Purification of highly intact plastids from various heterotrophic plant tissues: analysis of enzymic equipment and precursor dependency for starch biosynthesis

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Starting with a protocol originally developed for the purification of intact plastids from cauliflower buds [Journet and Douce (1985) *Plant Physiol.* **79**, 458–467] we have modified this method to obtain intact heterotrophic plastids from etiolated barley leaves (*Hordeum vulgare*) and pea (*Pisum sativum*) and maize (*Zea mays*) endosperm. Two subsequent centrifugation steps on Percoll gradients were performed, the first as an isopycnic, the second as zonal, centrifugation step in a swing-out rotor. Percoll density and centrifugation time were adjusted for the various tissues. The obtained plastid preparations are characterized by a low degree of contamination with other cellular components

and an intactness of at least 90%. In isolated maize endosperm amyloplasts, starch synthesis is driven by exogenously applied hexose phosphates (glucose 6-phosphate and glucose 1-phosphate) rather than by dihydroxyacetone phosphate. The hexose-phosphate-dependent starch synthesis is strictly dependent upon the intactness of the plastids and is increased up to 9-fold when ATP and 3-phosphoglyceric acid are added to the incubation medium. The occurrence of fructose-1,6-bisphosphatase and malate dehydrogenases in some plastid types is discussed in relation to their possible role in starch synthesis.

INTRODUCTION

Starch synthesis in plants is exclusively localized in the plastidic compartment (Preiss, 1982). In chloroplasts, the sources of carbon and energy for this process are generated by CO₂ assimilation and photophosphorylation respectively. Starch synthesis in plastids of heterotrophic origin depends upon the uptake of both carbon precursor and energy from the cytosol. A variety of different precursors have been proposed to enter the stroma for synthesis of starch. Mohabir and John (1988) claimed that application of triose phosphates leads to starch synthesis in isolated potato (*Solanum tuberosum*) tuber amyloplasts. These results were supported by Echeverria et al. (1988), who used enriched maize (*Zea mays*) endosperm amyloplast preparations. Those authors did not exclude the possibility that the applied triose phosphates are converted into other compounds by enzymes liberated from broken plastids or cytosolic contamination. Finally, etioplasts from barley (*Hordeum vulgare*) leaves import dihydroxyacetone phosphate (DHAP) and incorporate these carbon skeletons into starch (Batz et al., 1992). Isolated amyloplasts from wheat (*Triticum aestivum*) endosperm use solely glucose 1-phosphate (Glc1P) as carbon source for starch synthesis (Tyson and ap Rees, 1988), whereas amyloplasts isolated from both pea (*Pisum sativum*) endosperm and cauliflower (*Brassica oleracea*) buds use glucose 6-phosphate (Glc6P) most efficiently as a precursor for starch biosynthesis (Hill and Smith, 1991; Neuhaus et al., 1993). Pozueta-Romero et al. (1991) demonstrated that isolated amyloplasts from sycamore (*Acer pseudo-platanus*) suspension-culture cells are able to import ADP-glucose (ADPGlc) into the stroma and incorporate the glucose moiety into starch.

The ability of heterotrophic plastids to incorporate imported triose phosphates into starch is dependent upon the occurrence of a plastidic fructose-1,6-bisphosphatase (FBPase). However, in heterotrophic plastids contradictory data have been presented concerning both the presence and the absence of this enzyme (Journet and Douce, 1985; Echeverria et al., 1988; Entwistle and ap Rees, 1990).

There are two possible explanations for the contradictory results concerning the enzymic equipment and the precursor-dependency of plastidic starch synthesis: (1) the plastids from various tissues possess a specific set of enzymes and they differ in their ability to take up and use specific metabolic intermediates for starch synthesis; (2) the different methods for isolation of heterotrophic plastids applied in these studies yield preparations of variable purity and intactness and thus possibly have led to differing results.

The aim of this work was to develop a method suitable to prepare plastids of high purity and intactness from a range of heterotrophic tissues and to use them to determine the precursor-dependency and the rate of starch synthesis in maize endosperm amyloplasts. The plastids were also analysed for the presence of critical enzymes likely to be required for the postulated pathways.

MATERIALS AND METHODS

Source of plant material

Cauliflower (*B. oleracea* L., var. Prince de Bretagne) buds were purchased at the local farmers' market. The outermost layer of the bud (5–7 mm) was used for purification of plastids. Pea plants (*P. sativum* L., var. Kleine Rheinländerin) were grown on soil in the greenhouse for 6–8 weeks. Developing pea seeds were

Abbreviations used: ADPGlcPPase, ADP-glucose pyrophosphorylase; DHAP, dihydroxyacetone phosphate; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; MDH, malate dehydrogenase; OAA, oxaloacetate; PGA, 3-phosphoglyceric acid; PGM, phosphoglucomutase; PFP, pyrophosphate:fructose-6-phosphate phosphotransferase; UDPGlcPPase, UDP-glucose pyrophosphorylase.

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Table 1 Specific conditions for preparation of the various heterotrophic plastids and starch content of isolated plastids

The method described by Journet and Douce (1985) was modified as indicated. Results for starch contents are means ($\pm$ S.E.M.) for three independent preparations. Abbreviation: FW, fresh weight.

