Reconstitution of the hexose phosphate translocator from the envelope membranes of wheat endosperm amyloplasts

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Amyloplasts were isolated and purified from wheat endosperm and the envelope membranes reconstituted into liposomes. Envelope membranes were solubilized in n-octyl β-D-glucopyranoside and mixed with liposomes supplemented with 5.6 mol% cholesterol to produce proteoliposomes of defined size, which showed negligible leakage of internal substrates. Transport experiments with proteoliposomes revealed a counter-exchange of glucose 1-phosphate (Glc1P), glucose 6-phosphate (Glc6P), inorganic phosphate (P_i), 3-phosphoglycerate and dihydroxyacetone phosphate. The Glc1P/P_i counter-exchange reaction exhibited an apparent K_m for Glc1P of 0.4 mM. Glc6P was a competitive inhibitor of Glc1P transport (K_i 0.8 mM), and the two hexose phosphates could exchange with each other, indicating the operation of a single carrier protein. Glc1P/P_i antiport in proteoliposomes showed an exchange stoichiometry at pH 8.0 of 1 mol of phosphate transported per mol of sugar phosphate.

INTRODUCTION

The process of starch biosynthesis in the amyloplasts of non-photosynthetic plant tissues is dependent upon the import of ATP and a source of carbon skeletons [1]. Investigations in whole tissues from a variety of starch synthesizing crop plants have indicated that hexose units are taken up into the plastids as precursors for starch [2,3]. Studies with isolated amyloplasts have shown that the specific hexose units transported appear to be tissue/species-dependent. Starch synthesis in plastids isolated from pea cotyledons [4], pea roots [5–8] and cauliflower buds [9] was shown to be dependent on glucose 6-phosphate (Glc6P). However, glucose 1-phosphate (Glc1P), and not Glc6P, was able to support starch synthesis in amyloplasts isolated from wheat endosperm [10,11] and suspension-cultured soybean [12]. Amyloplasts isolated from potato have also been shown to synthesize starch from Glc1P [13], although in that study the capacity of Glc6P to support starch synthesis was not tested. Maize endosperm amyloplasts can synthesize starch from both Glc1P and Glc6P [14]. Recent findings have shown that some amyloplasts utilize Glc1P most effectively for starch biosynthesis, while Glc6P is the preferred substrate for carbohydrate oxidation via the oxidative pentose phosphate pathway, implying that both hexose phosphates can be transported across the amyloplast envelope [11,12].

Silicone oil centrifugal filtration studies with intact wheat endosperm amyloplasts have indeed shown that these organelles have the ability to transport both Glc1P and Glc6P [15]. However, these studies have been limited because metabolite transport using silicone oil centrifugal filtration in extremely fragile, starch-rich, organelles is problematical. In such studies a proportion of the organelle preparation will be ruptured, exposing labelled hexose phosphates in the assay medium to interconversion by plastidial phosphoglucomutase. This can lead to a misleading interpretation of the ability of substrates to cross the plastid envelope [5]. Isotope also adheres to naked starch grains, making rates of metabolite uptake difficult to determine [15]. Establishing the nature of carbon translocation into amyloplasts, and the characterization of the transporter(s) involved, is essential for a complete understanding of starch synthesis, influencing our view of the number of steps involved.

The problems of measuring transport in isolated organelles can be overcome by reconstituting membrane transporters into artificial membranes (liposomes) prepared by sonication of phospholipid mixtures [16,17]. Although such reconstituted systems cannot mimic physiological conditions, they offer a means of investigating transport parameters in isolation and in detail. One advantage of using a reconstituted system is that the transport of a metabolite is not influenced by its subsequent metabolism. Schünemann and Borchert [18] showed that, when the envelope proteins from tomato fruit chromoplasts were reconstituted into proteoliposomes, both Glc6P and Glc1P as well as other substrates could be transported and counter-exchange with P_i. Liposome preparations containing envelope proteins of amyloplasts from transgenic potato tubers have been reported to transport Glc6P, but not Glc1P [19].

The purpose of the present study was to analyse the transport of hexose phosphates, associated with the amyloplast membrane in wheat endosperm, with respect to substrate specificity and to determine the stoichiometry of exchange reactions. An efficient reconstitution protocol is an essential part of this approach, and we report a new procedure for incorporating functional amyloplast envelope proteins into liposomes.

EXPERIMENTAL

Materials

L-α-Phosphatidylcholine (PC) (Soy, Type II S), L-α-dipalmitoyl phosphatidylcholine (DPPC), cholesterol, L-α-phosphatidylinositol (PI), Sephadex G-50, sodium orthovanadate, sugar phosphates and the non-ionic surfactant n-octyl β-D-glucopyranoside (OBG) were purchased from Sigma Chemical Co., Poole, Dorset, U.K. 5-(*N*-2,3-Dihydroxypropylacetamido)-2,4,6-tri-

Abbreviations used: APPase, alkaline pyrophosphatase; CMC, critical micelle concentration; DHAP, dihydroxyacetone phosphate; DPPC, L-α-dipalmitoyl phosphatidylcholine; Glc1P, glucose 1-phosphate; Glc6P, glucose 6-phosphate; MLV, multilamellar vesicle; OBG, n-octyl β-D-glucopyranoside; PC, L-α-phosphatidylcholine; PI, L-α-phosphatidylinositol; SUV, small unilamellar vesicle.

