Different properties of the mitochondrial and cytosolic hexokinases in maize roots

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After tissue homogenization, 43 % of the total hexokinase activity found in maize radicles was recovered in the mitochondrial fraction and 35 % was soluble, in the cytosol. The maize sub-mitochondrial particles obtained after mitochondrial sonication retained a high hexokinase activity. The mitochondrial respiration (state 4 rate) was activated by glucose. This activation was blocked by carboxyatractyloside (0.5 mM) and by oligomycin (2 µg/ml). The affinities for ATP and glucose of both soluble and membrane-bound maize hexokinases are similar to those of yeast hexokinase. The K_m for ATP of these different forms of hexokinase varied between 0.15 and 0.37 mM, and the K_m for glucose between 0.05 and 0.13 mM. A major difference between the two maize hexokinase forms is that only

the mitochondrial enzyme was strongly inhibited by ADP (K_i 0.04 mM). The soluble forms of hexokinase found both in the cytosol of maize radicles and in yeast are not inhibited by ADP. In a previous report [de Meis, Grieco and Galina (1992) *FEBS Lett.* **308**, 197–201] it was shown that the mitochondrial F_1F_0 -ATPase can use glucose 6-phosphate and yeast hexokinase as an ATP regenerating system. We now show that the membrane-bound hexokinase and glucose 6-phosphate can also serve as an ATP regenerating system for the mitochondria of maize radicles provided that the ADP concentration is kept below 0.05 mM. Higher ADP concentrations inhibit the reverse reaction of the mitochondrial hexokinase.

INTRODUCTION

Hexokinase is an ubiquitous enzyme found in yeast [1,2], plants [3–7] and mammalian tissues [2,8–11]. This enzyme catalyses the reaction:

The glucose 6-phosphate formed provides a focal point for different pathways of carbohydrate metabolism [5]. In mammalian tissues there are four hexokinase isoenzymes, designated I, II, III and IV. The regulation of glucose 6-phosphate metabolism varies depending on the hexokinase form involved. Isoenzymes I–III have a high affinity for glucose (K_m 7×10^{-6} to 2×10^{-4} M) and are strongly inhibited by glucose 6-phosphate (K_i 2.5×10^{-5} M). These three isoenzymes are monomers of 100 kDa and all of them have a regulatory site for glucose 6-phosphate [12–15]. The type IV isoenzyme (glucokinase) is a monomer of 52 kDa, has a low affinity for glucose (K_m 1.2×10^{-2} M) and is not regulated by glucose 6-phosphate [2,16]. High ADP concentrations inhibit non-competitively [K_i $(1-5) \times 10^{-3}$ M] the type I hexokinase found in brain and muscle cells [9,17–21]. However, this low affinity for ADP raises doubts concerning the physiological regulation of hexokinase by ADP [21]. In contrast to the mammalian enzymes, the yeast hexokinase is a dimer in which the two polypeptide chains have the same molecular mass of 50 kDa. The yeast enzyme is not inhibited by glucose 6-phosphate or ADP [2]. Forms I and II of mammalian hexokinase are bound to the outer mitochondrial membrane and to different membranous structures of the microsomal fractions of brain, kidney, liver and tumour cells [2,22]. Four hexokinase isoenzymes have been identified in wheat-germ, all of them monomers: two with a molecular mass 52 kDa [3,5] and two of 100 kDa [3]. On the basis of amino acid composition

and molecular mass, it has been proposed that the form with a high affinity for glucose found in mammalian tissues may have arisen from an ancestral form similar to the glucokinase of yeast and wheat-germ by a process of gene duplication and fusion [3,19,23]. Higgins and Easterby [5], working with purified wheat-germ hexokinase, demonstrated non-competitive inhibition by ADP (K_i 10^{-3} M). The plant hexokinase was practically insensitive to glucose 6-phosphate inhibition. Based on these findings, it was suggested that the role of wheat-germ hexokinase is to favour the synthesis of carbohydrate polymers [5]. A substantial portion of the hexokinase activity in plants is associated with mitochondria and plastids [6,24–28]. The plastid hexokinase seems to be involved in the synthesis of starch and fatty acids [6,24,28]. The physiological role of the mitochondria-bound hexokinase in both animal and plant tissues is still controversial [21,22,25,26].