Purification step	Origin of plastids...	Etiolated barley leaves	Cauliflower buds	Pea endosperm	Maize endosperm
Homogenization					
FW used (g)		800	1500	400	1000
medium A:FW (ml/g)		3.5	2	1	2
Isopycnic centrifugation					
[Percoll] (%)		35	35	20	28
Centrifugation time (min)		30	25	30	25
Rate-zonal centrifugation					
[Percoll] (%)		35	35	25	28
Centrifugation time (min)		25	15	15	15
Final yield of plastid protein (mg/kg FW)		50	20–40	5–7	10
Starch content of isolated plastids (μ mol of Glc/mg of protein)		19.1 $\pm$ 1.5	1.3 $\pm$ 0.2	5.1 $\pm$ 0.1	4.3 $\pm$ 0.2

collected 2 weeks after anthesis and used after removal of the testa. Maize (*Z. mays* L., var. Challenger) was grown in a local field. Developing maize kernels were collected 3–5 weeks after anthesis. Only kernels that were still white were used for the isolation of plastids. Barley (*H. vulgare* L., var. Roland) was grown in the dark as described (Batz et al., 1992). After 8 days, the seedlings were harvested by cutting them 4 cm above the ground.

Isolation of plastids

All steps were performed at 0–4 °C. In general the plant material was homogenized for 3 $\times$ 5 s in medium A consisting of 0.3 M mannitol, 20 mM sodium pyrophosphate (adjusted to pH 7.6 with phosphoric acid), 4 mM cysteine, 1 mM EDTA, 0.2 % BSA using a Waring blender (model 34BL23). The homogenate was subsequently filtered through a 40 μ m-mesh-size nylon net. Starch and broken-cell material was removed by centrifugation for 10 min (170 g, Sorvall GS3 rotor). The crude plastid preparation was subjected to a second centrifugation step for 20 min (3200 g, Sorvall GS3 rotor). The pellet was resuspended in 100 ml of medium B consisting of 0.3 M sucrose, 10 mM potassium phosphate (adjusted to pH 7.2 with phosphoric acid), 1 mM EDTA and 0.1 % BSA. Starch grains released from broken plastids were removed by a further centrifugation step of 10 min (200 g, Sorvall SS34 rotor) and the plastids were sedimented by centrifugation of the supernatant for 20 min (3200 g, Sorvall SS34 rotor). The plastid pellet was resuspended in medium B and a gradient of 20–35 % Percoll in medium B was overlaid with the plastid suspension. The following isopycnic centrifugation was performed for 25–30 min in a Kontron ultracentrifuge using a Sorvall T 1250 rotor (50600 g). Intact plastids banded in the lower part of this self-generated gradient and could be removed with a plastic pasteur pipette. In order to remove excess Percoll this preparation was diluted 5-fold with medium B and centrifuged for 10 min (3200 g, Sorvall SS 34 rotor). The sedimented plastids were resuspended in 3 ml of medium B and layered on top of a solution of 25–35 % Percoll in medium B. A rate-zonal centrifugation was performed for 15–25 min in a Kontron ultracentrifuge using a Sorvall AH 629 swing-out rotor (13200 g). The highly purified plastids banded in a zone near the bottom and were collected by using a pierce unit (Kontron). In

order to remove excess Percoll the suspension was diluted 1:15 with medium C, consisting of 0.3 M sorbitol, 15 mM Hepes/KOH, pH 7.6, 1 mM EDTA, 2 mM MgCl₂ and the plastids were recovered by another centrifugation step (10 min, 3200 g, Sorvall SS34 rotor). The plastid pellet was resuspended in a small volume of medium C. After removal of an aliquot for protein determination, BSA was added to a final concentration of 0.1 %.

The detailed conditions for the preparations of plastids from each of the tissues are given in Table 1. Thus the ratio of homogenization medium to fresh weight, the density of the Percoll media and the centrifugation times were varied in each case in order to yield optimal results in terms of purity and intactness.

Protein determination

Total protein (membrane-bound and soluble) was estimated with a colorimetric assay using a bicinchoninic acid/CuSO₄ mixture (Sigma, Deisenhofen, Germany) according to the supplier's instructions. BSA served as a standard.

Enzyme assays

Enzyme activities were measured at 25 °C (if not indicated otherwise) using a Sigma–Eppendorf double-wavelength spectrophotometer (ZFP 22). Published methods were used for the enzyme assays: ADP-glucose pyrophosphorylase (ADPGlcPPase) (Sowokinos, 1976); UDP-glucose pyrophosphorylase (UDPGlcPPase) and phosphoglucomutase (PGM) (Bergmeyer, 1974); pyrophosphate:fructose-6-phosphate phosphotransferase (PFP) (Neuhaus et al., 1990); citrate synthase and α -mannosidase (Stitt et al., 1989); FBPase (Entwistle and ap Rees, 1988); NADP⁺- and NAD⁺-dependent malate dehydrogenase (NADP⁺-MDH and NAD⁺-MDH) (Scheibe and Stitt, 1988); NADP⁺-dependent glyceraldehyde-3-phosphate dehydrogenase (NADP⁺-GAPDH) (Holtum and Winter, 1982); NAD⁺-dependent GAPDH activity was measured by addition of NADH₂ instead of NADPH₂ into the assay medium.

