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Hexokinase activity alters sugar-nucleotide formation in maize root homogenates

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Abstract

Two pools of hexokinase activities differing in sensitivity to ADP inhibition were characterised in maize roots. In order to evaluate how glucose utilisation could be affected by these hexokinases, glucose-6-P and NDP-5′-sugar levels were measured after a D-[U-^{14}C]glucose pulse in root extracts in the presence of 0 or 1 mM ADP. Analysis of radio-labelled activated sugars by paper chromatography revealed that: (1) without ADP, nearly 20% of the ^{14}C appeared in NDP-5′-sugars; (2) 0.1 mM ADP inhibited ^{14}C-NDP-5′-sugar formation by 85%; and (3) with 1 mM ADP, ^{14}C- NDP-5′-sugars were undetectable, but substantial (14%) ^{14}C accumulated as glucose-6-P. Mannoheptulose, a hexokinase inhibitor, blocked the NDP-5′-sugar formation, but did not modify the amount of ^{14}C-glucose-6-P in root extracts either with or without ADP. The analysis of the hexokinase activities with 0.8 mM glucose in maize root extracts showed that: (1) mitochondrial hexokinase activity was totally inhibited by 30 mM mannoheptulose; and (2) the cytosolic hexokinase was inhibited by only 30%. These data suggest that NDP-5′-sugar synthesis is sensitive to ADP fluctuations and that mannoheptulose affects preferentially the mitochondrial-bound hexokinase, but the cytosolic form is less sensitive. We propose that the mitochondrial hexokinase is the main energy charge sensor in this pathway in maize.

Keywords: Zea mays L; Maize roots; Mitochondrial hexokinase; Sugar-nucleotide synthesis; Mannoheptulose; Glucose phosphorylation

1. Introduction

During maize seed germination, endosperm starch is broken down into free sugars and converted into sucrose (Thomas & Rodriguez, 1994). The initial metabolism of the incoming free sugars in the embryo axis may form (a) free glucose and fructose; or (b) uridine-5′-diphosphoglucose (UDP-glucose) and fructose (Duke, McCarty & Koch, 1991; Koch, Nolte, Duke, McCarty & Avigne, 1992; Bret-Harte & Silk, 1994; Delmer & Amor, 1995). Regardless of which route is followed, free hexoses are phosphorylated by different hexokinases [EC 2.7.1.1] present in plant tissues (Dennis & Turpin, 1990). Hexokinases catalyse the phosphorylation of hexose by MgATP, producing MgADP and hexose 6-P. Depending on the energy demands of the cell, the hexose-6-P may enter the glycolytic pathway or it may be converted into UDP-glucose as a glucosyl donor for the synthesis of cell-wall β-glucan (Raymond, Gidrol, Salon & Pradet, 1987; Dennis & Turpin, 1990; Ross, 1992). The mechanisms that control hexose-6-P utilisation in plants have not been fully established. The different isoforms of hexokinase have been classified according to their hexose preference and their location within the cell (Katzen & Schimke, 1965; Dennis & Turpin, 1990; Agius, 1994;

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Table 1
Mitochondrial location of maize particulate hexokinase activity[a]

Fractions	Total enzyme activities (nkat)			
	F_0F_1 ATPase	Cytochrome c oxidase	Hexokinase	Triose phosphate isomerase
Particulate pellet (10,000 × *g*/12 min)	607 ± 180	975 ± 238	1784 ± 350	6618 ± 2000
Percoll Gradient				
Fraction 1	121 ± 14	117 ± 28	164 ± 19	5824 ± 697
Fraction 2	479 ± 32	822 ± 57	1477 ± 127	728 ± 107

[a] The *F_0F_1 ATPase*, *cytochrome c oxidase*, *hexokinase* and *triose phosphate isomerase* activities were measured as described in Section 3. The reaction time was 15 min and the temperature was 35°C. The activities represent mean ± s.e. of 4 independent preparations.

Tsao, Burcelin & Charron, 1996). In pea seeds (Turner, Chensee & Harrison, 1977; Copeland, Harrison & Turner, 1978), maize endosperm (Cox & Dickinson, 1973; Doehlert, 1989) and wheat germ (Higgins & Easterby, 1974), hexokinases from the cytosol are inhibited by ADP ($K_i = 1$–5 mM) and glucose 6-P ($K_i = 15$–20 mM). However, the concentrations required for this are too high to effectively regulate hexokinase activity in vivo. On the other hand, cytosolic hexokinases partially purified from potato tubers are competitively inhibited with respect to ATP by low ADP concentrations ($K_i = 40$–400 μM) (Renz & Stitt, 1993; Renz, Merlo & Stitt, 1993), suggesting that the ATP/ADP ratio may regulate the hexokinase reaction. A control mechanism involving the ATP/ADP ratio has been proposed for the cytosolic hexokinase involved in glycolysis in maize root tips (Bouny & Saglio, 1996).

