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# Subcellular Localization of Hexose Kinases in Pea Stems: Mitochondrial Hexokinase

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## ABSTRACT

**The subcellular localization of hexose phosphorylating activity in extracts of pea stems has been studied by differential centrifugation and sucrose density gradient centrifugation. The hexokinase (EC 2.7.1.1) was associated with the mitochondria, whereas fructokinase (EC 2.7.1.4) was in the cytosolic fraction. Some properties of the mitochondrial hexokinase were studied. The enzyme had a high affinity for glucose ($K_m$ 76 micromolar) and mannose ($K_m$ 71 micromolar) and a relatively low affinity for fructose ($K_m$ 15.7 millimolar). The $K_m$ for MgATP was 180 micromolar. The addition of salts stimulated the activity of the hexokinase. $Al^{3+}$ was a strong inhibitor at pH 7 but not at the optimum pH (8.2). The enzyme was not readily solubilized but, in experiments with intact mitochondria, was susceptible to proteolysis. A location on the outer mitochondrial membrane is suggested for the hexokinase of pea stems.**

The phosphorylation of hexoses by ATP is usually the first reaction in the metabolism of these sugars. Two isoenzymes of hexokinase (ATP: D-hexose 6-Ptransferase, EC 2.7.1.1) have been characterized in yeast (4, 18). Mammalian tissues contain four forms of hexokinase which have been designated I, II, III, and IV in order of elution from a DEAE-cellulose column (4, 18). Hexokinases I and II, the predominant forms in brain and skeletal muscle, respectively, bind reversibly to mitochondria and are readily solubilized by glucose-6-P (20). It is thought that the bound hexokinase is the more active form (15). Hexokinase III does not associate with the particulate fraction (14).

Plant tissues contain several enzymes capable of phosphorylating hexoses. In mature pea seeds, there are two hexokinases and two fructokinases (ATP: D-fructose 6-Ptransferase, EC 2.7.1.4) (24): these have been termed hexokinase I, hexokinase II, fructokinase I, and fructokinase II. It has been suggested that hexokinases I and II are primarily involved in the phosphorylation of glucose and mannose, whereas fructokinase I and II are largely responsible for the phosphorylation of fructose (6, 24–26).

There is little information available on the intracellular distribution of the hexose kinases in plant tissues. In the present investigation, the subcellular localization of hexokinase and fructokinase in pea stems has been studied. Pea stems were used as they are more suited to subcellular fractionation than pea seeds. The hexokinase of pea stems was associated with the mitochondria, whereas the fructokinase was soluble. The hexokinase was not readily solubilized and appeared to be located on the outer mitochondrial membrane.

## MATERIALS AND METHODS

**Materials.** Mature peas (*Pisum sativum L.*, cv Progress No. 9) were obtained from Yates-Cooper, Auckland, New Zealand. Perlite was from Australian Gypsum Ltd., Sydney. BSA (fraction V) was obtained from Sigma Chemical Co.; trypsin (bovine pancreas), trypsin inhibitor (hen egg white), and other biochemicals were from Boehringer-Mannheim GmbH; and Miracloth was from Calbiochem-Behring Corp.

**Subcellular Fractionation of Pea Stem Extracts.** Pea seeds were surface-sterilized for 10 min in 0.7% (w/v) NaOCl, soaked in water for 5 h, and germinated in Perlite in the dark for 10 d at 20°C. The seedlings were chilled at 4°C, the apical hook removed, and the stems harvested and rinsed in distilled $H_2O$. All subsequent operations were carried out at 4°C. Chilled pea stems (100 g) were sliced into 2-cm lengths and suspended in an equal volume of 1 mM Tes-KOH buffer (pH 7.2) which contained 0.3 M mannitol, 1 mM EDTA, and 1 mM GSH. After adding 1.8 ml of 0.2 M KOH, the suspension was poured through a Braun juice extractor lined with two layers of Miracloth. The crude extract was passed through another layer of Miracloth, adjusted to pH 7.2, and centrifuged at 1,000*g* for 10 min. The pellet was suspended in 10 ml of 0.3 M mannitol, centrifuged at 1,000*g* for 10 min, and resuspended in 5 ml of 10 mM Tes-KOH buffer (pH 7.2) (buffer A). The 1,000*g* supernatant and the wash supernatant were combined and centrifuged at 10,000*g* for 10 min. The 10,000*g* pellet was washed in 10 ml of 0.3 M mannitol and resuspended in 5 ml of buffer A. The 10,000*g* supernatant and the 10,000*g* wash supernatant were combined and centrifuged at 100,000*g* for 60 min, and the pellet was washed in 12 ml of 0.3 M mannitol and resuspended in 5 ml of buffer A.