Analysis of purity and intactness

The purity of the plastid preparations was calculated after measuring marker-enzyme activities for the different cell compartments in crude extracts and in the purified plastid extracts. Based on the relative enzyme activities in the plastid preparation and in the crude extract, contaminations by mitochondria, by cytosol, and by vacuolar content were calculated. Crude extracts were prepared as described by Batz et al. (1992). The following marker enzymes were used: ADPGlcPPase for plastids, UDPGlcPPase and PFP for cytosol, citrate synthase for mitochondria and α -mannosidase for vacuolar contamination. Intactness of the isolated plastids was estimated by performing latency experiments by the method of Entwistle and ap Rees (1988) using ADPGlcPPase as an exclusively plastidic enzyme. The activity was determined in a sample of intact plastids (in the presence of 330 mM sorbitol in the assay medium) and in a sonicated aliquot in the same buffer.

Starch synthesis from ^{14}C -labelled substrates

Starch synthesis in isolated plastids took place under conditions described by Neuhaus et al. (1993) in a medium consisting of 300 mM sorbitol, 15 mM Hepes/KOH, pH 7.2, 1 mM EDTA and 2 mM MgCl_2 . [^{14}C]Glc6P and [^{14}C]Glc1P were applied at specific activities between 1 and 2 MBq/mmol. [^{14}C]Dihydroxyacetone phosphate (DHAP) was synthesized by the method of Tyson and ap Rees (1988) and was applied at a specific activity of 1.8 MBq/mmol. The incubation of the plastids with the radioactive precursors and unlabelled effectors or inhibitors was terminated as indicated by transfer to a 95 °C thermostatically controlled shaker. The resulting residue was washed and radioactivity was quantified, in some cases subsequent to further analysis as described below (Neuhaus et al., 1993).

Identification of the ^{14}C -labelled insoluble compound

To remove even small traces of unincorporated radioactivity still adhering to the pellet each sample was subjected to anion-exchange chromatography [for details, see Kruckeberg et al. (1989) modified as described by Neuhaus et al. (1993)]. In order to verify whether the radioactive product was really starch rather than another insoluble product, the solubilized glucose moieties liberated after hydrolysis which passed the first anion-exchange column were phosphorylated with hexokinase and ATP and subsequently applied to a second anion-exchange column (Neuhaus et al., 1993). Quantification of the radioactivity in the anionic fraction indicated the amount of ^{14}C -labelled glucose derived from starch. Both the phosphorylated and the unphosphorylated samples were subjected to h.p.l.c. [on an anion-exchange column (Partisil SAX10) with a potassium phosphate gradient at pH 2.8; Kontron, Munich, Germany] and co-migrated with authentic Glc6P and glucose respectively (results not shown).

Immunological detection of plastid enzymes

Comparable amounts of plastid samples were subjected to SDS/PAGE, transferred to nitrocellulose, which was subsequently incubated with the respective polyclonal antiserum at 1:400 dilution after blocking with gelatine. For detection, the sheet was incubated with goat anti-rabbit IgG (horseradish peroxidase conjugate) (Bio-Rad, Munich, Germany). The colour reaction was performed with 4-chloronaphthol and H_2O_2 as substrates. The serum against purified spinach chloroplast

FBPase has been shown to be monospecific when applied to crude extracts (R. Scheibe, unpublished work). The serum against pea chloroplast NADP⁺-MDH has been described elsewhere (Fickenscher and Scheibe, 1983).

RESULTS

Characteristics of the isolated plastids

We have used a method that has been originally developed to purify amyloplasts from cauliflower buds (Journet and Douce, 1985). Changes with respect to the density of the Percoll media and centrifugation times led to a method that can be applied to purify amyloplasts from different tissues, as described in Material and methods section (Table 1). All plastid types contain starch at levels between 1.3 and 19 μmol of glucose equivalent/mg of protein. Using this isolation technique we routinely obtained plastids with an intactness greater than 90 % from etiolated barley leaves, cauliflower buds, and pea and maize endosperm (Table 2). Other methods have generally yielded preparations of lower intactness. Tyson and ap Rees (1988) purified wheat endosperm amyloplasts of an intactness of 54 % by preparing protoplasts and subsequently applying a fractionation step on a Nycodenz gradient. An improved protocol for the purification of wheat endosperm amyloplasts within 2 h led to a similar degree of intactness of 55–65 % (Tetlow et al., 1993). Amyloplasts obtained from potato tubers by sedimenting plastids with 1 g had an intactness of only 16–27 % (Mohabir and John, 1988). Pea endosperm amyloplasts purified by using a Percoll gradient exhibited an intactness of > 70 % (Hill and Smith, 1991). Pea root plastids purified by applying a rate-zonal Percoll centrifugation exhibit an intactness of 73–84 % (Bowsher et al., 1989). Since a high degree of intactness is an important prerequisite in order to analyse the enzymic equipment and the physiological processes within the plastids, preparations purified according to the modified protocols described here appear to be suitable to study metabolic processes. The extremely low degree of cytosolic contamination in all plastid types that have been purified (Table 2) excludes misinterpretations concerning the presence of iso-enzymes in the plastid fractions. Measuring the activity of citrate synthase as a mitochondrial marker enzyme we estimated low contaminations of our preparations with this cellular compartment (Table 2). Obviously, owing to the number of centri-

Table 2 Intactness and contamination with cellular components of purified heterotrophic plastids

The degree of contamination was estimated by measuring the activity of compartment-specific enzymes in crude extracts and purified plastids. For pea endosperm amyloplasts the cytosolic contamination was estimated by measuring the activity of PFP. Intactness was determined by measuring the latent activity of ADPGlcPPase. Results are the means ($\pm$ S.E.M.) for three independent preparations. Abbreviation: n.d., not detectable.