It has been observed that the reversal of the reaction catalysed by yeast hexokinase:

can be used as an ATP regenerating system by different ion transporting ATPases such as the Ca^{2+} transport ATPase of skeletal muscle [29], the H^+ -ATPase found in the plasma membrane of plants [30] and the F_0F_1 complex of mitochondria [31,32]. The affinity of these enzymes for ATP is sufficiently high (K_a varying from 10^{-6} to 10^{-12} M) to permit formation of the enzyme–substrate complex even in the presence of the very low ATP and high ADP concentrations found in the medium after the reaction reaches equilibrium. Thus glucose 6-phosphate, a phosphate compound that has a low free energy of hydrolysis [$\Delta G^0 = -10.5$ kJ (-2.5 kcal/mol)], can be used to regenerate ATP, a molecule with a high energy of hydrolysis ($\Delta G^0 = -29.3$ kJ).

In this paper we have studied the distribution of hexokinases in cells of maize radicles, a non-green plant tissue with a high

Abbreviation used: CAT, carboxyatractyloside.

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proliferation rate. Two forms were found, one soluble in the cytosol and another bound to mitochondria. Unlike the soluble form, the membrane-bound hexokinase is strongly inhibited by ADP.

MATERIALS AND METHODS

Plant material

Maize (*Zea mays* L.) seeds were germinated in the dark at 28 °C on wet filter paper. The radicles were harvested after the 4th day of growth.

Isolation of cell fractions

Maize radicles (100 g) were cut into small pieces and homogenized three times in a Waring blender for 5 s with 2 vol. of a cold buffer containing 5 mM Tris/Hepes buffer, pH 7.4, 7 mM cysteine, 0.3 M mannitol, 1 mM EGTA, 0.25 mM PMSF and 0.2 g % (w/v) BSA. The homogenate was strained through eight layers of cheesecloth and centrifuged at 3000 *g* at 4 °C for 3 min. The supernatant was centrifuged at 12000 *g* at 4 °C for 10 min. The pellet was washed twice with 40 ml of buffer and resuspended in 10 ml of ice-cold buffer. The microsomal fraction was prepared using the supernatant from the first centrifugation at 12000 *g*. This was centrifuged at 100000 *g* at 4 °C for 60 min. The microsomes were suspended in a solution containing 1 mM Mes/Tris buffer, pH 6.5, 1 mM dithiothreitol and 20 % (v/v) glycerol to a final protein concentration of 7 mg/ml.

Mitochondrial respiration

Samples of washed mitochondria were resuspended in a small volume of buffer (50–60 mg of protein/ml), and oxygen consumption was monitored using a Clark-type electrode (Yellow Springs Instruments). Measurements were carried out at 20 °C in a glass vessel (final volume 2 ml) containing 0.3 M mannitol, 30 mM Mops/Tris buffer, pH 7.4, 30 mM KCl, 3 mM MgCl₂, 0.5 mM EGTA, 1 mg/ml defatted BSA, 6 mM potassium phosphate and 0.5–0.6 mg of protein/ml. The respiratory substrates used were: (a) 5 mM glutamate plus 5 mM malate; (b) 10 mM succinate and (c) 2 mM NADH. ADP/O ratios of 1.43, 1.45 and 2.48 were measured when succinate, NADH and glutamate plus malate respectively were used as respiratory substrates.

Self-generated Percoll gradient

Aliquots of washed mitochondria (3 ml samples; 50–60 mg of protein) were mixed with 28 % (v/v) Percoll buffer and centrifuged at 40000 *g* at 4 °C for 30 min. The Percoll buffer used was the same as previously described, except that 0.3 M mannitol was used instead of 0.3 M sucrose [33].

Sucrose gradient

The microsomal fraction was layered on top of a 32.5 % and 46.5 % (w/v) discontinuous sucrose gradient as previously described [34]. The gradient was centrifuged at 150000 *g* for 5 h in a Beckman SW 40 rotor. The two major bands that equilibrated at the top and at the interface between 32.5 % and 46.5 % sucrose were collected and assayed for enzyme activities.

Preparation of submitochondrial particles

Samples of washed mitochondria (5–10 ml; adjusted to a protein concentration of 10–20 mg/ml) were sonicated as described by

Martins et al. [35]. The sonication buffer contained 20 mM MgCl₂ [36]. The submitochondrial particles thus obtained retain a hexokinase activity that remained fully active after 2 months of storage in liquid nitrogen.

