Characterization of Hexokinase and Fructokinase from Suspension-Cultured *Catharanthus roseus* Cells*

Yūko Yamashita[1] and Hiroshi Ashihara

Department of Biology, Faculty of Science, Ochanomizu University, 2-1-1, Otsuka, Bunkyo-ku, Tokyo, 112, Japan

Z. Naturforsch. **43c,** 827–834 (1988); received June 28, 1988

Catharanthus roseus, Suspension Culture, Fructokinase, Glycolysis, Hexokinase

Two different hexose-phosphorylating enzymes, hexokinase and fructokinase, were partially purified from suspension-cultured *Catharanthus roseus* cells. One of the enzymes, hexokinase, catalyzed the phosphorylation of both glucose and fructose. The K_m values for glucose and fructose were 0.06 mM and 0.23 mM, respectively. The V_{max} of the enzyme with fructose was approximately three times higher than with glucose. This enzyme was specific in its requirement for ATP and its K_m value for ATP was 52 μM. The optimum pH was 8.0 and Mg^{2+} or Mn^{2+} was required for the activity. The activity was inhibited by considerably higher concentrations of ADP (*i.e.,* 4 mM ADP was required for 50% inhibition). The second enzyme, fructokinase, was specific for fructose, and no activity was detected with glucose as substrate. This enzyme used UTP or CTP as phosphate donor. The K_m values of this enzyme for fructose and UTP were 0.13 mM and 0.15 mM, respectively. The pH optimum was 7.2, and Mg^{2+} or Mn^{2+} was required for the activity. These divalent cations could be partially replaced by Ca^{2+}. The activity was inhibited non-competitively by ADP and AMP. 90% inhibition of the activity by 0.5 mM ADP was observed in the presence of 2 mM UTP and 5 mM $MgCl_2$. Fructose-2,6-bisphosphate, glucose-1,6-bisphosphate, glucose-6-phosphate, and fructose-6-phosphate had little or no effect on the activity of both the hexokinase and the fructokinase. Based on these results, a discussion is presented of the role of hexokinase and fructokinase and their involvement in the regulation of the metabolism of sugars in *Catharanthus* cells.

Introduction

Phosphorylation of free hexoses is the initial step in the incorporation of sugars into the glycolytic and into biosynthetic pathways in plant cells. This step is catalyzed by enzymes called hexose kinases. Hexose kinases are usually classified into three types of enzyme with respect to their substrate specificity: (a) hexokinase (EC 2.7.1.1) which catalyzes the phosphorylation of both glucose and fructose; (b) fructokinase (EC 2.7.1.4) which catalyzes the phosphorylation of fructose; and (c) glucokinase (EC 2.7.1.2) which catalyzes the phosphorylation of glucose. Extensive studies of hexose kinases from yeast and mammalian cells have been carried out [1–3], but hexose kinases have been investigated in only a limited number of plant materials [4–6]. Characterization of four types of hexose kinase in pea seeds has been performed by Turner and his coworkers [7–11]. Baldus *et al.* [10] found two soluble hexokinases and a particulate hexokinase in spinach leaves. In a study of cultured plant cells, Fowler and Clifton [11] reported both particulate and soluble hexokinases in sycamore cells. Furthermore, Huber and Akazawa [12] found both glucokinase and fructokinase activities in extracts of these cells.

In the course of our research into the mechanisms that regulate glycolysis in cultured *Catharanthus roseus* cells [13–15], we found that one of the most likely rate-limiting reactions in the flux of glycolysis is the phosphorylation of hexose. Thus, in the present study, our attention was focussed on hexose kinases in these cells. We purified two hexose phosphorylating enzymes from the cells and characterized their properties. From our analysis of the kinetic data, we are able to speculate on the possible role of these enzymes in the metabolism of carbohydrates in plant cells.

Abbreviations: FK, fructokinase (ATP:D-fructose-6-phosphotransferase, EC 2.7.1.4); HK, hexokinase (ATP:D-hexose-6-phosphotransferase, EC 2.7.1.1); UDPG, UDP-glucose; PPi, pyrophosphate.

* Part 29 of the series, Metabolic Regulation in Plant Cell Culture.

[1] Present address: Research Centre, Nippon Roche Co. Ltd., Fuji Building, 3-2-1, Marunouchi, Chiyoda-ku, Tokyo, 100, Japan.

