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REGULATORY KINETICS OF YEAST HEXOKINASE IN SITU

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SUMMARY

Purified yeast hexokinase P_{II} (EC 2.7.1.1) has previously been shown to exhibit negative cooperativity with MgATP and a burst-type of slow transient during assay below pH 7. To determine if these properties occur under conditions which are more nearly physiological, studies have been made of glucose-phosphorylating enzymes in situ. Yeast cells were made permeable to substrates by treatment with protamine (Schlenk, F. and Zydek-Cwick, C. R. (1970) Arch. Biochem. Biophys. 138, 220–225). These "ghost" cells were then assayed for hexokinase activity and showed both the negative cooperativity and the slow transient at pH 6.5 characteristic of purified hexokinase P_{II}. Kinetics at pH 8 were essentially linear. Although K_m values, cooperativity, ratio of fructose to glucose phosphorylating activity, and pH dependencies of ghost hexokinase were similar to purified hexokinase P_{II}, differences were observed in the ratio of initial to steady-state velocities, in the presence of negative cooperativity in the initial velocity, and in the degree of activation by citrate. These results suggest a difference in the initial ratio of interconvertible enzyme forms in situ as compared with purified enzyme. The data also suggest that there was a minor amount of an isozymic form of glucose-phosphorylating enzyme present. Since ghost hexokinase shows negative cooperativity and a slow transient, these properties probably occur physiologically, but their significance remains uncertain. The observation of these two kinetic properties in semi-permeable ghosts supports the slow transition model (Ainslie, G. R., Shill, J. P. and Neet, K. E. (1972) J. Biol. Chem. 247, 7088–7086) for cooperativity of yeast hexokinase.

INTRODUCTION

Levitzki and Koshland [1] found that many regulatory enzymes show negative cooperativity. It was later found that purified hexokinase (EC 2.7.1.1), a regulatory enzyme, also had this property [2, 3]. During assay purified hexokinase also shows a burst-type transient progress curve, i.e. an initial high activity followed by a slower steady state activity [3]. The possibility exists that one or both properties of hexokinase may be an artifact of purification or may be physiologically insignificant. This possibility has been examined by assaying hexokinase under conditions approximating

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the in vivo situation. A method developed by Schlenk and coworkers [4, 5] allows yeast enzymes to be assayed under conditions closer to physiological than a purified enzyme. With this method the yeast cells, now called "ghosts", are permeable to small molecular weight compounds, including hexokinase substrates. Kosow and Rose [2] reported that hexokinase was still susceptible to activation by citrate in such ghosts. We report here more extensive studies of the kinetic properties of hexokinase, in situ, within the more physiological but still altered milieu of the yeast ghost. Properties unique to the ghost system were also studied and their possible effect on the enzymatic properties are discussed.

MATERIALS AND METHODS

The Harden and Young strain of *Saccharomyces cerevisiae*, was studied unless otherwise mentioned. The yeast were stored on slants at 0–4 °C for three months. After three months the yeast were transferred to new slants, made of agar 2%, glucose 5%, Difco Bacto-peptone 0.5%, Difco yeast extract 1.0%, KH_2PO_4 0.9% and distilled water. The yeast were grown in media as described by Schlenk and Dainko [4]. The only variation from the Schlenk and Dainko method was that the length of the final incubation was varied between 8 and 48 h. Significant differences were found in the hexokinase activity for the two growth periods (see Results).

The ghosts were prepared from yeast by harvesting, washing in distilled water three times, and suspending the cells at 20 mg/ml (wet weight of packed cells). The ion-free suspension (10 ml) was placed in a 125-ml flask, 5 mg of protamine sulfate was added, and the flask put on a rotary shaker for 45 min [5]. The ghosts were then centrifuged, washed once and resuspended to a final volume of 2 ml, making a final concentration of ghosts of 100 mg/ml. The amounts assayed were computed from the original weight of yeast rather than from the weight of the ghosts, because of the difficulty in determining the weight of the wet ghosts. Assay procedures are described in the figure legends. Diffusion of substrates and products into and out of the ghosts has been shown to be rapid [4], not rate limiting and is substantiated by the linear progress curves observed here at pH 8. Each data point reported is the average of three or more assays with a standard deviation of less than 5% at high ATP concentrations and less than 10% low ATP concentrations.

