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DIFFERENCE IN PYRUVATE KINASE REGULATION AMONG THREE GROUPS OF YEASTS

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Summary

Yeast pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase EC 2.7.1.40) was classified into three groups based on the interaction with fructose-1,6-bisphosphate. The pyruvate kinases of *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* were activated by fructose 1,6-bisphosphate in the concentration range tested (up to 10 mM) of the substrate, phosphoenolpyruvate; the enzymes of "fermentative *Candida*" (*Candida tropicalis* and *Candida utilis*) were affected by fructose 1,6-biphosphate only when the substrate concentration was below 2 mM. Although the pyruvate kinase of *Candida lipolytica* (a yeast belonging to "oxidative *Candida*") was also affected by fructose 1,6-bisphosphate, the degree of the activation was extremely small as compared with the above four yeasts. The pyruvate kinase of *C. tropicalis* was inhibited by ATP more strongly in the absence of fructose 1,6-bisphosphate than its presence. In the case of the *C. lipolytica* enzyme, however, the enzyme was inhibited to a lesser extent by ATP, and fructose 1,6-bisphosphate did not reverse the inhibitory effect of ATP. Time course changes of the enzyme levels in the yeasts grown on glucose and on ethanol indicate that the pyruvate kinases of *S. cerevisiae* and *C. tropicalis* can be controlled both by an allosteric mechanism and by changes in the enzyme concentration, although a marked difference was observed in the susceptibility to the allosteric effect by fructose 1,6-biphosphate between these fermentative yeasts. On the other hand, that of *C. lipolytica* would be controlled only by the latter mechanism.

Introduction

Two mechanisms, which maintain enzyme activity at a certain low level under gluconeogenic conditions, have been presented with respect to the regu-

Abbreviation: Fru-1,6-P_2, fructose 1,6-bisphosphate.

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lation of pyruvate kinase (ATP : pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) in yeasts; one is the control of enzyme concentration and the other is the regulation of enzyme activity mediated by allosteric effectors such as fructose 1,6-bisphosphate (Fru-1,6-P_2) and ATP [1–4]. It has been proposed that the mode of the regulation of the enzyme is different depending upon the kind of yeasts. Gancedo et al. [2] reported that the pyruvate kinase of *Candida utilis* was not activated by Fru-1,6-P_2 and the enzyme level in this yeast was lowered markedly under gluconeogenic conditions. In contrast, the enzyme of *Saccharomyces cerevisiae* was activated by Fru-1,6-P_2 and was maintained at a relatively high level of activity even under gluconeogenic conditions. Thus, the former enzyme seemed to be controlled by change in the enzyme level and the latter by allosteric regulation. Barbalace et al. [5] have also described differences in pyruvate kinase regulation between oxidative yeasts and fermentative yeasts.

However, at least in some yeasts, the control of pyruvate kinase may be accomplished in a much more complicated manner; the two mechanisms mentioned above might operate simultaneously or in connection with each other. In this paper, we compared the kinetic properties and the levels of pyruvate kinases of five species of yeasts; two strains of *Saccharomyces* (fermentative), two strains of "fermentative *Candida*" and one strain of "oxidative *Candida*" [6], grown under glycolytic and gluconeogenic conditions. Significant differences were observed in the regulation of the enzymes of these yeasts: susceptibilities to Fru-1,6-P_2 activation and to ATP inhibition, and the changes of the enzyme levels depending upon carbon sources. Based on the results obtained, the differences in the regulatory mechanism of pyruvate kinase among these three groups of yeasts are discussed.

