^{14}C]ADP and [^{3}H]AMP were purchased from Amersham. Specific radioactivities used for uptake into proteoliposomes were between 5 and 10 MBq/mmol.

Linearity of uptake with time and increasing amounts of proteoliposome was demonstrated before the experiments (results not shown). All data presented were corrected for transport occurring on ice (or in the presence of the inhibitor mix; both treatments gave similar values), which was always less than 2 % of the observed rates.

RESULTS

Transport of Glc6P and other phosphorylated intermediates across the envelope of maize endosperm amyloplasts

Hexose phosphates and ADPGlc have both been discussed as potential substrates for starch biosynthesis imported into heterotrophic plastids. We recently demonstrated that both Glc6P and Glc1P can serve as precursors for starch synthesis in isolated maize endosperm amyloplasts [4]. To examine whether these compounds are transported by the action of a hexose phosphate/phosphate transporter, we reconstituted membrane proteins from isolated envelopes of maize endosperm amyloplasts into proteoliposomes. These proteoliposomes offer the advantage that they allow preloading with defined concentrations of potential counter-exchange substrates.

Table 1 Rates of [^{14}C]Glc6P or [^{32}P]P_i uptake into proteoliposomes containing reconstituted envelope membrane protein from maize endosperm amyloplasts preloaded with various phosphorylated intermediates

Proteoliposomes were prepared as given in the Materials and methods section. P_i or phosphorylated intermediates used for preloading were present internally at a concentration of 10 mM. Both [^{14}C]Glc6P and [^{32}P]P_i were added at a concentration of 200 μM. Uptake was allowed to take place for 2 min. Termination of uptake and quantification of transport was carried out as described in the Materials and methods section. Data represent means ± S.E.M. for three independent experiments.

Counter-exchange substrate	Rate of transport (nmol/h per mg of protein)			
	[^{14}C]Glc6P	% of exchange with P_i	[^{32}P]P_i	% of exchange with P_i
None	257.0 ± 18.4	16.3	330.0 ± 60.9	18.3
P_i	1581.0 ± 37.1	100.0	1807.0 ± 46.0	100.0
DHAP	1760.0 ± 17.7	111.3	1300.0 ± 57.0	71.9
Glc6P	1998.0 ± 40.1	126.4	861.0 ± 25.0	47.6
Glc1P	236.3 ± 7.4	14.9	111.0 ± 6.6	6.1
Fru6P	332.0 ± 64.5	21.0	185.0 ± 19.5	10.2
PEP	619.3 ± 34.4	39.2	1355.0 ± 48.0	75.0
Rib5P	362.5 ± 22.9	22.9	264.3 ± 1.2	14.6

Table 2 Rates of Glc6P- or ADPGlc-dependent starch synthesis in isolated maize endosperm amyloplasts

Glc6P-dependent starch synthesis was carried out at the following precursor and effector concentrations: [^{14}C]Glc6P, 2 mM; ATP, 2 mM; PGA, 0.5 mM. [^{14}C]ADPGlc-dependent starch synthesis was carried out at a substrate concentration of 1 mM. Sonication was performed on ice for 3 × 3 s. No heating of the sample was detectable during this treatment. Addition of 0.5% Triton X-100 reduces starch synthesis to similar values to those observed after sonication (results not shown). Data are means ± S.E.M. for three independent experiments.

Precursor/effectors	Rate of starch synthesis (nmol/h per mg of protein)
[^{14}C]Glc6P, ATP, PGA	16.8 ± 1.1
[^{14}C]ADPGlc	99.5 ± 3.2
[^{14}C]ADPGlc, sonicated	23.9 ± 1.4

As shown in Table 1, unloaded proteoliposomes are able to import [^{14}C]Glc6P at a rate of about 260 nmol/h per mg of protein. Preloading of proteoliposomes with 10 mM P_i, dihydroxyacetone phosphate (DHAP), Glc6P or phosphoenolpyruvate (PEP) stimulates strongly Glc6P import, demonstrating that it is catalysed in a counter-exchange mode. Glc6P transport in homo exchange with preloaded Glc6P exhibits the highest rate of transport (Table 1). In contrast, Glc1P, Fru6P and Rib5P do not promote [^{14}C]Glc6P import significantly above control values (no preloading) (Table 1). This observation demonstrates that the latter compounds do not act as counter-exchange substrates (Table 1).

To compare Glc6P-transport rates with rates of P_i transport, we analysed the effect of various phosphorylated intermediates on the import of [^{32}P]P_i. Proteoliposomes not preloaded with phosphorylated compounds import P_i at a rate of 330 nmol/h per mg of protein (Table 1). Preloading with 10 mM P_i induced the highest stimulation of P_i import to about 1800 nmol/h per mg of protein (Table 1). DHAP, Glc6P and PEP also act as counter-exchange substrates (Table 1), whereas Glc1P, Fru6P and Rib5P do not promote increased rates of transport. It is remarkable that [^{14}C]Glc6P/Glc6P homo exchange is only 25% faster than [^{14}C]Glc6P/P_i exchange (Table 1). In contrast, [^{32}P]P_i/P_i homo exchange is about twice as fast as [^{32}P]P_i/Glc6P exchange.