**Sucrose Density Gradient Fractionation of Pea Stem Extracts.** Pea stems (100 g) were placed in a glass tray with 50 ml of 1 mM Tes-KOH buffer (pH 7.2) containing 0.5 M mannitol, 1 mM EDTA, and 1 mM GSH. The stems were sliced into 1-mm sections and stirred briefly, the extract was filtered through Miracloth, and the pH was adjusted to 7.2. Pea stem extract (9 ml) was layered on top of gradients of 24 ml containing 1 mM Tes-KOH buffer (pH 7.2), 1 mM EDTA, and sucrose in concentrations which increased linearly from 20 to 55% (w/w). A 3-ml cushion of 60% (w/w) sucrose was placed at the bottom of the gradients. After centrifuging in a Beckman L2-65 Ultracentrifuge at 25,000 rpm for 3 h using an SW-27 rotor, fractions of 30 drops were pumped from the bottom of each gradient. The fractions corresponding to the soluble contents of the cell were dialyzed against five changes of buffer A over 24 h.

**Preparation of Washed Mitochondria from Pea Stems.** Pea stems (100 g) were sliced into 2-cm lengths and suspended in an equal volume of 1 mM Tes-KOH buffer (pH 7.2) containing 0.3 M mannitol, 1 mM EDTA, 3 mM cysteine-HCl, and 0.1% (w/v) BSA. After adding 1.8 ml of 0.2 M KOH, the suspension was homogenized in a Braun juice extractor and the pH adjusted to 7.2. The extract was centrifuged at 370*g* for 5 min, and the supernatant was decanted through Miracloth and centrifuged at 3,000*g* for 10 min. The 3,000*g* pellet was resuspended in 10 ml of 0.3 M mannitol and 0.1% (w/v) BSA and centrifuged at 370*g* for 5 min. The

supernatant was centrifuged at 3,000*g* for 10 min in a glass centrifuge tube. Inspection of the washed 3,000*g* pellet showed that there were three layers. After the loose yellow layer at the top of the pellet was removed by aspiration, the middle buff colored layer containing washed mitochondria was suspended in 2 ml of 0.3 M mannitol containing 0.1% (w/v) BSA. (This suspension will be referred to as washed mitochondria.) The bottom yellow-green layer was left undisturbed.

**Sucrose Density Gradient Centrifugation of Washed Mitochondria.** Washed mitochondria (0.3 ml) were diluted to 2 ml with 0.3 M mannitol, layered on top of a sucrose gradient as described above, and centrifuged at 25,000 rpm for 3 h. Fractions of 30 drops were pumped from the bottom of each gradient.

**Preparation of Mitochondrial Membranes.** Washed mitochondria were centrifuged at 3,000*g* for 10 min and osmotically shocked by resuspending in 10 ml of buffer A. The membranes from shocked mitochondria were sedimented at 9,000*g* for 10 min and resuspended in buffer A using 2 ml buffer/100 g tissue extracted. Preparations of this type were used to determine the kinetic constants of the pea stem mitochondrial hexokinase and to study the effect pH, metabolites, salts, and inhibitors on the enzyme.