Reprint requests to Dr. H. Ashihara.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/1100–0827 $ 01.30/0

Materials and Methods

Plant materials

Suspension cultures of *Catharanthus roseus* (L.) G. Don [= *Vinca rosea* L.] (strain B/TH) were maintained as described in an earlier report [14].

Purification of HK and FK

Catharanthus cells (50 g fresh weight) were harvested from 5-day-old cultures and washed with distilled water. The washed cells were homogenized in a Potter-Elvehjem type glass homogenizer with 70 ml of imidazole-HCl buffer (pH 7.6) that contained 2 mM $MgCl_2$, 1 mM sodium EDTA, and 0.1% 2-mercaptoethanol. The homogenate was centrifuged at $20{,}000 \times g$ for 30 min at 2 °C. The supernatant obtained was treated with finely ground, solid ammonium sulphate. The protein fraction precipitating between 40 and 60% saturation was collected by centrifugation, and dissolved in 2.5 ml of 0.2 M HEPES-NaOH buffer (pH 7.2). The fraction was desalted on a column of Sephadex G-25 (bed volume, 9.0 ml). The eluted protein fraction (approximately 3.5 ml) was filtered through a cellulose nitrate membrane disc (pore size 0.45 μm, Toyo Roshi Kaisha Ltd., Tokyo, Japan). A portion of the filtrate (3.0 ml) was loaded, for HPLC, on a anion-exchange column, Shodex IEC QA-824 (Showa Denko Co., Tokyo, Japan), equilibrated with 20 mM Tris-HCl buffer (pH 8.2) which contained 20 mM KCl. After the column was washed with 9 ml of the equilibration buffer over the course of 10 min, the enzymes were eluted over a period of 50 min with a linear gradient from 0.02–1.0 M KCl in the Tris-HCl buffer. The flow rate was 0.9 ml per min, and fractions of 0.9 ml were collected. HK and FK were eluted with 0.27–0.43 M KCl, but the separation of the two enzymes was not always complete. Therefore, active fractions that contained both HK and FK (usually fraction numbers 20–30) were pooled and treated again with solid ammonium sulphate. The protein fraction that precipitated at 70% saturation was collected and dissolved in 2 ml of 0.1 M sodium phosphate buffer (pH 7.0) that contained 0.6 M ammonium sulphate. The fraction was filtered through a cellulose nitrate membrane disc, and the filtrate was loaded onto a hydrophobic column, Shodex HIC PH-814 (Showa Denko Co., Tokyo, Japan), equilibrated with 0.1 M sodium phosphate buffer (pH 7.0) that contained 1.2 M ammonium sulphate. After the column was washed with 9 ml of the equilibrium buffer over the course of 10 min, HK and FK were eluted over a period of 80 min with a linear gradient from 1.2 M to 0 M ammonium sulphate in the sodium phosphate buffer. The flow rate was 0.9 ml per min, and fractions of 0.9 ml were collected. Active fractions (usually, fraction numbers 59–61 for HK and fraction numbers 64–66 for FK) were pooled and glycerol was added to a final concentration of 50% v/v. The preparation was stored at −20 °C and used for assays of enzymatic activity within three days.

Assays of enzymatic activities

The activities of HK and FK were measured spectrophotometrically by following changes in absorbance at 340 nm, at 30 °C, with a Hitachi double beam spectrophotometer, type U-3200, which was fitted with an accessory for enzymatic analysis.

The standard reaction mixture for the assay of HK contained 50 mM HEPES buffer (pH 7.2), 5 mM fructose, 2 mM ATP, 5 mM $MgCl_2$, 0.2 mM NAD^+, 1 U glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*) and 2.8 U phosphoglucoisomerase. When glucose was used as the substrate for HK, 5 mM glucose was substituted for 5 mM fructose, and phosphoglucoisomerase was omitted. The mixture for the assay of FK was the same as for HK, except that 2 mM ATP was replaced by 2 mM UTP. In the reaction mixtures for the determination of the kinetic properties of HK and FK, constituents of the mixtures were changed as indicated in the text. In the case of the determination of the effects of glucose-6-phosphate and fructose-6-phosphate on the activity, the reaction mixture was changed to the following: 50 mM HEPES buffer (pH 7.2), 5 mM fructose, 2 mM ATP (or 2 mM UTP), 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 3 U pyruvate kinase and 3 U lactate dehydrogenase.