Precursor-dependence of starch biosynthesis in isolated maize endosperm amyloplasts

We recently demonstrated that endosperm amyloplasts isolated from field-grown maize plants are able to use Glc6P as a precursor for starch biosynthesis [4]. Table 2 shows the rate of starch synthesis that could be sustained by various external substrates. The rate of Glc6P-dependent starch synthesis is about 17 nmol/h per mg of protein at a Glc6P concentration of 2 mM and this value is close to previously reported rates [4].

The identification of a cytoplasmic ADPGlcPPase led us to test whether maize endosperm amyloplasts are able to synthesize starch from exogenously added ADPGlc. ADPGlc present at a final concentration of 1 mM was sufficient to induce starch biosynthesis at rates of up to 100 nmol/h per mg of protein (Table 2). The observed rates of starch biosynthesis were dependent on intactness; disintegration of the organelles by ultrasonication before incubation with labelled ADPGlc reduced the rate of ADPGlc-dependent starch synthesis to about 25% of the control value (Table 2).

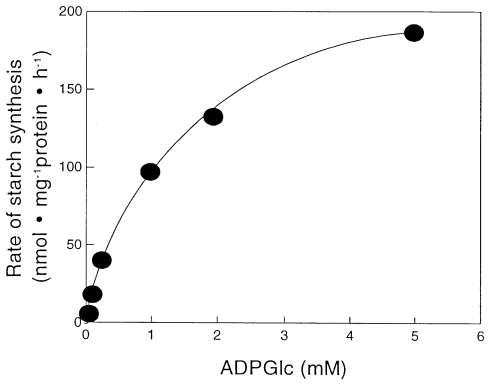


Figure 1 Effect of increasing substrate concentration on ADPGlc-dependent starch synthesis in isolated maize endosperm amyloplasts

Isolated maize endosperm amyloplasts were incubated for 30 min at 25° with increasing concentrations of [^{14}C]ADPGlc. Termination of the reaction and quantification of starch synthesis was carried out as described in the Materials and methods section. Results are means for three independent experiments. S.E.M. was less than 8% of each mean value.

The Glc6P concentration chosen for the experiments given in Table 1 is saturating for starch biosynthesis by isolated maize endosperm amyloplasts [4]. However, nothing is known about the substrate saturation of ADPGlc-dependent starch biosynthesis in isolated cereal endosperm amyloplasts. The dependence of the rate of starch synthesis on ADPGlc concentration is shown in Figure 1. Rates of starch synthesis were saturated at about 4 mM ADPGlc ($V_{max} \cong 200$ nmol/h per mg of protein).

Transport of ATP, ADP, AMP and ADPGlc across the envelope of maize endosperm amyloplasts

It has been speculated that ADPGlc is transported into plastids by the action of the ATP/ADP transporter [14]. To examine whether this is true for uptake of ADPGlc into maize endosperm amyloplasts, we examined the rate and counter-exchange-dependence of ATP, ADP, AMP and ADPGlc transport into proteoliposomes containing the reconstituted envelope membrane proteins.

Unloaded proteoliposomes transport [^{3}H]ATP at only 3.1% of the rate observed for homo exchange (proteoliposomes preloaded with 10 mM ATP) (Table 3). ATP and ADP both serve as effective counter-exchange substrates for ATP uptake reaching about 1000 nmol of [^{3}H]ATP/h per mg of protein (Table 3). Neither AMP nor ADPGlc promote the uptake of [^{3}H]ATP into proteoliposomes, indicating that this transporter is specific for ATP and ADP.

[^{14}C]ADP is also taken up into unloaded proteoliposomes (Table 3). Again, preloading with either ATP or ADP (each 10 mM) promotes the uptake of [^{14}C]ADP, which reaches a maximum of about 1100 nmol of [^{14}C]ADP/h per mg of protein (in the case of the homo exchange) (Table 3). Preloading with AMP or ADPGlc does not stimulate [^{14}C]ADP uptake above the rate observed for unloaded vesicles (Table 3).

[^{3}H]AMP uptake is not stimulated by preloading of proteoliposomes with ATP (Table 3). The presence of ADP in the proteoliposomes stimulates [^{3}H]AMP uptake to about 100 nmol/h per mg of protein, representing 80% of the rate of [^{3}H]AMP uptake in homo exchange (Table 3). ADPGlc, which does not promote [^{3}H]ATP or [^{14}C]ADP uptake, stimulates [^{3}H]AMP uptake significantly to about 70 nmol/h per mg of protein (Table 3).