**Assay of Enzyme Activity.** All enzyme assays were carried out at 30°C. Glucose phosphorylation was measured by coupling the production of glucose-6-P with the reduction of NADP using 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), 5.5 μmol $MgCl_2$, 5 μmol ATP, 0.34 μmol NADP, 50 μmol KCl, 5 μmol glucose, 0.6 μg glucose-6-P dehydrogenase, 30 μg 6-P-gluconate dehydrogenase, and an appropriate volume of enzyme. The change in *A* at 340 nm was followed. When the phosphorylation of fructose was measured, reaction mixtures contained 0.5 μmol fructose as substrate and 0.3 μg P-glucose isomerase was added. When mannose was used as substrate, 0.3 μg P-glucose isomerase and 0.8 μg P-mannose isomerase were added. For assays of hexokinase activity in mitochondrial membranes, 6-P-gluconate dehydrogenase was omitted. Low mol wt compounds were removed from crude homogenates and the 100,000*g* supernatant before assay by Sephadex G-25.

Other enzyme assays included succinate-Cyt *c* reductase and NADH-Cyt *c* reductase (9), triose-P isomerase (23), catalase (1), fumarase (16), and Cyt *c* oxidase (28). One mU[1] of enzyme activity is defined as the amount of enzyme which catalyzed the formation of 1 nmol of product/min. Protein concentration was measured by the dye binding method of Bradford (3).

**Electron Microscopy of Washed Mitochondria.** Washed mitochondria (0.1 ml) were fixed in 3% (w/v) glutaraldehyde at pH 7.2 for 2 h, pelleted at 100,000*g*, washed with 0.1 M sodium cacodylate (pH 7.2), and postfixed with 1% (w/v) $OsO_4$ for 2 h at room temperature. The pellet was dehydrated in an acetone series and embedded in Spurr's resin (22). Thin sections were cut from top to bottom through the pellet with a LKB Ultramicrotome, stained with 2% (w/v) aqueous uranyl acetate, followed by Reynolds' lead citrate reagent (19), and examined in a Phillips 400 electron microscope at 100 kv.

**Effect of Trypsin on Mitochondrial Hexokinase.** Washed mitochondria (equivalent to 15 μg protein) were incubated at 30°C in a mixture which contained, in a volume of 0.4 ml, 1 μmol Tes-KOH buffer (pH 7.2), 100 μmol mannitol, and 1 μl of a 1 mg/ml trypsin solution in 1 mM HCl. After 1 min, trypsin inhibitor (5 μg) was added. Control incubations which contained no trypsin, or where trypsin was added to the mitochondria after the trypsin inhibitor, were also carried out. Hexokinase activity was measured following the addition to incubation mixtures of 0.6 ml of a solution which contained 25 μmol Tris-HCl buffer (pH 8.2), 5 μmol glucose, 5 μmol ATP, 5.5 μmol $MgCl_2$, 0.34 μmol NADP, 50 μmol KCl, and 0.6 μg glucose-6-P dehydrogenase. The intactness of the outer mitochondrial membrane following the trypsin incubations was determined by measuring succinate-Cyt *c* reductase activity, both in reaction mixtures which contained 0.25 M mannitol and at a mannitol concentration which disrupted the mitochondria (0.1 M). A solution which contained, in a volume of 0.6 ml, 5 μmol Pi buffer (pH 7.2), 15 μmol sodium succinate, 0.05 μmol Cyt *c*, 1 μmol KCN and, for the higher mannitol concentration, 150 μmol mannitol, was added to incubation mixtures.

[1] Abbreviations: U, unit; *V*, maximum velocity.

## RESULTS

The phosphorylating activity of crude extracts of pea stems was approximately 1.5 times greater with fructose as substrate than with glucose (Table I). When crude extracts were fractionated by differential centrifugation, most of the glucose phosphorylating activity was associated with the particulate fractions. More than 50% of this activity sedimented at 10,000*g* together with over 80% of the Cyt *c* oxidase activity. Less than 5% of the glucose phosphorylating activity in the crude extract remained in the 100,000*g* supernatant, but essentially all of the fructose phosphorylating activity was soluble. It soon became clear that these activities were due to a hexokinase and a fructokinase, respectively, and this terminology will now be used (25).