In a previous study (Galina, Reis, Albuquerque, Puyou, Puyou & de Meis, 1995; Galina, Logullo, Souza, Rezende & da Silva, 1999), two hexokinase activities with distinct kinetic properties and subcellular locations were characterised in maize seedling roots. The mitochondria-bound hexokinase has greater affinity for glucose than for fructose, and is highly sensitive to ADP when using glucose as substrate. It is inhibited by low concentrations of ADP ($K_i = 20$–40 μM), and the inhibition is not competitive with respect to both ATP and glucose. In contrast, the soluble form is not sensitive to ADP concentrations up to 1 mM (Galina et al., 1995). The role of these ADP-sensitive (ADP-HK) and insensitive (HK) hexokinase activities in glucose metabolism of developing maize seeds is still unknown.

The aim of this work was to evaluate how glucose utilisation is affected by these two pools of hexokinase activities both by the presence of its natural modulator, ADP, and an inhibitor of glucose phosphorylation, the mannoheptulose. To perform this investigation, glucose utilisation was evaluated in homogenates of growing roots by measuring the synthesis of glucose-6-P and NDP-5′-sugars.

2. Results and discussion

2.1. Particulate distribution of hexokinase in maize radicles

Previous study has shown that maize hexokinase activity sedimenting after 10,000 × *g* centrifugation is bound to the mitochondria (Galina et al., 1995). To verify in which organelle the hexokinase is located, the activities of cytochrome oxidase and F_0F_1 ATPase, two enzymes found in mitochondria, triose phosphate isomerase, a plastid marker (Dennis & Green, 1975; Dennis & Miernyk, 1982) and hexokinase, were assayed in different particulate fractions separated in a self-generated Percoll gradient (Neuburger, Journet, Bligny, Carde & Douce, 1982) (Table 1). After Percoll gradient fractionation, 87% of triose phosphate isomerase activity was recovered in fraction 1 of the gradient (plastid fraction). The amounts of total particulate activities of cytochrome oxidase, F_0F_1 ATPase and hexokinase varied between 10 and 20% in plastid fraction. However, 83% of hexokinase activity was found in fraction 2 of the Percoll gradient (mitochondrial fraction). Practically the same enrichment was obtained with the mitochondrial enzymes in fraction 2 (79–84%). These data indicate that the high hexokinase activity found associated with mitochondria (Galina et al., 1995) is not related to a cross-contamination with plastid bound hexokinase. The amounts of total activity of hexokinase detected in maize plastid fraction is very close to that described for nonphotosynthetic plant tissues from different sources (Dennis & Miernyk, 1982; Borchert, Harborth, Schunemann, Hoferichter & Heldt, 1993).

Some evidence indicates that the particulate hexokinase activity may be associated with the outer mitochondrial membrane (Dry, Nash & Wikich, 1983; Tanner, Copeland & Turner, 1983). One possible explanation for the mitochondrial location of the hexokinase may be that it serves to maintain a close relationship with the ATP-generating system of oxidative phosphorylation. This would ensure that the

Table 2
Effect of ADP on hexokinase activity in the cytosolic and mitochondrial fractions[a]

Fraction	Specific activity (nkat mg of protein^{-1})		Inhibition (%)
	0 ADP (a)	+1 mM ADP (b)	(c)
Homogenate	0.66 ± 0.12	0.30 ± 0.06	54.6
Crude mitochondrial pellet (10,000 × *g*)	2.06 ± 0.68	0.46 ± 0.07	77.9
Washed mitochondrial pellet	3.08 ± 0.48	0.12 ± 0.09	96.0
Submitochondrial particles	11.56 ± 3.67	ND[b]	100
Cytosolic supernatant (10,000 × *g*)	0.54 ± 0.08	0.37 ± 0.08	30.9
Soluble supernatant (100,000 × *g*)	0.38 ± 0.17	0.43 ± 0.13	No inhibition

[a] Each fraction was assayed in two different reaction media (see Section 3) containing either 10 units/ml pyruvate kinase (0 ADP) or 1 mM ADP without pyruvate kinase. The reaction was started by the addition of protein fractions (30–150 μg/ml) and quenched by boiling after 10 min at 37°C. Adding glucose-6-P dehydrogenase and β-NAD^+ revealed the glucose-6-P. The percentage of inhibition by ADP is given by the relation $c = (1 - b/a) \times 100$. The values are mean ± s.e. of 3–21 independent preparations.
[b] ND: activity not detected.

hexokinase could respond rapidly to changes in the cellular demand for glucose-6-P, which is known to be a key intermediate in several metabolic pathways including glycolysis, sucrose synthesis, pentose phosphate pathway, and cellulose biosynthesis.

