Activators of Yeast Hexokinase*

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SUMMARY

The rate of the ATP:glucose transphosphorylase reaction catalyzed by yeast hexokinase, isozyme P\textsubscript{11}, is inhibited by lowering the pH of the reaction below 7.0, especially at suboptimal concentrations of ATP. This effect of acidity is largely overcome by activators such as orthophosphate, citrate, malate, 3-phosphoglycerate, and riboside triphosphates. Thus, in the acid range, ATP appears to serve both as an activator and a substrate with the result that 1/ν versus 1/[ATP] plots are nonlinear. It appears that yeast hexokinase may exist as two conformational isomers, an inactive form which is favored in the acid range and an active form favored by various polyonions or by alkaline pH.

The conversion of inactive to active enzyme by citrate is slow, requiring about 1 min at 25°C when citrate is added to the reaction mixture after the substrates. When citrate is added to the enzyme simultaneously with both substrates, the reaction begins at the fully activated rate indicating that only the ternary complex can undergo the conversion to inactive enzyme. Equilibrium isotope exchange experiments indicate that citrate activates the glucose → glucose 6-phosphate, ADP ↔ ATP, and glucose-6-P ↔ ATP exchanges equally. This is consistent with the idea that the ternary complex is undergoing the allosteric interconversions.

Native hexokinase assayed inside semipermeable yeast cells exhibits the same kinetic properties as the isolated native enzyme, indicating that the activation phenomena may be important in controlling the rate of hexokinase in vivo.

RESULTS

Effect of pH on MgATP Dependence of Yeast Hexokinase—Most previous reports on the MgATP dependence of yeast hexokinase have been limited to the alkaline range of pH where the activity is maximal and the initial velocity curves are strictly hyperbolic (1, 4–7). A recent report by Bohnensack and Hoffmann (13), in which measurements with commercially available enzyme were extended to pH 6.3, did not disclose any change from this pattern. However, as shown in Fig. 1A, there is a significant change to complex behavior when the pH is lowered in a study with the P\textsubscript{11} isozyme obtained from Dr. S. P. Colowick. Qualitatively similar results were obtained with the P\textsubscript{1} isozyme obtained from Dr. S. P. Colowick and with the enzyme obtained from Boehringer Mannheim. All experiments reported in this paper were performed with the P\textsubscript{11} isozyme. As seen in Fig. 1A, two "hyperbolic" regions were observed at pH 7.3 and below, presumably indicative of negative cooperativity (14). As expected, a double reciprocal plot of these data shown in Fig. 1B is nonlinear. It is evident from the double reciprocal plots shown in...
Fig. 1. Velocity of yeast hexokinase as a function of MgATP concentration. Each tube contained: yeast hexokinase (Prr), 60 ng; glucose, 1 mm; MgCl₂, 5 mm; TPN, 0.5 mm; glucose-6-P dehydrogenase, 0.5 unit; P-enolpyruvate, 1 mm; pyruvate kinase, 1.5 units; and buffer, 100 mM, in a final volume of 1 ml. Buffers used were 2-(N-morpholino)ethanesulfonic acid, pH 6.9 (○), or H-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.2 (□), pH 7.3 (△), and pH 7.9 (○).

Fig. 1B that in the low ATP region there is a constant slope which is indicative of a comparable effect of pH on the magnitude of the $K_m$ of ATP and the $V_{max}$ for the first hyperbolic region of the initial velocity plot (Fig 1A). Thus, in this region, where there is no demonstrable cooperativity, the effect of pH on the extrapolated low ATP $V_{max}$ values indicates the inhibition constant of H⁺ as an uncompetitive inhibitor with respect to ATP as the variable substrate. A plot of such values, $V'_{max}$, as a function of pH (Fig. 2A) over the range pH 6.6 to 8.0, indicates a rather sharp effect of pH. Fig. 2B is a replotted of these data in order to examine the sensitivity to H⁺ concentration, according to Footnote 1. The results clearly show that two protons are required for inhibition with a mean pH of 7.3. A working hypothesis would be that protonation of two sites on the enzyme causes a shift of one or more of the reaction intermediates to an inactive form and that this shift can be prevented by further addition of MgATP. The question then arises whether MgATP is acting as a substrate at a second reaction site, in which case the kinetics could be interpreted as an example of negative cooperativity, or whether the additional ATP functions as an activator. If the latter, then other activators might exist and a search for allosteric activators was undertaken.