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iodo-*N*,*N*′-bis-(2,3-dihydroxypropyl)isophthalamide (Nycodenz) was purchased from Nycomed (U.K.) Ltd., Birmingham, U.K. [^{3}H]DPPC, [^{14}C]Glc1P and [^{14}C]Glc6P were from Amersham International, Amersham, Bucks., U.K. [^{32}P]P_i was from ICN Pharmaceuticals Ltd., Thame, Oxon., U.K. Cellulose nitrate filters (0.2 μm pore size; 47 mm diameter) were purchased from Whatman International (Maidstone, Kent, U.K.). Anion-exchange resin (AG 1-X8) was purchased from Bio-Rad Laboratories Ltd. (Hemel Hempstead, Herts., U.K.). All other reagents were of analytical grade, and aqueous solutions were made up with double-distilled water.

Plant material and amyloplast envelope isolation

Spring wheat (*Triticum aestivum* L. cv. Axona) was grown in John Innes No.3 compost in a greenhouse at 15–25 °C under a 16 h photoperiod in daylight supplemented with sodium light (photosynthetically active radiation; 160 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Plants were fed weekly with 1.7 $g \cdot l^{-1}$ Phostrogen (Phostrogen, Corwen, Clwyd, U.K.). Amyloplasts were mechanically isolated from developing wheat endosperm dissected from grains taken from the mid-ear region of the head 7–11 days after anthesis (the first appearance of anthers). Between 1500 and 2000 grains of uniform size were used to prepare intact amyloplasts containing $(2–6) \times 10^{-2}$ units ($\mu mol \cdot min^{-1}$) of alkaline pyrophosphatase (APPase) activity $\cdot cm^{-3}$. The amyloplasts were purified by centrifuging twice through a layer of 2 % (w/v) Nycodenz as previously described [20]. The maximum cytosolic contamination of the plastids used was less than 0.5 %, based on recoveries of UDP-glucose pyrophosphorylase and pyrophosphate:fructose-6-phosphate 1-phosphotransferase (see below); recovery of the mitochondrial marker enzyme citrate synthase in this fraction was less than 0.2 %. Recovery of the amyloplast marker enzyme APPase in the pellet varied between 17 and 25 %. Amyloplasts were ruptured by three cycles of freezing (in liquid nitrogen) and thawing on ice followed by centrifugation at 13000 ***g*** for 2 min in a Beckman Microfuge E to remove starch grains. The supernatant was then centrifuged at 100000 ***g*** [207 kPa (30 p.s.i.)] for 15 min in a Beckman Airfuge. The resulting membrane pellet was rinsed three times in a buffer containing 100 mM Tricine/KOH, pH 8.0, 250 mM potassium gluconate, 2 mM dithiothreitol, 1 $mg \cdot cm^{-3}$ aqueous lipid mixture (see below) and 20 % (v/v) glycerol (resuspension buffer). The washed amyloplast envelope pellet was resuspended in resuspension buffer and stored on ice before solubilization (see below). Protein was determined using a detergent-compatible kit (Bio-Rad) based on the Lowry protein assay, using gamma globulin as the standard and following the manufacturer's instructions.

Enzyme assays

Sugar phosphatase activity was measured in amyloplast membrane preparations as the production of free sugar from Glc1P or Glc6P. Samples and substrates were prepared in 10 mM Mes/NaOH (pH 6.5). The reaction (in a total volume of 34 mm^3) was started by the addition of 4.5 mM [^{14}C]Glc1P or [^{14}C]Glc6P (0.05 μCi per sample) to the sample, followed by incubation at 25 °C for 1 h. The reaction was terminated by the addition of 0.5 cm^3 of an ice-cold solution of 0.5 mM sodium orthovanadate in distilled water and applying the mixture to an anion-exchange column (Bio-Rad AG 1-X8, acetate form, 100–200 mesh) pre-equilibrated in Mes/NaOH buffer (as above). Free sugar was measured as the radioactivity eluted with distilled water. Control reactions were stopped at zero time. Recovery of labelled free sugars was greater than 95 % in control experiments using glucose 6-phosphatase from rabbit microsomes (Sigma).

The following enzymes were assayed as previously described: APPase (EC 3.6.1.1) [20], phosphoglucomutase (EC 5.4.2.2) [21], cytochrome *c* oxidase (EC 1.9.3.1) [22], UDP-glucose pyrophosphorylase (EC 2.7.7.9), pyrophosphate:fructose-6-phosphate 1-phosphotransferase (EC 2.7.1.90) and citrate synthase (EC 4.1.3.7) [20].