2.2. Effect of ADP on maize root hexokinases

In the maize root homogenate, approximately 50% of the hexokinase activity was abolished when 1 mM ADP was included in the assay medium. Among the mitochondrial fractions (crude, washed, and SMP), the higher the specific activity of the hexokinase the greater was the magnitude of inhibition promoted by the nucleotide (Table 2). In submitochondrial particles, where the hexokinase activity was the highest, it was not possible to detect glucose-6-P formation in the presence of 1 mM ADP. In the Percoll purified mitochon-

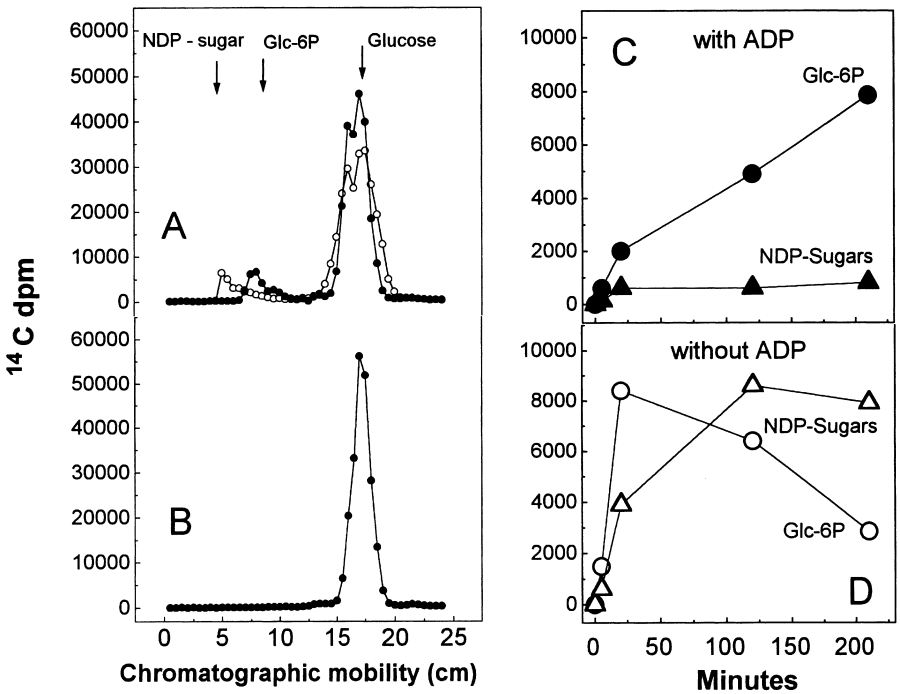

Fig. 1. [$U^{14}C$]glucose utilization in maize root extracts. Maize root homogenates were incubated at 35°C in two different reaction media containing 20 mM Tris–HCl pH 7.5, 0.8 mM [$U^{14}C$]glucose (240 μCi/μmol), 6 mM $MgCl_2$, 1 mM UTP, 1 mM ATP, 2 mM phosphoenolpyruvate and either 10 units/ml of pyruvate kinase (open symbols), or 1 mM ADP without pyruvate kinase (closed symbols). The reaction was started by adding maize extract to a final concentration of 0.9 mg of protein/ml, and stopped by heating to 100°C. After centrifugation the supernatant containing ^{14}C-sugars was resolved by paper chromatography, and the chromatogram was cut into strips for counting. In (A) the homogenate was incubated for 4 h. In (B) the homogenate was boiled before addition to the reaction medium. In (C) with 1 mM ADP; (D) with pyruvate kinase without ADP. (△, ▲) NDP-[^{14}C]-sugar; (○, ●) ^{14}C-glucose-6-P.

dria (fraction 2, Table 1) the associated hexokinase activity was completely inhibited by 1 mM ADP similarly as observed with submitochondrial particles (data not shown). In the cytosolic supernatant (10,000 × *g*), on the other hand, 1 mM ADP inhibited the activity by only 30% (Table 2). When the 100,000 × *g* supernatant was used, 1 mM ADP did not alter the hexokinase reaction. These data indicate that most of the HK activity in the mitochondrial fraction is ADP-sensitive (ADP-HK), whereas the cytosolic activity is mainly insensitive (HK), as demonstrated by lack of inhibition by ADP. These two kinds of hexokinases contribute equally to the total activity measured in maize root homogenates (Table 2). This fact may be related to different metabolic roles (Atkinson, 1968): (1) an energy-producing, glycolytic hexokinase in the cytosol; and (2) a biosynthetic hexokinase bound to mitochondria or microsomal membranes. It was suggested that the carbon demand would be satisfied if the sucrose transported were converted to free hexoses (Bret-Harte & Silk, 1994). Free hexoses arise from apoplastic (extracellular) route by the action of invertases bound to the cell wall in growing maize tissues (Robbins, 1958; Humphreys, 1974; Rovira & Davey, 1974).