**Effectors of Yeast Hexokinase**

Yeast hexokinase (75 ng) was incubated at 25° in a final volume of 1 ml with 2-(N-morpholino)ethanesulfonic acid, pH 6.5, or 2,2',2''-nitrilotriethanol, pH 8.0, 100 mM; glucose, 1 mM; ATP, 0.05 mM; MgCl₂, 5 mM; TPN, 0.5 mM; and glucose-6-P dehydrogenase, 0.5 unit.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Added (1 mM)</th>
<th>Relative rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>2-Phosphoglycerate</td>
<td></td>
<td>55.3</td>
</tr>
<tr>
<td>3-Phosphoglycerate</td>
<td></td>
<td>54.6</td>
</tr>
<tr>
<td>P-enolpyruvate</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>L-Malate</td>
<td>54</td>
<td>104</td>
</tr>
<tr>
<td>D-Malate</td>
<td>44.6</td>
<td>104</td>
</tr>
<tr>
<td>Succinate</td>
<td>17.6</td>
<td>96</td>
</tr>
<tr>
<td>Citrate</td>
<td>53.3</td>
<td>91</td>
</tr>
<tr>
<td>Tricarbarylactate</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>16</td>
<td>75.5</td>
</tr>
<tr>
<td>GTP</td>
<td>48</td>
<td>52.5</td>
</tr>
<tr>
<td>CTP</td>
<td>60.8</td>
<td>87.1</td>
</tr>
<tr>
<td>UTP</td>
<td>23.9</td>
<td>93.5</td>
</tr>
<tr>
<td>β,γ-Methylene ATP</td>
<td>34.3</td>
<td>76.5</td>
</tr>
<tr>
<td>Pi</td>
<td>27</td>
<td>91</td>
</tr>
<tr>
<td>SO₄</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>35</td>
<td>98</td>
</tr>
</tbody>
</table>

The ratio of the extrapolated maximum rate at pH 8.0, where, at saturating substrates $ES = K_{cat}$, to that at the lower pH, $ES = E_p + (ESH + ESP + ...)$ is given by the general equation:

$$\frac{V_{max}}{V_{max}} = 1 + \frac{(H^+)^2}{K_1} + \frac{(H^+)^3}{K_1K_2} + \cdots$$

where $K_1$, $K_2$, etc., are proton dissociation constants for inactive forms of $ES$. The data of Fig. 2A is well represented by a single line with a slope of 2 in Fig. 2B. This implies that $pK_1 < pH$ in the neutral region and that a second protonation step occurs such that $(K_1K_2)^+ = H_2$, where $H_2$ is the hydrogen ion concentration, pH 7.3, that gives 50% inhibition.
FIG. 3. Effect of pH on the activation of yeast hexokinase by Pi. Conditions were the same as those in Fig. 1, except that MgATP was held constant at 0.2 mM and the buffers were 2-(N-morpholino)ethanesulfonic acid, pH 6.6 (C); pH 7.0 (O); pH 7.18 (H); and pH 7.4 (D).

obtained at pH 8.0 without additions. Although Pi activates, SO4 does not; citrate activates but tricarballylate does not; 3-phosphoglycerate is more effective than 2-phosphoglycerate or P-enolpyruvate; and succinate is not an activator, while malate is one of the better activators.

CTP, at best, is a very poor phosphoryl donor for yeast hexokinase under conditions where it stimulates ATP-dependent phosphorylation of glucose. Thus CTP must be acting as an activator. It is reasonable to propose that the effect seen with high levels of ATP (Fig. 1) is an allostery one also.

Activation by Pi—The finding that Pi activates yeast hexokinase is of interest, since Pi has been shown to overcome glucose-6-P inhibition of mammalian hexokinases (15). In addition, Schulze and Colowiek (10) have shown an effect of Pi on the state of aggregation of the yeast enzyme. It was with these two facts in mind that an effect of Pi on the pH dependence of the yeast enzyme was tested.

Fig. 3 shows the effect of pH on the activation of yeast hexokinase by Pi. The $K_{activation}$ is dependent on the pH and increases from 1 mM to 4 mM as the pH decreases from 7.2 to 6.6, perhaps indicating that it is the dianion form of phosphate that binds to the activator site.

