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GLUCOSE-PHOSPHORYLATING ENZYMES OF *CANDIDA* YEASTS AND THEIR REGULATION IN VIVO

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Summary

Three glucose-phosphorylating enzymes having different specificities for glucose and fructose were separated from the cell-free extract of *Candida tropicalis* by means of ammonium sulfate fractionation and chromatography on DEAE-cellulose and Sephadex G-100. Two of them, which phosphorylated fructose 1.5 times faster than glucose, were designated as hexokinase I and II (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1), and the other with very low or no fructose-phosphorylating activity, as glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2). K_m values for glucose with both hexokinase I and glucokinase were 0.3 mM, and that for fructose with hexokinase I was 2.2 mM. Time-course changes in the levels of these enzymes in *C. tropicalis* growing on glucose and on *n*-alkane revealed that hexokinase was induced specifically by the sugars, while glucokinase was a constitutive enzyme. Addition of cycloheximide to the culture medium prevented the increase in the hexose-phosphorylating activity and in the Fru/Glu ratio (the ratio of enzymatic phosphorylation of fructose to that of glucose) in the cells. Although *Candida lipolytica* also contained hexokinase and glucokinase, both enzymes seemed to be constitutive.

Introduction

Since the existence of two native hexokinase isoenzymes (ATP : D-hexose 6-phosphotransferase, EC 2.7.1.1) [1,2] and glucokinase (ATP : D-glucose 6-phosphotransferase, EC 2.7.1.2) [3,4] in *Saccharomyces cerevisiae* was reported, the kinetic properties of these hexokinase isoenzymes have been studied extensively [5—7]. Although Mazón et al. [8] reported the constitutive

Abbreviation: Fru/Glu ratio, the ratio of enzymatic phosphorylation of fructose to that of glucose.

nature of both hexokinase and glucokinase synthesis in *Rhodotorula glutinis*, very little is known about the regulation of the levels of these enzymes in yeasts. In liver, glucokinase was reported to be an inducible enzyme with kinetic properties different from hexokinase [6].

During the comparative studies on the glycolytic enzymes of various kinds of yeasts, we observed that yeast pyruvate kinase was classified into three groups based on the interaction with fructose 1,6-bisphosphate [9]. The interesting differences in the levels of glucose-phosphorylating activity and Fru/Glu ratio (the ratio of enzymatic phosphorylation of fructose to that of glucose) were also observed among three kinds of yeasts, *Candida tropicalis*, *Candida lipolytica* and *S. cerevisiae*.

This paper deals with the separation of the distinct glucose-phosphorylating enzymes, hexokinase and glucokinase, from *C. tropicalis* and *C. lipolytica*. Regulation of the enzyme levels in these yeasts growing on different carbon sources is also described.

Materials and Methods

Yeast strains and cultivation. The yeast strains used in this study were *C. lipolytica* NRRL Y-6795, *C. tropicalis* (Castellani) Berkhout strain pK 233 and *S. cerevisiae* ATCC 7753. These yeasts were cultivated aerobically as described previously [9,10]. The *n*-alkane mixture used was composed of C_{10} to C_{13}.

Assay. The enzyme activity to phosphorylate glucose or fructose was measured by the spectrophotometric method of Maitra [3] with a slight modification. The reaction mixture for glucose-phosphorylating activity consisted of 25 mM D-glucose (Nakarai Chemicals Co., Japan), 2 mM ATP (Kyowa Hakko Kogyo Co., Japan), 1 mM NADP (Sigma, U.S.A.), 10 mM $MgCl_2$, 1 unit of glucose-6-phosphate dehydrogenase (Sigma, Type VII) and 50 mM triethanolamine/HCl buffer (pH 8.0) in a total volume, 2.0 ml. Fructose-phosphorylating activity was measured in the same system as that for glucose except that glucose was substituted by 25 mM D-fructose (Nakarai Chemical Co.) and 1 unit of phosphoglucose isomerase (Sigma, Grade III). The assay was done at 30°C. One unit of the enzyme activity was defined as that which converted 1 μmole of substrate per min. Protein was assayed by the method of Lowry et al. [11] using bovine serum albumin as a standard.

