Hexokinase II of Pea Seeds

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ABSTRACT

A second hexokinase (EC 2.7.1.1) was obtained from pea seed (Pisum sativum L. var. Progress No. 9) extracts. The enzyme, termed hexokinase II, had a high affinity (K_m, 48 micromolar) for glucose and a relatively low affinity (K_m, 10 millimolar) for fructose. The K_m for MgATP was 86 micromolar. MgATP was required for activity, but excess MgATP was inhibitory. MgADP inhibited hexokinase II. The addition of salts of monovalent cations increased hexokinase II activity. Al^3+ was a strong inhibitor of the enzyme at pH 6.6 but not at the optimum pH (8.2). Citrate and 3-phosphoglycerate activated pea seed hexokinase II at pH 6.6, probably by coordinating with aluminum present as a contaminant in commercial ATP. The properties of hexokinase II are compared with those of the other three hexose kinases obtained from pea seed extracts. The possible role of these enzymes in plant carbohydrate metabolism is discussed.

The first step in the metabolism of hexoses in most organisms usually involves phosphorylation of the sugar by ATP. There are two naturally occurring isozymes of hexokinase (ATP: d-hexose 6-phosphotransferase; EC 2.7.1.1) in yeast (3, 8). Four hexokinases have been found in mammalian tissues: three of these enzymes are termed hexokinases, while the fourth is a glucokinase (ATP: d-glucose 6-phosphotransferase; EC 2.7.1.2) (3, 8). Glucokinase was originally found in liver (5, 9, 15).

It now appears that the situation in plants in respect to the phosphorylation of hexoses may be analogous in some ways to that obtaining in mammalian tissues. A previous communication from this laboratory showed that the hexose kinases of pea seed extracts could be separated into four main fractions (13). These fractions were numbered I, II, III, and IV, in order of elution from a DEAE-cellulose column. The enzyme contained in fraction I had K_m values of 0.07 mm and 30 mm for glucose and fructose, respectively (13). Under in vivo conditions, this enzyme is unlikely to be of significance in the phosphorylation of fructose (13). Fraction III contained a fructokinase (ATP: d-fructose 6-phosphotransferase; EC 2.7.1.4), which had high activity with fructose as substrate but low activity with glucose (4). Fraction IV contained a different fructokinase which was essentially specific for fructose as substrate and which had very little activity with glucose and mannose (14). In the present investigation, the properties of the enzyme in fraction II have been studied. This enzyme has a high affinity for glucose and mannose and a relatively low affinity for fructose. Activity was increased by the addition of salinity of monovalent cations, and the enzyme was strongly inhibited at pH 6.6 by aluminum.

Now that information is available on the properties of the hexose kinases in all four fractions derived from the pea seed extracts, it is appropriate to review the nomenclature of these enzymes. The hexose kinases from fractions I, II, III, and IV will now be termed hexokinase I, hexokinase II, fructokinase I, and fructokinase II, respectively. In previous communications (4, 13, 14), the fraction I enzyme has been referred to as glucokinase and the fraction III and fraction IV enzymes as fructokinase (fraction III) and fructokinase (fraction IV), respectively.

MATERIALS AND METHODS

Materials. Mature pea seeds (Pisum sativum L. var. Progress No. 9) were obtained from F. Cooper Ltd., Wellington, New Zealand. Biochemicals were from Sigma Chemical Co. or Boehringer Mannheim GmbH. AGATP type 2 was from P-L Biochemicals Inc., Milwaukee, WI.