Materials and Methods

Cultivation

The yeast strains used in this study were as follows; *Saccharomyces cerevisiae* ATCC 7753, *Saccharomyces carlsbergensis* ATCC 9080, *Candida lipolytica* NRRL Y-6795, *Candida tropicalis* (Castellani) Berkhout strain pK 233 [7] and *Candida utilis* ATCC 9226. *Candida* yeasts were cultivated aerobically by the same procedure as reported previously [8]. The medium used was composed of 5.0 g $NH_4H_2PO_4$, 2.5 g KH_2PO_4, 1.0 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeCl_3 \cdot 6H_2O$, 1.0 g corn steep liquor and carbon source (see below) per liter of tap water (pH 5.2). When *n*-alkane was used as a substrate, 0.5 ml/l of Tween 80 was added to the medium. *Saccharomyces* yeasts were grown under the same condition except that corn steep liquor was replaced by a vitamin mixture (10 mg inositol, 5 μg biotin, 200 μg calcium pantothenate, 200 μg thiamin · HCl, 200 μg pyridoxine · HCl and 200 μg niacin per liter of medium). The carbon sources used were 1.65% (w/v) of glucose, 1.0% (v/v) of ethanol, 1.0% (v/v) of *n*-alkane (a mixture of C_{10-13}) and 1.36% (w/v) of sodium acetate.

Assay

Cells harvested by centrifugation were suspended in 0.05 M potassium

phosphate buffer (pH 7.2) and disrupted with a sonic disintegrator. Cell-free extracts obtained by centrifugation at 13 000 × *g* for 20 min were used for the assay of pyruvate kinase activity essentially according to the same method of Bücher and Pfleiderer [9]. In some cases, the cell-free extracts thus obtained were dialyzed against the buffer to eliminate effectors of low molecular weight. The assay mixture, unless stated otherwise, contained 1 mM phosphoenolpyruvate (Sigma), 1 mM ADP (Kyowa Hakko), 0.1 mM NADH (Kyowa Hakko), 1 mM Fru-1,6-P_2 (Sigma), 4 units of lactate dehydrogenase (Sigma, Type II), 10 mM $MgCl_2$, 0.1 M KCl and 50 mM triethanolamine · HCl (pH 7.4) in a total volume, 2 ml. All chemicals were reagent grade. Assay was done at 30° C, and one enzyme unit was defined as 1 μmol phosphoenolpyruvate decomposed per min. Protein was estimated by the method of Lowry et al. [10]. Residual glucose in the medium was determined according to the anthrone-H_2SO_4 method of Trevelyan and Harrison [11] with a slight modification.

Results

As shown in Table I, the pyruvate kinase levels of five yeasts grown on ethanol or acetate were lower than those of the respective glucose-grown cells. When *n*-alkane was used as carbon source (*C. lipolytica* and *C. tropicalis*), the enzyme levels were also as low as those of respective ethanol- or acetate-grown cells. *Saccharomyces* yeasts grown even under gluconeogenic conditions had much higher pyruvate kinase activities than *Candida* yeasts grown under glycolytic conditions.

When Fru-1,6-P_2 was omitted from the assay system, pyruvate kinase activities could hardly be detected in the cell-free extracts of *Saccharomyces* yeasts under the assay condition employed (phosphoenolpyruvate, 1 mM). On the other hand, the activities of *Candida* yeasts were scarcely affected by the

TABLE I

COMPARISON OF PYRUVATE KINASE ACTIVITIES OF VARIOUS YEASTS GROWN UNDER GLYCOLYTIC AND GLUCONEOGENIC CONDITIONS

Yeasts were harvested in the respective early stationary phases of growth on glucose (1.65%), ethanol (1.0%), sodium acetate (1.36%) or *n*-alkane mixture (C_{10-13}) (1.0%). The concentration of Fru-1,6-P_2 was 1 mM. Preparation of cell-free extracts and assay conditions were the same as described in Materials and Methods.

Yeast	Specific activity (units/mg protein)							
	Glucose-grown cells		Ethanol-grown cells		Acetate-grown cells		Alkane-grown cells	
	+ Fru-1,6-P_2	− Fru-1,6-P_2	+ Fru-1,6-P_2	− Fru-1,6-P_2	+ Fru-1,6-P_2	− Fru-1,6-P_2	+ Fru-1,6-P_2	− Fru-1,6-P_2
S. cerevisiae	5.44	<0.1	3.93	<0.1	3.91	<0.1	— *	— *
S. carlsbergensis	6.73	<0.1	3.64	<0.1	3.49	<0.1	— *	— *
C. tropicalis	1.45	1.43	0.47	0.45	0.42	0.44	0.30	0.30
C. utilis	1.97	1.85	1.15	1.00	0.77	0.63	— *	— *
C. lipolytica	0.76	0.75	0.22	0.21	0.32	0.32	0.37	0.38

* No growth.