Origin of plastids	Contamination with cellular components (%)			Intactness (%)
	Cytosol	Mitochondria	Vacuole	
Etiolated barley leaves	0.9 $\pm$ 0.1	2.0 $\pm$ 0.3	n.d.	92.0 $\pm$ 0.9
Cauliflower buds	0.8 $\pm$ 0.1	5.8 $\pm$ 0.4	n.d.	89.9 $\pm$ 1.2
Pea endosperm	1.5 $\pm$ 1.3	10.1 $\pm$ 0.7	1.9 $\pm$ 0.7	89.1 $\pm$ 5.4
Maize endosperm	0.6 $\pm$ 0.1	2.9 $\pm$ 0.5	n.d.	96.0 $\pm$ 2.5

Table 3 Precursor- and effector-dependency of starch synthesis in isolated maize endosperm amyloplasts

Starch synthesis in isolated amyloplasts was performed by the method of Neuhaus et al. (1993). Results are means ($\pm$ S.E.M.) for three independent samples.

¹⁴ C-labelled-precursor concn. (mM)		Effector concn. (mM)				Rate of starch synthesis (nmol/h per mg of protein)
Glc6P	Glc1P	PGA	ATP	DHAP	ADP	
5	—	2	2	—	—	26.6 $\pm$ 2.1
5	—	2	2	—	—	0.4 $\pm$ 0.7*
5	—	—	2	—	—	9.8 $\pm$ 0.6
5	—	2	—	—	—	3.0 $\pm$ 0.6
5	—	2	2	10	—	4.3 $\pm$ 1.0
5	—	2	2	—	10	2.9 $\pm$ 1.4
—	1	2	2	—	—	10.1 $\pm$ 0.1
—	1	2	2	—	—	0.7 $\pm$ 0.2*

* Sample was sonicated for 3 $\times$ 3 s before incubation.

Figure 1 Effect of hexose phosphate concentration upon the rate of starch synthesis in isolated maize endosperm amyloplasts

Plastids were incubated for 20 min in medium C containing, in addition Glc6P or Glc1P at the indicated concentrations, 2 mM ATP and 2 mM PGA. Results are the means ($\pm$ S.E.M.) for three independent samples.

fugation steps and the density of the Percoll solutions used, most of the mitochondria are removed. Only amyloplasts purified from pea endosperm exhibit a mitochondrial contamination of 10%. We have tried to modify our protocol in order to obtain also amyloplasts from potato tubers and from carrot roots. These preparations show low levels of cytosolic contamination, but still exhibit high mitochondrial contents (results not shown).

Incorporation of carbon precursors into starch in isolated plastids from maize endosperm and comparison with other tissues

Both hexose phosphates (Glc6P and Glc1P) can drive starch synthesis in isolated maize endosperm amyloplasts. The rate is strongly dependent upon the intactness of the plastids (Table 3). Glc6P, particularly at low concentrations, is more rapidly incorporated into starch than is Glc1P. This result confirms reports by Hill and Smith (1991) and by Neuhaus et al. (1993), who demonstrated Glc6P-dependent starch synthesis in pea

endosperm and cauliflower bud amyloplasts respectively. The hexose phosphate-dependent starch synthesis is strongly stimulated by addition of ATP and 3-phosphoglyceric acid (PGA) to the incubation medium (Table 3), whereas addition of either DHAP or ADP inhibits starch synthesis significantly (Table 3).

Tyson and ap Rees (1988) demonstrated that isolated wheat endosperm amyloplasts incorporate Glc1P into newly synthesized starch. These results are in contrast with other findings, where only low incorporation of Glc1P by amyloplasts purified from both pea endosperm (Hill and Smith, 1991) and cauliflower buds (Neuhaus et al., 1993) was observed. Plastids purified from pea roots exhibit almost no uptake of Glc1P (Borchert et al., 1989). However, maize endosperm amyloplasts incorporate both hexose phosphates (Glc6P and Glc1P) at comparable rates when applied at higher concentrations (Figure 1). To elucidate whether Glc1P is taken up directly or as Glc6P after mutation by an externally bound PGM that had been described as an envelope-associated protein in chloroplasts (Salvucci et al., 1990) we applied two approaches. The PGM activity was determined in crude extracts and in the intact plastid preparation. However, we failed to find any PGM activity in the intact plastid preparation. As the crude extract contained a PGM activity of 1.03 μ mol $\cdot$ min⁻¹ $\cdot$ mg of protein⁻¹, a cytosolic contamination of 0.6% would lead to a maximal PGM activity of 6.2 nmol $\cdot$ min⁻¹ $\cdot$ mg of protein in the plastid preparation. Under the conditions used for starch synthesis (20 min, 50 μ g of plastid protein in 0.3 ml) a concentration of only 20 μ M Glc6P could then be degenerated. This would be too low to drive starch synthesis at the observed rates (Figure 1). In addition, it was established that Glc1P-dependent starch synthesis is strictly linear with time for at least 60 min (Figure 2). This would not be the case if newly synthesized Glc6P drives starch synthesis, because the initial Glc6P concentration would be close to zero. Therefore it can be assumed that isolated maize amyloplasts are able to take up external Glc1P directly.