The preparation of glucose-6-phosphate dehydrogenase was obtained from Oriental Yeast Co., Tokyo, Japan, and the other enzymes were obtained from Boehringer Mannheim GmbH, Mannheim, F.R.G. The activity of these auxiliary enzymes is described in units (U), *i.e.*, μmol of substrate consumed per min at 30 °C. Glucose-6-phosphate, fructose-6-phosphate, glucose-1,6-bisphosphate, and fructose-2,6-bisphosphate were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., and nucleotides were from Kyowa Hakko Kogyo Co., Tokyo, Japan.

Kinetic experiments were carried out at least twice using different preparations of enzymes. Typical data are presented in tables and figures.

Results

Hexokinase activities in extracts

In preliminary experiments, extracts from 5-day-old cells from suspension cultures of *Catharanthus roseus* were treated with 70% saturated ammonium sulphate. After the precipitated protein fraction was desalted on Sephadex G-25, the protein was fractionated by HPLC on Shodex IEC QA-824 as described in Materials and Methods. When fractions were assayed for hexose kinase activity with glucose and ATP, two peaks were observed. In addition, a third peak appeared when the activity was assayed with fructose and UTP. Fig. 1 shows elution profiles of hexose kinases with fructose as the substrate and with ATP or UTP as the phosphate donor. For convenience, the peaks are designated Fractions I, II, and III, in order of elution. During these preliminary studies, outlines of the properties of these Fractions were elucidated. Fraction I phosphorylated both glucose and fructose and used ATP as phosphate donor. Fraction II had a relatively greater ability to phosphorylate fructose and used UTP rather than ATP as phosphate donor. Fraction III phosphorylated both glucose and fructose with ATP, but its activity was much lower than the activities of Fractions I and II. Therefore, we purified the activities in Fractions I and II and characterized them further. From the properties of the materials in these fractions, we refer to Fraction I as hexokinase (HK), and to Fraction II as fructokinase (FK).

Separation of HK from FK

HK and FK were partially separated from each other by HPLC on Shodex IEC QA-824 (Fig. 1). However, the separation of the two enzymes was incomplete, especially when large amounts of protein were loaded. Therefore, for kinetic studies, we followed the procedure described in Materials and Methods. Fig. 2 shows the elution profiles of HK and

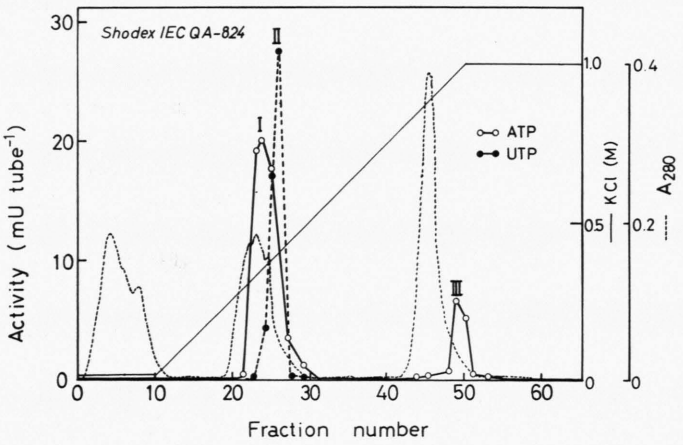

Fig. 1. Elution profiles of hexose-phosphorylating enzymes chromatographed on a column of Shodex IEC QA-824. The protein fraction precipitated at 70% saturation with ammonium sulphate was desalted and applied to the column. The enzymatic activities were assayed with 5 mM fructose and 2 mM ATP (○——○) or UTP (●----●).