The original experiments of Schlenk and Dainko [4] utilized RNAase instead of protamine as the basic protein that made yeast cells permeable. Ribonuclease treatment was therefore examined to determine if it was more effective than protamine. The hexokinase activity was not different with the RNAase treatment therefore protamine was used routinely.

The protamine sulfate was Sigma Grade A. The RNAase used was Calbiochem Grade A. All chemicals were of the highest possible commercial grade.

RESULTS

The major results of the experiments to be presented here are the observation of negative cooperativity and a slow transient process in the kinetics of hexokinase in semipermeable yeast ghosts. Related experiments on the amount of ghosts formed by protamine treatment, the yield of hexokinase at different stages of growth, and the

effect of ghost concentration on enzymatic activity are reported in order to better interpret the kinetic studies of hexokinase.

Amount of hexokinase assayed

Kosow and Rose [2] found that only about half of the yeast cells were affected by the protamine treatment, based upon the appearance of the preparation under a phase contrast microscope. If this is true and all the hexokinase in cells converted to ghosts is assayable, then interpretation of kinetic data is possible. This was checked by breaking up untreated yeast cells by use of the French press. The activity data for freshly broken whole cells and ghosts made the same day is given in Table I. These

TABLE I

COMPARISON OF GHOST HEXOKINASE WITH HEXOKINASE OF BROKEN YEAST CELLS

Activities expressed in μmoles/min per mg per ml ghost or whole cell $\times$ 10^3.

Cell type assayed	pH	Activity	
		Initial	Steady state
Ghosts	6.5	5.5	4.0
Ghosts	8.0	12.8	12.8
French pressed whole cells	6.5	11.4	*
French pressed whole cells	8.0	23.0	23.0
Untreated whole cells	8.0	No detectable activity	

* Approach to equilibrium, no linear portion.

results agree with earlier results [2, 4, 5]. For pH 6.5 and 8.0, the ghost preparation has about half of its possible activity. No hexokinase activity was found in washes during preparation of the ghosts.

It was possible to selectively stain ghosts by using trypan blue and observing the ghosts under a phase contract microscope. Untreated yeast cells did not take up

TABLE II

QUANTITATION OF NUMBER OF GHOSTS AS DETERMINED BY TRYPAN BLUE DYE

Unbuffered ghost preparations were stained on the slide with trypan blue solution and counted in a hemacytometer. Numbers represent totals of 5 (for 2 mg/ml) or 2 (for 20 mg/ml) different counts. The estimated error based on replicate counts is 5 to 10%.

	Concentration of ghosts (mg/ml)	
	2.0	20
Stained cell number	208	936
Unstained cell number	274	770

the dye, while for ghost preparations about 50% of the cells took up the dye (Table II). Clumping together of ghost cells was observed at the higher concentration of ghosts, (20 mg/ml) while there was no clumping at the lower concentration (2 mg/ml) or for the whole yeast cells. The results indicate that about half of the cells take up trypan blue (Table II) and are presumably ghosts, and that only half the total hexokinase is assayable (Table I). The simplest interpretation of these data are that nearly 100% of the hexokinase in each ghost cell is assayable (assuming all the hexokinase is assayable in the crude preparation used).

Time of growth versus specific activity of hexokinase

Ghosts from yeast with a final incubation period [4] of 48 h had a much greater specific activity (defined as μmole NADPH produced per min per mg of ghosts per ml) than the yeast grown for 8 h. The data are summarized in Table III. A final incubation of 40–48 h was used for all succeeding experiments.

TABLE III

EFFECT OF GROWTH TIME ON HEXOKINASE ACTIVITY

Assay procedure described in Fig. 1A. Specific activities expressed in μmoles/min per mg per ml ghost $\times 10^3$. The values presented are specific activities of a typical preparation. The variability of these numbers between ghost preparations was about ± 0.9 units for the 8-h incubation and ± 0.4 units for the 48-h incubation.