Preparation of Hexokinase II. Pea seeds were ground and defatted with ether, as described previously (12). All subsequent operations were carried out at 4 C. Ether-extracted pea powder (40 g) was suspended in 120 ml 50 mM NaHCO_3 and placed on a mechanical roller. After a 3-h rotation, the soluble fraction was separated by centrifugation at 20,000g for 10 min. Glucose and MgSO_4 were then added to give final concentrations of 0.1 mM glucose and 0.1 mM MgSO_4, and the preparation was allowed to stand for 10 min. The mixture was centrifuged at 20,000g for 10 min, and saturated (NH_4)_2SO_4 (pH 7.8) was added to 60% saturation. Glucose was added to maintain a concentration of 0.1 mM. The pellet obtained after centrifuging at 20,000g for 10 min was dissolved in H_2O and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA and then against 10 mM Tris-HCl buffer (pH 7.5) (buffer A). Dialyzed material containing 4000 to 5000 mU glucose-phosphorylating activity was placed on a DEAE-cellulose column (2.6 x 40 cm) previously equilibrated with buffer A and washed until unbound protein was removed. The hexose kinases were then eluted with a gradient obtained by introducing 550 ml of buffer A containing 0.4 mM KCl and 2 mM EDTA into 550 ml of buffer A. Fractions of 10 ml were collected. The tubes containing hexose kinase fractions II and III (13) were pooled, concentrated to approximately 20 ml in a Diaflo (Amicon Corp., Lexington, MA) apparatus (XM-50 membrane, 300 kPa/m^2 N_2 pressure), and dialyzed against 10 mM Tris-HCl buffer (pH 8.0) (buffer B). The dialyzed material was rechromatographed on a DEAE-cellulose column (1.5 x 30 cm) previously equilibrated with buffer B. The hexose kinases were eluted with a gradient obtained by introducing 200 ml of buffer B containing 0.3 mM KCl and 2 mM EDTA into 200 ml of buffer B. Fractions of 5 ml were collected. The fractions containing fraction II (hexokinase II) and residual fraction III (fructokinase I) were pooled and concentrated to approximately 15 ml. After dialysis against 10 mM Tris-HCl buffer (pH 8.0) containing 50 mM KCl (buffer C), the preparation was passed through a Sephadex G-200 column (2.6 x 70 cm) previously equilibrated in buffer C. The remaining fructokinase I was removed by affinity chromatography. MgCl_2 (0.4 mM) was added to the Sephadex G-200 eluent to give a final MgCl_2 concentration of 5 mM. The solution was then passed through a column (0.8 x 10 cm) of AGATP type 2. The column was washed with buffer C containing 5 mM MgCl_2 at the rate of approximately 2 ml/min to remove the additions.

Abbreviations: AGATP, agarose-hexane-adenosine-5'-triphosphate; mU, milliunit; V, maximum velocity.
0.3 ml per min until the A at 280 nm was less than 0.02. Fructokinase I was retained on the column, whereas hexokinase II was not bound. The eluate containing hexokinase II was dialyzed against buffer B to remove MgCl₂. The hexokinase II preparation was free from glucose-6-P dehydrogenase and 6-P-glucuronate dehydrogenase. The enzyme could be stored at -18°C with 10% loss of activity in 3 months.

**Assay of Hexokinase II Activity.** Enzyme activity was assayed by coupling the production of glucose-6-P with the reduction of NADP in the presence of excess glucose-6-P dehydrogenase. Reaction mixtures for the standard assay contained, in a total volume of 1 ml, 25 μmol Tris·HCl buffer (pH 8.2), 50 μmol KCl, 5 μmol glucose, 5 μmol ATP, 7 μmol MgCl₂, 0.33 μmol NADP, 0.6 μg glucose-6-P dehydrogenase, and an appropriate volume of the pea seed enzyme. When fructose was used as the substrate, 3 μg P-mannose isomerase was added, and, with mannose as substrate, 7 μg P-mannose isomerase and 3 μg P-glucose isomerase were added. Reaction mixtures were maintained at 30°C, and the increase in A at 340 nm was followed. One mU hexokinase II activity is defined as 1 nmol glucose-6-P produced per min.

When the phosphorylation of sugars other than glucose, fructose, or mannose was measured, the production of ADP was coupled with the oxidation of NADH using pyruvate kinase and lactate dehydrogenase. Reaction mixtures contained, in a total volume of 1 ml, 25 μmol Tris·HCl buffer (pH 8.2), 50 μmol KCl, 5 μmol ATP, 7 μmol MgCl₂, 1.5 μmol P-enolpyruvate, 0.2 μmol NADH, 8 μg pyruvate kinase, 16 μg lactate dehydrogenase, the sugar substrate, and an appropriate volume of the enzyme preparation. Controls without hexose were used to correct for phosphatase activity acting on P-enolpyruvate and ATP.