Fig. 4. Plot of the reciprocal of the activation by citrate versus the reciprocal of the citrate concentration. Activation is defined as $(v - v_0)/v_0$, where $v$ and $v_0$ are activity in the presence and absence of citrate, respectively. Each tube contained: PII, 60 ng; glucose, 1 mM; chelated ATP, 0.4 mM; MgCl₂, 5 mM; TPN, 0.5 mM; glucose-6-P dehydrogenase, 1 unit; and buffer, 100 mM. Buffers were H-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, pH 7.0 (C); 2-(N-morpholino)ethanesulfonic acid, pH 6.6 (O); pH 6.2 (O); and pH 5.9 (O).

Activation by Citrate—Citrate differs from Pi in that its $K_{activation}$ for citrate is 0.06 mM at 0.4 mM ATP in the pH range of 6.0 to 7.0 (Fig. 4). Since a linear double reciprocal plot is obtained, it appears that only 1 eq of activator is required to overcome the effect of two protons as in the case for Pi. Malate and 3-phosphoglycerate also have $K_{activation}$ of 0.06 mM over this pH range. Note that in Fig. 4 the maximum activation by citrate at a constant ATP decreases below pH 6.6 where one of the carboxyl groups of citrate becomes protonated. Fig. 5 shows that at pH 6.5, a double reciprocal plot with MgATP as the variable substrate becomes less biphasic as more citrate is added. At 1 mM citrate, the plot is nearly linear and the $K_m$ for MgATP is 0.22 mM, compared with the previously reported value of 0.29 mM at pH 8.0 (7).

Double reciprocal plots with glucose as the variable substrate are linear at all pH values that have been examined, that is, pH 6.0 to 8.0. Fig. 6 shows an experiment at pH 6.6 with and without 1 mM citrate. Almost no activation is seen at low glucose levels. This is consistent with the idea that the conversion to the inactive form with H⁺ requires that the enzyme be occupied by both substrates; thus, at glucose levels well below the $K_m$ at pH 6.6 there is little activation by citrate because very little of the enzyme is in the inhibited state. The fact that citrate raises both $V_{max}$ and $K_m$ of glucose to the same extent (6-fold) indicates that citrate increases the concentrations of both E and E·glucose to the same extent in the reaction path (16).

In order to test whether particular steps of the hexokinase reaction pathway were stimulated by activator, the isotope ex-
I. A. Rose

Effect of citrate on yeast hexokinase. Conditions were the same as Fig. 1, except that buffer was 2-(N-morpholino)ethanesulfonic acid, pH 6.6, and citrate (cit.) was added as indicated.

![Figure 5](image)

**Fig. 5.** Effect of citrate on yeast hexokinase. Conditions were the same as Fig. 1, except that buffer was 2-(N-morpholino)ethanesulfonic acid, pH 6.6, and citrate (cit.) was added as indicated.

![Figure 6](image)

**Fig. 6.** Velocity of yeast hexokinase as a function of glucose concentration. Conditions were the same as Fig. 1, except that MgATP was held constant at 0.05 mM and the buffer was 2-(N-morpholino)ethanesulfonic acid, pH 6.6.

TABLE II

Effect of citrate on equilibrium isotope exchange rates

The equilibrium was set up at pH 6.0 and 25°C (K_eq = 160 (17)) as follows, 2-(N-morpholino)ethanesulfonic acid, 50 mM at pH 6.0; MgCl_2, 20 mM; glucose-6-14C, 0.1 mM with 6.4 x 10^6 cpm per pmole; ATP, 0.1 mM labeled with 14C in the adenine portion, 10^6 cpm per pmole and 32P in the γ position, 10^6 cpm per pmole; ADP, 0.1 mM; and glucose-6-P, 16 mM. An amount of hexokinase P; was added which, when assayed in the absence of ADP and glucose-6-P, produced glucose-6-P at the rate of 5.6 x 10^4 amoles per min per ml. Citrate, 1 mM, was present as indicated. Samples were removed for acidification at two intervals during the early portion of the equilibration course, as well as a sample of 0', and another to which a large amount of hexokinase was added to obtain data for complete equilibrium. The specific activity of each component recovered on Dowex-1 (chloride) was determined and the rates of each exchange were calculated from the fraction (f) to which the components (say a and b) had proceeded toward isotopic equilibrium at the time of sampling, t.

\[ v_t = -2.3 \cdot \frac{(a)(b)}{(a + b)} \cdot \log (1 - f_t) \]