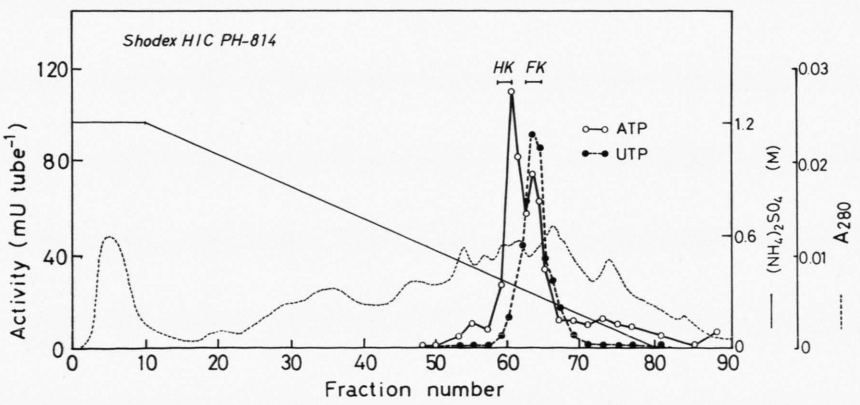

Fig. 2. Elution profiles of hexokinase and fructokinase chromatographed on a column of Shodex HIC PH-814. The active fractions eluted from the column of Shodex IEC QA-824 were desalted and applied to the column as described in Materials and Methods. The enzymatic activities were assayed with 5 mM fructose and 2 mM ATP (○——○) or UTP (●----●). The activity with UTP has been drawn reduced by a factor of four.

FK from Shodex HIC PH-814. HK and FK were almost completely separable from each other. In order to minimize cross-contamination, fractions indicated as bars in Fig. 2 were collected. Specific activities of HK and FK were approximately 800 and 2200 mU (nmol min^{-1} mg $protein^{-1}$), respectively, under optimal conditions. These values were higher than the specific activities of hexose kinases purified from pea seeds [7–9, 16].

Effect of concentration of hexose

The effects of various concentrations of glucose and fructose on the activity of HK and FK were determined and kinetic constants are summarized in Table Ia. The K_m value of HK for glucose was approximately 4-fold lower than that for fructose, but the V_{max} of this enzyme for fructose was 3 times higher than that for glucose. As a result, similar V_{max}/K_m values were obtained for glucose and fructose. In contrast, the activity of FK was strictly specific for fructose. The K_m value of FK for fructose was rather lower than that of HK for fructose.

Specificity and the effects of concentration of nucleoside triphosphates

The specificity of the nucleotide triphosphates required by HK and FK is shown in Table II. ATP was the preferred phosphate donor for HK. In contrast, the pyrimidine nucleotides, UTP and CTP, were better phosphate donors for FK than purine nucleotides. The effects of various concentrations of ATP and UTP on the activity of HK and FK were examined and kinetic constants are summarized in Table Ib. The activity of HK was better adapted to use of ATP than of UTP as phosphate donor. In fact, the value of V_{max}/K_m for ATP was more than 7-fold higher than that for UTP. By contrast, the V_{max} of FK was much higher in the presence of UTP, even though the K_m value of FK for UTP was approximately 3-fold higher than that for ATP.

Table I. Kinetic constants of hexokinase (HK) and fructokinase (FK) from suspension-cultured *Catharanthus roseus* cells.

	Hexokinase		Fructokinase	
a. Sugars	Fructose	Glucose	Fructose	Glucose
K_m [mM]	0.23	0.06	0.13	–
V_{max}	625	215	2200	0
V_{max}/K_m	2720	3580	16920	0
b. Nucleotides	ATP	UTP	ATP	UTP
K_m [mM]	0.052	0.288	0.050	0.151
V_{max}	625	468	690	2200
V_{max}/K_m	12020	1625	13800	14570

For determinations of the K_m values of HK for sugars and for nucleotides, saturating concentrations of ATP or fructose were used. For determinations of the K_m values of FK, saturating concentrations of UTP or fructose were used. V_{max} values are expressed as nmol of hexosephosphate formed per min per mg protein.

Effect of pH

The effect of pH on the activities of HK and FK is shown in Fig. 3. The optimum pH for HK was 8.0, while that for FK was 7.2.

Effects of divalent cations

The effect of Mg^{2+}, Mn^{2+}, and Ca^{2+} on the activity of HK and FK were investigated. Mg^{2+} was the most effective cation, and Mn^{2+} was also effective in promoting the activity of both HK and FK (Fig. 4 and unpublished results). In contrast, Ca^{2+} was effective only for the activity of FK (Fig. 4). The optimum concentration of Ca^{2+} for FK was higher than that of Mg^{2+}, and the maximum activity of FK with Ca^{2+} was approximately 2.5 times lower than that with Mg^{2+}.

Effects of nucleoside mono- and diphosphates

The effects of ADP, UDP, AMP, and UMP on the activity of HK and FK were investigated (Fig. 5). Each of these nucleotides inhibited the activity of both HK and FK, but the most significant inhibition was found in the case of FK. Among nucleotides

Table II. Nucleotide triphosphate specificity of hexokinase and fructokinase from suspension-cultured cells of *Catharanthus roseus*.