Maize soluble hexokinase

The extract of maize soluble hexokinase was prepared according to Crane and Sols [13]. The maize homogenate was centrifuged at 108000 *g* at 4 °C for 50 min. The supernatant was filtered through two layers of cheesecloth to remove the fatty layer formed. At this stage the specific activity of hexokinase was 0.08 ± 0.05 μmol of glucose 6-phosphate $\cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$ (mean $\pm$ S.E.M. of four preparations). To this supernatant was added, slowly and with stirring, a saturated solution of ammonium sulphate, pH 7.5, at 0 °C. The precipitate formed at 45 % was discarded by centrifugation. The remaining supernatant was brought to 55 % ammonium sulphate and the precipitate formed was collected by centrifugation, and dissolved in a small volume of 30 mM Tris/HCl buffer, pH 7.4, 5 mM MgCl₂ and 5 mM EDTA. The solution was dialysed against 1 litre of buffer. The specific activity of hexokinase in the extract was 1.20 ± 0.52 μmol of glucose 6-phosphate $\cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$ (mean $\pm$ S.E.M. of four preparations).

Protein determination and enzyme assays

The protein concentration was determined as described by Lowry et al. [37], with BSA as standard. The medium with Percoll was diluted to less than 0.5 % (v/v) to avoid interference with the method.

Hexokinase (ATP:D-hexose 6-phosphotransferase; EC 2.7.1.1) activity was assayed in a medium containing 50 mM Tris/HCl buffer, pH 7.4, 6 mM MgCl₂, 10 mM glucose, 1 mM ATP, 2 mM phosphoenolpyruvate and 0.2 mM of the adenylate kinase inhibitor *P*¹*P*⁵-diadenosine 5'-pentaphosphate. The reaction was started with maize submitochondrial particles, maize soluble extract or yeast hexokinase obtained from Sigma. When submitochondrial particles were used, 0.2–1.0 % Triton X-100 was included in the assay medium. The reaction was carried out for 5–10 min at 35 °C. The reaction was stopped by heating the mixture at 100 °C for 30 s, and then diluted 2-fold in the assay medium. When the effect of ADP was studied, the assay medium contained 50 mM Tris/HCl buffer, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 0.4 mM NADP⁺ and 1 unit/ml glucose 6-phosphate dehydrogenase (baker's yeast; Sigma). When the effect of glucose 6-phosphate was studied, the same buffer was used, but 0.25 mM NADH, 10 units/ml pyruvate kinase and 7 units/ml lactate dehydrogenase (Sigma) were used instead of NADP⁺ and glucose 6-phosphate dehydrogenase. The extent of glucose phosphorylation was determined spectrophotometrically at 340 nm.

Triose phosphate isomerase activity 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 [38].

Cytochrome oxidase activity was tested in different fractions in a medium containing 50 mM Tris/HCl buffer, pH 7.4, 0.25 M sucrose and 0.15 mM NADH. The reaction was started by addition of the protein (0.05–0.3 mg of protein/ml), and the rate of NADH oxidation was followed at 340 nm at room temperature. 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.

Lactate dehydrogenase and glucose 6-phosphate dehydrogenase activities were measured as previously described [39,40].

The enzymic unit is defined as the amount of enzyme needed to convert 1 μmol of substrate $\cdot \text{min}^{-1}$.

ATP and glucose 6-phosphate hydrolysis

This was determined by measuring the release of [^{32}P]P_i from either [γ - ^{32}P]ATP or glucose 6- [^{32}P]phosphate with the use of ammonium molybdate and a mixture of 2-methylpropan-1-ol and benzene as previously described [41]. The reaction was started by the addition of enzyme and quenched after different incubation intervals at 35 °C by adding trichloroacetic acid to a final concentration of 10 % (w/v). 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 F₀-F₁ complex. This activity is not impaired by 1 mM orthovanadate, 0.1 μM bafilomycin or 1 mM sodium molybdate, specific inhibitors of P-type ATPases, tonoplast ATPase and phosphatases respectively [42,43]. In the presence of 40 μM ADP, the azide-sensitive activity represented 30–50 % of the total activity. Soluble F₁-ATPase without inhibitory protein was prepared from bovine heart mitochondria as previously described [44].

RESULTS

Intracellular distribution of hexokinase in maize radicles

The hexokinase activity of the plant cell was found to be equally distributed between the cytosol and the mitochondrial fraction (Table 1). Triton X-100 was included in the medium used to assay the hexokinase activity. Triton doubled the activity found in the mitochondrial fraction but had practically no effect on the hexokinase activity measured in the 100 000 *g* supernatant (results not shown). The effect of the detergent is attributed to the solubilization of the organelle, which has the effect of fully exposing the hexokinase entrapped in the membrane [2,45,46].

The results in Table 2 indicate that the particulate activity is bound to the mitochondrial membrane. The specific activity of hexokinase increased when the mitochondria were washed twice (Table 2a). A further increase was observed when they were sonicated and the submitochondrial particles collected after high-speed centrifugation. Parallel to the increase in hexokinase activity, there was an enhancement of the mitochondrial markers (cytochrome oxidase and azide-sensitive ATPase activity). On the other hand, the triose phosphate isomerase activity, a plastid marker [47,48], decreased during washing and preparation of submitochondrial particles.