Results

Level of glucose-phosphorylating activity and Fru/Glu ratio in three kinds of yeasts

Table I shows that both the level of glucose-phosphorylating activity and the Fru/Glu ratio in *S. cerevisiae* were high, while those in *C. lipolytica* were low, irrespective of carbon sources used. On the other hand, those values in *C. tropicalis* changed markedly depending upon the growth substrates. That is, the cells grown under glycolytic conditions showed higher levels of glucose-phosphorylating activity and the Fru/Glu ratio than the cells grown under gluconeogenic conditions. Although two assay methods differing in their principles were employed, a similar trend in the activity and Fru/Glu ratio was obtained

except that the absolute values of the activity were different. This may be due to the differences in their assay temperature and pH.

Partial purification of hexokinase and glucokinase from C. tropicalis

C. tropicalis was cultivated on glucose for 16 h with shaking. About 30 g of dry cells harvested from 10 liters of the broth were suspended in 600 ml of 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA and 2 mM 2-mercaptoethanol (buffer A), disrupted with a Dyno-Mill (Willy A. Bachofen Manufacturing Engineers, Type KDL), and centrifuged at 13000 × *g* for 20 min. The supernatant obtained was treated with solid ammonium sulfate to bring the saturation degree as indicated in the first ammonium sulfate fractionation of Table II. pH was maintained at 6.5 during the fractionation. Precipitates obtained by centrifugation at 13000 × *g* for 20 min were dissolved in 50 mM potassium phosphate buffer (pH 6.5) containing 1 mM EDTA and 2 mM 2-mercaptoethanol (buffer B). As shown in Table II, the enzyme activity with a low Fru/Glu ratio was detected in the fraction between 0.7 and 1.0 saturation. The fraction between 0.4 and 0.7 saturation containing most of glucose-phosphorylating activity was dialyzed overnight against buffer B with several

TABLE I

COMPARISON OF GLUCOSE-PHOSPHORYLATING ACTIVITY AND Fru/Glu RATIO OF VARIOUS YEASTS GROWN UNDER GLYCOLYTIC AND GLUCONEOGENIC CONDITIONS

The yeasts were harvested in the respective early stationary phases of growth on glucose (1.65%), fructose (1.65%), ethanol (1.0%), sodium acetate (1.36%) or *n*-alkane mixture (C_{10-13}) (1.0%).

Yeast	Carbon source	Glucose-phosphorylating activity			
		Assay method (1) *		Assay method (2) **	
		Activity ***	Fru/Glu ratio	Activity ***	Fru/Glu ratio
Candida tropicalis	Glucose	0.82	0.99	0.47	1.0
	Fructose	0.83	0.96	—†	—
	Ethanol	0.31	0.46	0.11	0.62
	Acetate	0.39	0.38	0.17	0.34
	Alkane	0.28	0.36	0.11	0.34
Candida lipolytica	Glucose	0.28	0.27	0.096	0.37
	Fructose	0.21	0.35	—	—
	Ethanol	0.15	0.32	0.057	0.33
	Acetate	0.19	0.47	0.10	0.55
	Alkane	0.17	0.26	0.084	0.43
Saccharomyces cerevisiae	Glucose	1.0	1.0	0.39	1.0
	Ethanol	0.88	0.69	0.35	0.67
	Acetate	0.72	0.83	0.30	0.79

* Assay system was described in the text.

** The cresol red method as specified by Lazarus et al. [1] was employed. The assay mixture was composed of 26.7 mM D-glucose or D-fructose, 3.3 mM ATP, 0.3% (w/v) $MgCl_2$, 0.001% cresol red and 8.3 mM glycylglycine in a total volume, 3 ml. pH was adjusted to 8.5 with 0.1 N NaOH. Decrease in the absorbance at 574 nm was measured.

*** Activity was expressed as unit/mg protein.

† Not tested.

TABLE II

AMMONIUM SULFATE FRACTIONATION OF GLUCOSE-PHOSPHORYLATING ENZYME FROM GLUCOSE-GROWN *CANDIDA TROPICALIS*

Cell-free extract was subjected to the 1st ammonium sulfate fractionation. The proteins precipitated between 0.40 and 0.70 saturation of ammonium sulfate in the 1st fractionation were subjected to the 2nd fractionation. Details are described in the text.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fru/Glu ratio	Yield (%)
Cell-free extract	4380	4100	0.935	1.02	100
1st $(NH_4)_2SO_4$ Fractionation					
0—0.40	1760	422	0.240	1.03	10.3
0.40—0.70	2320	3250	1.40	1.05	79.4
0.70—1.0	212	358	1.69	0.145	8.74
2nd $(NH_4)_2SO_4$ Fractionation					
0—0.43	509	179	0.35	1.06	4.37
0.43—0.60	748	2280	3.05	1.24	55.7
0.60—0.65	94.3	193	2.05	0.796	4.71
0.65—1.0	271	557	2.06	0.228	13.6

changes. The protein concentration of this dialyzed solution was adjusted to 10—15 mg/ml with the same buffer. This solution was subjected to the second ammonium sulfate fractionation as indicated in Table II. Fru/Glu ratio of the fraction between 0.43 and 0.60 saturation increased upto 1.24, while the enzyme activity with the low Fru/Glu ratio was detected in the fraction between 0.65 and 1.0 saturation.