**Sucrose Density Gradient Fractionation.** The distribution after sucrose density gradient fractionation of the hexose kinases together with marker enzymes for certain organelles is shown in Figure 1. Glucose phosphorylating activity (hexokinase) occurred predominantly in a band of density equivalent to 1.16 g/ml, while the fructokinase activity remained at the top of the gradient (Fig. 1a). There was quantitative recovery of the hexose phosphorylating activity applied to the gradient. The distribution of hexokinase activity in the gradient corresponded closely with the mitochondrial marker fumarase (Fig. 1b) (5). The low level of fumarase activity at the top of the gradient indicated that little fumarase had been solubilized and that most of the mitochondria were isolated with inner membranes intact. NADH-Cyt *c* reductase which is located in mitochondria (10) and the ER (17) was found in several regions of the gradient (Fig. 1b), one of which (density 1.16 g/ml) corresponded to the mitochondrial marker fumarase. Hexokinase was clearly separated from the two regions of the gradient in which triose-P isomerase activity was recovered (Fig. 1c). Triose-P isomerase has been shown to occur in etioplasts and in the cytosol (21). Some catalase was associated with the mitochondrial fraction (Fig. 1c). Catalase normally occurs in microbodies in plant tissues but is easily solubilized due to the fragile nature of these organelles (2, 13). There was a small pellet which contained 2% of the total protein but no enzyme activity.

**Purity of Pea Stem Mitochondria.** The enzyme profile obtained when a preparation of washed mitochondria was centrifuged in a 20 to 55% (w/w) continuous sucrose density gradient is shown in Figure 2. Hexokinase activity was located in a sharp band (density 1.16 g/ml) which corresponded in the gradient to the mitochon-

Table I. *Subcellular Distribution of Hexose Kinases in Pea Stem Extracts*

Stems (100 g) of 10-d-old pea seedlings were homogenized and centrifuged as described in the text.

| Fraction | Glucose Phosphorylation | Fructose Phosphorylation | Cyt *c* Oxidase | Protein |
|---|---|---|---|---|
| | *mU* | *mU* | *U* | *mg* |
| Crude homogenate | 2,860 | 4,450 | 47.1 | 57.5 |
| 1,000*g* Pellet | 325 | 44 | 4.1 | 2.6 |
| 10,000*g* Pellet | 1,508 | 109 | 37.9 | 5.6 |
| 100,000*g* Pellet | 468 | 29 | 9.6 | 10.6 |
| 100,000*g* Supernatant | 125 | 3,193 | 0.1 | 35.6 |