In a second set of experiments (Table 2b) the mitochondria were purified in a self-generated Percoll gradient. Neuburger et al. [33] reported that heterotrophic plant tissues generate three bands in this gradient and that mitochondria are concentrated in the second band. Accordingly, after centrifugation we obtained three major bands, and both hexokinase and the mitochondrial markers were found to be enriched in the second band. The triose phosphate isomerase activity was enriched in the first band, which has a low mitochondrial content [33].

The activities of lactate dehydrogenase and glucose 6-phosphate dehydrogenase, two enzymes found in the cytosol, were assayed in the different cell fractions prepared as described in Table 2. In two experiments, the total activity of lactate dehydrogenase measured in the homogenate was 10.5 and 20.1 $\mu\text{mol} \cdot \text{min}^{-1}$ and that of glucose 6-phosphate dehydrogenase was 183.8 and 200.9 $\mu\text{mol} \cdot \text{min}^{-1}$. After cell fractionation, 75 % of the lactate dehydrogenase and 94.6 % of the glucose 6-phosphate dehydrogenase activity was recovered in the 12 000 *g* supernatant. The amounts of these two enzyme activities found in the washed mitochondria and submitochondrial particles varied between 0 and 5 %. These data indicate that the high hexokinase activity found associated with mitochondria in Table 2 is not related to a cross-contamination with cytosolic hexokinase.

Note in Table 1 that 12.2 % of the total hexokinase activity was recovered in the microsomal fraction, which is enriched with fragments of plasma membrane, membranes derived from the tonoplast and submitochondrial particles derived from the small part of the total mitochondrial population that is disrupted during tissue homogenization [34]. In order to evaluate whether hexokinase is also associated with the membranes of the cell and of the tonoplast, these were separated on a sucrose gradient as described by Serrano [34]. Two major bands are formed after centrifugation (Table 3). The specific activity of the vacuolar bafilomycin-sensitive H⁺-ATPase found in the top band was 4-fold higher, and that of the plasma membrane vanadate-sensitive H⁺-ATPase present in the second band was 2-fold higher, than that measured in the microsomes before centrifugation. The hexokinase activity, however, did not increase in the two fractions; it decreased 10-fold in the top band and was practically the same in band two, which in addition to the plasma membrane H⁺-ATPase also contains the mitochondrial azide-sensitive ATPase. This indicates that the activity measured in the microsomal fraction (Table 1) was not related to a possible association of hexokinase with the plasma membrane or the tonoplast membrane. It probably represents a cross-contamination with submitochondrial particles.

Table 1 Intracellular distribution of hexokinase activity in maize radicles

Cell fractionation and hexokinase activity measurements were performed as described in the Materials and methods section. The values represent means $\pm$ S.E.M. of three different preparations.

Fraction	Protein (mg)	Total activity		Specific activity ($\mu\text{mol} \cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$)
		($\mu\text{mol} \cdot 10 \text{ min}^{-1}$)	(%)	
Homogenate	1911 $\pm$ 300	490 $\pm$ 60	100.0	0.26 $\pm$ 0.02
Mitochondrial pellet (12 000 <i>g</i>)	240 $\pm$ 11	206 $\pm$ 30	42.5	0.86 $\pm$ 0.27
Microsomal pellet (100 000 <i>g</i>)	143 $\pm$ 20	60 $\pm$ 3	12.2	0.42 $\pm$ 0.03
Cytosolic supernatant (100 000 <i>g</i>)	1082 $\pm$ 85	174 $\pm$ 13	35.6	0.16 $\pm$ 0.02

Table 2 Enrichment of hexokinase in the mitochondrial fraction

Cell fractionation, Percoll gradient and the assay of the different enzymic activities were performed as described in the Materials and methods section. The values shown represent means $\pm$ S.E.M. of five different preparations.

Fraction	Protein (mg)	Specific activity ($\mu\text{mol} \cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$)			
		Hexokinase	Cytochrome oxidase	Azide-sensitive ATPase	Triose phosphate isomerase
(a)					
Mitochondrial pellet	305 $\pm$ 40	0.64 $\pm$ 0.10	0.20 $\pm$ 0.15	—	—
Washed mitochondria	49 $\pm$ 13	2.18 $\pm$ 0.32	1.05 $\pm$ 0.38	0.64 $\pm$ 0.22	8.10 $\pm$ 3.60
Submitochondrial particles	4 $\pm$ 1	7.6 $\pm$ 2.04	2.05 $\pm$ 0.31	1.77 $\pm$ 0.21	1.30 $\pm$ 0.80
(b)					
Percoll gradient					
Fraction 1	23 $\pm$ 2	0.43 $\pm$ 0.05	0.26 $\pm$ 0.05	0.28 $\pm$ 0.03	15.03 $\pm$ 0.90
Fraction 2	13 $\pm$ 1	6.72 $\pm$ 0.58	7.15 $\pm$ 0.50	3.92 $\pm$ 0.26	3.38 $\pm$ 0.50
Fraction 3	8 $\pm$ 1	1.03 $\pm$ 0.17	1.41 $\pm$ 0.08	0.90 $\pm$ 0.10	5.08 $\pm$ 0.37