The fraction between 0.43 and 0.60 saturation was dissolved in 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 2 mM 2-mercaptoethanol and 10% (v/v) glycerol (buffer C), and dialyzed overnight against the same buffer with several changes. The dialyzed solution was applied to DEAE-cellulose column (30 cm height and 150 ml packed volume) equilibrated previously with buffer C. The column was washed with 300 ml of this buffer and the enzyme was eluted with a linear gradient of NaCl (0 to 0.3 M) in 800 ml of the same buffer. Fractions of 10 ml each were obtained at 12-min intervals. In the DEAE-cellulose column chromatography, two distinct peaks and one shoulder of the activity were eluted. The Fru/Glu ratio of the first peak (around tube 65) was 1.4 to 1.6, and that of the second peak (around tube 75) was 1.1 to 1.3. After the peaks were eluted, the shoulder (around tube 85) could be observed, accompanied with a rapid decrease in the Fru/Glu ratio. These results indicated the existence of at least two isoenzymes of hexokinase having different mobility on anion-exchange chromatography, in addition to glucokinase. Hexokinase I (tubes 63—71) and hexokinase II (tubes 72—80) were collected separately, concentrated with a membrane filter (Amicon Co.), and applied to a Sephadex G-100 column (60 cm height and 300 ml packed volume) equilibrated previously with buffer A. When the preparation of hexo-

TABLE III
SUMMARY OF PURIFICATION PROCEDURES FOR HEXOKINASES FROM *CANDIDA TROPICALIS*
Details are described in the text.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)	Fru/Glu ratio
Cell-free extract	4380	4100	0.94	1	100	1.02
1st ammonium sulfate fractionation						
0.40—0.70 saturation	2320	3250	1.40	1.5	79.3	1.05
2nd ammonium sulfate fractionation						
0.43—0.60 saturation	748	2280	3.05	3.3	55.6	1.24
1st DEAE-cellulose column chromatography						
Hexokinase I	180	1140	6.36	6.8	27.8	1.43
Hexokinase II	128	760	5.94	6.4	18.6	1.25
Sephadex G-100 column chromatography						
Hexokinase I	50.4	780	15.5	16.6	19.0	1.44
Hexokinase II	10.1	165	16.3	17.4	4.0	1.46
2nd DEAE-cellulose column chromatography						
Hexokinase I	1.3	46	35.6	38.1	1.1	1.58

kinase I was subjected to the chromatography, glucokinase contaminated was eluted at first as fractions with a low Fru/Glu ratio, and then the major part of the activity was eluted with rapid increase in the Fru/Glu ratio to 1.4 to 1.6 (around tube 28). Fractions from tube 25 to 32 were collected, concentrated and dissolved in buffer C. This enzyme solution was re-chromatographed on DEAE-cellulose by the same procedure as described above. After the major peak with a Fru/Glu ratio of 1.4 to 1.6 was eluted, a shoulder with a little higher Fru/Glu ratio could be seen. A decrease in the Fru/Glu ratio with further elution was not observed. Sephadex G-100 column chromatography of hexokinase II showed a similar profile to that of hexokinase I except that the enzyme activity with the low Fru/Glu ratio was large in amount. The purification procedures of hexokinases are summarized in Table III.

The fraction between 0.65 and 1.0 saturation in the second ammonium sulfate fractionation was subjected to DEAE-cellulose column chromatography by the same procedures described above. After the fraction with the high Fru/Glu ratio were eluted (around tube 60), the major part of glucose-phosphorylating activity with the low Fru/Glu ratio was recovered around tube 80. Fractions from tubes 75 to 90 were collected and applied to Sephadex G-100 column. The active fractions with a very low Fru/Glu ratio were eluted around tube 21. The purification procedures of glucokinase are summarized in Table IV.