Table 3 Rates of nucleotide or ADPGlc uptake into proteoliposomes containing reconstituted envelope membrane protein from maize endosperm amyloplasts preloaded with various potential counter-exchange substrates

Proteoliposomes were prepared as given in the Materials and methods section. Nucleotides or ADPGlc used for preloading were present internally at a concentration of 10 mM. Radioactively labelled nucleotides or ADPGlc were added at a concentration of 50 μM. Since no [^{14}C]ADPGlc/ADPGlc homo exchange was measurable, we calculated the rate of homo exchange from the exchange rate of [^{14}C]ADPGlc import compared with ADP export (no net charge transfer). Uptake was allowed to take place for 2 min. Termination of uptake and quantification of transport was carried out as described in the Materials and methods section. Data represent means ± S.E.M. for three independent experiments. n.d., not detectable.

	Rate of transport (nmol/h per mg of protein)							
Counter-exchange substrate	[^{3}H]ATP	% of homo exchange	[^{14}C]ADP	% of homo exchange	[^{3}H]AMP	% of homo exchange	[^{14}C]ADPGlc	% of homo exchange
None	43.1 ± 7.1	3.1	49.2 ± 0.3	4.5	54.0 ± 0.8	44.6	15.6 ± 6.1	51.5
ATP	1402.4 ± 28.9	100.0	646.0 ± 17.7	58.6	45.2 ± 1.8	37.4	34.3 ± 14.8	113.2
ADP	979.5 ± 15.7	69.8	1101.6 ± 52.0	100.0	98.2 ± 3.7	81.2	30.3 ± 13.9	100.0
AMP	82.0 ± 6.9	5.8	100.1 ± 1.6	9.1	121.0 ± 0.9	100.0	95.5 ± 7.2	315.2
ADPGlc	88.2 ± 4.2	6.3	91.9 ± 35.2	8.3	70.3 ± 0.4	58.1	n.d.	n.d.

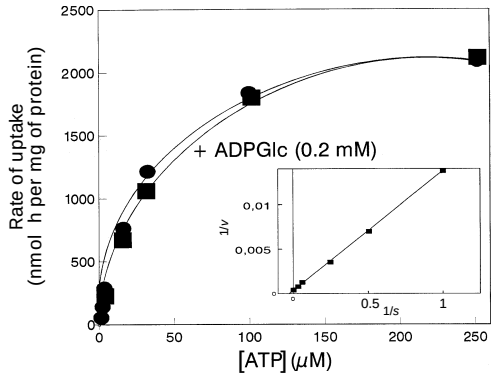


Figure 2 Substrate saturation of ATP uptake into proteoliposomes and effect of ADPGlc on ATP uptake

Uptake experiments were performed at 30 °C for 2 min. Termination of uptake and quantification of radioactivity passing through the anion-exchange column was carried out as described in the Materials and methods section. ●, Substrate saturation of ATP uptake in the absence of 0.2 mM ADPGlc; ■, substrate saturation of ATP uptake in the presence of 0.2 mM ADPGlc. The inset represents a double-reciprocal plot of ATP-uptake data in the absence of additional ADPGlc, indicating a K_m of 40.8 μM.

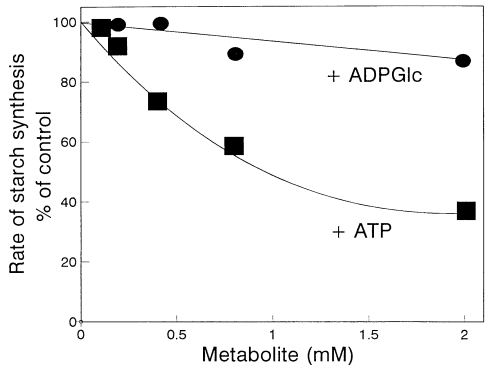


Figure 3 Effect of ATP on ADPGlc-dependent starch synthesis and effect of ADPGlc on Glc6P-dependent starch synthesis in isolated maize endosperm amyloplasts

Glc6P-dependent starch synthesis (●) was carried out at the following effector concentrations: Glc6P, 2 mM; ATP, 2 mM; PGA, 0.5 mM. ADPGlc-dependent starch synthesis (■) was carried out at a concentration of 2 mM. Starch synthesis was allowed to take place for 30 min at 25 °C. Termination of the reaction and quantification of starch synthesis was as described in the Materials and methods section. Data are means for three independent experiments. S.E.M. was less than 7% of the mean values.

[^{14}C]ADPGlc is transported at the lowest rate of all the nucleotides tested. Unloaded proteoliposomes import [^{14}C]ADPGlc at a rate of about 16 nmol/h per mg of protein. Preloading of liposomes with either ATP or ADP appears to stimulate [^{14}C]ADPGlc import (Table 3). However, the standard errors indicate that this stimulation is statistically not significant. In contrast, [^{14}C]ADPGlc import into proteoliposomes preloaded with 10 mM AMP is increased to about 95 nmol/h per mg of protein (Table 3). [^{14}C]ADPGlc uptake into proteoliposomes preloaded with ADPGlc was not detectable (Table 3).

