Starch Synthesis and Carbohydrate Oxidation in Developing Potato Tuber Amyloplasts

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Abstract: Starch, the major source of caloric intake in the human diet, is synthesised in the amyloplasts of non-photosynthetic tissues. To understand its synthesis, to determine which substrate is taken up by the organelle and which metabolite is broken down for the release of energy, the present studies were started from potato tuber (cv. Record). Glucose 1-phosphate (Glc1P) was the most effective substrate for starch synthesis in amyloplasts. The rates of incorporation of hexose (Glc1P) into starch were dependent on the presence of exogenous ATP and on the saturated by 5-10 mM Glc1P. The uptake of Glc1P was inhibited by the addition of exogenous glucose 6-phosphate (ADPG) also supported similar rates of starch synthesis, however, rate was saturated by 1mM ADPG. The import membrane. Carbohydrate oxidation in the amyloplast was stimulated by the addition of 2-oxoglutarate and phosphate. The implications of these results for our understanding of the pathway of starch synthesis and carbohydrate oxidation are discussed.

Keywords: Carbohydrate oxidation, amyloplast, 2-oxoglutarate, glutamine and Potato

Introduction
Atmospheric free carbon is stored in plants in the form of starch and makes up 50% or more of the dry weight of many storage organs (Burton et al. 1995). Amyloplasts are the plastids that accumulate starch. They are usually found in storage tissues i.e. endosperm, roots and tubers of plants. They lie in tissues that under normal conditions receive no light, which clearly shows that the enzymatic machinery of starch formation in these cells is independent of photosynthesis. Synthesis of starch in amyloplasts takes place after the translocation of sucrose from photosynthetic tissues. The amyloplast membrane is impermeable to sucrose and therefore it must be degraded into a form which can cross the amyloplast membrane. Starch synthesis in the amyloplasts of non-photosynthetic tissues depends upon the import of carbon compound(s) from the cytosol. Work with a variety of tissues has indicated that a six carbon compound enters the amyloplast for starch synthesis (Hatzfeld and Stitt, 1990; Viola et al., 1991). This is supported by work with amyloplasts isolated from wheat endosperm (Tyson and Rees, 1988) which preferentially incorporate 14C from glucose 1-phosphate into starch. However, the preferred substrate for starch synthesis and carbohydrate oxidation in amyloplasts isolated from pea embryos (Hill and Smith, 1991) and pea roots (Boswer et al., 1989) appears to be glucose 6-phosphate. It has also been suggested that some amyloplasts may take up ADP-glucose directly from the cytosol for starch synthesis (Pozueta-Romero et al., 1991).

Using a rapid method, we have isolated amyloplasts from the developing potato tuber (Naeem et al., 1997), allowing us to determine the substrates which are most likely to enter the amyloplast and support starch synthesis in vivo. An assessment of the impact of carbohydrate oxidation on starch synthesis within purified organelles has been carried out by supplying the substrate for the test amylase and stimulating the OPPP (Boswer et al., 1992).

Materials and Methods
Chemicals: Enzymes, Substrates and Co-factors were from Sigma (UK) or Boehringer Corp. (UK). Nycodenz was bought from Nycomed Co., UK.