When DHAP was applied to isolated maize amyloplasts, incorporation of radioactivity into an insoluble compound at a high rate was observed (Table 4). However, when the nature of the newly synthesized radioactive compound was analysed by phosphorylation of the hydrolytically liberated radioactivity, only in the case of the hexose phosphates was it found to be mostly starch. In contrast, the radioactivity obtained after feeding [¹⁴C]DHAP appeared to be largely of another nature that remains to be elucidated. Only 3.5% of the original rate was confirmed to lead to starch (Table 4). In order to compare the *in vitro* rate of starch synthesis in isolated maize endosperm amyloplasts with the *in vivo* starch content we determined the total starch of isolated maize amyloplasts. The equivalent of 4.28 $\pm$ 0.2 μ mol glucose units $\cdot$ mg of protein⁻¹ was found in three independent preparations. If starch synthesis *in vivo* proceeds at the rate determined *in vitro*, 6–7 days would be required to accumulate this amount. However, it can be assumed that the rate of starch biosynthesis *in vivo* might be higher than in isolated plastids as documented for amyloplasts from potato tubers (Mohabir and John, 1988) or barley etioplasts (Batz et al., 1992).

Comparison of FBPase activity present in the various plastids

When DHAP is imported and incorporated into starch, the conversion of triose phosphates into hexose phosphates depends upon the existence of a plastidic aldolase/FBPase reaction (Entwistle and ap Rees, 1988). Therefore analysis of the respective plastid preparation for a plastidic FBPase using both attempts, photometric and immunological detection, can further substantiate the observed differences of the precursor utilization.

Figure 2 Effect of incubation time upon Glc1P-dependent starch synthesis in isolated maize endosperm amyloplasts

Plastids were incubated in medium C containing in addition 1 mM Glc1P, 2 mM ATP and 2 mM PGA. Results are the means ($\pm$ S.E.M.) for three independent samples.

Table 4 Analysis of radioactivity incorporated into starch

Glc6P or DHAP were applied at a concentration of 5 mM. ATP (2 mM) and PGA (2 mM) were present in addition in both assays. Hydrolysis of the water-insoluble products, hexokinase-dependent phosphorylation and anion-exchange chromatography were performed as described in the Materials and methods section. Results are means ($\pm$ S.E.M.) for three independent samples.

Precursor	Rate of incorporation (nmol of C ₆ units/h per mg of protein)	Glucose units in product (%)
Glc6P	28.0 $\pm$ 2.2	99.0
DHAP	52.9 $\pm$ 4.8	3.5

The combination of two independent methods allows the detection of an enzyme protein even if its activity is drastically underestimated by omission of an unknown effector in the assay medium.

As expected, owing to their ability to incorporate DHAP much more efficiently into starch than the other plastid types, barley etioplasts contain a high FBPase activity of 120 nmol · mg of protein⁻¹ (Table 5). The presence of an appreciable amount of this protein was confirmed using the immunological approach. A strong reaction with the antibody raised against purified spinach chloroplast FBPase verifies the finding of a plastidic FBPase in

Table 5 Activity of stromal enzymes in purified heterotrophic plastids

Values are the mean of at least three independent preparations with an S.E.M. of less than 8%.

Type of plastid	Enzyme activity (nmol/min per mg of protein)					
	FBPase (reduced)	NADP ⁺ - MDH (reduced*)	NADP ⁺ - MDH (oxidized)	NAD ⁺ - MDH	NADP ⁺ - GAPDH	NAD ⁺ - GAPDH
Barley etioplasts	120.0	412.0	6.9	672.0	141.0	145.0
Cauliflower amyloplasts	3.8	369.0	24.0	2304.0	8.4	79.0
Pea endosperm amyloplasts	n.d.	730.0	289.4	3650.0	21.0	680.0
Maize endosperm amyloplasts	0.5	18.0	20.0	1157.0	12.0	41.0

* This activity is not corrected for unspecific activities (see Scheibe et al., 1990).