Bouny and Saglio (1996) observed that during anoxic conditions the formation of lactate and ethanol was reduced in maize root tips, and they postulated that the low ATP/ADP ratio and low pH inhibited the step catalysed by cytosolic hexokinase. However, the cytosolic hexokinase activity is practically insensitive to 1 mM ADP (Table 2). It may be that the inhibition of glycolytic flux during anoxia is due mainly to low cytoplasm pH. In the following experiments, ADP was used as a natural inhibitor to distinguish the activity of HK and ADP-HK.

2.3. Conversion of glucose into activated sugars by maize root extracts

Upon incubation of the maize root extracts with D-[U^{14}C]glucose, about 20% of ^{14}C radioactivity was converted into two activated sugar species, [U^{14}C]-glucose-6-P and NDP-5′-[^{14}C]sugars (Fig. 1(A)). In the absence of ADP (open circles), the ^{14}C was incorporated into NDP-sugar and low amounts of [^{14}C]glucose-6-P were detected. When ADP was included in the medium (closed circles), the ^{14}C accumulated as glucose-6-P and no NDP-5′-[^{14}C]sugar was detected. The time course of these reactions after a [U^{14}C]-glucose pulse is shown in Fig. 1(C) and (D). In the presence of ADP (Fig. 1(C)), the [^{14}C]-glucose-6-P increases steadily over the first 4 h and practically no NDP-5′-[^{14}C]sugar is formed. In the absence of ADP (Fig. 1(D)), the [^{14}C]-glucose-6-P accumulates immediately in the first 20 min and then decreases sharply, whereas

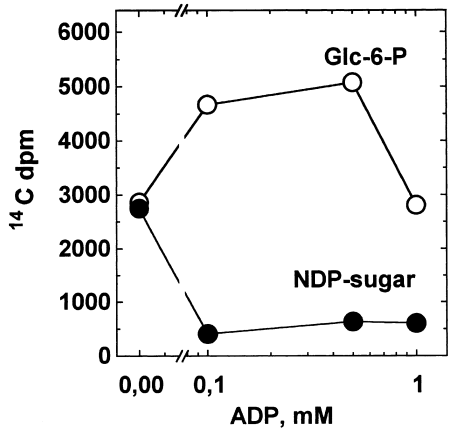

Fig. 2. ADP concentration dependence for formation of [^{14}C]-activated sugars in maize root extracts. Maize root homogenate was incubated in reaction media containing 20 mM Tris–HCl pH 7.5, 0.8 mM [U^{14}C]glucose (240 μCi/μmol), 6 mM $MgCl_2$, 1 mM UTP, 1 mM ATP, 2 mM phospho-enolpyruvate and either 10 units/ml of pyruvate kinase (0 ADP), or the ADP concentrations shown on the abscissa without pyruvate kinase. The reaction was started by adding maize extract to a final concentration of 1.3 mg protein/ml. After 2 h at 35°C, the reaction was quenched by boiling and the samples were separated by paper chromatography as described in Section 3 to determine the amount of radioactivity incorporated into (●) NDP-[^{14}C]sugar and (○) [^{14}C]glucose 6-P.

the amount of NDP-5′-[^{14}C]sugar increases in the first 2 h up to a plateau value that is much higher than that seen with ADP. These data suggest that in the absence of ADP, glucose-6-P is used to form NDP-sugar.

Gibeaut and Carpita (1993) have ascertained that membranes of the Golgi apparatus and crude mitochondrial fraction from maize coleoptiles are capable of synthesising in vitro the cell wall (1 → 3)(1 → 4) β-D-glucans from UDP-glucose. According to our observations, the mitochondrial fraction contains the ADP-sensitive hexokinase (Tables 1 and 2). This association would favour the channelling of glucose toward polysaccharide synthesis when the mitochondria are in a phosphorylating state (ADP-removing system and high ATP/ADP ratio). Under anaerobic conditions (low ATP/ADP ratio) (Hooks, Shearer & Roberts, 1994), the synthesis of polysaccharide would be downregulated by the inhibitory effect of ADP on the membrane-bound hexokinase. In fact, Roberts (1986a, 1986b) has shown by in vivo ^{31}P-NMR spectra of root tips that in extreme anoxia, the UDP-glucose peak disappears.

The effect of ADP was concentration-dependent (Fig. 2). The inclusion of 0.1 mM ADP was sufficient to lower the NDP-5′-[^{14}C]sugar formed in 2 h to a basal level, while the amount of [^{14}C]-glucose-6-P increased. This activation was no longer observed with 1 mM ADP. Higher ADP concentrations are usually not detected in roots. In fact, Hooks et al. (1994)

Table 3
Activity of enzymes involved in NDP-sugar synthesis in maize root homogenates[a]