Plants. All experiments were performed using developing potato tubers (Solanum tuberosum L. cv. Record). Plant stock was maintained by culturing tissue in a regular order. Each sample was grown in 500 ml jars (50 mm x 95 mm) containing 25 ml nutrient medium at 20 °C and 18 h photoperiod. Sub-culturing was carried out in an air flow chamber at 15 °C. During culturing, aseptic technique was practised (Naeem et al., 1997). The plants were placed in pots containing mixture of Levington M2 compost and Silvaperi in a 1:1 ratio. Tubers developed after three months from initial culturing. Uniform tubers approximately 3-4 cm across were used as the material for amyloplast isolation. Fresh tubers were harvested on the day of the experiment. The tubers were peeled to remove the skin / pigmment. The washed and peeled tubers were sliced into small discs. During preparation sliced discs were placed on ice in wash buffer until all the tubers were sliced. The wash buffer was then removed and replaced with 100 ml extraction buffer (Tietlow et al., 1993). Sliced potato discs were kept in extraction Buffer for at least 30 minutes. The extraction buffer was removed and the tissues were rapidly chopped within 5 minutes with a sharp razor blade until pieces were less than 1 mm square size in 2 ml extraction buffer. The homogenate was filtered through 6 layers of muslin, previously dampened with the extraction buffer. The crude extract produced contained a mixture of intact amyloplasts, starch grains, cytosolic and mitochondrial enzymes and cellular debris. All enzyme assays were performed on UV spectrophotometer (Cecil 5000 series UK.) at 25 °C. For determination of organelle distribution after passing through Nycodenz the amyloplasts were freeze-thawed three times with liquid nitrogen and the contents were centrifuged at 10,000g for 5 minutes. The supernatant was used as a source for enzyme assays. (Tietlow et al., 1993). The purified amyloplasts were used to study the incorporation of 14C-labelled substrate into starch. Metabolism of 14C-labelled substrate. Using purified amyloplasts incorporation of radioactivity from 14C-labelled substrate into starch was assayed in the following medium: 50 mM Hepes (pH 7.5), 1 mM Sucrose or Sorbitol, 1 mM KCl, 2 mM MgCl2, 1 mM EDTA, 0.1% (w/v) bovine serum albumin (BSA), 5 mM 14C-labelled substrate. In some experiments 1 mM ATP was added. Assays contained 1 ml plastid cuvette in a final volume of 500 ml. Reactions were initiated by the addition of 14C-labelled substrate (3.7-7.4 kBq per sample), incubated at 25 °C for 60 minutes and mixed by gentle inversion after every 20 minutes. Controls contained boiled plastid and or were stopped within 15s of starting the incubation by the addition of 1 ml 75% (v/v) aqueous methanol-1% (w/v) KCl (methanol-KCl). Values for incorporation into starch were corrected by the values obtained in the zero-time boiled controls. Controls using lysed plastid were prepared by subjecting samples to three cycles of freezing in liquid nitrogen and thawing at 25 °C. For each assay triplicate samples of intact and ruptured amyloplasts were used. Reactions were stopped by the addition of 1 ml methanol-KCl and left to stand for 15 minutes. Samples were then centrifuged at 10,000g for 5 minutes. The supernatant was discarded and the starch containing pellet was washed a total of three times by resuspension in 1 ml water. This was then taken up in 500 mM 50 ml sodium acetate (pH
Results & Discussion

To determine which metabolite(s) may act as substrates supporting starch synthesis, intact and ruptured (freeze-thawed 3 times) plastids were incubated with ¹⁴C-labelled metabolites in the absence and presence of ATP. Whereas, the net value is that which is dependent upon the integrity of the organelles.

Table 1 shows the amount of label appearing as released carbon dioxide from plastid supplied with varied combinations of substrates for 1h at 25°C. Significant net release of labelled carbon dioxide was only observed when plastid were incubated with Glc1P, ATP, glutamine and oxoglutarate. There was some evidence that CO₂ from Glc1P plus ATP was observed in intact and ruptured plastid but the values in ruptured plastid were higher than those from intact plastid. When [U-¹⁴C]Glc6P was used as a substrate with ATP and with or without glutamine and 2-oxoglutarate, no CO₂ evolution was detected above the background value for the time zero control.

The pathway of carbon from translocated sucrose to storage starch in non-photosynthetic cells of potato tuber is controversial. One of the most important unanswered questions is which compounds cross the amyloplast envelope to support starch synthesis therein? There is no doubt about the sucrose of carbon and energy for starch synthesis in chloroplast, but starch synthesis in amyloplasts depends on the uptake of both carbon precursor and energy from the cytosol.

Historically, different views have been put forward about the precursors which enter the amyloplast stroma for the synthesis of starch. Proposed precursors have included triose-phosphate, hexose-phosphate and ADP-glucose.

Of the metabolites tested, it appears that Glc1P is transported into the amyloplasts where it acts as a substrate for the synthesis of starch, a process which also depends on exogenous ATP. Thus the present investigations reveal one possible route for the synthesis of starch. Sucrose is degraded into Glc1P, which is transported via a phosphate-translocase type protein into the amyloplast. In the amyloplast, Glc1P is converted into ADPG and then into starch, and PPI is formed as a by-product.