To our knowledge the data give in Table 3 represent the first kinetic data for the ATP/ADP transporter from cereal endosperm amyloplasts. To analyse the kinetic properties of this transport protein in more detail we performed substrate-saturation experiments. Figure 2 shows that increasing concentrations of ATP induce a rapid increase in ATP uptake, reaching apparent saturation above 100 μM ATP. The inset in Figure 2 allows an estimation of the affinity constant of the plastid ATP/ADP transporter, giving an apparent K_m of 40 μM. The presence of 0.2 mM ADPGlc does not affect ATP uptake into proteoliposomes (Figure 2).

Interaction of Glc6P- and ADPGlc-dependent starch synthesis

From the data presented above (Table 1, Figure 1) and in a recent publication [4] it is clear that isolated maize endosperm amyloplasts are able to use both Glc6P and ADPGlc as precursors for starch biosynthesis. However, we have not analysed to what extent the two metabolic pathways interact. To analyse this interaction we have examined how changing ADPGlc concentrations act on Glc6P-dependent starch synthesis and how changing ATP concentrations act on ADPGlc-dependent starch synthesis. In previous experiments we have shown that neither Glc6P nor PGA (both of which are required for Glc6P-dependent starch synthesis [4]) inhibit ADPGlc-dependent starch synthesis (results not shown).

The data presented in Figure 3 show that Glc6P-dependent starch synthesis is not inhibited by increasing concentrations of ADPGlc. In contrast, ADPGlc-dependent starch synthesis is inhibited by increasing concentration of exogenous ATP. When ADPGlc and ATP are present at equal concentration (2 mM), the resulting rate of starch synthesis is reduced to about 35 % of the control value (no ATP) (Figure 3).

DISCUSSION

Transport of Glc6P, P_i and other phosphorylated intermediates across the envelope of maize endosperm amyloplasts

In contrast with leaf mesophyll chloroplasts [21], most heterotrophic plastids are able to transport hexose phosphates across the inner envelope membrane [20,22]. However, there is a debate about the molecular nature of hexose phosphates imported into heterotrophic plastids and utilized for anabolic reactions. For example, plastids isolated from pea embryos or cauliflower buds import Glc6P more or less exclusively as a precursor for starch biosynthesis [10,11], whereas amyloplasts purified from wheat endosperm or from a soya-bean cell suspension culture use Glc1P as the most efficient precursor for starch biosynthesis [7–9]. Here we report that maize endosperm amyloplasts are able to transport Glc6P in counter exchange with P_i or other phosphorylated intermediates (Table 1). Interestingly, Glc1P does not act as a counter-exchange substrate for Glc6P or P_i uptake (Table 1). The latter result may be interpreted as contradicting previous data from our group with which we demonstrated that isolated maize endosperm amyloplasts are able to use both Glc6P and Glc1P as precursors for starch biosynthesis [4]. However, the demonstration that Glc1P can be used for starch synthesis in a heterotrophic plastid does not necessarily mean that such transport occurs *in vivo*. For the following reasons we can exclude any contribution of Glc1P import to carbohydrate supply of maize endosperm amyloplast. (1) Glc1P is not a counter-exchange substrate for the amyloplast hexose phosphate transporter.This transport protein allows high rates of metabolic flux (Table 1). (2) We have recently demonstrated that the rate of Glc6P-dependent starch synthesis by isolated maize endosperm amyloplasts is saturated at much lower external substrate concentrations than Glc1P-dependent starch synthesis [4]. Moreover, the Glc6P/Glc1P ratio in both crude extracts of whole maize endosperm tissue and enriched plastid fractions is in the range 45–55 [23]. This high Glc6P/Glc1P ratio makes it unlikely that Glc1P uptake contributes substantially to anabolic reactions in maize endosperm amyloplasts.

Nevertheless, we have to ask how Glc1P enters the plastid compartment? The capability of Glc1P-dependent starch synthesis has also been demonstrated for isolated cauliflower bud amyloplasts [20]. Detailed analysis, however, indicated that, under physiological concentrations, Glc6P is the most likely precursor for starch biosynthesis [12]. In the case of amyloplasts purified from cauliflower buds, Glc1P is not imported via a hexose phosphate translocator but by another so far unidentified membrane protein [20]. Obviously, the same holds true for Glc1P transport across the envelope of isolated maize endosperm amyloplasts.