Enzymes and reactions	Specific activity (nkat mg of protein^{-1})	
	0 ADP	+1 mM ADP
AGPase		
(a) ADP-Glc + $PP_i \rightarrow$ Glc-1P + ATP	ND[b]	ND[b]
(b) Glc-1P + ATP $\rightarrow$ ADP-Glc + $PP_i \rightarrow 2\ P_i$	ND[b]	0.02 ± 0.01
UGPase		
(c) UDP-Glc + $PP_i \rightarrow$ Glc-1P + UTP	0.18 ± 0.02	0.15 ± 0.02
(d) Glc-1P + UTP $\rightarrow$ UDP-Glc + $PP_i \rightarrow 2\ P_i$	1.03 ± 0.02	1.02 ± 0.01
PGM		
(e) Glc-1P $\leftrightarrow$ Glc-6P	0.60 ± 0.24	0.57 ± 0.08

[a] The *phosphoglucomutase* (PGM), *ADP-glucose pyrophosphorylase* (AGPase) and *UDP-glucose pyrophosphorylase* (UGPase) activities were measured as described in Section 3. When the reaction was measured in the direction of glucose-1-P ("a" and "c"), 1.5 mM PP_i, 1 mM NDP-glucose, 0.3 mM NAD^+, 2 units/ml of *phosphoglucomutase*, and 1 unit/ml *glucose-6-P dehydrogenase* were added. When the reaction was measured in the direction of NDP-glucose ("b" and "d"), 1 mM Glc-1P, 1 mM ATP or UTP and 2 units/ml of yeast *pyrophosphatase* were added. The P_i released was measured colorimetrically. The reaction time was 15 min and the temperature was 35°C. The activities represent mean ± s.e. of 4–7 independent preparations.

[b] ND: activity not detected.

observed that even in severe anoxia the ADP concentration in maize roots did not exceed 0.3 mM. The data showed that low concentrations of ADP may play a critical role in directing glucose toward the synthesis of NDP-5′-sugars and that the mitochondria-bound hexokinase is coupled to NDP-5′-sugar formation in maize root homogenates.

2.4. Enzymatic steps in NDP-sugar synthesis

The decrease in NDP-5′-sugars formed from glucose in the presence of ADP (Figs. 1 and 2) suggested that one or more of the reversible steps involved in the conversion of glucose-6-P into NDP-sugar might be affected by ADP. To test this possibility, the effects of ADP on the activity of phosphoglucomutase (PGM), ADP-glucose pyrophosphorylase (AGPase) and UDP-glucose pyrophosphorylase (UGPase) in maize root homogenates were measured (Table 3). In the absence of ADP, it was not possible to detect AGPase activity in either the forward or reverse reactions, and when ADP was present, only a low activity of AGPase was measured in the direction of ADP-glucose formation (Table 3, reaction b). In contrast, the UGPase activity was substantial in both directions (reactions c and d), and there was no effect of ADP in either case. The PGM activity was also not affected by ADP. The distribution of these activities among the subcellular fractions of maize roots revealed that 86–96% of the UGPase and PGM activities were present in the supernatant and 4–14% remained in the $10{,}000 \times g$ pellet (data not shown). A similar distribution has been found in rice (Kleczkowski, 1994) and in animal cells (Persat, Azzar, Martel & Got, 1983). ADP (Table 3) does not alter the activities of phosphoglucomutase and UDP-glucose pyrophosphorylase, two enzymes that are further along in this pathway. A similar observation has been reported for the enzyme from calf liver (Roach, Warren & Atkinson, 1975).

2.5. Effect of mannoheptulose on distribution of ^{14}C radioactivity

The data of Table 3 and Fig. 1 indicate that ADP affects primarily the reaction catalysed by hexokinases, and has little effect on the steps involved in the conversion of glucose-6-P to NDP-sugar. In additional exper-

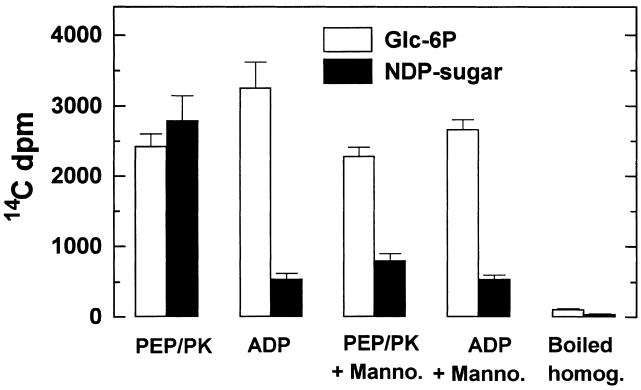

Fig. 3. Effect of mannoheptulose on formation of [^{14}C]-activated sugars in maize root extracts. Maize root homogenate was incubated in reaction media containing 20 mM Tris–HCl pH 7.5, 0.8 mM [U^{14}C]glucose (240 μCi/μmol), 6 mM $MgCl_2$, 1 mM UTP, 1 mM ATP, 2 mM phospho-enolpyruvate and either 0 ADP and 10 units/ml pyruvate kinase ("PEP/PK"), or 1 mM ADP without pyruvate kinase. When mannoheptulose was added, the final concentration was 20 mM. The reaction was started by adding maize extract to a final concentration of 1.1 mg protein/ml. After 2 h at 35°C, the reaction was quenched by boiling and the distribution of ^{14}C was determined by paper chromatography. Filled bars, NDP[^{14}C]-sugar; open bars, [^{14}C]-glucose 6-P.