Table 3 Specific activity of hexokinase and enzyme markers in microsomal fractions of maize roots

The values shown represent means $\pm$ S.E.M. of three different preparations. Azide-sensitive ATPase activity was assayed with and without 5 mM sodium azide in an assay medium containing 50 mM Tris/HCl, pH 7.5, 5 mM MgCl_2 , 30 mM KCl, 10 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and 2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Vanadate-sensitive ATPase activity was assayed with and without 1 mM vanadate in medium containing 50 mM Mops/Tris, pH 6.5, 5 mM MgCl_2 , 30 mM KCl, 0.2 mM sodium molybdate, 10 μM FCCP and 2 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Bafilomycin-sensitive ATPase activity was assayed with and without 0.1 μM bafilomycin in the same medium as for vanadate-sensitive activity, except that 50 mM Tris/HCl, pH 7.5, was used instead of 50 mM Mops/Tris, pH 6.5. The temperature was 30 $^\circ\text{C}$.

Fraction	Hexokinase ($\mu\text{mol} \cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$)	ATPases ($\mu\text{mol} \cdot 10 \text{ min}^{-1} \cdot \text{mg}^{-1}$)			
		Inhibitor ...	Azide	Vanadate	Bafilomycin
Crude microsome	0.42 $\pm$ 0.04		0.08 $\pm$ 0.04	3.39 $\pm$ 1.64	0.19 $\pm$ 0.09
Band 1	0.04 $\pm$ 0.02		0.10 $\pm$ 0.04	2.33 $\pm$ 0.73	0.73 $\pm$ 0.08
Band 2	0.34 $\pm$ 0.07		0.35 $\pm$ 0.06	7.19 $\pm$ 3.24	0.15 $\pm$ 0.06

Figure 1 Effect of glucose on maize mitochondrial respiration

The assay mixture composition and other conditions were as described in the Materials and methods section. The reaction was started by the addition of 5 mM glutamate plus 5 mM malate (Glu/Mal). In (a), 245 nmol of ADP and 0.5 mM CAT were added at the points indicated. In (b), 180 nmol of ADP and 2 $\mu\text{g}/\text{ml}$ oligomycin were added at the points indicated. In both experiments, 10 mM glucose was added.

Mitochondrial respiration

In animal tissues it has been shown that glucose stimulates the respiration of mitochondria that have a membrane-bound hexokinase. This activation is not observed in hexokinase-free mitochondria [49,50]. We now show that the respiration (state 4) of the maize radicle mitochondria was also activated by glucose (Figure 1). The enhancement of O₂ consumption promoted by glucose was impaired by carboxyatractylolide (CAT), a specific inhibitor of the mitochondrial ATP-ADP translocase (Figure 1a), and by oligomycin, a specific blocker of the F₀-F₁ complex (Figure 1b).

Kinetic properties of soluble and mitochondria-bound hexokinase

The kinetic properties of the soluble and membrane-bound maize hexokinases were compared with those of the yeast hexokinase (Table 4 and Figures 2 and 3). These experiments revealed the following information. (a) The apparent K_m values for glucose were similar in all three forms (Table 4). (b) The K_m for ATP of the yeast hexokinase was two to three times higher than that of the two forms of maize hexokinase (Table 4 and Figure 2). (c) In contrast to the hexokinases found in mammalian

Table 4 Kinetic parameters of maize submitochondrial particles (SMP), maize soluble hexokinase and yeast hexokinase

The values are means $\pm$ S.E.M. of at least three different preparations. The inhibition observed in the presence of ADP was non-competitive with regard to both ATP and glucose. The K_i value for the non-competitive inhibition observed in Figure 2 was calculated according to the following equation: $K_i = (V_{\max,i}/(V_{\max} - V_{\max,i}))$. The $V_{\max}$ value is expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$. The K_m value for substrates in the presence and in the absence of the inhibitors were practically the same for a given hexokinase form. The $V_{\max,i}$ value is expressed as $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ in the presence of the inhibitor. The K_m and $V_{\max}$ values were calculated as described by Florini and Vestling [64]. Abbreviation: G-6-P, glucose 6-phosphate.