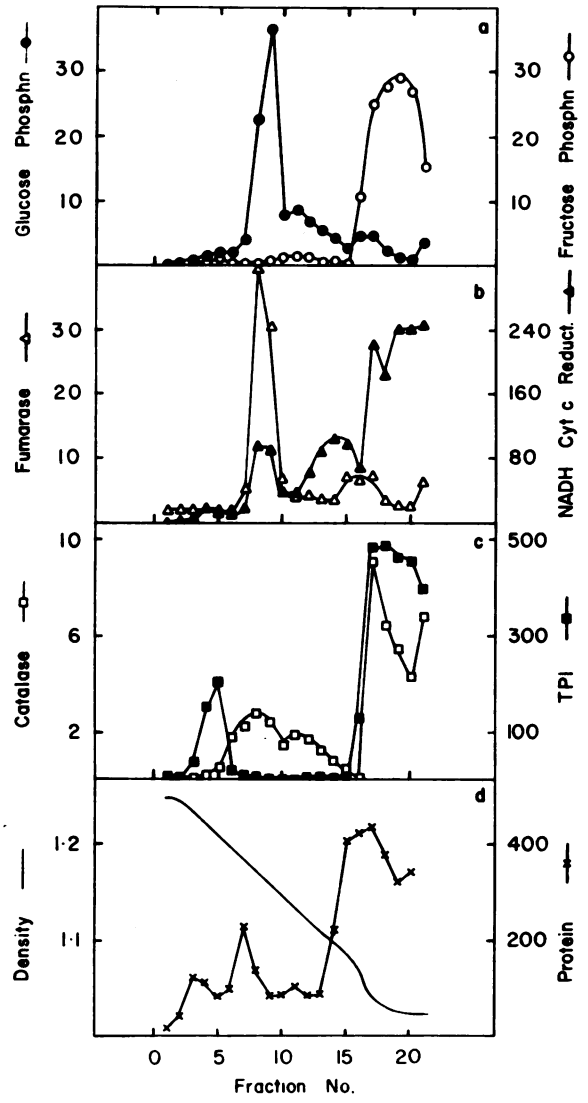


FIG. 1. Distribution of enzymes of a crude pea stem extract in a sucrose density gradient. A crude extract of pea stems was fractionated in a sucrose gradient as described in the text. a, Glucose and fructose phosphorylation; b, fumarase and NADH-Cyt *c* reductase; c, catalase and triose-P isomerase; and d, protein content and density. Enzyme activities are in mU/fraction with the exception of catalase, which is shown in U/fraction. Density is given as g/ml and protein as μg/fraction.

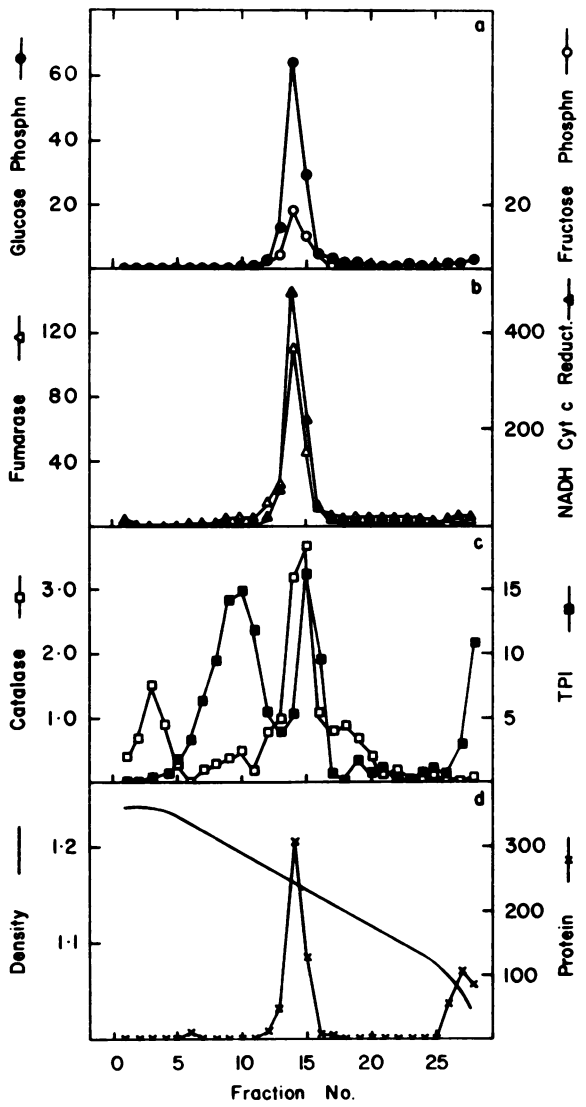


FIG. 2. Determination of purity of pea stem mitochondria by sucrose density centrifugation. The washed mitochondrial preparation was centrifuged in a sucrose density gradient as described in the text. a, Glucose and fructose phosphorylation; b, fumarase and NADH-Cyt *c* reductase; c, catalase and triose-P isomerase; and d, protein content and density. Enzyme activities are in mU/fraction with the exception of catalase, which is shown in U/fraction. Density is given as g/ml and protein as μg/fraction.