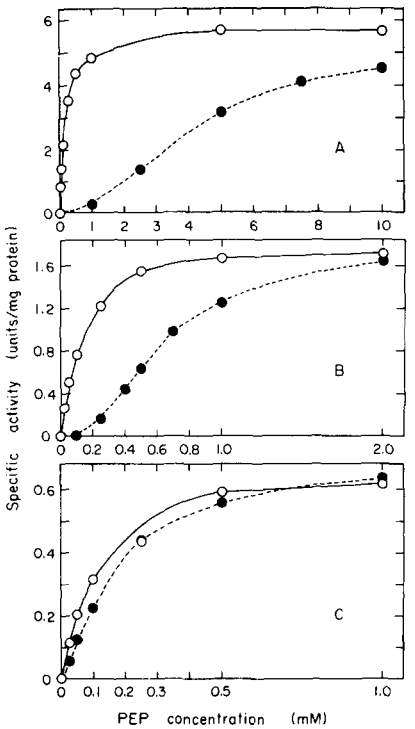

Fig. 1. Comparison of the effect of phosphoenolpyruvate (PEP) concentration on the pyruvate kinase activities of *S. cerevisiae* (A), *C. tropicalis* (B) and *C. lipolytica* (C) in the presence and absence of Fru-1,6-P_2. Cell-free extracts of the glucose-grown cells of these yeasts were dialyzed overnight against 0.05 M potassium phosphate buffer (pH 7.2). The enzyme of *S. cerevisiae* was assayed using potassium phosphate buffer (pH 6.2) instead of KCl plus triethanolamine buffer and those of *Candida* were assayed as described in the text. The enzyme activities were measured with 1 mM of Fru-1,6-P_2 (○———○) or without the activator (●- - - - -●).

omission of the allosteric activator (Table I). When the concentration of the substrate, phosphoenolpyruvate, was reduced, however, the activation of pyruvate kinase by Fru-1,6-P_2 was clearly observed even in the case of *C. tropicalis* (Fig. 1B). These results indicate that pyruvate kinases of fermentative *Candida* are also subject to an allosteric-type activation by Fru-1,6-P_2, but the activation occurs only at approximately one tenth of the concentration range of phosphoenolpyruvate compared with the enzyme of *S. cerevisiae* (Fig. 1A) and *S. carlsbergensis* [4]. Fig. 1C shows that the pyruvate kinase of *C. lipolytica* was similarly activated by Fru-1,6-P_2 at a low concentration of phosphoenolpyruvate, but the degree of this activation was very much smaller than the cases of fermentative yeasts tested.

Table II shows the $K_{0.5}$ values for phosphoenolpyruvate (the concentrations of phosphoenolpyruvate required for the half maximal velocity) of the pyruvate kinases of various yeasts grown on glucose. When assayed in the presence of Fru-1,6-P_2, the enzymes of five yeasts tested showed nearly equal $K_{0.5}$ values. However, the increase of $K_{0.5}$ values caused by the omission of the effector from the reaction mixture differed markedly among these yeasts: *Saccharomyces*, about 30 times; fermentative *Candida*, 4—5 times; *C. lipo-*

TABLE II

COMPARISON OF AFFINITIES FOR PHOSPHOENOLPYRUVATE OF PYRUVATE KINASES OF VARIOUS YEASTS IN THE PRESENCE AND ABSENCE OF Fru-1,6-P_2

Yeasts were harvested in the respective early stationary growth phases on glucose. Cell-free extracts were dialyzed overnight against 0.05 M potassium phosphate buffer (pH 7.2). The assay conditions of the pyruvate kinase activities of *Candida* yeasts are described in the text. The enzymes of *Saccharomyces* yeasts were assayed using potassium phosphate buffer (pH 6.2) instead of KCl plus triethanolamine buffer. The concentration of Fru-1,6-P_2 was 1 mM. $K_{0.5}$ for phosphoenolpyruvate was calculated from the plots such as those in Fig. 1.