Hexokinase	Substrates	Inhibitors	$V_{\max}$	K_m (μM)	$V_{\max,i}$	K_i (μM)
Maize SMP	Glucose	—	2.04 ± 0.04	129 ± 50	—	—
	ATP	—	2.10 ± 0.05	156 ± 56	—	—
	—	40 μM ADP	—	—	0.94 ± 0.07	34 ± 10
	—	10 mM G-6-P	—	—	2.01 ± 0.06	No inhibition
Maize soluble	Glucose	—	0.12 ± 0.03	49 ± 25	—	—
	ATP	—	0.09 ± 0.03	167 ± 84	—	—
	—	1 mM ADP	—	—	0.10 ± 0.03	No inhibition
	—	10 mM G-6-P	—	—	0.11 ± 0.02	No inhibition
Yeast	Glucose	—	150 ± 10	107 ± 35	—	—
	ATP	—	154 ± 8	356 ± 95	—	—
	—	1 mM ADP	—	—	138 ± 22	No inhibition
	—	10 mM G-6-P	—	—	152 ± 17	No inhibition

Figure 2 ADP inhibition of mitochondrial hexokinase

The assay mixture composition was 50 mM Tris/HCl, pH 7.5, 6 mM MgCl_2 , 150 mM KCl, 10 mM glucose, 1 mM ATP, 0.2 mM P^1P^5 -diadenosine 5'-pentaphosphate and 2 mM phosphoenolpyruvate. Triton X-100 to a final concentration of 0.1% (v/v) was included in the assay medium when submitochondrial particles were used. When glucose was varied, the ATP concentration used was 0.3 mM, and when ATP was varied, the glucose concentration used was 10 mM. In (a), 0.02 mg/ml maize submitochondrial particles were used. For measurements in the absence of ADP (○) pyruvate kinase (10 units/ml) was included in the media. For 50 μM ADP (□) and 200 μM ADP (△), pyruvate kinase was not included. In (b), 0.01 mg/ml yeast hexokinase was used. The assay temperature was 35 °C. For △ and □, the concentration of ADP has been corrected for the amount calculated as being formed by hexokinase reaction.

tissues [12–15], the rate of glucose phosphorylation by ATP catalysed by each of the three forms of hexokinase tested in Table 4 was not altered by high concentrations (10 mM) of glucose 6-phosphate. (d) The new finding is that only one of the two hexokinase forms found in maize radicles is strongly inhibited by ADP (Table 4 and Figure 2 and 3). The inhibition of the mitochondrial hexokinase by ADP was non-competitive towards ATP and glucose (Figure 2). This effect was observed with both the submitochondrial particles and particles solubilized with 1% (v/v) Triton X-100. The same inhibition profile was observed with 10 mM glucose (Figure 2) and 0.1 mM glucose (results not shown). Only ADP was able to impair the mitochondrial hexokinase: no inhibition was observed in the presence of 0.4 mM IDP, CDP, GDP, UDP, AMP, cyclic AMP, IMP, CMP, GMP or UMP (results not shown). The rate of glucose

phosphorylation by ATP catalysed by both the soluble maize form and the yeast hexokinase was not decreased by 1 mM ADP. The ADP inhibition of the membrane-bound hexokinase was reversed when the excess ADP added was transformed into ATP. This is shown in Figure 3(b), where pyruvate kinase and phosphoenolpyruvate were added to the assay medium. The non-competitive kinetics observed in Figure 2 suggest that the mitochondrial hexokinase might have a regulatory site that binds ADP but not ATP.

Use of glucose 6-phosphate and hexokinase as an ATP regenerating system

The membrane-bound hexokinase found in maize mitochondria can catalyse the synthesis of ATP from ADP and glucose 6-

Figure 3 Effect of ADP on glucose phosphorylation activity by hexokinase isoforms