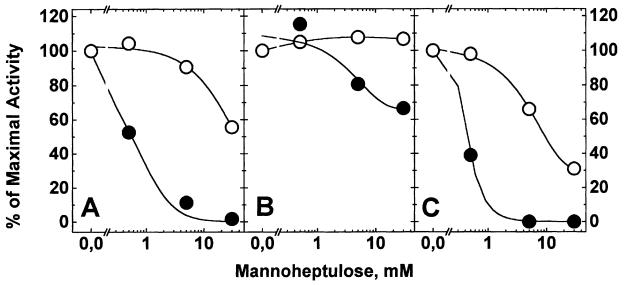

Fig. 4. Effects of mannoheptulose on hexokinase activities from maize mitochondria, cytosol and yeast. The hexokinase activities were assayed at 35°C in the presence of 10 units/ml pyruvate kinase and either (○) 0.1 mM or (●) 10 mM glucose. The reaction was started by the addition of (A) maize sub-mitochondrial particles (20 μg protein/ml); (B) 10,000 × *g* cytosolic supernatant (see Section 3) (100 μg protein/ml); (C) baker's yeast hexokinase Sigma Chemical Co., (0.2 units/ml); and the reaction times were (A) 4 min; (B) 30 min; and (C) 1 min. The reaction was quenched by heating (1 min at 100°C) and the glucose-6-P formed was measured as described in Section 3. The maximal activity (100%) for each assay (in nkat. mg of protein^{-1}) was A: (○) 7.5 and (●) 13.8; B: (○) 0.15 and (●) 0.18 and C: (○) 300 and (●) 967.

iments, the maize root homogenates were incubated with D-[U^{14}C]glucose and mannoheptulose, a specific inhibitor of both glucokinase and hexokinase (Coore & Randle, 1964; Ashcroft & Randle, 1970). This inhibitor was used to demonstrate whether or not the formation of [^{14}C]glucose-6-P or NDP-5′-[^{14}C]sugar is dependent on the hexokinases. The distribution of ^{14}C into [^{14}C]glucose-6-P and NDP-5′-[^{14}C]sugar after 2 h of incubation is shown in Fig. 3. Mannoheptulose (20 mM) caused a pronounced inhibition of NDP-sugar formation in the absence of ADP, but there was no additional inhibition when ADP and mannoheptulose were both present. These data indicate that the effects of ADP and mannoheptulose were not accumulative.

2.6. Effect of mannoheptulose on maize hexokinases

Mannoheptulose has been described as a competitive inhibitor with respect to glucose in hexokinases (Coore & Randle, 1964; Ashcroft & Randle, 1970). However, in the experiment shown in Fig. 3, mannoheptulose caused a low inhibition (~16%) of the formation of [^{14}C]glucose-6-P, an expected product of the hexokinase reaction. Therefore, the effect of mannoheptulose was also tested on the glucose-6-P formation by maize subcellular hexokinases and yeast hexokinase activities (Fig. 4). The maize mitochondrial and yeast hexokinases were inhibited by increasing concentrations of mannoheptulose (Fig. 4(A) and (C)). In the presence of 0.1 mM glucose as substrate, the $K_{0.5}$ values for inhibition by mannoheptulose were approximately 0.5 mM for both. The inhibition by mannoheptulose was greatly reduced by 10 mM glucose. Much higher mannoheptulose concentrations were required to inhibit the maize cytosolic hexokinase form at 0.1 mM glucose (Fig. 4(B)), and the inhibition was completely eliminated when the glucose concentration was raised to 10 mM (Fig. 4(B)). These results demonstrate that glucose-6-P formation by maize cytosolic hexokinase is much less sensitive to mannoheptulose than either yeast hexokinase or the maize mitochondria-bound hexokinase (Fig. 4(A) and (C)). These results indicate that the soluble form has a different sugar specificity or different affinity for mannoheptulose. In fact, several fructokinases have been purified from the cytosol of different plant sources, including maize kernels (Copeland et al., 1978; Baysdorfer, Kremer & Sicher, 1989; Doehlert, 1990; Gardner, Davies & Burch, 1992; Renz et al., 1993). In addition, a glucokinase was partially purified from young tomato fruits, which exhibits a very low affinity for the mannoheptulose (Randall & Martinez-Barajas, 1998). The question of whether maize cytosolic hexokinase properties are similar to those found in young tomato fruit has to be further investigated.