Yeast	$K_{0.5}$ for phosphoenolpyruvate	
	+ Fru-1,6-P_2	−Fru-1,6-P_2
S. cerevisiae	0.15	4.50
S. carlsbergensis	0.14	4.20
C. tropicalis	0.13	0.60
C. utilis	0.14	0.62
C. lipolytica	0.12	0.16

lytica, an oxidative yeast, scarcely affected. The pyruvate kinases of ethanol-grown *C. tropicalis* and *C. lipolytica* showed almost the same affinity for phosphoenolpyruvate as those of the glucose-grown cells, irrespective of the presence or absence of Fru-1,6-P_2. Although the $K_{0.5}$ value for phosphoenolpyruvate of the pyruvate kinase from ethanol-grown cells of *Saccharomyces* was not determined in this study, Barwell and Hess [12] have observed that the activation of the enzyme by Fru-1,6-P_2 and the affinity for phosphoenolpyruvate was independent of the growth substrate (glucose and ethanol).

Fig. 2 shows the stability of the pyruvate kinases of the glucose-grown yeasts at 0 or 23° C and the effect of Fru-1,6-P_2 at the former temperature. Although the pyruvate kinase of *C. tropicalis* was more labile at 0 than at 23° C and Fru-1,6-P_2 enhanced the inactivation (Fig. 2B), the effect of Fru-1,6-P_2 on

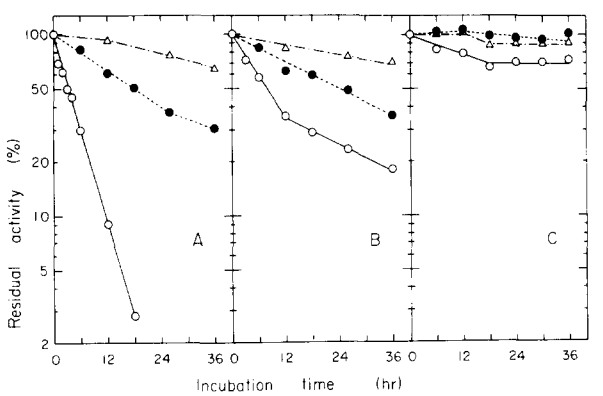

Fig. 2. Comparison of the stability of the pyruvate kinases of *S. cerevisiae*. (A), *C. tropicalis* (B) and *C. lipolytica* (C) in the presence and absence of Fru-1,6-P_2. The enzymes from respective glucose-grown cells were stood at 0° C with 1 mM of Fru-1,6-P_2 (○———○), at 0° C without Fru-1,6-P_2 (●- - - - -●) or at 23° C without Fru-1,6-P_2 (△— - — - —△).

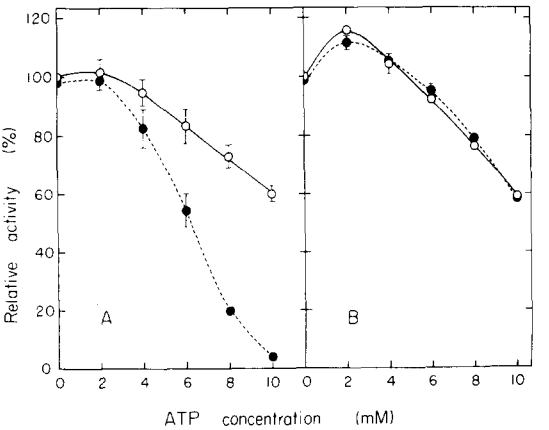

Fig. 3. Effect of ATP on the pyruvate kinase activities of *C. tropicalis* (A) and *C. lipolytica* (B) in the presence and absence of Fru-1,6-P_2. The enzymes were obtained by the same procedure as described in Fig. 1. The enzyme activities were measured with 1 mM of Fru-1,6-P_2 (○———○) or without Fru-1,6-P_2 (●- - - - -●).