The assay mixture composition was 50 mM Tris/HCl, pH 7.5, 6 mM MgCl₂, 150 mM KCl, 10 mM glucose, 1 mM ATP, 0.2 mM *P*¹*P*⁵-diadenosine 5'-pentaphosphate and 2 mM phosphoenolpyruvate. Triton X-100 to a final concentration of 0.1% (v/v) was included in the assay medium when submitochondrial particles were used. In (a), 10 units/ml pyruvate kinase was added only in the assay referred to in the figure as zero ADP. ●, 0.05 mg/ml maize submitochondrial particles; ○, 0.15 mg/ml maize soluble hexokinase prepared as described in the Materials and methods section; △, 0.06 mg/ml yeast hexokinase. The 100% values for glucose phosphorylation activities were 1.47 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (●), 0.097 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (○), and 150 $\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$ (△). The temperature was 35 °C. The concentrations of ADP have been corrected for the amount formed by the hexokinase reaction. In (b), the reaction medium composition was as in (a), but with 2 units/ml glucose-6-phosphate dehydrogenase, 0.5 mM NADP⁺, 0.1% (v/v) Triton X-100 and 0.03 mg/ml maize submitochondrial particles. The reaction was started by the addition of ATP; ADP was added before starting the reaction with ATP: A, no ADP; B, 0.1 mM ADP; C, 0.4 mM ADP. The arrows indicate the addition of 10 units/ml pyruvate kinase (PK). The reaction was assayed at room temperature.

Figure 4 Effect of ADP on azide-sensitive glucose 6-phosphate cleavage mediated by maize or yeast hexokinase

The assay medium composition was 50 mM Mops/Tris buffer, pH 6.5, 1 mM MgCl₂, 20 mM KCl, 15 mM glucose 6-phosphate, 0.2 mM *P*¹*P*⁵-diadenosine 5'-pentaphosphate, 1 mM NaVO₄ (monovanadate), 50 mM NaNO₃, 0.3 mg/ml maize submitochondrial particles (0.3 unit/ml maize hexokinase) and, when needed, 5 mM NaN₃. (a) ▲, Control; ●, plus 20 $\mu\text{g}/\text{ml}$ beef heart F₁-ATPase. In (b), the assay media were as in (a) plus 5 units/ml yeast hexokinase without (■) or with (▼) 20 $\mu\text{g}/\text{ml}$ beef heart F₁-ATPase. The points and bars correspond to means $\pm$ S.E.M. of three experiments.

phosphate. However, because of the ADP inhibition observed in Figures 2 and 3, the membrane-bound hexokinase is much less effective than the soluble hexokinases in promoting the synthesis of ATP. This is shown in Figure 4 using glucose 6-[³²P]phosphate and maize submitochondrial particles. There was no azide-sensitive production of [³²P]P_i when ADP was not included in the assay medium (Figure 4a). Formation of [³²P]P_i was observed in the presence of 40 μM ADP. The rate of [³²P]P_i production represents the balance of two activities: the rate at which the mito-

chondrial hexokinase catalyses the synthesis of [³²P]ATP from glucose 6-[³²P]phosphate and ADP, and the rate of [³²P]ATP cleavage by the submitochondrial particle F₀-F₁-ATPase. In the presence of 40 μM ADP, the rate of [³²P]ATP synthesis was faster than the rate of its hydrolysis. This was determined by enhancing the rate of cleavage by adding an excess of soluble F₁-ATPase to the medium (Figure 4a). The rate of synthesis was no longer faster than that of hydrolysis when the ADP concentration was raised to 400 μM . In this case, the azide-sensitive [³²P]P_i

Figure 5 Inhibition of azide-sensitive glucose 6-phosphate cleavage by glucose in maize submitochondrial particles at low ADP concentration

The reaction mixture was as in Figure 4, but in the presence of 40 μ M ADP, 0.6 mg/ml maize submitochondrial particles and the glucose concentrations shown. The reaction was started by the addition of maize protein. The temperature was 35 °C and the reaction time 40 min.

production was slower than that measured with 40 μ M ADP, and the rates measured with and without the addition of an excess of soluble F_1 -ATPase were practically the same. This decrease is probably related to the ADP inhibition of hexokinase observed in Figures 2 and 3. A different result was observed when soluble hexokinase was included in the assay medium together with the submitochondrial particles (Figure 4b). In this case, the rate of [32 P] P_i production increased as the ADP concentration in the medium was raised [29–32], even when the ATPase activity of the system was enhanced by adding extra amounts of F_1 -ATPase. In the presence of 400 μ M ADP, the membrane-bound hexokinase was inhibited but the ADP-insensitive soluble hexokinase added ensured the resynthesis of ATP (Figure 4b).

The concentration of ATP which can be formed at equilibrium from glucose 6-phosphate and ADP decreases when the glucose concentration of the medium is raised [29–32]. Accordingly, Figure 5 shows that the cleavage of glucose 6-phosphate catalysed by the submitochondrial particles is inhibited by glucose. This inhibition was not observed when 3 mM ATP was used as substrate (results not shown and [31]). The findings of Figures 4 and 5 indicate that the true substrate of the F_0 - F_1 -ATPase was the very small amount of ATP formed from glucose 6-phosphate and ADP in a reaction catalysed by the membrane-bound hexokinase found in the maize submitochondrial particles.