<table>
<thead>
<tr>
<th>Additions</th>
<th>glucose-6-P</th>
<th>ATP â†’ ADP</th>
<th>ATP â†’ glucose-6-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.75</td>
<td>8.0</td>
<td>12.65</td>
</tr>
<tr>
<td>+ Citrate</td>
<td>16.75</td>
<td>16.6</td>
<td>22.1</td>
</tr>
<tr>
<td>- Citrate</td>
<td>1.72</td>
<td>1.86</td>
<td>1.75</td>
</tr>
</tbody>
</table>

earlier conclusion of alternate products release pathways in which the rates of release cannot be considered rapid compared with the phosphoryl transfer step. It will be noted that the exchange rates reported in Table II exceed the initial rate of forward reaction measured under the same conditions except for the absence of ADP and glucose-6-P. This result, which was repeatedly observed, is interpreted to indicate activation by the high concentration of glucose-6-P necessary to establish the equilibrium.

Activators as Metal Chelators—The activation by EDTA seen in Table I naturally suggests that the removal of an inhibitory metal ion may be responsible for the effect of the activators. On the other hand, EDTA may be acting as a poor analogue of malate or citrate by virtue of its polycarboxylate structure. A number of experiments were designed to evaluate the first possibility. Several metal ions at 0.1 mM were tested for inhibition of the assay at pH 6.6 in the presence of glucose (1 mM), ATP (0.05 mM), and MgCl_2 (5 mM). No inhibition greater than 20% was evident with Co²⁺, Ca²⁺, Cd²⁺, Ni²⁺, Zn²⁺, Cu²⁺, and Fe²⁺, while Mn²⁺ gave a 50% inhibition. None of these effects is sufficient to explain the over 3-fold stimulation of the hexokinase rate obtained in the presence of citrate, 3-phosphoglycerate, malate, or GTP (Table I). A number of procedures lead to a doubling of the unactivated rate at pH 6.6 compared to the control but have no effect at pH 8.0; these include treatment of all the chemical reagents except MgCl_2 with Chelex-100 or with 8-hydroxyquinoline according to the procedure described under "Methods" or simply upon purification of the ATP on a DEAE-IEC column (18). However, in all these cases, further activation was obtained with 3-phosphoglycerate, citrate, or EDTA.
TABLE III

Effect of 8-hydroxyquinoline on activation

All reagents, including the glucose-6-P dehydrogenase and MgCl₂, were treated with 8-hydroxyquinoline as described under "Materials and Methods." Where indicated, hexokinase was also treated. Each tube contained in a final volume of 1 ml, 2-(N-morpholino)ethanesulfonic acid, pH 6.5, 100 mM; glucose, 1 mM; ATP, 0.4 mM; MgCl₂, 5 mM; TPN, 0.5 mM; and glucose-6-P dehydrogenase, 0.5 unit. \( V_A \) and \( V_0 \) are velocities in the presence and absence of activator, respectively. The decrease in velocity with the 8-hydroxyquinoline-treated enzyme is the result of shaking the hexokinase solution with chloroform.

<table>
<thead>
<tr>
<th>Hexokinase</th>
<th>((V_A - V_0)/V_0)</th>
<th>( V_0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 ng/ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>4.0</td>
<td>2.0</td>
</tr>
<tr>
<td>8-Hydroxyquinoline-treated</td>
<td>4.5</td>
<td>3.0</td>
</tr>
</tbody>
</table>

In the experiment summarized in Table III, where all reagents, including the enzymes, were treated with 8-hydroxyquinoline, there was good activation by citrate or EDTA. These results, the fact that neither o-phenanthroline nor tricarbalylate activate, and that such weakly complexing agents as 3-phosphoglycerate and malate have activation constants of \( 6 \times 10^{-5} \) M, indicate that the activation is not due to removal of a metal ion inhibitor by the activator.