We should also keep in mind that the specific use of Glc1P as a precursor for starch biosynthesis has been demonstrated for amyloplasts purified from wheat endosperm tissue [8]. In addition, Glc1P is the sole carbon source for starch synthesis in amyloplasts enriched from soya or potato cell suspension cultures [9,24]. It was recently demonstrated that Glc1P transport occurred in a 1:1 stoichiometry with P_i after reconstitution of envelope proteins from wheat endosperm amyloplasts in proteoliposomes [25]. Therefore we have to question why closely related amyloplasts (both maize and wheat are cereals) possess such different transport properties? Interestingly, a similar situation has been demonstrated for other closely related types of plastid. Schünemann and Borchet [26] showed that tomato fruit chloroplasts and chromoplasts are able to transport Glc1P in counter exchange with P_i. In contrast, chloroplasts and chromoplasts purified from sweet pepper fruits (both species belong to the *Solanaceae*) were not able to import Glc1P *via* a hexose phosphate transporter ([13]; H. E. Neuhaus, B. Camara, W. P. Quick, and T. Möhlmann, unpublished work). Obviously, much more work will be necessary before we understand the physiological reason for the presence of different types of hexose phosphate translocator in closely related types of plastid.

Analysis of the rate of transport of [^{14}C]Glc6P and [^{32}P]P_i into proteoliposomes shows that the rate of [^{32}P]P_i/P_i homo exchange is about twice that of [^{32}P]P_i/Glc6P exchange. In contrast, the rate of [^{14}C]Glc6P/Glc6P homo exchange is only about 20 % faster than that of [^{14}C]Glc6P/P_i hetero exchange. Such a discrepancy has also been observed for Glc6P and P_i transport cross the envelope of chromoplasts from sweet pepper fruits [27]. As demonstrated for the latter type of plastid, two different phosphate transporters (one specific for P_i, DHAP and PGA, and one that also transports Glc6P) are present simultaneously [27]. The observed discrepancies between the rates of homo and hetero exchange indicate that the same may hold true for maize endosperm amyloplasts. The assumption that different types of phosphate translocator can be present in the same envelope membrane is further supported by the recent observation that after detached spinach and potato leaves had been fed with glucose, chloroplasts with new transport properties were isolated [28]. In contrast with control chloroplasts, the organelles isolated from the glucose-fed leaves had gained the ability to import Glc6P. This suggests that a new type of hexose phosphate translocator was induced in addition to the typical triose phosphate translocator found in typical chloroplasts [28].

It is remarkable that there is a substantial difference between the rates of Glc6P and ADPGlc uptake into proteoliposomes and the rates of Glc6P- and ADPGlc-dependent starch synthesis by isolated maize endosperm amyloplasts (Tables 2 and 3). This discrepancy cannot be fully explained, but Glc6P might be used in maize endosperm amyloplasts mainly for glycolysis and the oxidative pentose phosphate pathway. It has been demonstrated that the oxidative pentose phosphate pathway at least is highly active in other cereal amyloplasts such as wheat endosperm plastids [8].

Interestingly, PEP induces increased rates of [^{14}C]Glc6P and [^{32}P]P_i import (Table 1). The stimulatory effect of PEP on P_i import has already been demonstrated for isolated pea root and embryo plastids [29,30], and for envelope membranes from tomato fruits [26]. It still remains unclear whether there is a specific metabolic requirement for PEP/P_i exchange in heterotrophic plastids (e.g. to supply intermediates to the shikimate pathway) or whether PEP transport is causally linked to Glc6P transport. The observation that preloading of proteoliposomes stimulates P_i import much more than Glc6P import may also be explained by the presence of a specific PEP translocator. In such a case, maize endosperm amyloplasts would contain at least three different types of phosphate translocator, namely a triose phosphate, a hexose phosphate and a PEP translocator.

It has recently been demonstrated that exogenously supplied Rib5P can support the oxidative pentose phosphate pathway in heterotrophic plastids [32,32]. In the present paper, we show that Rib5P is not taken up by the action of a phosphate translocator,

as it does not promote import of either [^{14}C]Glc6P or [^{32}P]P_i. It remains to be demonstrated how Rib5P enters the plastid.

Precursor-dependence of starch biosynthesis

We have demonstrated that 2 mM Glc6P induces substantial rates of starch synthesis in isolated maize endosperm amyloplasts provided that ATP and PGA are present [4]. It is remarkable, that 1 mM ADPGlc induced a rate of starch synthesis that was about six times faster than that of Glc6P-dependent starch synthesis (Table 2), even though the latter was conducted under saturating substrate conditions [4]. The demonstration that sonication of the plastid suspension before the start of the reaction reduces starch synthesis to 25% of the control values shows that the process is dependent on the physiological intactness of the organelles.

The observation that concentrations of ADPGlc above 2 mM are required to produce maximum rates of starch synthesis (Figure 1) agrees with previous measurements of ADPGlc-dependent starch synthesis in intact amyloplasts isolated from an *Acer pseudoplatanus* cell suspension culture [33]. In contrast, the low rate of ADPGlc-driven starch synthesis in isolated cauliflower amyloplasts is saturated at concentrations below 1 mM [12]. The cytosolic concentration of ADPGlc in maize endosperm is not known, but it is clear from the results given in Table 2 and Figure 1 that ADPGlc-dependent starch synthesis by isolated maize endosperm amyloplasts can be much faster than the rate of Glc6P-dependent starch synthesis [4].