DISCUSSION

There is an overexpression of hexokinase in tissues that under aerobic conditions sustain high rates of glycolysis (the Warburg effect), such as highly malignant tumour cells [51] and brain [21]. In these tissues, 50–80 % of the total hexokinase activity is found attached to the outer mitochondrial membrane [22]. Wilson [52], Vallejo et al. [53] and Mayer and Hubscher [54] presented evidence that hexokinase may also be found attached to the inner mitochondrial membrane. Bessman and co-workers [55,56] reported that glucose activates the respiration of tumour cell mitochondria, which have a membrane-bound hexokinase. This activation is not observed in mitochondria which do not have a

membrane-bound hexokinase. According to Arora and Pedersen [50] the mitochondrial hexokinase promotes the transfer of the phosphate of ATP to glucose on the outer surface of the mitochondrial membrane. The ADP produced would then be rapidly transferred back to the mitochondrial matrix, while the glucose 6-phosphate is released into the cytosol. Kabir and Nelson [57] reported that the mitochondrial hexokinase of rat brain binds preferentially the ATP found in the cytosol and not the intramitochondrial ATP. In agreement with Arora and Pedersen [50], we found that the respiration of maize mitochondria is activated by glucose and that this activation is arrested when the return of the ADP to the mitochondrial matrix is blocked by CAT, or when the ATP synthesis on F_0 - F_1 -ATP synthase is impaired by oligomycin (Figures 1a and 1b).

We were unable to strip off the outer membrane of the maize mitochondria with digitonin as described by Arora et al. [22] for mammalian tissues. Thus we were not able to ascertain whether the hexokinase is bound to the outer or inner mitochondrial membrane. After sonication, the outer membrane should sediment together with the submitochondrial particles (Table 2a).

The new finding is that in the maize radicles there is a major kinetic difference between the hexokinase that is bound to the mitochondria and the form that is soluble in the cytosol. As shown in Figures 2 and 3, only the form bound to mitochondria is inhibited by ADP. In mammalian tissues the kinetic properties of the soluble and mitochondrial hexokinases are practically the same [58]. Different forms of mammalian hexokinases are inhibited by ADP but in different manners. The rat brain, mammalian muscle and heart hexokinases have a low affinity for ADP (K_i varying from 1 to 5 mM) and the mechanism of inhibition is non-competitive [17–19]. The hexokinase found in ascites tumour cells is inhibited by low ADP concentrations (K_i 50 μ M); however, in contrast to the maize hexokinase (Figures 2 and 3), ADP competes with ATP for the hexokinase catalytic site [59]. The very low K_i for ADP detected in maize radicles indicates that the mitochondrial hexokinase is not suitable as an ATP regenerating system, but it could be involved in the regulation of glucose 6-phosphate in the cell. In developing plant cells there is a large requirement for polysaccharides to form cell walls [5]. In this case, mitochondrial respiration and the bound hexokinase would favour the accumulation of glucose 6-phosphate to be used for synthesis of carbohydrate polymers. The mitochondrially bound enzyme would only favour the accumulation of glucose 6-phosphate when the adenylate energy charge is high. When the ADP concentration in the cell rises, then the membrane-bound hexokinase would be impaired and the ATP synthesized by the mitochondria would be used exclusively to maintain the adenylate energy charge of the cell. Unlike the mitochondria-bound hexokinase, the soluble hexokinase is fully active even in the presence of high ADP concentrations, and may be used as an ATP regenerating system to sustain the function of several essential ion transport ATPases [29–31].

The physiological importance of having two differently regulated forms of hexokinase in plants becomes apparent in conditions where there is a large shift of the ATP/ADP ratio in the cell, such as that observed in germinating seeds and during anoxia in maize root tips. In the early stages of seed hydration, the mitochondria are functionally deficient and the adenylate energy of the cell is low [60–62]. In parallel with the uptake of water by the seed, there is an increment in oxidative phosphorylation activity and an enhancement of both the adenylate energy charge and the anabolic activity needed for the development of the plant. Hooks et al. [63] observed that anoxia in maize root tips promotes a large decrease in the ATP/ADP ratio. In both conditions, while the adenylate energy charge is

low, the mitochondrial hexokinase would be inhibited and both glucose 6-phosphate and the soluble hexokinase could be used as an emergency ATP regenerating system. When the adenylate energy charge increases and growth is initiated, as in the root tips and in hydrated seeds, then the mitochondrial hexokinase is activated and glucose 6-phosphate would not be used to regenerate ATP, but for the synthesis of polysaccharides.

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