Preparation and characterization of liposomes

Liposomes (vesicles) were prepared by sonicating aqueous lipid mixtures. Stock solutions of lipids were prepared by dissolving 1.5 g of acetone-washed soy PC, 60 mg of PI, 10 mg of DPPC and 42 mg of cholesterol in a 10 cm^3 mixture of distilled chloroform/methanol [4:1, v/v; stored with sodium-alumino silicate pellets (molecular sieve Type 4A; BDH, Poole, Dorset, U.K.)]. The solution was divided into two portions, each in 100 cm^3 round-bottomed flasks; to one of these was added [^{3}H]DPPC (10 μCi). The solvent was removed by rotary evaporation at 60 °C to leave a thin, even, lipid film to which was added 15 cm^3 of a buffer containing 100 mM Tricine/KOH (pH 8.0) and 250 mM potassium gluconate (buffer A) at 60 °C. In some experiments the Tricine in buffer A was replaced by 100 mM Mes and adjusted to pH 5.0. The suspension was vigorously mixed on a vortex mixer to form multilamellar vesicles (MLVs) and stored at 4 °C. Aliquots of MLVs were diluted 10-fold in buffer A including internal substrates (as noted in the text) to give a final lipid concentration of 14 mM. Control liposomes were diluted in buffer A only. Unilamellar vesicles (SUVs) were formed by sonicating 0.5–2.0 cm^3 of the MLVs in a bath-type sonicator (Decon, FS100) at room temperature for 20 min in a Pyrex test tube (25 mm × 150 mm) under a constant stream of nitrogen until the solution cleared. The sonicator bath contained 1 % (v/v) Decon detergent (Decon Laboratories Ltd., Hove, E. Sussex, U.K.), and the level in the bath was adjusted to give maximal agitation of the solution [23]. Liposome size distribution after preparation, during the interaction with OBG, and after freezing and thawing was determined by photon correlation spectroscopy using a Malvern Autosizer (model RR146). Turbidity measurements of liposome/proteoliposome solutions during interactions with OBG were made by measuring absorption at 560 nm on a Cecil (CE5501W) spectrophotometer. The assays were carried out in triplicate and the results given are the means of those obtained.

Solubilization experiments

Washed amyloplast envelope membrane proteins (1.2–1.5 mg of protein $\cdot cm^{-3}$) were solubilized with various concentrations of OBG dissolved in resuspension buffer (above) for 5 min on ice. Samples were then centrifuged at 100000 ***g*** for 15 min, and aliquots of the supernatant were assayed for protein to determine the extent of solubilization. The protein content of the pellet (washed three times in resuspension buffer) was also determined, and total protein values were used to determine recovery in each fraction.

Reconstitution of amyloplast membrane proteins

Envelope membrane proteins (100 mm^3) from wheat endosperm amyloplasts were solubilized in 4 % (w/v) OBG (136.8 mM) dissolved in resuspension buffer (see above) for 5 min on ice. Sonicated liposomes (SUVs; 700 mm^3) were added to the solubilized proteins (0.6–1.2 mg of protein $\cdot cm^{-3}$ of SUVs) so that the resulting OBG concentration in the mixture was 0.5 % (w/v) (17.1 mM). This dilution brought the OBG below its critical micelle concentration (CMC) of 0.73 % (w/v) (23 mM), so that

proteoliposomes formed as detergent dispersed in the aqueous phase. The mixture was quickly frozen in liquid nitrogen for at least 3 min before being thawed on ice [23]. Proteoliposomes were then sonicated for 20 s and subsequently kept on ice. Unentrapped material was removed by passing 500 mm^3 aliquots through a 5 cm^3 column of Sephadex G-50 medium resin (pre-equilibrated in buffer A) by centrifugation at 270 ***g*** (MSE 3000i Benchtop) for 4 min at 4 °C.

Metabolite transport experiments

Transport experiments were initiated by adding proteoliposomes, preincubated at 20 °C for 2–3 min, to labelled substrates, usually potassium dihydrogen orthophosphate ([^{32}P]P_i; 0.1 $\mu Ci \cdot sample^{-1}$) or ^{14}C-labelled sugar phosphates (0.2 $\mu Ci \cdot sample^{-1}$) at 1 mM final concentration. In some experiments labelled internal substrates (usually 10 mM) were used (for details, see the Results section). Uptake was stopped at timed intervals by filtration of the proteoliposomes on presoaked cellulose nitrate filters followed by washing with 10 cm^3 of buffer A. Radioactivity on washed filter discs was then measured with a Packard TriCarb (300C) liquid scintillation counter incorporating a dual-label program to count samples containing both ^{14}C and ^{32}P. Transport reactions stopped at zero time were used as controls for all experiments described. Recovery of proteoliposomes by the filtration method was determined using liposomes supplemented with [^{3}H]DPPC [24].

Leakage experiments

Liposomes lacking amyloplast membrane proteins were prepared as described above with or without cholesterol (5.6 mol %) and preloaded with ^{14}C-labelled Glc1P (10 mM; 2 $\mu Ci \cdot cm^{-3}$) or [^{32}P]P_i (1 $\mu Ci \cdot cm^{-3}$). After sonication, unentrapped material was removed by passage through Sephadex G-50 medium resin by column chromatography (see above). Liposomes (1.2 cm^3) were immediately placed inside 20 mm-diameter dialysis tubing ('Spectrapor 3' molecular porous membrane; molecular mass cut-off 3500 Da; Spectrum Medical Industries Inc.), sealed and briefly rinsed for 10 s in buffer A before being placed in 15 cm^3 of buffer A and gently stirred at 4 °C. Aliquots (100 mm^3) were removed at specific time intervals and radioactivity was determined by liquid scintillation counting.

RESULTS

Liposome characterization

The size distribution of a population of PC/PI/DPPC/cholesterol liposomes is shown after bath sonication of MLVs (Figure 1). Before sonication, liposomes were multilamellar (possessed more than one lipid bilayer) and covered a wide range of sizes. After sonication, the liposomes were a relatively homogeneous population of SUVs, having an average diameter of 101.0 ± 5.0 nm ($n = 10$ preparations). The freeze–thaw procedure during the reconstitution process produced a more heterogeneous population of liposomes as they fused together (Figure 1). Brief sonication after freezing and thawing resulted in a more homogeneous population of liposomes, so that proteoliposomes made by incorporation of amyloplast membrane proteins into SUVs showed a similar size distribution to the SUVs, having an average diameter of 110.2 ± 7.3 nm ($n = 10$ preparations). Scintillation counting before and after SUV formation showed that greater than 99 % of the [^{3}H]DPPC from the multilamellar vesicles was incorporated into the SUVs.