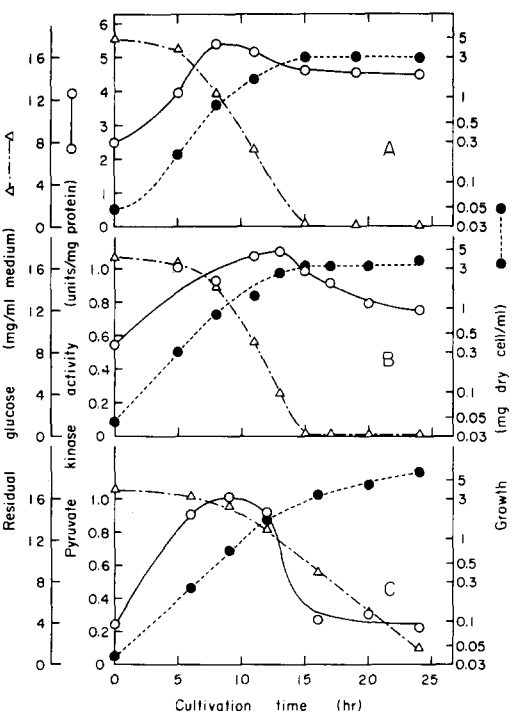

Fig. 4. Time course changes of the pyruvate kinase activities of *S. cerevisiae* (A), *C. tropicalis* (B) and *C. lipolytica* (C) during cultivation on glucose. Yeasts precultured on ethanol were cultivated in the glucose media mentioned in the text with shaking at 30° C. ○———○, specific pyruvate kinase activity; ●- - - - -●, growth; and △— · — · —△, residual glucose.

the enzyme was less than that on the *S. cerevisiae* enzyme (Fig. 2A). The enzyme of *C. lipolytica* was rather stable at 0°C. Fru-1,6-P_2 also stimulated inactivation of this enzyme in cold state, but the effect was very little as seen in Fig. 2C. These results also suggest the difference in interaction with Fru-1,6-P_2 among the pyruvate kinases of *Saccharomyces*, fermentative *Candida* and oxidative *Candida* tested.

The pyruvate kinase of *C. tropicalis* was inhibited by higher concentration of ATP, but this inhibition was significantly reduced by the addition of Fru-1,6-P_2 (Fig. 3A). On the other hand, the enzyme of *C. lipolytica* was not strongly inhibited even by the highest concentration of ATP tested (up to 10 mM), and Fru-1,6-P_2 did not show any reverse effect against the inhibitor action of ATP (Fig. 3B). In both yeasts, ATP activation of the enzymes was observed at a relatively low concentration of ATP (about 2 mM), similarly to the enzymes of *S. carlsbergensis* reported by Haeckel et al. [4]. There might be a possibility that the noted effect with ATP could be due, at least in part, to removal of Mg^{2+} through chelation with ATP, although Haeckel et al. [4] suggested that the action of ATP and Mg^{2+} on pyruvate kinase could not be explained by a simple competition or chelation mechanism. In this study, we did not examine the effect of ATP on the *Saccharomyces* enzyme, since the