Figure 3 Immunodetection of (a) FBPase and (b) NADP⁺-MDH in purified heterotrophic plastids of different origin

A 50 μ g sample of plastid protein was applied to each lane. Lane 1, etioplasts; lane 2, cauliflower bud plastids; lane 3, pea endosperm amyloplasts; and lane 4, maize endosperm amyloplasts.

these heterotrophic plastids (Figure 3). Neuhaus et al. (1993) have demonstrated that isolated cauliflower bud plastids incorporate DHAP into starch with an efficiency of 18% as compared with Glc6P as a precursor. The reason for this low efficiency might be the comparably low activity of the plastidic FBPase (Table 5 and Figure 3). The low activity of FBPase in cauliflower plastids has been already pointed out by Journet and Douce (1985), but these authors suggested that this activity would be sufficient to catalyse the conversion of triose phosphates into hexose phosphates *in vivo*. Echeverria et al. (1988) claim that 74% of the total FBPase activity is localized in the amyloplastic compartment in maize endosperm. The maize endosperm plastid preparations, prepared by the method described here, contain hardly any FBPase activity (Table 5) or protein (Figure 3). The extremely low FBPase activity measured in the plastid sample represents only 0.8% of the total, and is probably due to the cytosolic contamination, which is of about the same order of magnitude (0.6%; Table 2). This result confirms observations made by Entwistle and ap Rees (1990) using crude extracts from maize endosperm. We therefore suggest that DHAP-dependent starch synthesis in amyloplasts isolated from the maize endosperm can only represent a minimal (or no) portion of the total starch biosynthesis.

Comparison of dehydrogenase activities involved in generation and export of reducing equivalents

When etioplasts from barley leaves import DHAP as a precursor of starch synthesis, one third of these molecules should be oxidized for the generation of ATP (Batz et al., 1992). Therefore

all purified plastid types were analysed for NAD⁺- and NADP⁺-dependent activities of MDH and GAPDH (Table 5). Since the activity of the NADP⁺-dependent isoenzymes is known to be dependent upon preincubation with a reducing agent, such a treatment was also applied. In the case of NADP⁺-MDH the activity of the untreated oxidized enzyme form is given in addition. An unspecific activity of the NAD⁺-MDH with NADPH₂ of about 1:500 had been demonstrated previously (Scheibe et al., 1990) and should be considered, when the true activity of an NADP⁺-dependent plastid enzyme is to be established. The presence of dithiothreitol in the assay medium for GAPDH described by Holtum and Winter (1982) yields a fully active enzyme.

Interestingly, all plastid types contain a relatively high activity of NAD⁺-MDH; however, the high activity of 3.65 units/mg of protein in the pea endosperm amyloplasts (Table 5) might be due to mitochondrial contamination of around 10% (Table 2). When the NAD⁺-dependent activities are correlated with the activities of the oxidized NADP⁺-MDH, in all cases the coenzyme ratio is higher (1:100 and 1:10) than expected. Upon reduction the basic activity of the NADP⁺-MDH from maize endosperm amyloplasts is not increased (Table 5). The absence of a specific plastid NADP⁺-MDH in this tissue is also confirmed by the immunological approach shown in Figure 3. The other plastid types contain substantial amounts of NADP⁺-MDH activity, on a protein basis even more than mesophyll chloroplasts, which exhibit an activity of about 200 nmol/h per mg of protein (Scheibe et al., 1990).

GAPDH activity in chloroplasts is specific for both NADH and NADPH as coenzyme (Yonushot et al., 1970). For plastids of other origin, only limited information is available. The NAD⁺-dependent activities determined for the plastids of heterotrophic origin prepared as described in the Materials and methods section are higher than the NADP⁺-dependent ones, or at least as high in the case of barley etioplasts (Table 5).

DISCUSSION

Plastids obtained according to our protocols exhibit high degrees of intactness and purity (Table 2). These plastids were used to analyse the precursor-dependency for starch biosynthesis and to localize enzymes supposed to be involved in the utilization of imported substrates.

It is shown that starch synthesis in isolated amyloplasts from maize endosperm is driven by both Glc6P and Glc1P (Figure 1). Therefore it has to be assumed that the phosphate translocator involved in the transport of hexose phosphates has some affinity to transport Glc1P, at least when present at higher concentrations. Performing randomization experiments, Hatzfeld and Stitt (1990) proposed that starch synthesis in maize endosperm is driven by uptake of hexoses into the amyloplasts. However, using this experimental attempt it is not possible to gain information about the chemical nature of this hexose (e.g. free hexose, hexose phosphate). The finding that isolated maize endosperm amyloplasts use Glc1P for starch synthesis is in contrast with data from Borchert et al. (1993), who demonstrated that amyloplasts purified from pea roots do not transport Glc1P. The competence to import Glc6P and Glc1P could be a unique feature of amyloplasts purified from tissues of monocotyledonous origin.

Despite of the fact that maize amyloplasts are able to incorporate Glc1P into starch, we assume that Glc6P is the predominant precursor for starch biosynthesis. There is only a little information about the hexose phosphate content of the

cytoplasm of heterotrophic tissues. Hill and Smith (1991) calculated a Glc6P content of 4.5 mM in the cytoplasm of pea endosperm. For developing maize endosperm a cytosolic Glc1P content of 1–2 mM was calculated (Dancer, 1987). This concentration would lead to a Glc6P level between 20 and 40 mM on the basis of the thermodynamic equilibrium of the PGM reaction (Bergmeyer, 1974). Up to now we have no information about the competitive behaviour of these two hexose phosphates at the hexose phosphate translocator. But even under such high hexose phosphate levels, it can be supposed that Glc6P is the major carbon source for starch synthesis in maize endosperm (Figure 1).