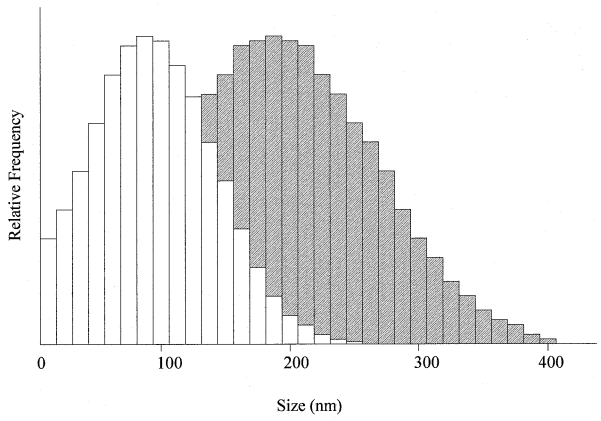

Figure 1 Size distributions of liposomes produced by sonication of MLVs used for the reconstitution of amyloplast membrane proteins

The liposome populations shown are the result of freezing and thawing during the reconstitution process (shaded) followed by brief sonication of this heterogeneous population to form a more homogeneous population of liposomes (unshaded). Liposome composition was PC/PI/DPPC/cholesterol (molar ratio 1:0.04:0.006:0.03). Liposome size distribution was determined by photon correlation spectroscopy at 25 °C.

Solute leakage from SUVs

When studying metabolite transport in reconstituted systems, it is important to minimize the loss of liposome contents by diffusion/leakage. This was investigated using liposomes in which amyloplast envelope proteins had not been incorporated.

The leakage of [^{14}C]Glc1P from PC/PI/DPPC SUVs was determined at 4 °C in buffer A (Figure 2). During the time course of typical transport experiments (from the gel-filtration step to the termination of transport on the membrane filters), which was between 20 and 30 min, up to 13 % of the initial amount of ^{14}C

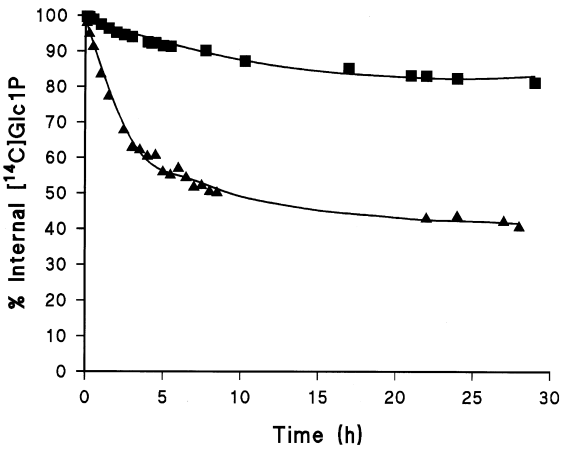

Figure 2 Leakage of entrapped solutes from liposomes

Liposomes composed of PC/PI/DPPC (▲) and similar liposomes supplemented with 5.6 mol% cholesterol (■) were preloaded with 10 mM [^{14}C]Glc1P, placed in dialysis bags at 4 °C and the external medium gently stirred. Leakage was measured as the recovery of ^{14}C in the external medium over the time course shown, and is expressed as a percentage of the [^{14}C]Glc1P in the dialysis bag at zero time.

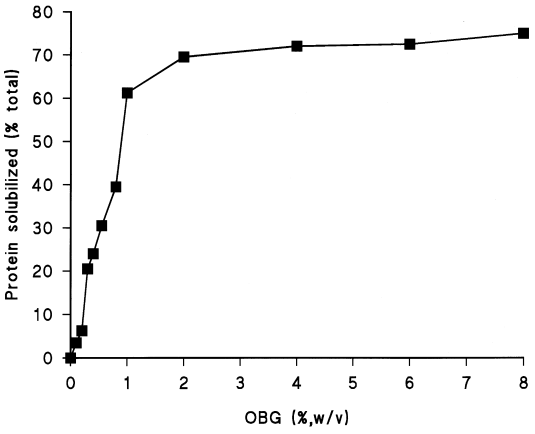

Figure 3 Solubilization of amyloplast membrane proteins by OBG

Amyloplast membrane proteins (1.2–1.5 mg · cm^{-3}) were treated with various concentrations of OBG for 5 min on ice. Protein solubilized by OBG was that amount recovered from the supernatant after the mixture had been centrifuged at 100000 ***g*** for 15 min.