Length of second incubation (h)	Specific activity		
	pH 6.5		pH 8.0
	Initial*	Steady state**	Steady state
8	2.5	2.0	3.4
48	6.3	3.8	25

* Tangent at zero time to the progress curve (product versus time).
** Linear portion (after 2–3 min) of progress curve.

Ghost concentration versus activity

In searching for an adequate concentration of ghosts to work with, it was observed that with a higher ghost concentration, hexokinase with a lower specific activity was obtained (Fig. 1). Malate dehydrogenase was also assayed to determine if this activity drop was a property of the ghosts or of the hexokinase in the ghosts. The specific activity of malate dehydrogenase also decreased with increasing ghost concentration in a similar pattern to hexokinase. Thus, it is unlikely that hexokinase, itself, was the main factor in producing a specific activity drop with ghost concentration. Increasing the concentration of coupling enzyme had no effect on the hexokinase activity.

If the activity decrease with ghost concentration was caused by the depletion of one substrate, then it should not be seen at a pH where the activity is substantially less than the pH where the decrease is observed. Although hexokinase assayed at pH 6.5 and pH 8.0 has an activity ratio of about 0.1, the activity decrease with ghost concentration is proportionally the same at both pH values. Therefore, the activity decrease is not caused by depletion of one substrate.

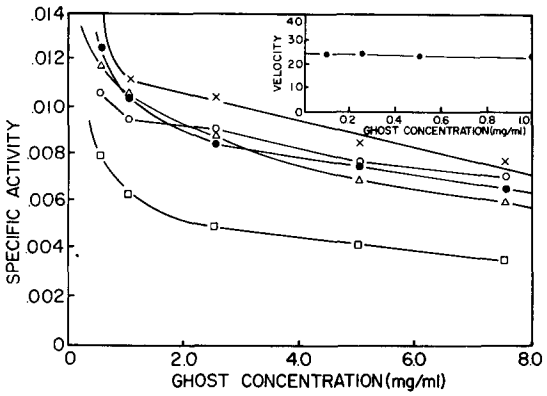

Fig. 1. A), Specific activity versus ghost concentration under different conditions. Spectrophotometric assay. Specific activity is defined as the μmoles of NADPH formed per min per mg of ghosts per ml of assay solution. The assay mix consists of: 0.1 or 0.5 M triethanolamine hydrochloride, 0.5 mM NADP, 5 mM glucose, 10 mM $MgCl_2$, 2 mM ATP and 0.5 to 5.0 units of glucose-6-phosphate dehydrogenase (coupling enzyme) in a final pH of 8.0. The reaction was initiated by addition of the ghosts, and then monitored spectrophotometrically at 340 nm, on a Gilford 2000 continuous recorder. In this experiment the ghosts were stored in varying concentrations of buffer at 0 °C and the concentration of buffer in the assay solution was varied. The concentration of triethanolamine hydrochloride was in assay (in ghost storage): ×—×, 0.1 M (none); ●—●, 0.1 M (0.5 M); △—△, 0.5 M (none); ○—○, 0.1 M (0.1 M); □—□, 0.5 M (0.1 M). B), Inset: pH stat-assay. The velocity is in terms of arbitrary units (divisions on the recording paper per min per mg of ghosts per ml of assay solution). The assay mix consists of: 1.0 mM triethanolamine hydrochloride, 2.0 mM ATP, 10.0 mM $MgCl_2$, 5.0 mM glucose (pH 8). The reaction was initiated by addition of glucose since there was a pH change with the addition of the ghosts. The ghosts were preincubated with the assay solution minus glucose for 10 min.