drial marker fumarase (Fig. 2, a and b). Only one band of NADH-Cyt *c* reductase activity was observed also at density 1.16 g/ml (Fig. 2b). The absence of NADH-Cyt *c* reductase activity at lower densities indicated that the washed mitochondrial preparation was not contaminated by ER. The triose-P isomerase and catalase activities in the washed mitochondrial preparation represented less than 5% of the total activity of these enzymes in crude extracts. The presence in the gradient of catalase at a density of 1.24 g/ml (Fig. 2c) and triose-P isomerase at a density of 1.21 g/ml (Fig. 2b) suggested the mitochondrial preparation contained a small proportion of microbodies and etioplasts, respectively. No hexose phosphorylating activity was found in the gradient fractions coinciding with either of these organelles. The major protein peak in the gradient was associated with the mitochondrial fraction (Fig. 2d). Negligible amounts of protein coincided with the etioplast and microbody markers. Most of the soluble protein in the gradient could be accounted for by the addition of BSA to the medium used to prepare the mitochondria. Examination of the washed mitochondrial preparation in the electron microscope showed over 90% condensed mitochondria which had both inner and outer membranes intact. Etioplasts (less than 5%) were the main contaminant of the preparation.

Table II. *Effect of Trypsin on Pea Stem Mitochondrial Hexokinase*
Experimental details were as described in the text.

| Addition | Hexokinase | Succinate-Cyt *c* Reductase | |
|---|---|---|---|
| | | 0.25 M Mannitol | 0.1 M Mannitol |
| | *mU/mg protein* | *nmol/min·mg protein* | |
| None | 433 | 98 | 633 |
| Trypsin | 2.9 | 87 | 503 |
| Inhibited trypsin | 422 | 77 | 493 |

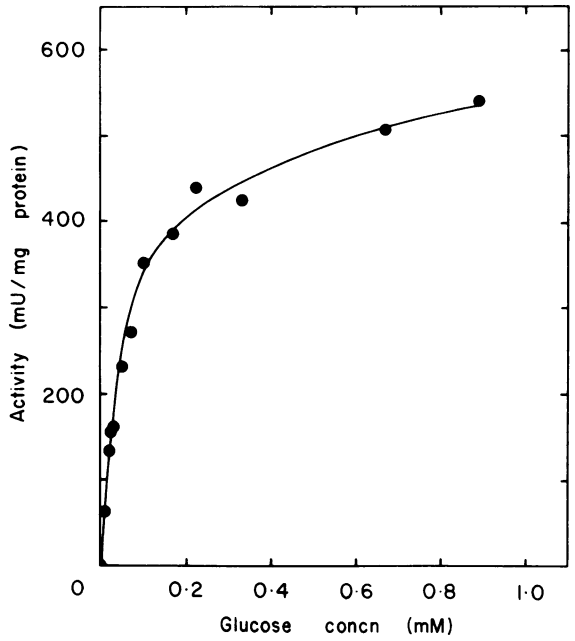


FIG. 3. Effect of glucose concentration on hexokinase of pea stems. Reaction mixtures were of the composition described for the standard assay with the concentration of glucose varied as shown.

Table III. *Kinetic Constants of Pea Stem Mitochondrial Hexokinase*

Reaction mixtures were of the composition described for the standard assay, with the concentration of hexose varied.

| Hexose | $k_m$ | $V$ | $V/K_m$ |
|---|---|---|---|
| | *mM* | *mU/mg protein* | *ratio* |
| D-Glucose | 0.076 | 580 | 7,632 |
| D-Mannose | 0.071 | 326 | 4,592 |
| D-Fructose | 15.7 | 633 | 40.3 |