**Time-dependence of Activation**—At acidic pH, the conversion of inactive to active enzyme by citrate is slow, requiring about 1 min to reach completion. However, if substrates are not present until citrate is added, then the reaction begins at the high fully activated rate as shown in Fig. 7. This suggests that the presence of substrates is required for the low pH effect. That both substrates are required for the low pH effect was demonstrated by previously incubating the enzyme at pH 6.7 with either MgATP or glucose before the simultaneous addition of citrate and the second substrate. In both cases, the reaction began at the fully activated rate without a detected lag. The addition of 15% glycerol increases the lag seen when citrate is added following the substrates to about 3 min (Fig. 7). Similarly, when inactive enzyme is converted to active enzyme by raising the pH from 6.7 to 8.0, there is a lag of about 2 min before optimal activity is obtained. Fifteen percent glycerol increases this lag to over 8 min, as seen in Fig. 8A. In contrast to these results, when the pH is lowered from 8.0 to 6.5 in the presence of both substrates, the decrease in activity is too rapid to follow by the experimental method used (Fig. 8D). Inspection of Fig. 7 reveals that 15% glycerol inhibits the assay about 55% at pH 6.7 and that this inhibition is decreased to 20% by the addition of 0.5 mM saturating citrate. In studies performed at pH 8.0, 15% and 30% glycerol gave 20% and 50% inhibition, respectively, and this inhibition was not reversed by citrate in contrast to the results obtained at low pH. That the observed lags are due to a slow change in aggregation of the hexokinase is ruled out by the fact that enzyme activity with and without citrate is linear with respect to enzyme concentration at pH 5.9 to 8.0 and from 60 to 600 ng of hexokinase per ml (Fig. 9). Thus, it appears that a slow conformational change of the acidic, inactive form to an active form upon the addition of citrate or...
Hexokinase Activity in Semipermeable Yeast Cells—It was considered important to determine whether the kinetic properties demonstrated with the isolated yeast hexokinase isoenzyme might indicate the behavior of hexokinase at its concentration in the milieu of the yeast cell. This question was approached by use of yeast cell "ghost", prepared by the method of Schlenk and Zydek-Cwick (11) in such a way as to be freely permeable to substrate molecules. In this preparation the yeast proteins are retained within a cell wall envelope (19) in what is presumed to be a normal concentration and condition. Preliminary experiments indicated that there was free diffusion of hexokinase substrates and products in and out of the ghosts since no delay in establishing a linear assay rate in the glucose-6-P dehydrogenase-coupled system could be observed. At least half the total hexokinase of the preparation was assayable in this way, as indicated by assays before and after sonic oscillation. The failure to assay all the hexokinase results from incomplete conversion of cells to the "ghost," as shown with the phase contrast microscope. The hexokinase remained in the ghosts during the assay period (10-15 min); however, longer periods of incubation caused leakage of the enzyme. Most of the activators previously studied were tested with the semipermeable yeast preparation. As noted in Table IV, the behavior of the hexokinases of this preparation was very similar to the highly dilute enzyme studied in Table I; citrate and 3-phosphoglycerate were among the best activators; neither SO₄²⁻ nor tricarboxylate activated, and activation occurred only at acid pH. The concentration of hexokinase in the semipermeable yeast cells can be calculated from a determination of the total activity of enzyme per ml of packed cells and assuming an average specific activity of 500 units per mg of pure enzyme (10). A value of 44 μg per ml of cells was found. This is about 100 times the highest concentration used in the in vitro studies with purified enzyme.

An alternative explanation for the effect of activators such as citrate in the experiment with the semipermeable yeast might be that normally a portion of the glucose-6-P formed by hexokinase reacts with endogenous phosphoglucose isomerase and then with phosphofructokinase in competition with the external glucose-6-P dehydrogenase. The effect of citrate in inhibiting the phosphofructokinase would be seen as an increase in TPN⁺ reduction.
and hence as an apparent activation of hexokinase. A further workup was done that is not subject to this possibility; 2-deoxyglucose-14C was incubated with yeast ghosts as in Table V. The extent of phosphorylation by the ghosts was determined as radioactivity that was retained on an anion exchange column after removal of cells and ghosts by centrifugation. As was also seen with purified hexokinase, activations by citrate and 3-phosphoglycerate are apparent only at the higher concentrations of sugar substrate. Since identical results were obtained with both the isotopic and spectrophotometric assays, it is evident that in the ghosts, citrate is activating hexokinase directly.

**DISCUSSION**

Two central questions arise from the present studies: what is the mode of action of the several activators at the molecular level and what is the importance of these effects in the normal function of hexokinase in the cell? With respect to both questions, further work is certainly needed. Attempts to show direct interaction of citrate with yeast-hexokinase by equilibrium dialysis have not been successful. However, the inability to carry out prolonged dialysis in the presence of both glucose and ATP limits the value of this experiment. Likewise, the failure to observe fluorescence changes with citrate and 3-phosphoglycerate is inconclusive and the implication from the kinetic studies that the activators interact with an intermediate of the reaction sequence limits the possibilities for a number of comparable tests such as effects of activators on the stability of the enzyme toward thermal or proteolytic denaturation.