A cytosolic location of ADPGlcPPase in maize endosperm was suggested by Hannah et al. [34] and recently confirmed by an immunological approach [17]. The latter group demonstrated that between 85 and 95% of the total ADPGlcPPase activity in barley or maize endosperm is extraplastidic [16,17]. Here we demonstrate ADPGlc-dependent starch biosynthesis in isolated maize endosperm amyloplasts. This is the first direct demonstration that a cereal amyloplast isolated from an endosperm tissue possessing an extraplastidic ADPGlcPPase takes up this nucleotide sugar and uses the glucose moiety with high efficiency for starch synthesis. Tetlow et al. [8] observed that ADPGlc induces high rates of starch synthesis in isolate wheat endosperm amyloplasts. As two groups had reported that in wheat endosperm most (if not all) of the ADPGlcPPase is located in the stroma [7,8], they came to the conclusion that, in amyloplasts from wheat endosperm, ADPGlc does not contribute to starch biosynthesis. In addition, Tetlow et al. [8] observed that the presence of ATP in the incubation medium stimulated ADPGlc-dependent starch synthesis. This result underlines the finding that starch synthesis in wheat endosperm amyloplasts is different from starch synthesis in isolated maize endosperm amyloplasts since, in the latter type of plastid, ATP strongly inhibits ADPGlc-dependent starch biosynthesis (Figure 3).

Until now it was not known how ADPGlc enters the plastid compartment. Pozueta-Romera et al. [14] speculated that the plastid ATP/ADP transporter, which was recently discovered at the molecular level [35,36], was responsible for ADPGlc transport. This assumption is contradicted by kinetic data on the properties of the ATP/ADP transporters from spinach mesophyll chloroplasts or pea root plastids. In both cases the plastid ATP/ADP transporter is unable to import radioactively labelled ATP in counter exchange with ADPGlc after reconstitution of the envelope proteins in proteoliposomes [37]. However, as both organelles were derived from tissues that probably lack cytosolic ADPGlcPPase, one might argue that the inability to transport ADPGlc *via* the plastid ATP/ADP transporter was due to the presence of a different translocator, as previously described by Pozueta-Romera et al. [14]. The data given in Table 3 clearly support the conclusion of Schünemann et al. [37] that the plastid ATP/ADP transporter is not able to transport ADPGlc.

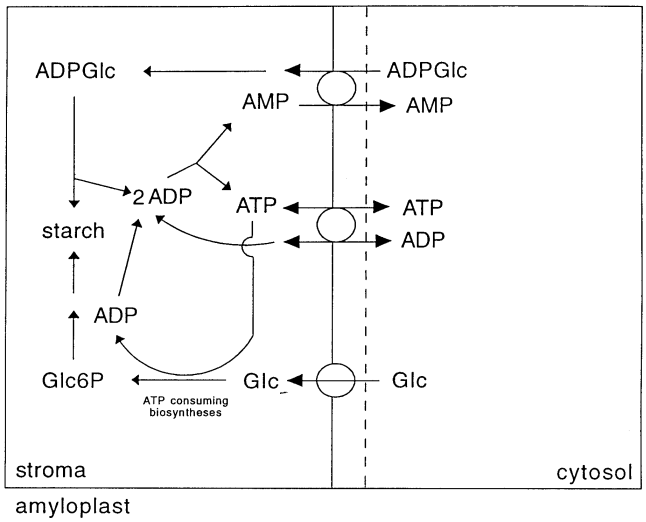
Interestingly, AMP uptake is stimulated by preloading proteoliposomes with ADPGlc (Table 2). Moreover, ADPGlc uptake occurs at the highest rate into vesicles preloaded with AMP (Table 3). This interaction indicates that ADPGlc transport is mediated by a ADPGlc/AMP transporter that is distinct from the plastid ATP/ADP transporter. In fact, the observation that nucleotide sugar transport occurs in counter exchange with the corresponding nucleotide monophosphate has been demonstrated in other systems. The transport of UDP-glucose or UDP-*N*-acetylglucosamine into endoplasmic reticulum purified from animal tissues is mediated in counter exchange with UMP (for a review see [38]). It remains to be analysed whether the ADPGlc/AMP transporter possesses structural similarities to the transporter described for the endoplasmic reticulum membranes.