TABLE IV

SUMMARY OF PURIFICATION PROCEDURES FOR GLUCOKINASE FROM *CANDIDA TROPICALS*

Details are described in the text.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)	Yield (%)	Fru/Glu ratio
Cell-free extract	4380	4100	0.935	1	100	1.02
1st ammonium sulfate fractionation 0.40—0.70 saturation	2320	3250	1.40	1.50	79.3	1.05
2nd ammonium sulfate fractionation 0.65—1.0 saturation	271	557	2.06	2.20	13.6	0.228
DEAE-cellulose column chromatography	38.7	243	6.28	6.72	5.93	0.080
Sephadex G-100 column chromatography	6.48	152	23.5	25.1	3.71	0.042

Some kinetic properties of hexokinase and glucokinase

The specific activity and Fru/Glu ratio of the partially purified glucose-phosphorylating enzymes were listed in Tables III and IV. Some kinetic properties of hexokinase I and glucokinase were determined (Table V), whereas the amount of hexokinase II was too low to be purified and examined. Sugar-saturation curves for both enzymes were of the Michaelis-Menten type. K_m for glucose with hexokinase I was 0.3 mM and that for fructose was 2.2 mM. The Fru/Glu ratio of hexokinase I varied with the initial concentration of sugars, giving a maximum value of 1.8. When the Fru/Glu ratio was measured under the standard assay conditions employed (sugar at 25 mM), the ratio (1.6) was

TABLE V

SOME KINETIC PARAMETERS OF HEXOKINASE AND GLUCOKINASE FROM *CANDIDA TROPICALIS*

The assay conditions except for the sugar concentrations were the same as those described in the text. The preparations at the respective last step of purification procedures (Tables III and IV) were used as the enzyme sources.

Parameter	Value	
	Hexokinase I	Glucokinase
K_m (glucose)	0.34	0.29
K_m (fructose)	2.2	—
Fru/Glu (sugar at 0.5 mM)	0.67	—
Fru/Glu (sugar at 1.0 mM)	0.72	—
Fru/Glu (sugar at 10 mM)	1.4	—
Fru/Glu (sugar at 25 mM)	1.6	0.04
$\frac{V \text{ (fructose)}}{V \text{ (glucose)}}$	1.8	—

TABLE VI

LEVELS OF HEXOKINASE AND GLUCOKINASE ACTIVITIES IN *CANDIDA TROPICALIS* GROWN ON VARIOUS CARBON SOURCES

Hexokinase and glucokinase activities were calculated from the results shown in Table I on the basis of the assumption that hexokinase had Fru/Glu ratio of 1.5 and glucokinase, 0.

Carbon source	Specific activity (units/mg protein)	
	Hexokinase	Glucokinase
Glucose	0.54	0.28
Fructose	0.53	0.30
Ethanol	0.10	0.21
Acetate	0.10	0.29
Alkane	0.07	0.21

close to the ratio of V values of the enzyme on the two sugars (1.8). The K_m value of glucokinase for glucose was similar to that of hexokinase (Table IV).

Regulatory properties of hexokinase and glucokinase in C. tropicalis

C. tropicalis grown on the sugars showed a higher level of hexokinase activity than the cells grown on the gluconeogenic compounds, while glucokinase activity was almost at the same level in the cells grown on different carbon sources (Table VI). When *n*-alkane-grown cells were transferred to the glucose medium and cultivated, glucose-phosphorylating activity and the Fru/Glu ratio increased along with the yeast growth, reached the maxima at the late exponential growth phase, and then decreased (Fig. 1A). As shown in Fig. 1B, these increases in glucose-phosphorylating activity and Fru/Glu ratio were derived from the inductive nature of hexokinase synthesis and the constitutive nature

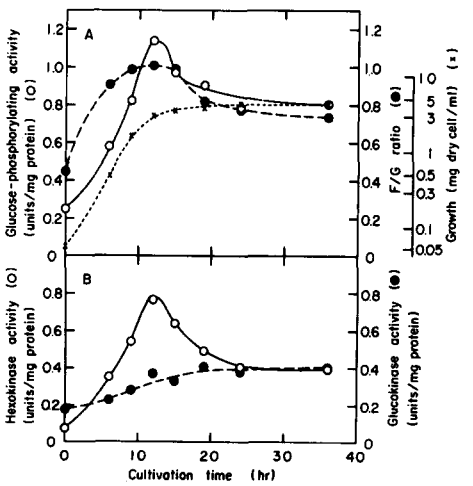

Fig. 1. Time-course changes in hexokinase and glucokinase activities of *Candida tropicalis* during cultivation on glucose. The yeast precultured on *n*-alkane was cultivated in the glucose medium with shaking at 30°C. Hexokinase and glucokinase activities were calculated as described in Table VI. (A) ○———○, glucose-phosphorylating activity; ●- - - - -●, Fru/Glu ratio; X- - - - -X, growth. (B) ○———○, hexokinase activity; ●- - - - -●, glucokinase activity.

