Relaxation Spectra of Yeast Hexokinases. Isomerization of the Enzyme†

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ABSTRACT: Yeast hexokinase isozymes P_I and P_{II} exhibit a pH dependent, rapid relaxation process at 15 °C at enzyme concentrations of 100–474 μM and over a pH range of 6–8. The process was detected by equilibrium temperature jump spectroscopy using the indicator probe phenol red. The value of $1/\tau$ varies from about 6 ms^{-1} at pH 8 for both isozymes to 50 ms^{-1} for P_I and 85 ms^{-1} for P_{II} at pH 6. The data are consistent with a mechanism involving an enzyme isomerization coupled to an ionization. The forward rate constant for the isomerization of the proposed mechanism varies between 3 and 7 ms^{-1}; the ratio of the reverse rate constant to the ionization K_a is between 0.5 and 2×10^{11} M^{-1} s^{-1}; the estimated pK_a varies between 5.5 and 6.1. The ranges of values in rate constants and pK_a represent variations observed between preparations of the same isozyme and between isozymes. The isomerization rate is at least 50 times faster than catalysis under all conditions and the pK_a is lower than that controlling activity. The rate of isomerization is unchanged by addition of sugar and nucleotide ligands, but the amplitude of the process is perturbed. These data imply that isomerizing and ionizing forms are sensitive to events at the active site. These equilibria between forms of hexokinase are fast enough, and have the right properties, to be important to the mechanism and regulation of the enzyme.

Hexokinase, the first enzyme on the glycolytic pathway, catalyzes the transphosphorylation of the 6 position of glucose (and other hexose sugars) by MgATP. The steady-state kinetics and the mechanism of action of hexokinase derived from baker's yeast have been investigated by a number of laboratories (Kosow and Rose, 1971; Noat et al., 1968; Noat and Ricard, 1968; Hammes and Kochavi, 1962; Purich et al., 1973; Danenberg and Cleland, 1975). However, little information is available in the literature concerning the direct determination of rates of individual steps.

Yeast hexokinase has been shown to have interesting regulatory properties at suboptimal, physiological pH values (below 7). In particular, Shill and Neet (1975) have shown that hexokinase exhibits a slow transient in enzyme progress curves during assay of enzyme activity, and Kosow and Rose (1971) have shown that hexokinase can be activated by a variety of small dianionic molecules. Both laboratories reported the existence of non-Michaelis-Menten steady-state kinetics with MgATP as the variable substrate. The relationship between these regulatory properties and the mechanism of action of the enzyme is not clear at this time.

Two isozymic forms of yeast hexokinase, designated P_I and P_{II}, have been shown to exist (Rustum et al., 1971; Schulze et al., 1966). Under normal conditions, both isozymes exist as dimers of molecular weight 100 000, and both forms appear to have the same mechanism of action. Distinct differences in catalytic and physical properties exist between the two forms, however. Hexokinase P_I has a much lower (three- to fivefold) specific activity towards glucose than does hexokinase P_{II}, and the two forms exhibit different ratios of activity with different sugar substrates. Amino acid analysis of the two isozymes established that the two forms are different polypeptides and not interconvertible forms (Gazith et al., 1968).

We have initiated an investigation of the kinetics of the elementary steps involved in the catalytic and regulatory mechanisms of yeast hexokinase isozymes for the purpose of expanding the kinetic information and differentiating between mechanistic possibilities. Our studies should also define more precisely the mechanistic similarities and differences of the isozymic forms of this enzyme, and relate these properties to the three-dimensional structure of hexokinase currently under investigation in Steitz's laboratory (Anderson et al., 1974; Anderson and Steitz, 1975; Fletterick et al., 1975). This paper reports the observation and characterization of a rapid isomerization of hexokinase P_I and of hexokinase P_{II} using the technique of temperature jump spectroscopy.

Methods and Materials

Enzyme Preparation and Characterization. Hexokinase P_I was purified to isozymic homogeneity from Red Star compressed baker's yeast by combining parts of the methods of Rustum et al. (1971) and Womack et al. (1973). Several hundred milligrams of hexokinase P_I of greater than 90% homogeneity (as determined by 7.5% acrylamide gel electrophoresis (Davis, 1964)) was isolated. The specific activity of enzyme prepared in this way was 150–200 units/mg at 25 °C.