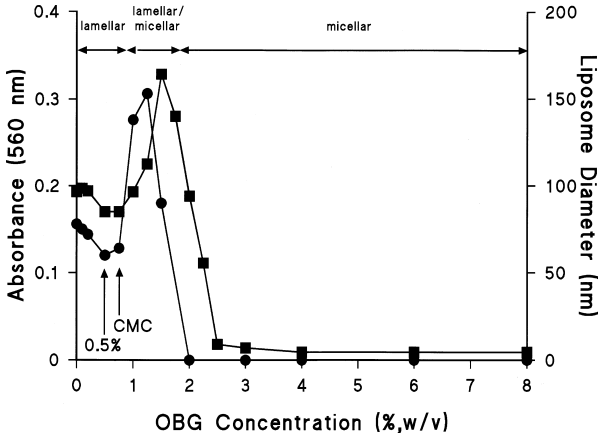

Figure 4 Stability of liposomes in OBG

The integrity of samples of liposomes at various concentrations of OBG was assessed by measuring the average diameter of the sample population using photon correlation spectroscopy (●) and the light scattering of the sample at 560 nm (■). Liposomes were mixed with the indicated concentrations of OBG and incubated for 5 min at 4 °C before measurements were taken. After reconstitution of amyloplast membranes into liposomes, the resulting proteoliposomes contained 0.5% (w/v) OBG, which is less than the CMC of the detergent (0.73%, w/v), as indicated by the arrows. The various transition structural stages of the liposomes from lipid bilayers (lamellar) to mixed micelles (micellar) are indicated.

loaded was lost from the liposomes. The addition of cholesterol (5.6 mol%) during SUV formation resulted in a considerable reduction in [^{14}C]Glc1P leakage (Figure 2). PC/PI/DPPC/cholesterol SUVs lost less than 1% of the initial amount of [^{14}C]Glc1P loaded over a 45 min period. Similar results were obtained in leakage experiments with SUVs loaded with [^{32}P]P_i (results not shown). All subsequent experiments were therefore performed with SUVs and proteoliposomes supplemented with 5.6 mol% cholesterol.

Solubilization of amyloplast membrane proteins by OBG

Prior to solubilization and reconstitution, washed envelope membranes from purified wheat endosperm amyloplasts were assayed for phosphoglucomutase and cytochrome *c* oxidase activity. In membrane samples containing up to 2.5 mg of protein · cm^{-3}, neither enzyme activity could be detected. Hydrolysis of Glc1P and Glc6P by phosphatase activity was not detected in amyloplast membrane preparations (results not shown).

Washed amyloplast membranes (1.2–1.5 mg of protein · cm^{-3}) were solubilized in increasing concentrations of OBG for 5 min on ice. Amyloplast membrane proteins were solubilized at relatively low OBG concentrations (Figure 3), with solubilization reaching a maximum of about 70% of total protein at OBG concentrations of 2% (w/v) and higher. Longer periods of solubilization on ice of up to 30 min did not increase the amount of membrane protein solubilized at any given OBG concentration (results not shown). Higher temperatures for solubilization were not used. Recovery of amyloplast protein in the supernatant and pellet fractions from the solubilization experiments was $90 \pm 6.3\%$ ($n = 5$). Transport activity of solubilized amyloplast membrane proteins reconstituted into liposomes (judged by Glc1P/P_i exchange; see below) was measurable in samples solubilized in concentrations of OBG of 2% (w/v) and greater; transport rates could not be increased in samples solubilized in up to 8% (w/v) OBG.

Stability of liposomes in OBG

It was important to determine whether or not preformed liposomes maintained their structural integrity during the reconstitution procedure. Consequently, the stability of liposomes at various OBG concentrations was assessed using light scattering at 560 nm [25,26] and by measurement of liposome diameters. Measurement of light scattering at 560 nm was a more convenient means of assessing liposome stability than the size distribution measurements, and closely resembled the curve for liposome diameter measurements (Figure 4). The data in Figure 4 show the solubilization curve of liposome suspensions arising from the addition of increasing amounts of OBG, and illustrate the different transition structural stages from lipid bilayers to mixed micelles. At low surfactant concentrations an initial increase in light scattering was observed. This is due to the incorporation of surfactant molecules into the bilayer, which causes an increase in liposome diameter, maximum values being reached for bilayer saturation [about 1.8% (w/v) OBG]. Increased surfactant led to a fall in light scattering and liposome diameter until a low constant value for bilayer solubilization was reached. The data in Figure 4 show the range of final OBG concentrations that can be used in the reconstitution process to maintain structurally stable liposomes. In the optimized reconstitution assay, OBG was diluted to 0.5% (w/v), which is below the detergent CMC and low enough to maintain the stability of the proteoliposome bilayer (Figure 4).

Transport measurements

The transport characteristics of reconstituted wheat endosperm amyloplast membrane proteins were determined by measuring the antiport of Glc1P/P_i using liposomes preloaded with P_i. Figure 5 shows that transport of [^{14}C]Glc1P into proteoliposomes depends strictly on the presence of internal substrate (P_i). Maximal transport rates were reached when proteoliposomes contained 10 mM P_i or more; these rates were 17-fold greater than when proteoliposomes contained no internal P_i.