**RESULTS**

**Effect of Concentration of Hexose.** Under standard assay conditions, the activity of hexokinase II increased until the glucose concentration was approximately 0.5 mM (Fig. 1). Further increase in glucose concentration did not affect the enzyme activity. The Kₘ value for glucose (determined from double reciprocal plots) was 48 μM. In separate experiments, it was found that mannose was also an effective substrate, and the Kₘ for this sugar was 76 μM. Pea seed hexokinase II phosphorylated fructose, but relatively high concentrations of this sugar were required for substantial rates of phosphorylation (Fig. 2). The Kₘ for fructose (10 mM) was much higher than the values for the aldose sugars. Some of the kinetic constants of hexokinase II with these hexose sugars are shown in Table I. Decreasing the pH of the reaction mixtures to 6.6 did not affect the Kₘ for glucose. Hexokinase II also phosphorylated D-glucosamine and 2-deoxy-D-glucose and, to a small extent, D-galactose (Table II).

**Effect of Concentration of Mg²⁺ and ATP.** In the presence of the standard assay concentration (5 mM) of ATP, increasing the MgCl₂ concentration increased the rate of hexokinase II activity (Fig. 3). Maximum activity was obtained when the MgCl₂ concentration was approximately equal to that of ATP, and further increase in MgCl₂ resulted in inhibition of the enzyme. When the concentration of MgATP was varied, the activity of hexokinase II increased until the MgATP concentration was 1 to 2 mM (Fig. 4). Further increase in the MgATP concentration did not affect the reaction velocity. The Kₘ for MgATP was 86 μM.

**Effect of pH.** The activity of pea seed hexokinase II in a series of Tris-acetate buffers is shown in Figure 5. The optimum pH was approximately 8.2, and activities of 90% or more of the peak activity were obtained between pH 7.5 and pH 9.5. The activity at pH 6.6 was approximately 52% of that obtained at the optimum pH.
Effect of ADP. Hexokinase II was inhibited by ADP, and the extent of this inhibition was dependent on the concentration of MgATP. With reaction mixtures of the composition used for the standard assay (5 mM MgATP), the addition of 0.5 mM and 1.0 mM MgADP resulted in inhibitions of 36 and 66%, respectively. When the MgATP concentration for the reaction mixtures was reduced to 1 mM, the addition of 0.5 mM and 1.0 mM MgADP gave inhibitions of 60 and 72%, respectively. Decreasing the glucose concentration in the standard mixtures had little effect on the extent of the MgADP inhibition.

Effect of Salts of Monovalent Cations. The addition of chlorides of monovalent cations produced some stimulation of hexokinase II activity. In experiments where the effects of added salts were studied, the enzyme preparation and the coupling enzymes were dialyzed against 10 mM Tris-HCl buffer (pH 8.2) for 90 min. There was little difference in the stimulation given by KCl, NaCl, LiCl, RbCl, and NH₄Cl. The percentage increase in hexokinase II activity produced by these salts averaged 24% at 60 mM and 41% at 120 mM.

Effect of Aluminum. Al³⁺ salts had essentially no effect on the activity of pea seed hexokinase II under the standard assay conditions (pH 8.2) (Fig. 6). When the pH of the reaction mixtures was lowered to 6.6 (Mes buffer), Al³⁺ became a strong inhibitor (Fig. 6). At this lower pH, 50% inhibition of enzyme activity was...
given by approximately 2 \mu M Al^{3+} (added as KAl[SO_4]_2-12H_2O) when the MgCl_2 concentration was 1.5 \text{ mm}. The inhibition was reduced when the concentration of MgCl_2 was increased, e.g. with 2.5 \text{ mm} MgCl_2, approximately 6 \mu M Al^{3+} was required for 50% inhibition.