If Glc1P does enter the amyloplast for starch synthesis via 1:1 counter exchange with Pi then the concentration of Pi inside the organelle will still increase because pyrophosphate, a by product
Substrates for starch biosynthesis in purified amyloplasts from potato tuber. Each replicate consisted of three samples of intact and three ruptured (freeze-thawed three times) amyloplasts prepared from the same preparation. Amyloplasts were incubated for 1h at 25°C with labelled sugars and sugar phosphates (5 mM) with or without 1 mM ATP. Incorporation of isotope into starch was determined. Bars shown are the net rate of incorporation (intact minus ruptured) of labelled substrate into starch. Each value is the mean ± S.E. of the number of independent amyloplast preparations.

Table 1: Carbohydrate oxidation in amyloplasts isolated from developing potato tuber. Intact and ruptured (freeze-thawed 3 times) preparations of amyloplasts were incubated with either 5 mM Glc1P or Glc6P with 1 mM ATP and in the presence or absence of 5 mM 2-oxoglutarate and 5 mM glutamine (2OG+Gln). The data presented give the amount of 14CO2 from the labelled substrates recovered as evolved 14CO2 after 1h incubation at 25°C. Each value is the mean ± S.E. of three replicated experiments. The P-value indicates any significant statistical difference between intact and ruptured amyloplasts within different treatments.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>CO2 (nmol CO2/unit APPase)</th>
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<tbody>
<tr>
<td></td>
<td>Intact</td>
</tr>
<tr>
<td>Glc1P + ATP + 2OG + Gln</td>
<td>1333.9 ± 265.7</td>
</tr>
<tr>
<td>Glc6P + ATP + 2OG + Gln</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glc1P + ATP</td>
<td>200.0 ± 82.8</td>
</tr>
<tr>
<td>Glc6P + ATP</td>
<td>N.D.</td>
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N.D. = Not detectable

Effect of elevated concentrations of Glc6P and 5 mM 3PGA on incorporation of [U-14C]-Glc1P (5mM) into starch. Assays were performed in the presence of 1mM ATP. Bars shown are the difference between the values obtained with intact and ruptured preparations incubated at 25°C. The reactions were terminated by the addition of 1 ml methanol/KCl. Results are the mean of 3 experiments ± S.E. 1, 10 mM Glc6P + 5 mM Glc1P + 1 mM ATP; 2, 20 mM Glc6P + 5 mM Glc1P + 1 mM ATP; 3, 5 mM 3PGA + 5 mM Glc1P + 1mM ATP; 4, 5 mM Glc1P + 1 mM ATP.

of starch synthesis is cleaved into 2 Pi (Preiss, 1993). Under such conditions, presumably another mechanism must operate to remove the additional phosphate. However, Ambudkar et al. (1986) demonstrated a variable stoichiometry of Pi/Glc6P in Streptococcus lactis. They observed 2:1 stoichiometry of Pi/Glc6P instead of 1:1 in the membrane vesicles of S. lactis and further revealed that the exchange stoichiometry was dependent on pH. On the other hand, pH had no effect on the stoichiometry of the exchange catalysed by the reconstituted wheat amyloplast translocator (Tetlow et al., 1996).

Therefore, it is argued that another mechanism must operate for the removal of the additional Pi. Neuhaus and Maaß (1996) while they were working on cauliflower bud amyloplasts, reported the unidirectional transport of Pi from the amyloplasts. But it is unclear in the case of potato tuber amyloplasts, whether this is a function of the bi-directional hexose-phosphate translocator or a separate protein, though Schwartz et al. (1994) have demonstrated that the triose-phosphate translocator of spinach chloroplast possesses this ability.

The plastid used in the present study were unable to support relevant rates of starch synthesis from Glc1P in the absence of exogenous ATP. Dependence of starch synthesis on exogenous ATP has been shown in plastid from many species (Hill and Smith, 1991; Neuhaus et al., 1993; Tetlow et al., 1994). Starch synthesis from Glc1P in the absence of ATP is likely to involve a reversal of the starch phosphorylase reaction, which is generally regarded as having a role in starch breakdown (Smith et al., 1995).