Jang and Sheen (1994), Koch (1996) and Jang, Leon, Zhou and Sheen (1997), have shown in maize protoplasts and *Arabidopsis* that hexokinase is involved in gene repression. 2-Deoxy-D-glucose (2-dG), a potent inhibitor of glycolysis (Wick, Drury, Nakada & Wolfe, 1957), also repressed these genes, whereas mannoheptulose antagonised this effect. Downstream glycolytic intermediates (including glucose-6P) had no effect on the hexokinase-mediated sugar-sensing pathway. These data were taken as evidence that the sugar signalling is uncoupled from sugar metabolism in plants. However, the formation of 2-dG-containing oligosaccharides has been reported in plants (Barber, 1959; Fischer & Weidemann, 1966), bacteria (Krieglstein & Fischer, 1967), and yeast (Biely, Kratky & Bauer, 1971), and UDP-2-dG and GDP-2-dG have been identified as precursors in a variety of cells (Biely & Bauer, 1968; Schmidt, Schwarz & Scholtissek, 1974). These studies showed that 2-dG does not alter the glucosyl-transfer reactions. The data shown in the present study are consistent with a complex role for hexokinases. It may be that the ADP-sensitive hexokinase function as an energy-charge sensor and is integrated with the sugar sensor activity (Jang & Sheen, 1994) by some metabolic intermediate of polysaccharide synthesis, or by formation of an ADP: hexokinase complex in the maize intracellular membranes.

3. Experimental

3.1. Preparation of plant material

Maize seeds (*Zea mays* L.) F-352 was a generous gift from *Sementes Agroceres* S.A., a Brazilian agricul-

tural company in the state of São Paulo. Maize seeds were surface-sterilised with sodium hypochlorite (~10 min in a 3% solution) and then washed several times with sterile water. Radicles were harvested from seeds allowed to germinate for 3 days on wet filter paper in the dark at 28°C.

3.2. Isolation of cell fractions

Maize root homogenates were obtained as previously described (Galina et al., 1995) using a cold extraction buffer containing: 5 mM Hepes/Tris pH 7.4; 0.3 mM mannitol; 7 mM cysteine; 1 mM EGTA; 1 mM PMSF and 0.1 g% (w/v) BSA. The "*crude mitochondrial pellet*" was obtained from the first centrifugation (10,000 × *g* for 12 min). The supernatant of the first 10,000 × *g* centrifugation was used for enzyme assays and throughout the text we refer to this fraction as "*cytosolic supernatant*". After centrifugation for 100,000 × *g* for 50 min, the pellet was discarded and the supernatant ("*soluble supernatant*") was used for enzyme assays. The "*washed mitochondrial pellet*" was obtained by washing the first pellet three times with 40 ml of buffer and resuspending in 10 ml of ice-cold buffer (~10 mg of mitochondrial protein/ml). The submitochondrial particles were obtained as previously described (Galina et al., 1995).

3.3. Self-generated Percoll gradient

Aliquots of washed mitochondria (3 ml samples; 50–60 mg of protein) were added to 28% (v/v) Percoll buffer and centrifuged at 40,000 × *g* for 30 min. The Percoll buffer used was the same as previously described, except that 0.3 M mannitol was employed instead of 0.3 M sucrose (Neuburger et al., 1982).

3.4. Hexokinase activity in maize roots

Roots from 30 seeds were homogenised by grinding in an ice-cold mortar and pestle with 3 ml extraction buffer containing 50 mM potassium phosphate pH 8.0, 1.4 mM β-mercaptoethanol and 0.1 g of PVP 40,000. The homogenate (7–10 mg protein/ml) was filtered through gauze and centrifuged at 10,000 × *g* for 10 min. The pellet (3–5 mg mitochondrial protein/ml) was resuspended in 2 ml extraction buffer and the supernatant (3–5 mg of cytosolic protein/ml) was stored for assay of enzyme activities. Each fraction was assayed for hexokinase activity in: 20 mM Tris–HCl pH 7.5, 6 mM $MgCl_2$, 5 mM glucose, 1 mM ATP, 2 mM PEP, 0.1% (v/v) Triton X-100 and either (a) 10 units/ml pyruvate kinase (total hexokinase activity); or (b) 1 mM ADP without pyruvate kinase (ADP-insensitive HK activity, HK). The difference between the activities measured in media (a) and (b) represents the hexokinase activity that is not-competitively inhibited by ADP (ADP-HK) (Galina et al., 1995). The reaction was started by adding protein fractions (30 to 150 μg protein/ml) and was quenched after 5–10 min at 35°C by heating (1 min at 100°C). The glucose-6-phosphate formed was measured by adding an equal volume of a solution containing 20 mM Tris–HCl pH 7.5, 6 mM $MgCl_2$, 1 unit/ml *glucose-6-phosphate dehydrogenase* (*Leuconostoc mesenteroids*) (Sigma Chemicals) and 0.3 mM β-NAD^+. The absorbance was read at 340 nm using a molar extinction coefficient of 6.22 M^{-1}. In all cases, activities were linear up to 30 min and linear with amount of extract added. The enzymic unit nkatal (nkat) is defined as the amount of enzyme needed to convert 0.06 μmol of substrate min^{-1}.