Transport activities of Glc1P/P_i exchange were linear with increasing protein/lipid ratios up to 0.6 mg of protein · cm^{-3} liposomes (results not shown). All subsequent transport experi-

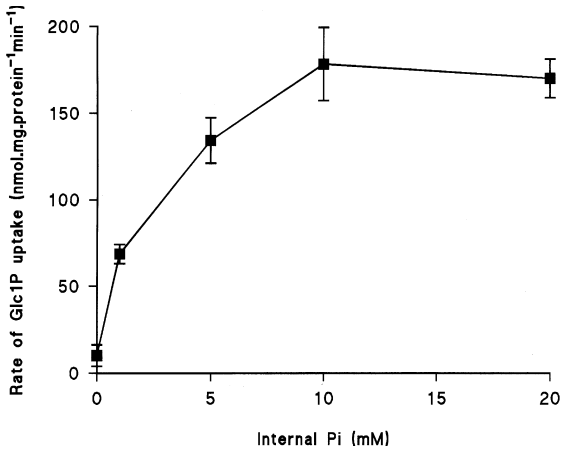

Figure 5 Dependence of Glc1P uptake into proteoliposomes upon the internal liposome concentration of P_i

Amyloplast envelope membranes were solubilized in 4% (w/v) OBG and added to liposomes containing increasing P_i concentrations (112 μg of protein · cm^{-3} liposomes). External medium was removed by gel filtration and transport initiated by the addition of 1 mM [^{14}C]Glc1P. The reaction was stopped after 60 s by filtration and washing of the proteoliposomes on cellulose nitrate filters. Control samples were stopped at zero time. Results are the means ± S.E.M. of three replicate experiments.

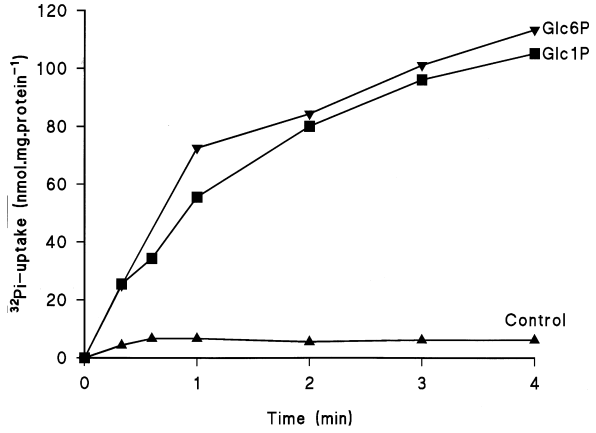

Figure 6 Time course of hexose phosphate/P_i exchange in proteoliposomes containing reconstituted amyloplast membrane proteins

Proteoliposomes were preloaded with 10 mM Glc1P or Glc6P; controls were preloaded with buffer A. Transport, measured at 20 °C, was initiated by addition of 1 mM [^{32}P]P_i. Results are the means of at least three separate experiments.

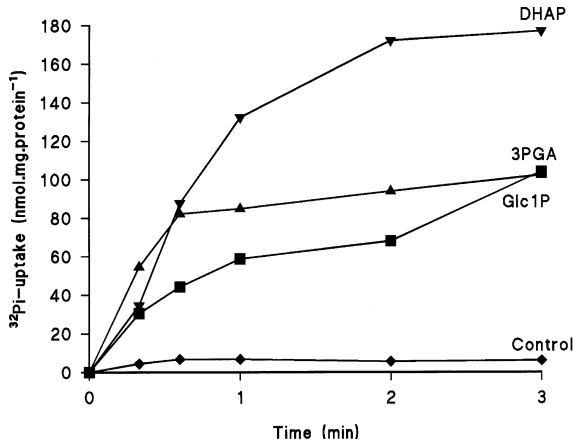

Figure 7 P_i exchange in proteoliposomes containing reconstituted amyloplast membrane proteins preloaded with various metabolites

Transport at 20 °C was initiated by addition of 1 mM [^{32}P]P_i to the proteoliposomes containing various metabolites (10 mM) as indicated. Control proteoliposomes contained buffer A. Reactions were stopped by washing the sample on filter discs (see the Experimental section). Results are the means of three separate experiments. Abbreviation: 3PGA, 3-phosphoglycerate.

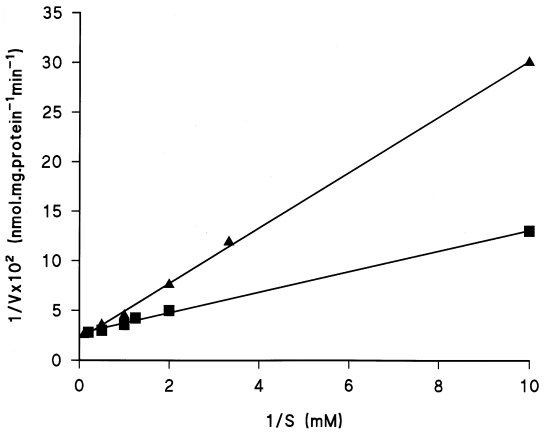

Figure 8 Inhibition of Glc1P/P_i exchange by Glc6P

Proteoliposomes containing reconstituted amyloplast membrane proteins were preloaded with 10 mM P_i. Rates of transport were estimated at 20 °C over 1 min with the stated concentrations of [^{14}C]Glc1P in the presence (▲) or absence (■) of unlabelled Glc6P (10 mM). Results are the means of five independent experiments.

ments were performed on proteoliposomes with a protein/lipid ratio of 112 μg of protein · cm^{-3} liposomes.

The time-dependent uptake of [^{32}P]P_i into proteoliposomes preloaded with various substrates was used to determine the specificity of the antiport mechanism on reconstituted amyloplast membranes. For the experiments shown in Figure 6, the time-dependent uptake of [^{32}P]P_i into liposomes preloaded with Glc1P and Glc6P was linear for about 60 s. The rate of uptake of both hexose phosphates was similar over the time course of the experiment. Proteoliposomes containing potassium gluconate buffer (buffer A) showed no appreciable uptake of [^{32}P]P_i which, together with results in Figure 5, shows that transport of hexose phosphate in amyloplast membranes requires counter-exchange with P_i. [^{32}P]P_i uptake was also supported in proteoliposomes preloaded with 10 mM 3-phosphoglycerate and 10 mM dihydroxyacetone phosphate (DHAP) (Figure 7).