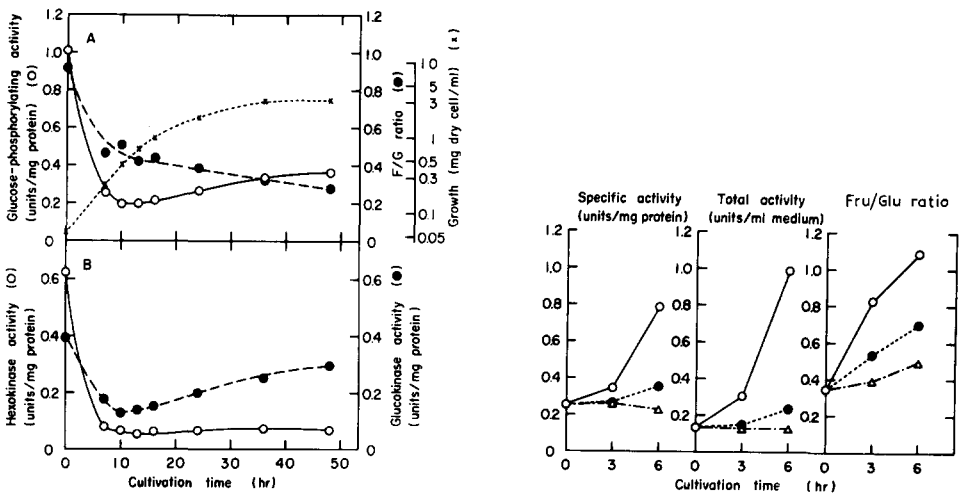

Fig. 2. Time-course changes in hexokinase and glucokinase activities of *Candida tropicalis* during cultivation on *n*-alkane. The yeast precultured on glucose was cultivated in the *n*-alkane medium with shaking at 30°C. Hexokinase and glucokinase activities were calculated as described in Table VI. (A) ○———○, glucose-phosphorylating activity; ●- - - - -●, Fru/Glu ratio; X- - - - -X, growth. (B) ○———○, hexokinase activity; ●- - - - -●, glucokinase activity.

Fig. 3. Effect of cycloheximide on synthesis of glucose-phosphorylating enzyme in *Candida tropicalis* growing on glucose. The yeast precultured on *n*-alkane was transferred to the glucose media without cycloheximide (○———○), with 1 μg/ml of cycloheximide (●- - - - -●) and with 10 μg/ml of cycloheximide (△— - —△).

of glucokinase synthesis, respectively. Fig. 2A shows the time-course changes in glucose-phosphorylating activity and Fru/Glu ratio in *C. tropicalis* growing on *n*-alkane. When the cells grown on glucose were used as the seed culture, glucose-phosphorylating activity decreased rapidly until the middle exponential growth phase on *n*-alkane, and then increased gradually. The Fru/Glu ratio decreased throughout the cultivation (Fig. 2A). Although both hexokinase and glucokinase activities decreased rapidly until the middle exponential growth phase, the rate of the decrease in hexokinase activity was larger than that in glucokinase activity (Fig. 2B). Thereafter, hexokinase activity was maintained at a low level, while glucokinase activity increased gradually. The cells precultured on *n*-alkane were transferred to the glucose medium containing cycloheximide (1 and 10 μg/ml), and cultivated for 6 h. As shown in Fig. 3, addition of cycloheximide prevented the increases in glucose-phosphorylating activity and the Fru/Glu ratio, although significant growth of the yeast was observed under these conditions.