A concentration dependent interaction between ghosts might contribute to the decreasing specific activity of hexokinase. Increasing the ionic strength by increasing buffer amounts in the assay can distinguish between two possible types of interactions, hydrophobic and hydrophilic. By varying the buffer concentration of the ghost suspension, information on the strength of the interaction can be obtained. Fig. 1 gives the results of these experiments. The buffered ghosts assayed in the high ionic strength assay were inhibited the most (□—□). The unbuffered ghosts in the lowest ionic strength assay were inhibited the least (×—×). Preincubation in 0.5 M (●—●) or 0.1 M buffer (○—○) followed by assay in 0.1 M buffer did not return to the level of the unbuffered ghosts (×). These results indicate that the interaction is strong and partially irreversible under the conditions used.

This was further tested by using an assay technique which utilizes a very low buffer concentration (Fig. 1 inset). The specific activity versus ghost concentration was constant up to 1 mg/ml, supporting the hypothesis that the interaction is very sensitive to ionic strength.

The kinetics of hexokinase at pH 8.0 were studied to determine if the V or the K_m changed with concentration of ghosts (Fig. 2). The specific V values were different but the K_m values for MgATP did not vary significantly, 0.4 $\pm$ 0.2 mM, over the concentration range studied. These results are an indication of some type of interaction of ghosts which reduces the availability of the total amount or total activity of enzyme but does not affect its substrate binding properties.

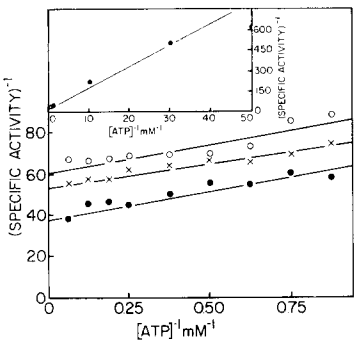

Fig. 2. Study of V and K_m values for MgATP at pH 8 to determine the nature of ghost interactions. Specific activity and the assay solution (0.1 M triethanolamine hydrochloride) were defined in the legend for Fig. 1A. In all kinetic assays MgATP was varied and there was always 10 mM $MgCl_2$ in solution in addition to the Mg^{2+} varied with the ATP. The data for 5.0 mg/ml exhibit a great deal of scattering because of the non-linearity of the recorder tracing. These readings were further complicated by the rise to nearly 2.0 A units to blank out the ghost absorbance, which should have no effect on relative velocities at a singly ghost concentration, but may make the true initial velocities difficult to obtain. Differences in V are not due solely to this effect. The following ghost concentrations were used: ●—●, 0.5 mg/ml; ×—×, 2.0 mg/ml; ○—○, 5.0 mg/ml. Inset: The low ATP concentration region expanded.

Observation of a burst-type transient

A burst-type transient has been observed previously in the isolated enzyme system at pH 6.5 but is absent at pH 8.0 [3, 6]. These properties have now been observed for hexokinase in the yeast ghost. Hexokinase in the ghosts of a yeast haploid strain TW42 (*α* 2, *α* 6, *lys* 2, *trp* 5-2, *ural* 1) were also found to have the transient at pH 6.5 and no transient at pH 8.0. This strain of yeast was grown with and without bacto-peptone in the media. The yeast harvest from the latter media was smaller, but both media produced yeast that showed the burst at pH 6.5 and linear progress curves at pH 8.0. Therefore, the transient at pH 6.5 is a property of hexokinase obtained from different yeast and under slightly different growth conditions.

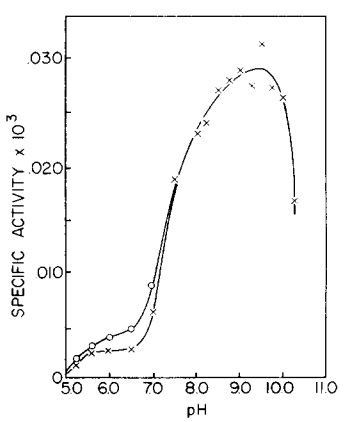

Fig. 3. The pH profile of ghost hexokinase activity. Spectrophotometric assay (0.1 M triethanolamine hydrochloride as in Fig. 1A). ×—×, steady state activity; ○—○, initial activity. Final ghost concentration was 1 or 2 mg/ml in the assay.