The conclusion that ADPGlc is transported in counter exchange with AMP receives further support from several observations made by us and other groups. (1) ATP uptake into proteoliposomes containing the reconstituted envelope protein from maize endosperm amyloplasts is not inhibited by the simultaneous presence of ADPGlc (Figure 2). (2) ADPGlc is identified as a strong inhibitor of AMP uptake into isolated amyloplasts from an *Acer pseudoplatanus* cell culture [14]. (3) Beside the plastid ATP/ADP transporter, a second inner envelope protein has been identified that probably represents the plastid ADPGlc transporter. Molecular analysis of the maize *brittle1* mutation clearly shows that this gene encodes an inner-envelope membrane protein with significant homology to several mitochondrial transporters [39,40]. Moreover, since ADPGlc concentration in maize endosperm tissues from *brittle1* mutants is several times higher than in the wild-type, an involvement of the Brittle1 protein in ADPGlc transport is strongly indicated [41]. In addition, isolated amyloplasts from *brittle1* endosperm exhibits substantially reduced capacity for ADPGlc-dependent starch synthesis, as proposed by Liu et al. [42].

Interaction between Glc6P- and ADPGlc-dependent starch synthesis

As demonstrated above, both Glc6P and ADPGlc can be used as carbon sources for starch biosynthesis (Table 1) [4]. Therefore it was of interest to analyse the potential competition between the two metabolic pathways involved. Figure 3 illustrates that rising concentrations of ADPGlc do not inhibit Glc6P-dependent starch synthesis even though it is well documented that Glc6P-dependent starch synthesis is totally dependent on the uptake of ATP into the amyloplast [4]. This result confirms recent results from our group showing that Glc6P-dependent starch synthesis in isolated amyloplasts from cauliflower buds is barely inhibited by increasing concentrations of ADPGlc in the incubation medium [12]. The low degree of inhibition induced by ADPGlc agrees with the inability of ADPGlc to interfere with the plastid ATP/ADP transporter (Table 3) [37], and is probably due to a minor contamination of commercially available ADPGlc with ADP [37], acting as a substrate for the plastid ATP/ADP transporter (Table 3) [37]. The inability of ADPGlc to reduce the rates of Glc6P-dependent starch synthesis indicates that starch synthase and branching enzyme activities do not restrict starch synthesis under the conditions chosen.

In contrast with the effect of ADPGlc on the rate of Glc6P-dependent starch synthesis, the rate of ADPGlc-dependent starch synthesis is considerably reduced by the simultaneous presence



Scheme 1 Carbon and adenylate metabolism in maize endosperm amyloplasts during starch synthesis

of ATP in the incubation medium (Figure 3). This observation confirms our previous characterization of ADPGlc-dependent starch synthesis in isolated cauliflower bud amyloplasts [12]. The observation of this inhibitory effect on starch synthesis in maize endosperm amyloplasts is the first demonstration of an interaction between ADPGlc-dependent starch synthesis in cereal plastids and other metabolites and might be important for our understanding of starch biosynthesis in this tissue. The ATP/ADP ratio in maize endosperm is about 2 [41]. Since both metabolites are largely located outside the amyloplasts [23] and as the mitochondrial ATP/ADP ratio is low [43], we can assume that the rate of ADPGlc-dependent starch synthesis *in vivo* is inhibited by the available ATP in the cytosol.

At present, we do not know the exact mechanism by which ATP inhibits ADPGlc-dependent starch synthesis. As ATP and ADPGlc do not share the same transport protein for uptake into the plastid (Table 3) [37], some other mode of inhibition is likely. In control experiments, we demonstrated that the amyloplast starch synthase activity is not inhibited by ATP (results not shown). We propose two other possible modes of inhibition of ADPGlc-dependent starch synthesis by exogenous ATP. (1) The presence of ATP in the extraplastidic compartment is coupled to rapid export of ADP (see Table 3) [4], which inhibits the synthesis of AMP by the plastid myokinase (Scheme 1). As AMP represents the counter-exchange substrate for ADPGlc uptake, reduced import of ADPGlc would be the result. (2) We have demonstrated that externally supplied ATP stimulates starch degradation in heterotrophic and autotrophic plastids [44,45]. There is a substantial body of evidence that, in both chloroplasts and amyloplasts, simultaneous synthesis and degradation of starch occurs [46,44]. If we assume that the same holds true for maize endosperm amyloplasts, increased concentrations of ATP might promote starch degradation, leading to a release of radioactivity previously bound in starch. Interestingly, Tetlow et al. [8] observed a similar effect in isolated wheat endosperm amyloplasts, where high concentrations of ATP strongly inhibit the rate of starch synthesis.