Figure 8 shows the concentration-dependence of the [^{14}C]Glc1P/P_i counter-exchange in proteoliposomes containing reconstituted amyloplast membrane proteins. Transport shows saturation kinetics, with a K_m for Glc1P of 0.40 ± 0.11 mM (n = 5) and a V_{max} of 193 ± 34 nmol · mg of protein^{-1}. The addition of unlabelled Glc6P competitively inhibited Glc1P/P_i exchange, with a K_i of 0.8 mM.

[^{14}C]Glc1P was able to counter-exchange with Glc6P-preloaded proteoliposomes (Figure 9). Rates of uptake of [^{14}C]Glc1P into Glc6P-loaded proteoliposomes were comparable with rates of Glc1P/P_i exchange shown in Figure 5. Transport of Glc1P was also possible into proteoliposomes preloaded with

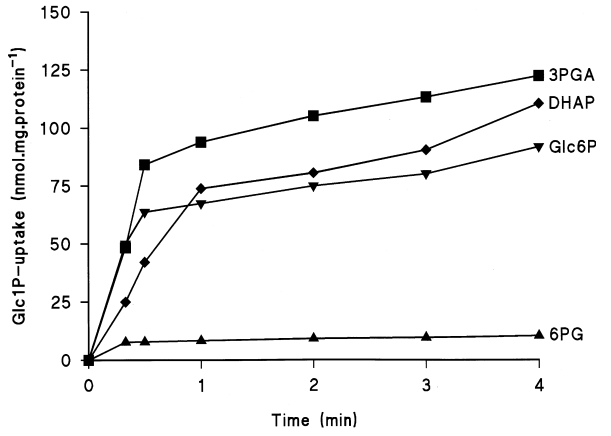

Figure 9 Glc1P exchange in proteoliposomes containing reconstituted amyloplast membrane proteins preloaded with various metabolites

Transport was initiated by the addition of 1 mM [^{14}C]Glc1P to the proteoliposomes, which were preloaded with various metabolites at 10 mM as indicated. Results are the means of three independent experiments. Abbreviations: 3PGA, 3-phosphoglycerate; 6PG, 6-phosphogluconate.

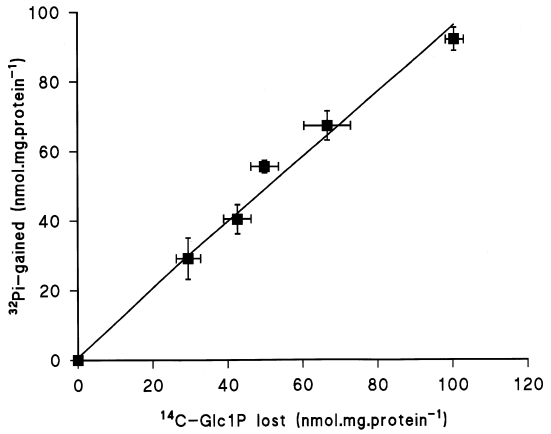

Figure 10 Stoichiometry of Glc1P/P_i exchange in reconstituted amyloplast membranes at pH 8.0

Proteoliposomes were preloaded with 10 mM [^{14}C]Glc1P and incubated with 1 mM [^{32}P]P_i at 20 °C for various times. Simultaneous measurements of [^{32}P]P_i uptake into and [^{14}C]Glc1P loss from the proteoliposomes at various time intervals are shown as the means ± S.E.M. of at least three independent experiments. The line has a slope of 0.98 as determined by linear regression analysis.

both DHAP and 3-phosphoglycerate, but not 6-phosphogluconate (Figure 9).

The data in Figure 10 are from dual-label experiments in which the uptake of 1 mM [^{32}P]P_i into proteoliposomes was monitored simultaneously with the export of [^{14}C]Glc1P from the preparation. The stoichiometry under the conditions tested was 0.99 ± 0.06 mol of phosphate taken up per mol of sugar phosphate exported. Similar results were obtained when monitoring Glc1P uptake and P_i export (results not shown). Stoichiometry determination was also conducted at pH 5.0, and in these experiments a 1:1 exchange of Glc1P/P_i also prevailed (results not shown).

DISCUSSION

A protocol has been developed for the reconstitution of functional wheat endosperm amyloplast membrane proteins in a bilayer system. The lipid composition of the liposomes was an important factor when considering the reconstitution protocol that was adopted. Leakage and fusion of SUVs is caused by lattice defects in the membrane; vesicles prepared in the presence of detergents as well as those undergoing a freeze–thaw cycle are particularly prone to this problem. Aggregation and fusion of lipids containing neutral lipid was overcome by including a small quantity of negatively charged PI in the lipid mixture to maintain colloidal stability [27]. Previous studies with reconstituted amyloplast transporters have used only PC in the preparation of liposomes [18,19,28]. The introduction of 5.6 mol % cholesterol into the lipid mixture considerably reduced leakage from liposomes preloaded with P_i and hexose phosphates, by introducing a more rigid and saturated membrane (Figure 2). This is particularly important since leakage by proteoliposomes would bias measurements of uptake and stoichiometry. Cholesterol has also been shown to catalyse spontaneous protein incorporation into vesicles at concentrations as low as 0.1 mol % [29], possibly by causing packing defects in the bilayer [17].