Hexose phosphate-dependent starch synthesis in maize endosperm amyloplasts is strongly stimulated by the addition of ATP and PGA to the incubation medium. The stimulatory effect of ATP on starch synthesis appears to be a general feature of most amyloplasts analysed so far (Tyson and ap Rees, 1988; Echeverria et al., 1988; Hill and Smith, 1991; Neuhaus et al., 1993). In agreement with our results on starch synthesis in isolated intact cauliflower-bud amyloplasts we observed a stimulatory effect of exogenously applied PGA. The reason for this stimulation could be the activation of ADPGlcPPase by a rising PGA/P_i ratio (Neuhaus et al., 1993). Akazawa's group (Pozueta-Romero and Akazawa, 1993) recently observed a stimulatory effect of PGA on hexose phosphate-dependent starch synthesis in isolated amyloplasts from sycamore suspension-culture cells. Glc6P-dependent starch synthesis in isolated maize endosperm amyloplasts is strongly inhibited by addition of either DHAP or ADP to the incubation medium (Table 3). This finding is in agreement with the results obtained for Glc6P-dependent starch synthesis in isolated cauliflower bud plastids (Neuhaus et al., 1993). DHAP is known as a competitive inhibitor of Glc6P uptake in isolated pea root plastids (Borchert et al., 1989). ADP

Table 6 Comparison of precursor- and effector-dependency of starch synthesis in various heterotrophic plastids

Notes: ^adata for DHAP-dependent starch synthesis in isolated barley etioplasts are taken from Batz et al. (1992). Hexose phosphate-dependent starch synthesis in etioplasts was performed $\pm$ ATP and PGA as effectors (by the method of Neuhaus et al., 1993), but we failed to detect a significant incorporation (n.d., not detectable); ^bdata for cauliflower bud amyloplasts are taken from Neuhaus et al. (1993); ^cdata for pea endosperm are taken from Hill and Smith (1991); the rate of incorporation is expressed as nmol/h per unit of ADPGlcPPase; ^ddata for wheat endosperm are taken from Tyson and ap Rees (1988); the rate of incorporation is expressed as nmol/h per unit of alkaline pyrophosphatase.

Type of plastid	Precursor	Effector(s)	Rate of starch synthesis (nmol of C ₆ /h per mg of protein)
Barley etioplasts ^a	Glc6P	$\pm$ PGA/ATP	n.d.
	Glc1P	$\pm$ PGA/ATP	n.d.
	DHAP	None	114.0
Cauliflower bud amyloplasts ^b	Glc6P	PGA/ATP	127.5
	Glc1P	PGA/ATP	20.0
	DHAP	PGA/ATP	17.8
Pea endosperm amyloplasts ^c	Glc6P	ATP	1400.0
	Glc1P	ATP	38.3
	DHAP	ATP	13.9
Maize endosperm amyloplasts	Glc6P	PGA/ATP	26.6
	Glc1P	PGA/ATP	20.8
	DHAP	PGA/ATP	1.9
Wheat endosperm amyloplasts ^d	Glc6P	None	61.0
	Glc1P	ATP	248.0
	DHAP	None	31.5

acts as a competitive inhibitor of ATP uptake into cauliflower-bud plastids (Neuhaus et al., 1993), thus causing a decrease of the ATP/ADP ratio within the plastids.

Isolated maize amyloplasts convert exogenously applied DHAP into a water-insoluble neutral product. As demonstrated, maize endosperm amyloplasts contain hardly any FBPase. As a consequence the conversion of imported triose phosphate into hexose phosphate, as required for starch biosynthesis, should be excluded. In fact the labelled insoluble compound was found not to be starch (Table 5). Therefore it appears to be essential to analyse any labelled product of precursor incorporation and confirm its nature as starch.

In Table 6 the precursor-dependency of starch synthesis in plastids isolated from various heterotrophic tissues is compared. Obviously the ability to use external triose phosphate for plastidic starch synthesis is correlated with the presence of an FBPase (Table 5). Only etioplasts appear to use DHAP as a substrate at a substantial rate. The rate of starch synthesis (114 nmol/per mg of protein) is much lower than the maximal activity of the plastidic FBPase. Therefore the enzyme does not need to be fully activated *in vivo* to catalyse the required metabolic flux. In contrast with the redox-modulated enzymes in chloroplasts which are activated by the ferredoxin/thioredoxin system [for a review, see Scheibe (1990)], the information about the regulatory properties of these enzymes in heterotrophic tissues is scarce and needs to be elucidated.

According to the data in Table 6, most plastids use Glc6P as the most effective precursor for starch synthesis. This is in contrast with the findings of Tyson and ap Rees (1988), who used isolated amyloplasts from wheat endosperm. They demonstrated that this type of plastid most efficiently incorporates Glc1P into starch. However, the ability to use a certain external substrate for starch biosynthesis apparently depends upon the addition of various effectors (e.g. ATP and PGA; Table 6). We therefore suggest that using other combinations of precursors and effectors might lead to differing results.