3.5. Other enzyme assays

Roots from 30 seeds were homogenised in a mortar with 3 ml of the same extraction buffer described for hexokinase. The total homogenate (10 ± 2 mg protein/ml) was filtered through gauze. For *ADP-glucose pyrophosphorylase* and *UDP-glucose pyrophosphorylase* (Borchert et al., 1993), the reaction medium contained 80 mM HEPES–Tris pH 7.5, 10 mM $MgCl_2$, 2 mg/ml BSA, 2 mM 3-phosphoglycerate, 2 mM PEP, 30 mM $NaNO_3$, 5 mM NaN_3 and 50 μM Ap5A. When the reaction was measured in the direction of glucose-1-P formation, 1.5 mM PP_i, 1 mM ADP-glucose or UDP-glucose, 0.3 mM NAD^+, 2 units/ml *phosphoglucomutase*, and 1 unit/ml *glucose-6-P dehydrogenase* were added. The NADH was read spectrophotometrically at 340 nm. When the reaction was measured in the direction of NDP-glucose formation, 1 mM Glc-1P, 1 mM ATP or UTP and 2 units/ml *yeast pyrophosphatase* were added to ensure that the reverse reaction would not occur. The P_i released (Fiske & Subbarow, 1925) was linear with time. The reaction temperature was 35°C. For *phosphoglucomutase*, the activity was measured according to Simcox, Reid, Canvin & Dennis (1977), in a medium containing 20 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 0.3 mM β-NAD^+, 2 mM PEP, 5 mM glucose 1-P, 0.3% (v/v) Triton X-100 and 1 unit/ml *glucose-6-P dehydrogenase* (*Leuconostoc mesenteroids*). The formation of NADH was read spectophotometrically at 340 nm.

Triose phosphate isomerase was measured spectrophotometrically in the direction of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate by coupling the product to α-glycerophosphate dehydrogenase (Sigma), as previously described (Rozacky, Sawyer, Barton & Gracy, 1971). *Cytochrome oxidase* activity was tested in different fractions as previously described (Galina et al., 1995). The rate of NADH oxidation was followed at 340 nm. After 2 min, 0.2 mM KCN was added and the difference in the rate was taken as

the KCN-sensitive cytochrome c NADH oxidation. The *ATPase* activity was determined by measuring the release of $[^{32}P]P_i$ from [γ-^{32}P]ATP as previously described (de Meis, 1988). In these experiments the production of $[^{32}P]P_i$ was measured both in the absence and in the presence of 5 mM sodium azide. The difference between the two activities is referred to as the azide-sensitive activity and is related to the ATPase activity of the maize F_0F_1 ATPase complex (Galina et al., 1995).

3.6. Pulse label with ^{14}C-glucose in maize roots

We opted for a mild, cell-free extract that would allow us to manipulate the ADP and inhibitor levels and observe how they affect glucose utilisation. Fresh homogenate was prepared from 3-day germinated seeds. The 15–20 roots were removed, put immediately in an ice-cold mortar with 1–2 ml of cold buffer and cut in small fragments of 3–4 mm. The buffer contained 20 mM potassium pyrophosphate pH 8.0, 0.3 M mannitol, 1.4 mM β- mercaptoethanol, 7 mM cysteine, 1 mM EDTA, 0.1% BSA, 1 mM PMSF and 0.1 g PVP 40,000/g of tissue. The root pieces were very mildly ground in a mortar for one round of 30 to 60 s or 10 s with Polytron yielding the same results. The pH was checked periodically and, when necessary, adjusted with a few microliters of 1 M KOH solution, never dropping below pH 6.0. The homogenate was filtered through nylon mesh and then incubated in a basic reaction medium containing: 20 mM Tris–HCl pH 7.5, 0.8 mM [U^{14}C]glucose (240 μCi/μmol), 6 mM $MgCl_2$, 1 mM UTP, 1 mM ATP, 2 mM PEP, 50 μM Ap5-A, 0.5 mM KCN and 0.5 mM carboxyatractyloside. The KCN and carboxyatractyloside were added to abolish the competition for ADP between mitochondria with the ADP-draining system of PEP and pyruvate kinase. Adding maize extract (0.9 to 2 mg protein/ml) started the reaction. After 2–5 h at 35°C, the reaction was quenched by boiling. The coagulated precipitate was sedimented in an Eppendorf centrifuge and the supernatant mixtures containing the ^{14}C-activated sugars were applied to Whatman No 1 paper and developed by descending chromatography in isobutyric acid: 1 N NH_4OH (5:3 v/v) for 24 h. The chromatograms were cut transversely into 3 mm wide strips, which were added to 5 ml of 0.5% PPO/toluene solution and counted in a liquid scintillation counter. The relative mobilities were compared to those of standard sugars run in parallel.

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