	Hexokinase		Fructokinase	
Nucleotides	Concentration [μM]			
	50	100	50	100
ATP	299 (100)	458 (100)	363 (66)	391 (45)
GTP	57 (19)	128 (28)	303 (55)	382 (44)
UTP	69 (23)	179 (39)	550 (100)	868 (100)
CTP	105 (35)	183 (40)	561 (102)	799 (92)

The values are expressed as mU (*i.e.*, nmol of hexose phosphate formed per min) per mg protein and % of control values (*i.e.*, the velocities in the presence of ATP for hexokinase and in the presence of UTP for fructokinase).

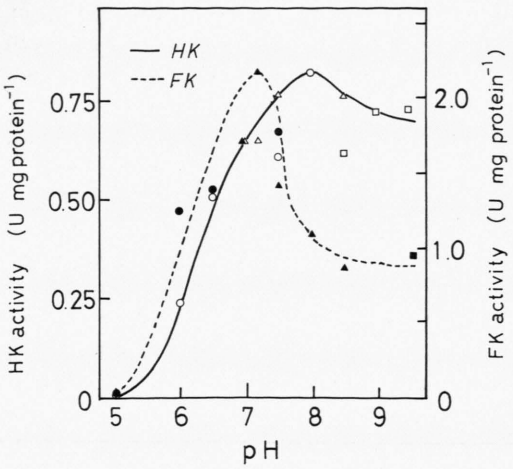

Fig. 3. Effects of pH on the activity of hexokinase (open symbols with a solid line) and fructokinase (closed symbols and a dashed line). The following buffers were used at a concentration of 50 mM: ○, MES-NaOH; △, HEPES-NaOH; □, glycine-NaOH.

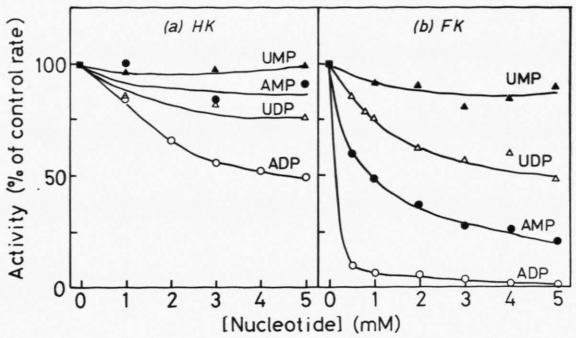

Fig. 5. Effects of ADP, AMP, UDP, and UMP on the activities of hexokinase and fructokinase. ○, ADP; ●, AMP; △, UDP; ▲, UMP.

tested, ADP was the most potent inhibitor of FK; more than 90% inhibition was found in the presence of 0.5 mM ADP. Fig. 6 shows the effects of nucleoside triphosphates on the inhibition by ADP and UDP. The V_{max} values of HK and FK were significantly reduced by ADP and UDP. Therefore, this inhibition seems to be non-competitive. The effect of pH on the inhibition of FK by 0.3 mM ADP and 3 mM UDP was also examined. Little effect of pH on the inhibition was found when the activity was measured at pH 6.0, 6.5, 7.2 and 8.0 (data not shown).

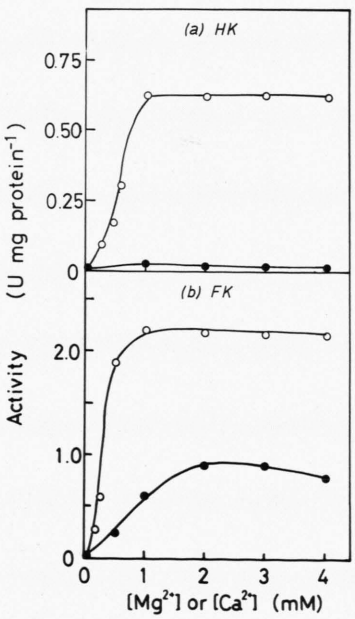

Fig. 4. Effects of Mg^{2+} and Ca^{2+} on the activities of hexokinase (a) and fructokinase (b). ○——○, activity with Mg^{2+}; ●——●, activity with Ca^{2+}.