Hexokinase P_{II} was obtained either by isolation from Red Star baker's yeast, as described above, or obtained from Worthington (Lot N4E534). The commercial enzyme was further purified by column chromatography on Whatman DE-52 with a linear pH gradient similar to that used by Rustum et al. (1971). The enzyme was treated with phenylmethanesulfonyl fluoride to protect against proteolysis. The enzyme was homogeneous on sedimentation velocity centrifugation and 7.5% acrylamide gel electrophoresis. The average specific activity of the enzyme used here was 575 units/mg at 25 °C.

Enzyme solutions were stored at concentrations greater than 5 mg/ml in a solution containing 1 M NaCl, 0.1 M succinate,

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and 2 mM EDTA[1] at pH 5.5 (Rustum et al., 1971). The EDTA in the storage buffer was present to remove traces of undesired metal ions.

One unit of activity is defined as 1 μM of product formed per minute as assayed spectrophotometrically at 25 °C by coupling the reaction with glucose-6-phosphate dehydrogenase and observing the increase in absorbance at 340 nm due to NADPH formation. The assay mix contained 20 mM glucose, 2 mM MgATP, 8 mM $MgCl_2$, 0.5 mM NADP, 1–2 units of glucose-6-phosphate dehydrogenase, and 0.1 M triethanolamine hydrochloride, all at pH 8.0. The presence of 0.1 M KNO_3 in the assay reduced the activity of hexokinase by about 25%, compared to the salt-free assay medium. Since the same reduction is observed with 0.1 M NaCl or KCl, we attribute it to a nonspecific salt effect. Full activity was restored upon dialysis into storage buffer.

Enzyme solutions containing 5–23.7 mg/ml of hexokinase were prepared for temperature jump experiments by dialysis for at least 24 h at 4 °C vs. 0.1 M KNO_3 and 20–160 μM phenol red indicator at the approximate pH desired. Enzyme activities were checked on solutions before and after temperature jumps; more than 40 jumps have been performed in a single day on a single enzyme solution without affecting the specific activity of the enzyme. Following temperature jump experiments, the enzyme was dialyzed vs. the storage buffer for 1 or 2 days and stored at 4 °C. Enzyme solutions which had been used for temperature jump experiments were periodically checked for homogeneity by 7.5% acrylamide gel electrophoresis; the enzyme band remained homogeneous throughout use. After prolonged storage and use, the hexokinase solutions eventually lost activity. Enzyme solutions with a specific activity below 100 units/mg for P_I and below 400 units/mg for P_{II} were discarded. When enzyme solutions became too dilute to use in the temperature jump spectrometer, active samples were pooled, concentrated (Rustum et al., 1971), and used for further experiments.

In some cases, a complete pH-dependence curve was obtained from a single enzyme solution. For such experiments, the pH of the enzyme solution was adjusted by adding microliter quantities of 0.1 M KOH or 0.1 M HNO_3. If solutions became turbid after pH adjustment, they were filtered through 5 μm or smaller Millipore filters before being returned to the temperature jump cell.

Chemicals. All supplies were of the highest purity commercially available. ATP, ADP, and D-(+)-mannose were from Sigma, D-glucose was from Fischer Scientific, and the $MgCl_2$ and KNO_3 were from Baker. Phenol red, obtained from Sigma and Fischer Scientific, was shown by thin-layer chromatography to be free from impurities. $Cr(NH_3)_2ATP$ was prepared by the method of DePamphilis and Cleland (1973).

Instrumentation. Kinetic data were obtained at 15 °C on a temperature jump spectrometer (Messanlagen Studiengesellschaft) connected to a Biomation 802 transient recorder. Perturbation of the equilibrium distribution of enzyme species following a temperature jump was coupled to the pH indicator phenol red; the temporal response of the indicator probe was monitored by optical transmittance at 520–580 nm. Prior to a kinetic determination, the temperature jump cell compartment was equilibrated to 10 °C. At approximately 20-min intervals, the solution was jumped 5 ± 0.3 °C by means of a calibrated high-voltage discharge (30.5 kV) and the resultant relaxation trace was stored in the Biomation recorder. The data were recorded either by plotting the Biomation output via a strip chart recorder or by photographing an oscilloscope display of the stored data.

pH measurements were obtained from a Sargent digital or a Beckman expandomatic pH meter and a Corning semimicro combination electrode. pH values were obtained both before and after a series of temperature jumps on a given solution; measurements were either made at 4 °C and corrected to 15 °C or made at 15 °C. In either case, the pH meter was calibrated with standard Sargent–Welch pH 4 and 7 buffers, which were maintained at the same temperature as the enzyme solutions.