An important question is whether the present rates of starch synthesis observed in vitro are relevant to the rates of starch synthesis in vivo. Comparison of these entities is somewhat difficult at this stage due to the lack of sufficient information in the literature. However, ap Rees and Morrall (1990) reviewed that ADPglucose starch synthase, ADPglucose and alkaline are present in sufficient amounts to mediate the rates of starch synthesis in vivo, and are confined to the amyloplast. Other than Glc1P, the other substrate which seems to be able to support starch synthesis is ADPglucose (ADPG). In this way sucrose is degraded into ADPG outside the amyloplast membrane. ADPG thus formed from cytosolic AGPase crosses the organelle membrane. If this is the route of starch synthesis then it raises the question whether there is enough activity of AGPase present outside the amyloplast in vivo. It has been suggested by Villand and Kleczkowski (1994) that AGPase is extra-amyloplastic in barley seed endosperm. These workers proposed that ADPG formed by the extra-amyloplastic enzyme is transported to the amyloplasts via an ADPglucose carrier in the plastid membrane, and then utilized by the starch synthesising machinery of these organelles.
In addition to the extra-amyloplastic form of AGPase, barley endosperm contains also a second isozyme of AGPase, located in the amyloplasts. There is now substantive evidence in support of this, in which plastidic form and a distinct cytosolic isozyme have been demonstrated in barley endosperm (Thorbjørnsen et al., 1996).

Similarly, immunoblotting of gels of endosperm and plastid extracts from barley and maize also indicated that the enzyme is both inside and outside the plastid (Denyer et al., 1996). Their results suggest that there are distinct plastidial and cytosolic forms of AGPase in these species which are composed of different subunits. The cytosolic AGPase is likely to be involved in the provision of ADPglucose for starch synthesis. Its activity is far in excess of the plastidial form is only slightly greater (barley) or less (maize) than the rate of starch synthesis. However, if AGPase is present outside the amyloplast then this will affect the turnover of Ppi. Kléczkowski (1994) has suggested that activity of a cytosolic AGPase could be coupled to that of UDPglucose pyrophosphorylase (UGPase) through production and consumption of pyrophosphate and Glc1P, potentially allowing integration of the breakdown of sucrose and the synthesis of starch. Similarly, parallel to formation of starch, the possibility that the extra-plastidial AGPase provides ADPGlucose for some other process, for example the synthesis of the considerable amounts of 6-glucans found in barley endosperm (Bhatty et al., 1991) can not be ruled out.

The synthesis of starch from Glc6P was not measurable and supports the previous observations of Coates and ap Rees (1994) and Tetlow et al., 1994 etc. that Glc1P and not Glc6P is the preferred hexose phosphate entering the organelle for starch synthesis. The fact that Glc6P was not able to act as a substrate for starch synthesis also reveals that there is little interconversion between Glc6P and Glc1P outside the organelle by contaminating phosphoglucomutase, which is either released from broken organelles or present as a cytosolic contaminant (Borchert et al., 1993).

In addition to the enzyme capacities of starch synthesis in potato tuber amyloplasts, the first two enzymes of the oxidative pentose phosphate pathway (OPPP), glucose 6-phosphate and 6-phosphogluconate dehydrogenase are present in potato tuber amyloplasts (Naem et al., 1997). This is consistent with other studies which indicate that amyloplasts contain significant capacity for carbohydrate oxidation as well as starch synthesis (Tetlow et al., 1994; Coates and ap Rees, 1994).

The stimulation of CO2 evolution from Glc1P brought about by the combined presence of glutamine and 2-oxoglutarate is evidence that the OPP is responding to a demand for reductant from glutamate synthesis (Bowsher et al., 1992). It has previously been observed by Tetlow et al. (1991) that when Glc1P plus ATP were supplied to amyloplasts of wheat endosperm, rates of starch synthesis were reduced by 75% when carbohydrate oxidation was driven by the glutamate synthase reaction, which implies that the need for hexose phosphates to sustain carbohydrate oxidation in wheat endosperm amyloplasts had the potential to act as a considerable drain on carbon for starch synthesis may involve a reduction in starch synthesis in potato. Besides being involved in the synthesis of starch in potato tuber amyloplasts, Glc1P also acts as a substrate for the OPPP. This contrasts with other studies which have demonstrated that Glc6P is the best substrate for oxidation in amyloplasts of plant species (Bowsher et al., 1992; Tetlow et al., 1994; Coates and ap Rees, 1994). Though Glc6P was unable to act as a substrate for oxidation, the pathway could not proceed without synthesis of Glc6P. Therefore, it must be concluded that in amyloplasts there is enough activity of phosphoglcomutase present, able to isomerise Glc1P to Glc6P and hence complete the OPPP.

References


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