The choice of surfactant used for amyloplast membrane solubilization was an important consideration in the reconstitution protocol. OBG is a commonly used detergent in many transporter reconstitution studies ([17], and references therein). To effect reconstitution, detergent is diluted below its CMC. Detergents such as OBG with high CMC values are favoured for this reason. Another non-ionic detergent commonly used in reconstitution studies is Triton X-100 [polyoxyethylene-(9,10)-*p*-t-octophenol], but there are a number of recorded instances of enzyme activation with this agent (e.g. glucose-6-phosphatase [30], wheat endosperm amyloplast APPase [20] and ATPase-active P-glycoprotein [31]), suggesting it may have effects on biological activity. OBG was therefore used, at a concentration that caused maximum solubilization of functional transporter activity, and in proteoliposomes was diluted to maintain the liposomes structurally intact. Since the transport of Glc6P is suggested to occur in a number of plastids, it is particularly important to minimize the possibility of hydrolysis by phosphatases. Experiments where the uptake of [^{32}P]P_i by proteoliposomes preloaded with Glc6P is being investigated could be misinterpreted if the latter is hydrolysed to glucose and unlabelled P_i by the preparation, allowing counter-exchange of unlabelled P_i within the preparation for external labelled P_i.

Amyloplast envelope membranes used in the reconstitution experiments contained no phosphoglucomutase activity, excluding the possibility of interconversion of hexose phosphates prior to transport into proteoliposomes [6]. Metabolite transport in proteoliposomes can be attributed solely to the activity of amyloplast translocators; this is borne out by the absence of mitochondrial membranes in the amyloplast membrane preparation, as judged by cytochrome *c* oxidase measurements. Further, amyloplast transporters must be utilizing phosphorylated hexose units in these reactions, since no Glc1P or Glc6P phosphatase activity could be detected in amyloplast membrane preparations.

Previous studies with non-photosynthetic plastids have shown that hexose phosphates are transported in exchange for P_i [5]. Recent studies with reconstituted membranes from non-photosynthetic potato tuber amyloplasts have shown their ability to transport P_i, DHAP, 3-phosphoglycerate and Glc6P, but not Glc1P [19]. In the present study, reconstituted wheat endosperm amyloplast membranes were capable of transporting both Glc1P

and Glc6P with equal efficiency in P_i-loaded proteoliposomes. The K_m determined for Glc1P transport in Figure 8 (0.4 mM) is close to that determined for Glc6P transport in reconstituted potato amyloplast membranes (0.43 mM) [19] and pea root plastids (0.5 mM) [6]. Unlabelled Glc6P competitively inhibited Glc1P transport, indicating the presence of a single carrier system for hexose phosphates on the wheat endosperm amyloplast membrane. This finding is supported by the fact that Glc1P could counter-exchange with Glc6P preloaded into proteoliposomes, in contrast with transport measurements made in cauliflower-bud amyloplasts suggesting that Glc1P and Glc6P are transported by separate carrier proteins [28]. The specificity of the antiport mechanism from wheat endosperm amyloplast membranes was determined by measuring uptake of [^{32}P]P_i in vesicles preloaded with various substrates. The transporter on the membrane of wheat endosperm amyloplasts appears to be able to exchange P_i, 3-phosphoglycerate, DHAP, Glc6P and Glc1P. That reconstituted amyloplast membranes can transport Glc1P and Glc6P is consistent with the finding that Glc1P and Glc6P support starch synthesis and carbohydrate oxidation respectively in isolated wheat endosperm amyloplasts [11]. The specificity of the metabolite exchange reactions is shown by the negligible rates of metabolite exchange with buffer A (potassium gluconate) and 6-phosphogluconate.

Initial rates of [^{14}C]Glc1P uptake into P_i-loaded proteoliposomes were the same as values derived for P_i uptake into Glc1P-loaded proteoliposomes, suggesting a 1:1 exchange stoichiometry for these metabolites. This was confirmed directly by dual-label measurements. During starch biosynthesis, PP_i, which is formed as a result of ADP-glucose pyrophosphorylase activity, is cleaved to 2 mol of P_i by plastidial APPase. If the stoichiometry for P_i/Glc1P exchange in proteoliposomes containing reconstituted wheat endosperm amyloplast membranes prevails *in vivo*, then a net increase in the amyloplast P_i concentration would occur, which would inhibit ADP-glucose pyrophosphorylase activity [32] and starch synthesis. Membrane vesicles from *Streptococcus lactis* exhibit a variable stoichiometry of P_i/Glc6P exchange from 1:1 to 2:1 depending on pH [33]. However, pH had no effect on the stoichiometry of the exchange catalysed by the reconstituted wheat amyloplast translocator. The implication of this is that another mechanism must operate for the removal of the additional P_i. Neuhaus and Maass [34] have recently reported the unidirectional transport of P_i from cauliflower-bud amyloplasts. Whether this is a function of the bidirectional hexose phosphate translocator or of a separate protein is unclear, although Schwartz et al. [35] have demonstrated that the triose phosphate translocator of spinach chloroplasts possesses this ability.

In summary, we have reconstituted the hexose phosphate translocator of wheat endosperm amyloplasts and demonstrated, for the first time, the stoichiometry of Glc1P/P_i counter-exchange. Future studies will be concerned with the structural basis of the substrate specificity of this transporter in relation to others.

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