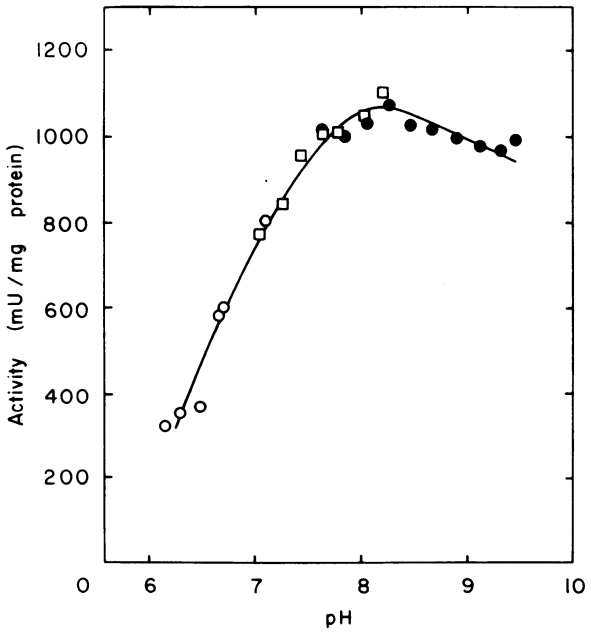


FIG. 4. Effect of pH on hexokinase of pea stems. Reaction mixtures were of the composition described for the standard assay except that 25 mM Mes-KOH (○), 25 mM Hepes-KOH (□) or 25 mM Tris-HCl (●) buffers were used.

**Structural Integrity of Pea Stem Mitochondria.** A measure of the degree of intactness of the outer membrane of the washed mitochondria was obtained by determining the reduction of exogenous Cyt *c* with succinate as described by Douce *et al.* (9). The ratio of the rates of Cyt *c* reduction by disrupted and intact mitochondria was usually between 6 and 15 indicating that over 80 to 90% of the mitochondria had intact outer membranes (8).

**Effect of Proteolysis on Mitochondrial Hexokinase.** Hexokinase associated with mitochondria of pea stems was readily attacked by trypsin (Table II). The rates of reduction of Cyt *c* by succinate in the trypsin-treated mitochondria, determined at mannitol concentrations of 0.25 and 0.1 M, are also shown in Table II. A comparison of these rates indicates that at least 80% of the trypsin-treated mitochondria had intact outer membranes after hexokinase had been completely inactivated (8, 9).

**Solubilization of Mitochondrial Hexokinase.** Washed mitochondria were incubated at 4°C for 20 min in 100 mM Tris-HCl buffer (pH 8.4) containing 0.25 M mannitol and 1 mM EDTA, and centrifuged at 7,000*g* for 15 min. Approximately 20% of the total hexokinase activity was released into the supernatant. The addition glucose-6-P (final concentration 5 mM) to the incubation mixture did not increase the amount of hexokinase activity released into the supernatant. Addition of detergents solubilized hexokinase activity was released into the supernatant. The addition of glucose-6-P (final concentration 5 mM) to the incubation mixture did not increase the amount of hexokinase activity released into the supernatant. Addition of detergents solubilized sodium deoxycholate solubilized 90% of the hexokinase, but the solubilized enzyme rapidly lost activity. The enzyme associated with mitochondrial membranes was more stable and retained over 95% of activity after 3 h at 4°C. Membranes of mitochondria could be stored frozen at −15°C with no detectable loss of hexokinase activity in 2 months.

**Effect of Concentration of Hexose.** The effect of increasing the concentration of glucose on the activity of the mitochondrial hexokinase is shown in Figure 3. The $K_m$ value for glucose was 76 μM (determined from double reciprocal plots). In separate experiments, it was found that the enzyme also had a high affinity for mannose as substrate ($K_m$ 71 μM). Fructose was phosphorylated effectively only when present in the reaction mixtures at relatively high concentrations, and the $K_m$ for this sugar was 15.7 mM. Some kinetic constants of the mitochondrial hexokinase for glucose, mannose, and fructose are given in Table III.

**Effect of Concentration of $Mg^{2+}$ and ATP.** The mitochondrial hexokinase required $Mg^{2+}$ for activity. Maximum activity was obtained when the concentration of $Mg^{2+}$ and ATP were approximately equal. The enzyme was not inhibited by excess $Mg^{2+}$, but excess ATP was inhibitory. When the concentration of MgATP was varied in the presence of a fixed excess (0.5 mM) of $MgCl_2$, the $K_m$ for MgATP was 0.18 mM.