pH optimum of ghost hexokinase

Comparison of the pH profile of ghost hexokinase (Fig. 3) to purified hexokinase P_{II} [6] showed almost identical behavior. The sharp transition of the ghost hexokinase profile between pH 7.0 and 8.0 is very likely due to the ionization of a single type of amino acid residue in hexokinase. Therefore only one or two enzymes with identical titration curves are causing the rising curve above pH 6.5. From the pH profile, it is also likely that the plateau below pH 6.5 is also caused by one form (or multiple forms with identical pH behavior) of hexokinase in the ghosts since this plateau region is also seen for hexokinase isozyme P_{II} [6]. The initial and the steady-state velocities show similar pH behavior where they both occur.

Kinetics and negative cooperativity of ghost hexokinase

Negative cooperativity for purified hexokinase at pH 6.5 has been reported for isozyme P_{II} [2, 3]. Kinetic studies of ATP with hexokinase in ghosts were undertaken even though there may be difficulty in interpreting kinetic experiments on a

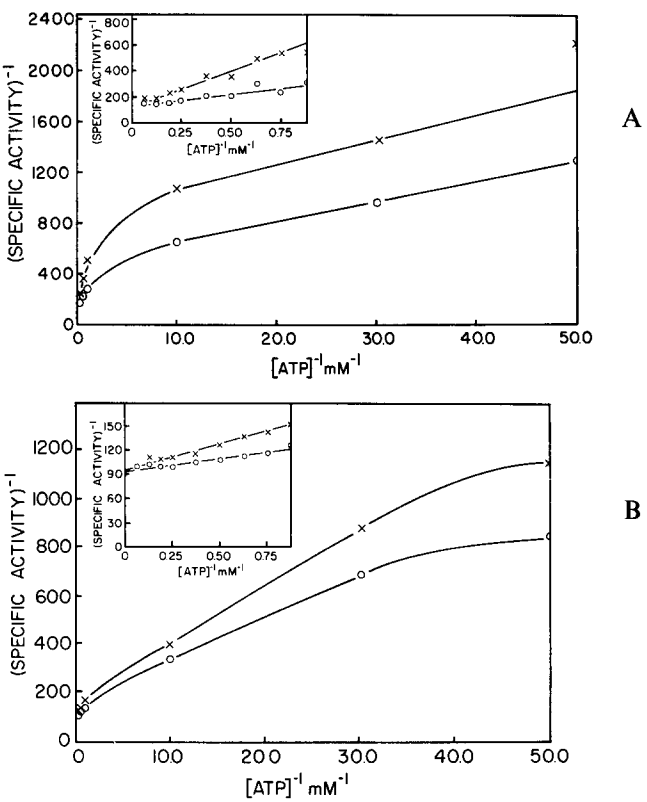

Fig. 4. A. Double reciprocal plots of specific activity and ATP concentration at pH 6.5. Spectrophotometric assay (0.1 M triethanolamine hydrochloride) as in Fig. 1A. Final ghost concentration of 1.0 mg/ml in the assay. Inset: The region of high ATP concentration expanded. B. Double reciprocal plots of specific activity and ATP concentration with citrate activation at pH 6.5. Spectrophotometric assay (0.1 M triethanolamine hydrochloride) as in Fig. 1A. Citrate was in a final concentration of 5 mM. ×—×, initial activity; ○—○, steady state activity. Final ghost concentration was 1.0 mg/ml in the assay. Inset: The high ATP concentration region expanded.

heterogeneous system such as yeast ghosts. Apparent negative cooperativity was demonstrated in the ghosts by the non-linearity of Lineweaver–Burke plots at pH 6.5 (Fig. 4A). The graphs can be interpreted as having two linear regions per curve: one in the ATP concentration range of 1–16 mM, the other in the range of 0.02–0.1 mM. The high concentration range is shown in the inset to Fig. 4A. These two parts of the kinetic curve may not have any real basis in structure but may be a result of complex interactions.