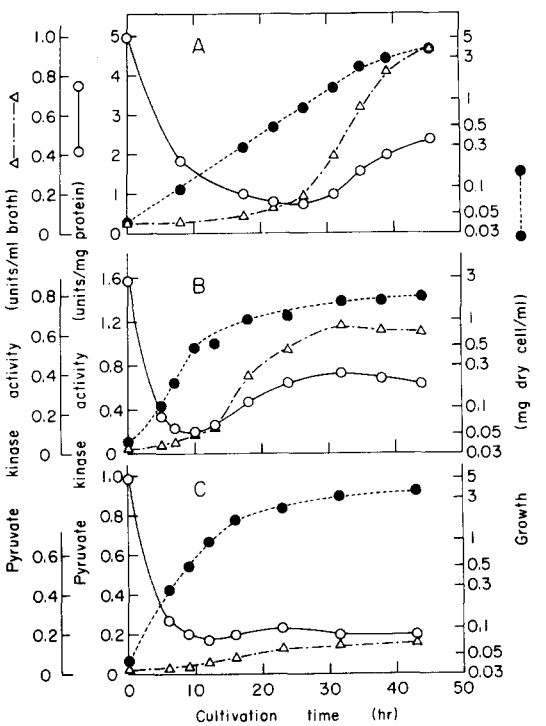

Fig. 5. Time course changes of the pyruvate kinase activities of *S. cerevisiae* (A), *C. tropicalis* (B) and *C. lipolytica* (C) during cultivation on ethanol. Yeasts precultured on glucose were cultivated in the ethanol media mentioned in the text with shaking at 30°C. ○———○, specific pyruvate kinase activity; △— - — - —△, total pyruvate kinase activity; and ●- - - - - -●, growth.

enzyme was far less active in the absence of Fru-1,6-P_2 in a probable physiological concentration range of phosphoenolpyruvate.

Three characteristic yeasts, *S. cerevisiae*, *C. tropicalis* and *C. lipolytica* belonging to different groups were cultivated on both glucose and ethanol, and the time course changes of the enzyme levels were investigated. When the cells precultured on ethanol were grown on glucose, the pyruvate kinase activities of all the yeasts increased along with the growth, attained the maximum levels at the middle to late exponential phases of growth, and then decreased (Fig. 4). Despite the fact that a greater part of glucose remained in the medium, the specific activity of the *C. lipolytica* pyruvate kinase began to decrease and the rate of decrease was much larger (Fig. 4C) than that in the other two yeasts (Fig. 4A and 4B). Fig. 5A shows the time course change of the pyruvate kinase in *S. cerevisiae* growing on ethanol. When the cells precultured on glucose were used as inoculum, the initial specific activity decreased rapidly until the middle exponential growth phase on ethanol, and then increased gradually. Although similar patterns were observed in the other two yeasts, the enzyme level in *C. tropicalis* was far lower than that in *S. cerevisiae* (Fig. 5B). The enzyme in *C. lipolytica* was at the lowest level among the yeasts tested throughout the cultivation (Fig. 5C).

Discussion

In this paper we compared the levels and sensitivities toward the allosteric effectors, Fru-1,6-P_2 and/or ATP, of the pyruvate kinases of four fermentative yeasts and one oxidative yeast [6] grown under glycolytic and gluconeogenic conditions, respectively. From the results presented in Fig. 1 and Table II, the yeast pyruvate kinases studied here would be classified into three groups with respect to the susceptibilities to Fru-1,6-P_2 activation. Although all of the enzymes of four fermentative yeasts were subject to an allosteric-type activation by Fru-1,6-P_2, the $K_{0.5}$ values for phosphoenolpyruvate of the *Candida* enzymes tested were about one tenth those of the *Saccharomyces* enzymes in the absence of the allosteric effector. The pyruvate kinase of *C. lipolytica*, an oxidative yeast, was scarcely subject to this type of the activation. Although Gancedo et al. [2] reported that the pyruvate kinase of *C. utilis*, a fermentative yeast, was not activated by Fru-1,6-P_2, we observed that the enzyme of *C. utilis* ATCC 9226 was activated by this effector at the lower concentration range of phosphoenolpyruvate.

Taking into consideration the report of Imamura et al. [13] that rat tissues contained three types of pyruvate kinase having different kinetic and immunological properties, M_1-, M_2- and L-type, it is interesting that three kinetically different enzymes were also observed in different kinds of yeasts.

Kuczenski and Suelter [14—16] observed that the pyruvate kinase highly purified from baker's yeast was cold-labile and that the addition of Fru-1,6-P_2 markedly enhanced the inactivation rate. They suggested that Fru-1,6-P_2 bound to the enzyme might cause a conformational change. The results presented in Fig. 2 coincide with the degree of susceptibility to the activation by Fru-1,6-P_2 of the enzymes as shown in Fig. 1 and Table II.