**Effect of pH.** The mitochondrial hexokinase of pea stem showed a broad pH optimum when assayed in a series of Mes-KOH, Hepes-KOH, and Tris-HCl buffers (Fig. 4). The optimum pH was 8.2, and 90% or more of the maximum activity was obtained between pH 7.5 and pH 9.5.

**Effect of ADP.** Pea stem mitochondrial hexokinase was inhibited by ADP. Under standard assay conditions (5 mM ATP, 5.5 mM $MgCl_2$), 50% inhibition of activity was obtained with 0.4 mM MgADP. When the concentrations of ATP and $MgCl_2$ in the reaction mixtures were reduced to 1 and 1.5 mM, respectively, the inhibition of hexokinase activity was more marked, and 0.15 mM MgADP gave 50% inhibition.

**Effect of Salts.** The addition of chlorides of monovalent cations stimulated hexokinase activity. In experiments involving the effect of salts, the coupling enzyme was dialyzed against 1 mM Tris-HCl buffer (pH 8.2) and the concentration of ATP was reduced to 1 mM with 1.5 mM $MgCl_2$. Under these conditions, the concentra-

tions of $Na^+$, $K^+$, and $NH_4^+$ in the reaction mixtures was 7.4, 1.0, and 2.5 mM, respectively. The addition of 60 mM KCl, NaCl, RbCl, LiCl, or CsCl to reaction mixtures stimulated activity by approximately 100%. Increasing the concentration of the salts to 120 mM produced no further increase in activity.

**Effect of $Al^{3+}$.** At the optimum pH (8.2), $Al^{3+}$ salts had no significant effect on the activity of the mitochondrial hexokinase from pea stems. When the pH was lowered to 7 using Mes-KOH buffer, $Al^{3+}$ became a potent inhibitor. At pH 7, 50% inhibition of activity was given by 10 $\mu$M $Al^{3+}$. The effect of $Al^{3+}$ on the mitochondrial hexokinase was very similar to that previously observed with pea seed hexokinase II (25).

## DISCUSSION

Subcellular fractionation by differential centrifugation and sucrose density gradient centrifugation indicated that the fructokinase activity of pea stems was located exclusively in the cytosol. Fractionation of crude pea stem extracts by sucrose density gradient centrifugation showed that essentially all of the hexokinase activity coincided with enzyme markers for the mitochondria. The hexokinase remained with the washed mitochondria which were structurally intact and substantially free of contamination by other organelles. There was no evidence of hexokinase activity associated with other subcellular organelles or in the cytosol.

The preferred substrate of the pea stem hexokinase was glucose and the enzyme had a low affinity for fructose. The properties of the pea stem mitochondrial hexokinase were in many ways similar to the properties of hexokinase II of mature pea seeds (25). Both hexokinase II and the pea stem hexokinase were sensitive to inhibition by MgADP, and both enzymes were also strongly inhibited by $Al^{3+}$ at pH values close to 7 (25).

The pea stem hexokinase was not readily solubilized from the mitochondria. Glucose-6-P was not effective in releasing the enzyme. This contrasts with the situation in mammalian tissues where hexokinase bound to mitochondria can be readily solubilized by salts and low concentrations of glucose-6-P (12, 20).

In experiments with intact mitochondria, the hexokinase was sensitive to inactivation by trypsin. The attack by trypsin on hexokinase occurred without major disruption of the outer mitochondrial membrane indicating that the hexokinase was accessible to the medium. This suggests that the enzyme was located on the outer surface of the outer mitochondrial membrane. Hexokinase has been shown to bind to the outer membrane of mitochondria from brain (7) and a specific hexokinase-binding protein has been characterized from the outer membrane of rat liver mitochondria (11).

Mammalian hexokinases are subject to regulation by glucose-6-P (27), but little evidence has been found for fine control mechanisms for pea seed hexose kinases or pea stem hexokinase. It is possible that enzyme activity is limited mainly by substrate availability. Assuming the hexokinase in pea stems is located on the outer membrane, then the proximity of the enzyme to a supply of ATP from the mitochondria would tend to keep the glucose concentration in the cytosol at a very low level.

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