The kinetics at pH 8.0 show remarkable similarity between the purified [6] and yeast ghost (Fig. 2, inset) hexokinase. Both show essentially linear double reciprocal plots, and a K_m value for MgATP near 0.5 mM. The linearity of these double reciprocal plots indicate either that one form of the glucose phosphorylating enzymes is the major contributor to activity at pH 8.0 or that the different glucose phosphorylating enzymes do not differ significantly in their K_m values at pH 8.0.

Kinetics of citrate activation

Citrate is an activator of hexokinase (2) and eliminates the curvature in the double reciprocal plots of ATP concentration versus specific activity at pH 6.5 [2, 6]. In the ghost hexokinase system, activation by citrate occurs, in agreement with Kosow and Rose [2], and the curvature of the ATP plots is reduced but not eliminated when 5 mM citrate is used at pH 6.5 (Fig. 4B). Increasing the amount of citrate does not activate hexokinase further, but rather produces inhibition. This latter effect may be due to chelation of Mg^{2+}, an inhibition due to increased ionic strength, or an alteration in ghost permeability rather than a direct interaction of hexokinase and citrate.

Hexokinase assays at various citrate concentrations at pH 6.5 and 8.0 were run to test these hypotheses. The results are given in Table IV. These experiments show that there is some chelating of Mg^{2+}, but this is not the sole effect of increased amounts of citrate since added $MgCl_2$ did not remove the inhibition. Fig. 1 showed experiments where increasing the buffer concentration five times, from 0.1 M

TABLE IV

CITRATE EFFECT ON HEXOKINASE ACTIVITY OF YEAST GHOSTS

Citrate (mM)	$MgCl_2$*	pH	ATP (mM)	Specific activity × 10^3	
				Initial	Steady state
5	—	6.5	0.2	2.2	1.8
10	—	6.5	0.2	1.8	1.5
25	—	6.5	0.2	0.7	0.7
50	—	6.5	0.2	0.3	0.3
5	5	6.5	0.2	3.4	2.6
10	10	6.5	0.2	2.4	2.1
25	25	6.5	0.2	1.1	1.0
50	50	6.5	0.2	0.25	0.25
0	—	8.0	2.0	11.7	11.7
5	—	8.0	2.0	10.6	10.6
50	—	8.0	2.0	1.1	1.1

* Concentration of $MgCl_2$ added in addition to the amounts in Fig. 1A. Increasing $MgCl_2$ above these levels, in the absence of citrate, had no effect.

to 0.5 M, decreased the activity by about a factor of two. Increasing citrate from 5 mM to 50 mM decreased the activity by a factor of ten. Thus, the effect of ionic strength of the 50 mM citrate experiment should only be a factor of less than two. It is concluded that high concentrations of citrate directly inhibit hexokinase or alter its accessibility in the ghost system. An inhibition by citrate was not seen in the purified hexokinase system up to levels of 10 mM [6].

The activation at pH 6.5 by citrate (5 mM, Table V) is primarily due to a lower-

TABLE V

THE EFFECT OF CITRATE ON THE APPARENT K_m FOR ATP OF GHOST HEXOKINASE

K_m values (mM) calculated graphically from double reciprocal plots. V was unchanged in the presence of citrate.

	K_m (high)*	K_m (low)**
pH 6.5 (no citrate)		
from initial velocity	2.1	0.023
from steady-state velocity	5.4	0.030
pH 6.5 (5 mM citrate)		
from initial velocity	0.48	0.060
from steady-state velocity	0.69	0.10

* High ATP concentration region of 1–16 mM.
** Low ATP concentration region of 0.02–0.1 mM.

ing of the apparent K_m in the high ATP concentration region. This effect on the steady-state velocity is virtually the same as with the purified enzyme P_{II} [6]. In contrast, the initial velocity of the yeast ghost hexokinase is also affected by citrate (Fig. 4) whereas that of the purified enzyme is not [6]. This unexpected difference will be discussed later.

For Figs 2, inset and 4B, the point representing 0.02 mM ATP deviates below a straight line through the higher concentration points. While there may be some error in reading the progress curves, since small errors at low substrate concentration are magnified when reciprocal values are used, it is interesting to note the deviations are all downward in relation to the graphs. This may well indicate the presence of a small amount of an additional glucose phosphorylating enzyme with a lower K_m.