During gluconeogenesis pyruvate kinase must be maintained at a certain

low level or be inhibited to avoid the degradation of phosphoenolpyruvate synthesized from oxaloacetate by yeast phosphoenolpyruvate carboxykinase [17]. As shown in Table I, the enzyme level in ethanol-grown or acetate-grown cells was highest in *Saccharomyces* and lowest in *C. lipolytica*. This result strongly suggests that the allosteric control mechanism plays an important role, especially in the case of *Saccharomyces* and fermentative *Candida* containing high levels of the enzyme, when these yeasts were cultivated under the gluconeogenic conditions. In fact, it has been reported that there was a marked difference in the intracellular contents of Fru-1,6-P_2 between the *S. cerevisiae* cells growing under glycolytic and gluconeogenic conditions [2]. Furthermore, from the studies on the intracellular levels of glycolytic intermediates, adenine nucleotides and citrate in the resting cells of *S. cerevisiae*, it was suggested that Fru-1,6-P_2 would act as the primary regulator of pyruvate kinase [12,18,19]. However, the importance of ATP in pyruvate kinase regulation would not be excluded, although Barwell and Hess [12] revealed that the difference in the intracellular concentration of ATP was not observed between glycolytic and gluconeogenic conditions. ATP inhibited the enzyme activity of *C. tropicalis* more strongly in the absence of Fru-1,6-P_2 than in the presence of the effector (Fig. 3A). This fact suggested that, under the conditions where the intracellular concentration of Fru-1,6-P_2 was low, i.e. gluconeogenic conditions, ATP would play an important role for the reduction of pyruvate kinase activity, and thus the degradation of phosphoenolpyruvate to pyruvate would be prevented. This control mechanism probably operates in *C. tropicalis*. It is very interesting that such a type of the pyruvate kinase regulation seems unlikely to be conducted in *C. lipolytica* (Fig. 3B). Sodium citrate, which inhibited the pyruvate kinase of *S. carlsbergensis* [4], did not inhibit the enzymes of *C. tropicalis* and *C. lipolytica* at 10 mM concentration both in the presence and absence of Fru-1,6-P_2, but slightly activated in the case of the *C. lipolytica* enzyme. Thus, we could not find any inhibitor for the *C. lipolytica* enzyme in this study. However, the possibility that another potent inhibitor may be involved in the regulation of the pyruvate kinase in *C. lipolytica* cannot be excluded.

When the yeasts were cultivated in media containing glucose, a decrease in the enzyme activities occurred along with the exhaustion of glucose (Fig. 4). This phenomenon in fermentative yeasts is probably due to the depletion of glucose and the utilization of ethanol accumulated at the early growth phase [20]. In the case of *C. lipolytica*, a rapid and remarkable reduction in activity was observed even when a greater part of glucose was still remaining in the medium. This implies that the condition in cells may change from glycolytic one to gluconeogenic one even when glucose still remains in the medium. The reason of this phenomenon, however, cannot be explained at the present stage of this study. As shown in Figs 4 and 5, not only *C. lipolytica* but also *S. cerevisiae* and *C. tropicalis* involve the regulation mechanism mediated by changes in the enzyme levels depending on environmental conditions. However, the enzyme level of *C. lipolytica* was controlled more strictly than those of the other two yeasts tested.

In conclusion, the pyruvate kinases of *S. cerevisiae* and *C. tropicalis*, fermentative yeasts, would be controlled by both allosteric mechanism and changes of the enzyme concentration, whereas that of *C. lipolytica*, an oxida-

tive yeast, would be regulated only by the latter mechanism. However, it should be noted that there is a remarkable difference in the interaction with Fru-1,6-P_2 between the pyruvate kinases of *Saccharomyces* and those of fermentative *Candida*, such as *C. tropicalis* and *C. utilis*.

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