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REFERENCES

1 Preiss, J. (1982) Annu. Rev. Plant Physiol. **33**, 431–454
2 Ziegler, P. and Beck, E. (1989) Annu. Rev. Plant Physiol. Plant Mol. Biol. **40**, 95–117
3 Entwistle, G. and ap Rees, T. (1990) Biochem. J. **271**, 467–72
4 Neuhaus, H. E., Thom, E., Batz, O. and Scheibe, R. (1993) Biochem J. **296**, 395–401
5 Keeling, P. L., Wood, J. R., Tyson, R. H. and Briggs, I. G. (1998) Plant Physiol. **87**, 311–319
6 Hatzfeld, W. D. and Stitt, M. N. (1990) Planta **180**, 198–204
7 Tyson, R. H. and ap Rees, T. (1988) Planta **175**, 33–38
8 Tetlow, I. J., Blisset, K. J. and Emes, M. J. (1994) Planta **194**, 454–460
9 Coates, S. A. and ap Rees, T. (1994) Phytochemistry **35**, 881–883
10 Hill, L. M. and Smith, A. M. (1991) Planta **185**, 91–96
11 Neuhaus, H. E., Henrichs, G. and Scheibe, R. (1993) Plant Physiol. **101**, 573–578.
12 Batz, O., Scheibe, R. and Neuhaus, H. E. (1994) Biochim. Biophys. Acta **1200**, 148–154
13 Batz, O., Scheibe, R. and Neuhaus, H. E. (1995) Planta **196**, 50–57
14 Pozueta-Romero, J., Frehner, M., Viale, A. M. and Akazawa, T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5769–5773
15 Okita, T. W. (1992) Plant Physiol. **100**, 560–564.
16 Thorbjørnsen, T., Villand, P., Denyer, K., Olsen, O.-A. and Smith, A. M. (1996) Plant J. **10**, 243–250
17 Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P. and Smith, A. M. (1996) Plant Physiol. **112**, 779–785.
18 Alban, C., Joyard, J. and Douce, R. (1988) Plant Physiol. **88**, 709–717
19 Batz, O., Scheibe, R. and Neuhaus, H. E. (1993) Biochem J. **294**, 15–17
20 Möhlmann, T., Batz, O., Maaß, U. and Neuhaus, H. E. (1995) Biochem J. **307**, 521–526
21 Fliege, R., Flügge, U. I., Werdan, K. and Heldt, H. W. (1978) Biochim. Biophys. Acta **502**, 232–247.
22 Borchert, S., Große, H. and Heldt, H. W. (1989) FEBS Lett. **253**, 183–186
23 Liu, T.-T. Y. and Shannon, J. C. (1981) Plant Physiol. **67**, 525–529
24 Kosegarten, H. and Mengel, K. (1994) Physiol. Plant. **91**, 111–120
25 Tetlow, I. J., Bowsher, C. G. and Emes, M. J. (1996) Biochem. J. **319**, 717–723
26 Schünemann, D. and Borchert, S. (1994) Bot. Acta **107**, 461–467
27 Quick, W. P. and Neuhaus, H. E. (1996) Biochem. J. **320**, 7–10
28 Quick, W. P., Scheibe, R. and Neuhaus, H. E. (1995) Plant Physiol. **109**, 113–121
29 Borchert, S., Harborth, J., Schünemann, D., Hoferichter, P. and Heldt, H. W. (1993) Plant Physiol. **101**, 303–312
30 Hill, L. M. and Smith, A. M. (1995) J. Plant Physiol. **146**, 411–417
31 Bowsher, C. G., Boulton, E. L., Rose, J., Nayagam, S. and Emes, M. J. (1992) Plant J. **2**, 893–899
32 Hartwell, J., Bowsher, C. G. and Emes, M. J. (1996) Planta **200**, 107–112
33 Pozueta-Romero, J. and Akazawa, T. (1993) J. Exp. Bot. **44**, (Suppl.) 297–306
34 Hannah, L. C., Giroux, M. and Boyer, C. (1993) Sci. Hortic. **55**, 177–197
35 Kampfenkel, K., Möhlmann, T., Batz, O., van Montagu, M., Inzé, D. and Neuhaus, H. E. (1995) FEBS Lett. **374**, 351–355
36 Neuhaus, H. E., Thom, E., Möhlmann, T., Steup, M. and Kampfenkel, K. (1996) Plant J. **11**, 73–82
37 Schünemann, D., Borchert, S., Flügge, U. I. and Heldt, H. W. (1993) Plant Physiol. **103**, 131–137
38 Abeijon, C. and Hirschberg, C. B. (1992) Trends Biol. Sci. **17**, 32–36
39 Sullivan, T. D., Strelow, L. I., Illingworth, C. A., Phillips, R. L. and Nelson, O. E. (1991) Plant Cell **3**, 1337–1348
40 Sullivan, T. D. and Kaneko, Y. (1995) Planta **196**, 477–484
41 Shannon, J. C., Pien, F.-M. and Liu, K. C. (1996) Plant Physiol. **110**, 835–843
42 Liu, T.-T. Y., Boyer, C. and Shannon, J. C. (1992) Plant Physiol. **99**, (Suppl.) 39
43 Klingenberg, M. (1989) Arch. Biochem. Biophys. **270**, 1–14
44 Neuhaus, H. E., Henrichs, G. and Scheibe, R. (1995) Planta **194**, 454–460
45 Neuhaus, H. E. and Schulte, N. (1996) Biochem. J. **318**, 945–953
46 Stitt, M. and Heldt, H. W. (1981) Biochim. Biophys. Acta **638**, 1–11