Effect of Other Metabolites. Under the standard assay conditions (pH 8.2), the following metabolites (final concentration 1 \text{ mm}) had no effect on hexokinase II activity: glucose-1-P; 2-P-glycerate; 3-P-glycerate; 2,3-P2-glycerate; P-enolpyruvate; pyruvate; lactate; ethanol; UDP-glucose; ADP-glucose; AMP; malate; succinate; citrate; and Pi. At pH 6.6 (Mes buffer), 3-P-glycerate (1 \text{ mm}) and citrate (1 \text{ mm}) stimulated the activity of pea seed hexokinase II by 2- to 6-fold. The extent of the stimulation depended, in a large part, upon the particular sample of ATP used in the preparation of the reaction mixtures. The addition of 1 \text{ mm} 3-P-glycerate or citrate also relieved the inhibition produced by 50 \mu M Al^{3+}.

**DISCUSSION**

In the present investigation, a second hexokinase, hexokinase II, has been obtained from pea seeds. This enzyme is distinct from the fraction I enzyme, hexokinase I, described previously (13). Glucose was the preferred substrate of hexokinase II, and the enzyme had low affinity (K_m, 10 \text{ mm}) for fructose. It seems possible that in vivo hexokinase II may be mainly concerned with the phosphorylation of glucose rather than with that of fructose.

It is useful, at this stage, to compare some of the properties of the four hexose kinases obtained from pea seed extracts and to consider the relevant phosphorylation coefficients (Table III). The phosphorylation coefficient, introduced by Sols and Crane (10), is thought to give a good indication of the physiological suitability of a substrate for hexokinase (16). The expression

\[
\text{Phosphorylation coefficient} = \frac{V (\text{substrate})}{V (\text{glucose})} \times \frac{K_m (\text{glucose})}{K_m (\text{substrate})}
\]

was originally applied to brain hexokinase, for which glucose is the physiological substrate (10). This equation has been used in Table III for hexokinase I and hexokinase II. For fructokinase I and fructokinase II, the expression has been changed to

\[
\text{Phosphorylation coefficient} = \frac{V (\text{substrate})}{V (\text{fructose})} \times \frac{K_m (\text{fructose})}{K_m (\text{substrate})}
\]

in view of the probable physiological role of the fructokinases. It is apparent, from Table III, that glucose is the preferred substrate for hexokinase I and hexokinase II and that, as a consequence of relatively high K_m values for fructose, the phosphorylation coefficient for glucose of fructokinase I and fructokinase II is low, but this is due to a lower V for glucose than for fructose. Metabolic specificity in the pea seed hexose kinases may therefore be achieved either by (a), a K_m value for one hexose so high that in vivo utilization is unlikely; or, (b), a lowered V for the hexose not favored. This may mean that, in pea seeds, there are two enzymes, hexokinase I and hexokinase II, the primary role of which is the phosphorylation of glucose and that there are two other enzymes, fructokinase I and fructokinase II, which are primarily involved in the phosphorylation of fructose.

Pea seed hexokinase II was inhibited by relatively low concentrations of ADP and, in this respect, was similar to hexokinase I and fructokinase I (4, 13). Form P-II of yeast hexokinase was also inhibited by ADP (3, 7).

Aluminum was a very strong inhibitor of pea seed hexokinase II at pH 6.6. Aluminum toxicity has been observed in a number of plant species, and a variety of physiological effects of aluminum have been reported (6). It has been suggested that one effect of aluminum may be to decrease the utilization of ATP in glucose phosphorylation (1, 2). This would be consistent with the results of the present investigation. Citrate and 3-P-glycerate stimulated the activity of pea seed hexokinase II when the pH of the reaction mixtures was 6.6. There was no stimulation at the optimum pH (8.2). It is probable that the activation of the enzyme at pH 6.6 by these metabolites was due to coordination with aluminum which was present in the reaction mixtures (17). Aluminum has been shown to be a contaminant in commercial preparations of ATP (11, 17).

**LITERATURE CITED**

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