In order to ensure that the processes observed were due to hexokinase, indicator systems were tested independently and did not show relaxation effects in the time regions where the enzyme relaxation effects were observed. The indicator (up to 20 μM) was shown to have no effect upon enzyme activity under assay conditions nor was there any other indication of indicator binding. A wide range of indicator concentrations at constant enzyme concentration (e.g., 60–185 μM phenol red at 100 μM hexokinase P_I, pH 6.3 and 40–160 μM phenol red at 263 μM hexokinase P_{II}, pH 7.4) and a wide range of enzyme concentrations at constant indicator concentration (see Figure 2) were used for temperature jump experiments; the relaxation effects showed no dependence upon the indicator concentration under either set of conditions.

Data Treatment. Relaxation curves obtained from the Biomation were manually digitized and fit by a nonlinear regression program to a least-squares fit of an exponential curve. Each data point in Figures 2 and 3 is the average of at least three reciprocal relaxation times, $1/\tau$, obtained for the same solution. The error bars represent the standard deviation of the average value of $1/\tau$.

Results

Relaxation data were obtained over the pH range from 6.0–8.1 and at enzyme concentrations of 100–474 μM. Photographs of typical relaxation effects for hexokinase P_I or P_{II} are shown in Figure 1. The initial transmittance change corresponds to rapid proton transfer equilibria involving enzyme and indicator systems, while the slower process is not itself a proton transfer. The initial proton transfer processes, which relax in 20 μs or less, were too fast for detailed study. The initial rapid steps, resulting in a net increase in pH and a corresponding decrease in transmittance, are followed by the slower process, which relaxes in the opposite direction. This latter process, referred to as the enzyme relaxation effect, was characterized as a function of enzyme concentration and solution pH. We have found that the enzyme relaxation effect varies somewhat in pH behavior from one preparation to another, as well as from the P_I to the P_{II} isozyme.

French and Hammes (1965) list four criteria for detecting possible enzyme–indicator interactions, which in principle could lead to observable relaxation effects. By these criteria, we were able to eliminate the possibility that such interactions were responsible for the observed effects. Approximate experimental conditions are given in parentheses. First, we found that there was no detectable difference in indicator concentration inside or outside a dialysis bag containing the enzyme (20 μM phenol red, 240 μM hexokinase P_I, pH 6.5). Second, cuvettes containing indicator and indicator plus enzyme showed no difference spectrum (30 μM phenol red, 60 μM

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; $Cr(NH_3)_2ATP$, chromium diammine adenosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, reduced NADP.

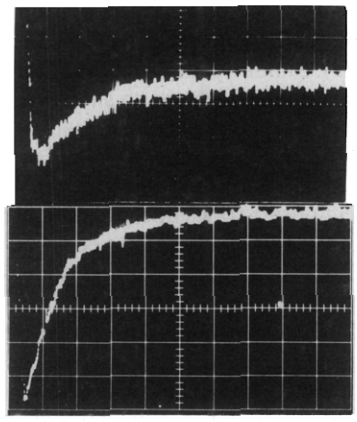

FIGURE 1: Representative relaxation effects for hexokinase P_I (preparation 1) in 0.1 M KNO_3 and 60 μM phenol red. The observation wavelength was 558 nm. The vertical scale is in arbitrary transmittance units. Upper trace: 244 μM hexokinase, pH 8.13; the horizontal scale is 100 μs/large division. The value of $1/\tau$ for this relaxation process is 6.7 ± 0.3 ms^{-1}. Lower trace: 252 μM hexokinase, pH 6.36; the horizontal scale is 50 μs/large division. The value of $1/\tau$ for this relaxation process is 21.4 ± 0.5 ms^{-1}.