Fructose to glucose ratio

Ramel et al. [7] have demonstrated that different enzyme forms of hexokinase have differing activities towards phosphorylation of fructose and glucose (see ref. 8) for details concerning the assay procedure). The only modification required for assaying ghosts was initiation of the reaction by sugar rather than by the enzyme preparation. All the assays were linear, (pH 8), and the values of fructose and glucose activity are the averages of five assays. The ratio of fructose to glucose activity was 1.18, indicating a predominance of hexokinase P_{II}, but suggesting also that some hexokinase P_I and possibly other glucose phosphorylating enzymes are present. Additional support for this conclusion comes from the apparent non-linearity of the last point of Figs 2, inset and 4B.

DISCUSSION

Late stationary activity versus early stationary activity

The ghosts from late stationary phase yeast had a 10-fold greater activity at pH 8 than did those from late log phase yeast (Table III). The magnitude of this effect suggests that the differences are not due to differences in membrane resistance, cell weight, or protamine interaction. The amounts of hexokinase in cells harvested in the stationary and log phase may not be equivalent which suggests that during different phases of growth different phosphorylating enzymes are preferentially synthesized. For example, the enzymes studied in this project may be synthesized only in the late stationary phase.

Cooperativity and transients

Ainslie et al. [9] have demonstrated that cooperativity and transients may be a result of a slow reaction step such as a conformational change and Shill and Neet [6] have interpreted the kinetics of purified yeast hexokinase P_{II} in this manner. The slow transition mechanism [8] assumes two forms of the enzyme, E and E′. For hexokinase, the negative cooperativity and the burst-type transient can be explained by hypothesizing that the E form of the enzyme binds substrate better and is favored by low concentration of substrate. The E′ from binds substrate less well and is favored by high concentration of substrate. The negative cooperativity of the steady state velocity can be explained by this hypothesis.

The burst-type transient is produced by a slow conformational change during assay. For the isolated enzyme P_{II}, the ratio of the initial velocity to the steady-state velocity tends to be larger than for the ghost system. During purification, hexokinase may be converted entirely to the E form and then when it is assayed an $E \rightleftharpoons E'$ equilibrium can take place, producing more E′ form of the enzyme and resulting in a decreased specific activity in the steady state. However, in the ghosts, the ratio of E to E′ may be lower and may be the normal distribution in the cell. Therefore, there is more of the less active form of the enzyme in the ghost initially and the initial velocity relative to the steady-state velocity is less than for the purified enzyme. The same ratio of initial to steady state velocity was obtained for TW 42 yeast and the Harden and Young strains.

A difference in the initial $E \rightleftharpoons E'$ equilibrium would also explain the apparent negative cooperativity of the initial velocity which would reflect a simple mixture of enzyme forms, with different K_m values, before they had interconverted. The negative cooperativity of the steady state would be due to the dependence of the interconversion on MgATP concentration, as with the isolated hexokinase P_{II} [6]. Thus, the slow transition mechanism [6, 9] can account for the differences observed with the ghost hexokinase in the apparent negative cooperativity of the initial velocity, the lower ratio of initial to steady state velocity, and the lack of elimination of the cooperativity by citrate on the basis of an initial equilibrium of $E \rightleftharpoons E'$ different from the purified enzyme.

The nonlinearity of the double reciprocal plots for the initial velocities in the ghosts is also explainable on the basis of two separate kinetic forms (isozymes) being present at the start of the assay. When the substrate level is high, the high K_m isozyme would be kinetically important, while the low K_m isozyme will predominate enzymatic-

ally at low substrate levels. Even though the ratio of enzyme forms will be constant, the substrate level will determine the initial velocity and therefore the apparent negative cooperativity. Such an hypothesis would require that the K_m of both the initial and the steady state forms of the isozymes be different at pH 6.5 but the same at pH 8. For the isolated enzyme system only the more active form is initially present, thus the double reciprocal plots for the initial velocity is linear [6]. Thus, the requirements for the isozyme interpretation appear to be more complex than for the slow transition mechanism.