Hexokinase and glucokinase in C. lipolytica

Purification of glucose-phosphorylating enzymes from *C. lipolytica* was carried out by the same procedures described above with a slight modification. Cell-free extract obtained from 80 g dry cells of glucose-grown *C. lipolytica* showed 0.20 unit/mg protein of specific activity and 0.23 of Fru/Glu ratio. This extract containing 3570 units of activity and 18.2 g of protein was treated

TABLE VII

LEVELS OF HEXOKINASE AND GLUCOKINASE ACTIVITIES IN *CANDIDA LIPOLYTICA* GROWN ON VARIOUS CARBON SOURCES

Hexokinase and glucokinase activities were calculated from the results shown in Table I on the basis of the assumption that hexokinase had Fru/Glu ratio of 1.5 and glucokinase, 0.

Carbon source	Specific activity (units/mg protein)	
	Hexokinase	Glucokinase
Glucose	0.05	0.23
Fructose	0.05	0.16
Ethanol	0.03	0.11
Acetate	0.06	0.13
Alkane	0.03	0.15

with solid ammonium sulfate. In the fraction between 0.7 and 1.0 saturation, 24% of the activity with 0.04 of the Fru/Glu ratio was recovered. The fraction between 0.4 and 0.7 saturation containing 54% of the activity was subjected to the second ammonium sulfate fractionation. Fru/Glu ratio of the fraction between 0.45 and 0.55 saturation increased upto 1.1 with 10% yield of the activity. This fraction was subsequently applied to a DEAE-cellulose column and eluted with a linear gradient of KCl (0 to 0.3 M). Hexokinase having a maximal Fru/Glu ratio of 1.5 was eluted as a shoulder earlier than the peak of glucokinase. The results obtained revealed that at least two glucose-phosphorylating enzymes, hexokinase and glucokinase, were present in *C. lipolytica*, as the case of *C. tropicalis*. Further purification could not be performed because of a low content of hexokinase in this yeast. The levels of hexokinase and glucokinase in *C. lipolytica* were not affected by the growth substrates used, and the level of glucokinase was much higher than that of hexokinase in all cases (Table VII).

Discussion

The results obtained in the purification of the enzymes revealed that *C. tropicalis* contained at least two hexokinase isoenzymes and a glucokinase. Hexokinase isoenzymes showed different mobility on anion-exchange chromatography, but gave a similar Fru/Gru ratio at the present stage of purification (Table III). Glucokinase preparation obtained on Sephadex G-100 column chromatography phosphorylated glucose 25 times faster than fructose. This observed activity on fructose might be due to the contamination of a small amount of hexokinase, although glucokinase purified from a hexokinase-less mutant of *S. cerevisiae* was demonstrated to have a measurable activity on fructose [3].

Hexokinase and glucokinase in *C. tropicalis* were regulated in different ways when the yeast was grown on various carbon sources (Table VI, Figs. 1 and 2). The level of hexokinase activity changed depending upon the carbon sources used, showing an inductive nature of its synthesis by sugars (Fig. 3). In the meantime, the constitutive nature of glucokinase synthesis was demonstrated. A change in the Fru/Glu ratio in cell-free extracts of *C. tropicalis* was not

observed during the storage at 0°C for one week, indicating lack of interconversion of hexokinase and glucokinase in vitro. It is interesting that the regulation of the enzyme level in *C. tropicalis* was quite opposite to that in liver. The level of liver glucokinase, but not that of hexokinase, changed depending upon hormonal and dietary conditions, and the glucokinase induction was responsible rather specifically to the glucose concentration [12—14]. It was also demonstrated that liver glucokinase showed a higher K_m for glucose than hexokinase, and that the former was insensitive to glucose 6-phosphate, while the latter was inhibited by this compound [12,15,16]. Viñuela et al. [12] suggested that glucokinase would be involved preferentially in the first step of glycogen synthesis at a high concentration of glucose, because of its insensitivity to glucose 6-phosphate and high K_m value. Different from the liver enzymes, hexokinase and glucokinase from *C. tropicalis* revealed a same level of K_m value for glucose.

Although the physiological significance is not clear at the present stage, the regulation of hexose-phosphorylating activity observed in *C. tropicalis* has never been demonstrated in any yeasts. *C. lipolytica* contained hexokinase and glucokinase, but these two enzymes seemed to be constitutive (Table VII), similarly to *R. glutinis* [8]. The existence of two native hexokinase isoenzymes having a different Fru/Glu ratio was also reported in *S. cerevisiae*. However, as the Fru/Glu ratio in this yeast grown on various carbon sources did not change (Table I), changes in the levels of hexokinase isoenzymes and glucokinase might not be involved in the regulation of hexose-phosphorylating activity in *S. cerevisiae*.

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