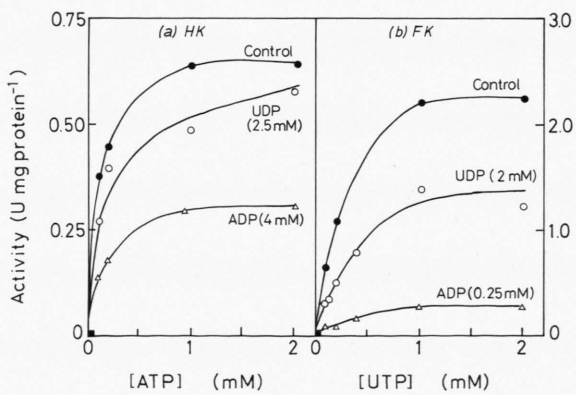

Fig. 6. Effects of various concentrations of ATP and UTP on the activities of hexokinase (a) and fructokinase (b) when inhibited by ADP or UDP. ●, control; △, ADP (4 mM for hexokinase and 0.25 mM for fructokinase); ○, UDP (2.5 mM for hexokinase and 2.0 mM for fructokinase).

Effect of metabolites

Under the standard assay conditions, little or no effect of glucose-6-phosphate (1 mM), fructose-6-phosphate (1 mM), glucose-1,6-bisphosphate (2 mM) and fructose-2,6-bisphosphate (2 μM) was found on the activities of HK and FK (data not shown).

Discussion

In heterotrophic liquid cultures of *Catharanthus roseus,* the sucrose in the medium is almost completely hydrolyzed to glucose and fructose by the wall-bound and/or extracellular invertase ([15] and K. Sagishima and H. Ashihara, unpublished data). Thus, the metabolism of the carbohydrates that are taken up by the cells from the medium seems to be initiated by the phosphorylation of glucose and fructose.

Several hexose kinases with different substrate specificities have been reported in mature pea seeds [7–9, 16, 17], endosperms and scutella of maize [18], developing endosperms of *Ricinus communis* [19], spinach leaves [10], the plant cytosolic fraction of soybean nodules [20], and cultured sycamore cells [12]. These data indicate that patterns of hexose kinases are different in different plant tissues and organs as well as in different plant species.

Our present study indicates that two hexose kinases, namely HK and FK, which have distinct different properties, are present in cultured *Catharanthus roseus* cells. HK from *Catharanthus* cells can phosphorylate both glucose and fructose (Table I). The K_m values of HK from *Catharanthus* for glucose (0.06 mM) and fructose (0.23 mM) are almost the same as the corresponding values for soluble hexokinase (isozyme IV) from spinach leaves (0.07 mM for glucose and 0.21 mM for fructose). Furthermore, similar to the *Catharanthus* enzyme, the spinach enzyme is also much more active with fructose than with glucose [10]. In contrast, FK from *Catharanthus roseus* was highly specific for fructose (Table I). A fructokinase with a high degree of substrate specificity, like the enzyme from *Catharanthus roseus,* has thus for been reported only in the plant cytosolic fraction of soybean nodules [20].

Catharanthus HK has a specific requirement for ATP, and the K_m value of this enzyme for ATP is similar to that of many other plant hexokinases [7, 9, 16, 19]. By contrast, FK from *Catharanthus* cells has a specific requirement for UTP and CTP. Pyrimidine nucleotide specificity has not been reported for any other FK from higher plants. The concentration of cytosine nucleotides in *Catharanthus roseus* cells appears to be much lower than that of uracil nucleotides [21, 22]. Thus, the most likely phosphate donor for FK *in vivo* seems to be the UTP in the cells.

The optimum pH of *Catharanthus* HK is 8.0 and the activity was higher at alkaline pH (7.5–9.5) than at neutral or acidic pH (Fig. 3). Similar pH optima were also reported in hexokinases from *Loranthus* leaves [23], the plant cytosolic fraction of soybean nodules [20], garlic bulbs [24], and pea seeds [7, 16]. In contrast, the pH optimum of FK from *Catharanthus* cells is 7.2 (Fig. 3). The optimal pH values for FK from various other plant materials are, however, above 8.0 [8, 17, 20].

The activities of HK and FK from *Catharanthus* cells were inhibited by nucleoside di- and monophosphates at pH 7.2 (*i.e.,* at physiological pH). The estimated concentrations of ATP, ADP, AMP, UTP, UDP, and UMP in 1-day-old *Catharanthus* cells are 2.0, 0.35, 0.32, 1.1, 0.59, and 0.65 mM, respectively, when the volume of cytoplasm is assumed to be 5% of the volume of the cells [21]. Therefore, inhibition by ADP and probably by AMP of the activity of FK seems likely to occur *in vivo.*

Glucose-6-phosphate and glucose-1,6-bisphosphate, inhibitors of mammalian hexokinases [3, 25] had no effect on HK and FK from *Catharanthus roseus*. This result indicates that regulation of hexokinase activities in plant cells is different from that in mammalian cells.