Citrate eliminates the negative cooperativity with purified hexokinase [6]. In the ghost system, we were not able to observe this due to inhibition at high citrate concentrations (either by ionic strength effects, allosteric inhibition of hexokinase, permeability changes, or a stronger ghost–ghost interaction). However, since the citrate concentration needed to observe the citrate inhibition was very high and probably unphysiological, it can be concluded that citrate does not eliminate the nonlinear Lineweaver–Burk plots in the ghosts. This may mean that citrate does not eliminate negative cooperativity in vivo. However, other interpretations are possible. Since other yeast enzymes are present, the actual citrate concentration inside the ghosts may be much less than that in the assay mixture. The other enzymes may bind or utilize citrate. The elimination of the negative cooperativity cannot be directly interpreted in the ghost system because of this ambiguity. Citrate does, however, decrease the extent of the cooperativity.

The effect of citrate on the initial velocity is to decrease the high K_m (Table V), consistent with an hypothesis that citrate can shift the $E \rightleftharpoons E'$ equilibrium towards the E form. Citrate would then have no effect on the initial velocity of purified enzyme, as observed, if the purified enzyme was already predominantly in the E form as discussed previously.

The validity and utility of the ghost system

Serrano et al. [10] have recently reported studies on hexokinase and pyruvate kinase of yeast cells made permeable by toluene treatment. Our studies have been done under more nearly physiological conditions, pH less than 7, and with mild treatment to produce semi-permeable ghosts, i.e. protamine. Their studies [10] were done under conditions in which no allosteric properties of hexokinase are observed. No direct comparison of the advantages and disadvantages of yeast cells made permeable by toluene vs protamine treatment has been made. Limited comparison of their results and ours indicate similar availability and properties of hexokinase when assayed at pH greater than 7.

The experiments measuring activity versus ghost concentration (Fig. 1) indicated a ghost–ghost interaction and depression of specific activity; the dye experiments gave an indication that the higher the ghost concentration the greater the ghost aggregation or clumping. Only about half of the protamine treated cells were stained with trypan blue and only about half of the potential hexokinase was assayable in the ghost system. This implies that nearly 100% of the hexokinase in the cells that are converted to ghosts is being assayed. Regardless of these complications, the ghost system is capable of giving unambiguous results for certain kinds of studies. The negative cooperativity, the burst-type transient, and citrate activation were observed both for the purified enzyme and the ghost enzyme. Therefore, it is very unlikely that

the ghosts would exhibit the same regulatory properties unless these properties were present under physiological conditions. The hexokinase properties observed [2, 3, 6, 7] are therefore very likely to have physiological significance.

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REFERENCES

1 Levitzki, A. and Koshland, D. (1969) Proc. Natl. Acad. Sci. 62, 1121–1128
2 Kosow, D. P. and Rose, I. A. (1971) J. Biol. Chem. 246, 2618–2625
3 Shill, J. P. and Neet, K. E. (1971) Biochem. J. 123, 283–285
4 Schlenk, F. and Dainko, J. L. (1965) J. Bacteriol. 89, 428–436
5 Schlenk, F. and Zydek-Cwick, C. R. (1970) Arch. Biochem. Biophys. 138, 220–225
6 Shill, J. P. and Neet, K. E., in preparation
7 Ramel, A. H., Rustum, Y. M., Jones, J. G. and Barnard, E. A. (1971) Biochemistry 10, 3499–3508
8 Darrow, R. A. and Colowick, S. P. (1962) Methods in Enzymology (Colowick, S. P. and Kaplan, N.O. eds), Vol. V, pp. 226–228, Academic Press, New York
9 Ainslie, G. R., Shill, J. P. and Neet, K. E. (1972) J. Biol. Chem. 247, 7088–7096
10 Serrano, R., Gancedo, J. M. and Gancedo, C. (1973) Eur. J. Biochem. 34, 479–482