However, the argument that these interactions are indirect, through the chelation of a metal ion inhibitor, does not seem strong; the activation is slow and the lag in attaining the activated rate is not shortened by prior incubation of the enzyme with activators. Previous treatment of all reagents and enzymes with Chelex or 8-hydroxyquinoline did not eliminate the activator effects, although a 2-fold enhancement of the unactivated rate was seen by either of these treatments or by purifying the commercial ATP on DEAE columns. On the other hand, several chelating agents such as tricarbalylate and e-phenanthroline do not activate, while some poor chelating agents such as 3-phosphoglycerate and malate are excellent activators at 10−5 M. Finally, the activators affect the Km of both glucose and MgATP, as well as the observed velocity, so that activation is not simply a matter of making more enzyme available in active form. Possibly the E-glucose-ATP-Mg complex in the acid form interacts with a tightly bound metal ion already present on the enzyme and this is prevented by interaction of the bound metal, the enzyme, or both with the activator. Although the specificity studies imply unusual variety among the activators, ATP, citrate, malate, Pi, and 3-phosphoglycerate, this is not without previous example, as in the case of phosphofructokinase, where a similar range in the nature of effectors is known.

Scheme 1 represents the observations of the effects of pH and activators on the enzyme-substrates ternary complex. A represents activators; B, active enzyme with both substrates bound; C, inactive enzyme with both substrates bound. The equilibrium for the allosteric conversion (Step 1) is far toward the inactive form as judged from the fact that in the unactivated low pH condition, the enzyme has only a small fraction of the activity that it has at pH 8.0 (Fig. 2A). Activators react only, or much more strongly, with the minor, active component of the equilibrium of acid forms. This interaction is not competitive with protons, as would appear if A reacted only with B since KA of citrate is independent of pH. Glycerol inhibits the enzyme at all pH values, but part of the inhibition seen at acid pH seems to be reversed by activators. Thus, the effect of glycerol appears to be to shift the allosteric equilibrium toward inactive form. Such an effect may be interpreted to indicate that this form has more of its polar groups buried (20). The loss of some functional groups on the enzyme in the inactive form would be consistent with its failure to interact as well with the polar activators.

The variety in the nature of the activators suggests that different activator sites may be involved for each class of compound, exemplified perhaps by Pi, 3-phosphoglycerate, malate, citrate, and ATP. This possibility requires further study. pH is important in determining the range of effectiveness of the various activators. Pi shows a pH-dependent KA, whereas citrate does not. However, in the range where citrate becomes protonated (pKa = 6.4), the extent of activation (VA − VI/VI) falls to low values, suggesting that the dianion form binds but does not activate.

The kinetic behavior of the activators has been interpreted to mean that they interact favorably only with the active form of the enzyme. The allosteric conversion to inactive form does not occur at the free enzyme stage, as shown by the fact that when either one or both substrates and activator are added together to enzyme that has been at pH 6.5, the initial rate is the same as that of the fully activated enzyme, whereas if both substrates are added prior to activator this rate is achieved only after a considerable lag. Thus only the ternary complex can undergo conversion to the inactive form.

The question of physiological role is one that is appropriate in view of the recent report that phosphofructokinase of yeast does not show the typical inhibition by ATP at acid pH values (21) which correspond to those for the yeast cytoplasm. As the present study shows, the unactivated rate of hexokinase falls rapidly with [H+]2 in the neutral region (Fig. 2). Thus it may be considered that in yeast, hexokinase as well as phosphofructokinase is a target for multiple control. It may be of further interest to recall that Rothman and Cabib (22) have demonstrated that various allosteric inhibitors of yeast glycogen synthetase are much more effective at pH 0 than at pH 7.5.

A role of Pi concentration in the control of yeast glycolysis was postulated long ago on the basis of its elevation during the aerobic to anaerobic transition (23-25) when glucose utilization is increased. However, more recent concepts of the control of fermentation in yeast favor a limitation in glucose transport rather than at the phosphorylation step, based largely on the very low concentration of intracellular glucose found in cells under steady state condition (26, 27). Both the very high KA of Pi under acid conditions (Fig. 3) and the insensitivity to
activators at low glucose concentration (Fig. 6) tend to discourage the elaboration of simple control schemes at this time. However, the use of the semipermeable yeast cell preparation of Schlenk and co-workers (11, 19) provides an ideal means of determining the kinetic properties of enzymes in concentrations and milieu approaching those of the intact cell, and may contribute further insights into the behavior of hexokinase in the cell.

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