Fig. 7 shows a scheme for the possible metabolic route of carbohydrates in suspension cultures of *Catharanthus roseus* grown with sucrose as the source of carbon. As mentioned above, sucrose seems to be hydrolyzed extracellularly (step 1), and glucose and fructose enter into the cells and are phosphorylated. ATP-linked HK may participate in this step (step 2). A portion of glucose-6-phosphate and fructose-6-phosphate may enter the glycolytic pathway, but another portion of these sugar phosphates may be utilized for the reformation of sucrose by sucrose phosphate synthetase (step 4) and sucrose phosphate phosphatase (step 5). In fact, when the cells are incubated with [U-^{14}C]glucose or [U-^{14}C]-fructose, a significant amount of radioactivity is incorporated into sucrose (K. Sagishima and H. Ashihara, unpublished results). Transiently accumulated sucrose may be utilized as an energy source

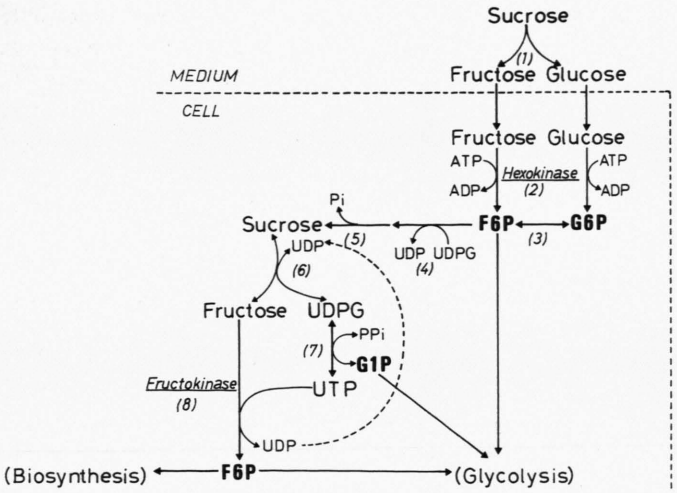

Fig. 7. Possible roles of hexokinase and fructokinase in the metabolism of carbohydrates in suspension cultures of *Catharanthus roseus*. The numbers represent enzymes as follows. (1) invertase (EC 3.2.1.26); (2) hexokinase (2.7.1.1); (3) phosphoglucoisomerase (5.3.1.9); (4) sucrose-6-phosphate synthase (2.4.1.14); (5) sucrose-6-phosphate phosphatase (3.1.3.22); (6) sucrose synthase (2.4.1.13); (7) UDPG-pyrophosphorylase (2.7.7.9); (8) fructokinase (2.7.1.4). F6P, fructose-6-phosphate; G1P, glucose-1-phosphate; G6P, glucose-6-phosphate; UDPG, UDP-glucose.

and for the biosynthesis of cell constituents in accordance with the demands of the cells. Sucrose synthase (step 6) and UDPG-pyrophosphorylase (step 7) may be responsible for the degradation of sucrose, as suggested by Copeland *et al.* [9], Echeverria and Humphreys [26], Edwards and ap Rees [27], and Huber and Akazawa [12]. FK in *Catharanthus* cells may participate in the phosphorylation of fructose which is split off from sucrose by the reactions of sucrose synthase. UTP used as phosphate donor for FK may be generated from UDPG by the reaction of UDPG-pyrophosphorylase. Thus, phosphorylated sugars, glucose-1-phosphate and fructose-6-phosphate, may be produced by the cycling of uridine nucleotides and the consumption of PPi. Possible routes for the supply of PPi in the cells have been discussed in a previous paper [14]. The different properties of HK and FK confirmed in the present study tend to support the hypothesis described above. However, further study is necessary to establish the actual functions of HK and FK and their involvement in the regulation of carbohydrate metabolism in cultured plant cells.

Acknowledgement

This research was supported in part by a Grand-in-Aid for Scientific Research (No. 60480010) from the Ministry of Education, Science and Culture, Japan.

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