As demonstrated, most plastids contain appreciable amounts of NADP⁺- and NAD⁺-MDH (Figure 3 and Table 5). The occurrence in heterotrophic plastids of enzymes which were formerly supposed to be solely localized in chloroplasts is not new (Bradbeer, 1976; El-Shora and ap Rees, 1991). Etioplasts purified from dark-grown barley leaves contain both types of MDH (Figure 3 and Table 5). The physiological function of these enzymes could be the removal of reducing equivalents generated during plastidic starch synthesis, a process which depends upon the oxidation of DHAP in order to yield ATP (Batz et al., 1992). Kleppinger-Sparace et al. (1992) demonstrated that amyloplasts purified from pea roots incorporate [¹⁴C]acetate into fatty acids. Their synthesis is strongly activated by the addition of oxaloacetate (OAA) and DHAP to the incubation medium. This group therefore proposed that the oxidation of

DHAP leads to the formation of ATP and NAD(P)H₂, the latter being removable by the reduction of OAA to malate via a plastidic NAD⁺- or NADP⁺-MDH. This observation supports the view that acetate-dependent fatty acid synthesis, which is driven by DHAP and OAA, involves the co-operative action of plastidic GAPDH and MDH. As discussed for the plastidic FBPase, the activation of these redox-modulated enzymes is unknown in non-green plastids.

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REFERENCES

- Batz, O., Scheibe, R. and Neuhaus, H. E. (1992) *Plant Physiol.* **100**, 184–190
- Bergmeyer, H. U. (1974) *Methoden der enzymatischen Analyse*, VCH-Verlag, Weinheim
- Borchert, S., Gross, H. and Heldt, H. W. (1989) *FEBS Lett.* **253**, 183–186
- Borchert, S., Harborth, J., Schünemann, D., Hoferichter, P. and Heldt, H. W. (1993) *Plant Physiol.* **101**, 303–312
- Bowsher, C. G., Huckleby, D. P. and Emes, M. J. (1989) *Planta* **177**, 359–366
- Bradbeer, J. W. (1976) in *Perspectives in Experimental Biology*, vol. 2 (Botany), Pergamon Press, Oxford and New York
- Dancer, J. E. (1987) Ph.D. Thesis, University of Cambridge
- Echeverria, E., Boyer, L. D., Thomas, P. A., Lin, K. C. and Shannon, J. C. (1988) *Plant Physiol.* **86**, 786–792
- El-Shora, H. and ap Rees, T. (1991) *Planta* **185**, 362–367
- Entwistle, G. and ap Rees, T. (1988) *Biochem. J.* **255**, 391–396
- Entwistle, G. and ap Rees, T. (1990) *Biochem. J.* **271**, 467–472
- Fickenscher, K. and Scheibe, R. (1983) *Biochim. Biophys. Acta* **749**, 249–254
- Hatzfeld, W. D. and Stitt, M. (1990) *Planta* **180**, 198–204
- Hill, L. M. and Smith, A. M. (1991) *Planta* **185**, 91–96
- Holtum, J. A. M. and Winter, K. (1982) *Planta* **155**, 8–16
- Journet, E.-P. and Douce, R. (1985) *Plant Physiol.* **79**, 458–467
- Kleppinger-Sparace, K. F., Stahl, R. J. and Sparace, S. A. (1992) *Plant Physiol.* **98**, 723–727
- Kruckeberg, A. L., Neuhaus, H. E., Feil, R., Gottleib, L. D. and Stitt, M. (1989) *Biochem. J.* **261**, 457–467
- Mohabir, G. and John, P. (1988) *Plant Physiol.* **88**, 1222–1228
- Neuhaus, H. E., Krause, K.-P. and Stitt, M. (1990) *Phytochemistry* **29**, 3411–3415
- Neuhaus, H. E., Henrichs, G. and Scheibe, R. (1993) *Plant Physiol.* **101**, 573–578
- Pozueta-Romero, J., Frehner, M., Viale, A. M. and Akazawa, T. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5769–5773
- Pozueta-Romero, J. and Akazawa, T. (1993) *J. Exp. Bot.* **44** (Suppl.), 297–306
- Preiss, J. (1982) *Annu. Rev. Plant Physiol.* **33**, 431–454
- Salvucci, M. E., Drake, R. R., Haley, B. E., Hanson, K. R. and Hale, N. A. (1990) *Plant Physiol.* **93**, 105–109
- Scheibe, R. (1990) *Bot. Acta* **103**, 327–334
- Scheibe, R. and Stitt, M. (1988) *Plant Physiol. Biochem.* **26**, 473–481
- Scheibe, R., Reckmann, U., Hedrich, R. and Raschke, K. (1990) *Plant Physiol.* **93**, 1358–1364
- Sowokinos, J. R. (1976) *Plant Physiol.* **57**, 63–68
- Stitt, M., Lilley, R. McC., Gerhardt, R. and Heldt, H. W. (1989) *Methods Enzymol.* **174**, 518–552
- Tetlow, I. J., Blisset, K. J. and Emes, M. J. (1993) *Planta* **189**, 597–600
- Tyson, R. H. and ap Rees, T. (1988) *Planta* **175**, 33–38
- Yonuschot, G. R., Ortwerth, B. J. and Koepp, O. J. (1970) *J. Biol. Chem.* **245**, 4193–4198