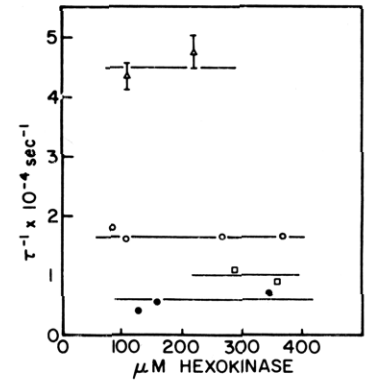

FIGURE 2: Dependence of $1/\tau$ on hexokinase concentration at three pH values for hexokinase P_I and one pH value for hexokinase P_{II}. Phenol red at 60 μM was used as the pH indicator. The isozyme and pH values for which data are presented are: (●) P_I, pH 7.33; (□) P_I, pH 6.80; (○) P_{II}, pH 6.71; (△) pH 6.02.

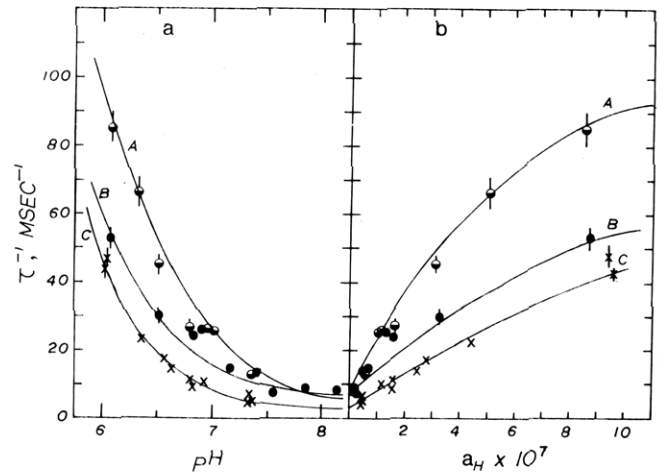

FIGURE 3: Dependence of $1/\tau$ on pH (3a) and a_H (3b) for two preparations of hexokinase P_I and one preparation of hexokinase P_{II}. In all cases, the experimental data, with standard deviations, are represented by circles or x's, and theoretical fits to the data (based on eq 1) are represented by solid lines. X's represent data from hexokinase P_I preparation 1; solutions contained 100–355 μM hexokinase and 60 μM phenol red. Parameters for the theoretical line are: k_1, 3 ms^{-1}; k_{-1}, 170 ms^{-1}; pK_a, 5.5. Solid circles represent data from hexokinase P_I preparation 2; solutions contained 170–474 μM hexokinase and 40 μM phenol red. Parameters for the theoretical line are: k_1, 7 ms^{-1}; k_{-1}, 130 ms^{-1}; pK_a, 5.8. Half-filled circles represent data for hexokinase P_{II}; solutions contained 267 μM hexokinase and 160 μM phenol red. Parameters for the theoretical line are: k_1, 6 ms^{-1}; k_{-1}, 156 ms^{-1}; pK_a, 6.1.

hexokinase P_{II}, pH 6.3). Third, the amplitude of the relaxation effect decreased in the presence of phosphate buffer; no relaxation effect was observed in the presence of 10 mM buffer (50 μM phenol red, 93 μM hexokinase P_{II}, pH 6.6). Fourth, the indicator had no discernible effect upon the activity of the enzyme (20 μM phenol red, hexokinase P_I, standard assay mix). These results, together with the observation that τ is independent of indicator concentration, support the conclusion that the relaxation effect is not due to an enzyme–indicator interaction.

Concentration Dependence. The reciprocal relaxation times obtained for hexokinases P_I and P_{II} over a range of enzyme concentrations and for several pH values is shown in Figure 2. At constant pH values, $1/\tau$ is concentration independent over an enzyme concentration range of ca. 86–400 μM.

Sedimentation velocity studies under the same conditions indicated that hexokinose P_I sedimented as a dimer, even at the most dilute concentration of enzyme (60–80 μM) used in the rapid kinetic studies.

pH Dependence. The data of Figure 3a demonstrate that the enzyme relaxation effect has a strong pH dependence. Plots of $1/\tau$ vs. pH for two preparations of hexokinase P_I and one preparation of hexokinase P_{II} are shown in Figure 3a. Although there are some differences among the three curves, all samples of hexokinase demonstrate the same functional form with a strong dependence on pH near pH 6.

Proposed Mechanism. The concentration independence and the pH dependence, taken together, are consistent with an isomerization process coupled to a proton transfer on the enzyme. Each of the three sets of data was found to be consistent with the following mechanism:

$$EH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EH' \overset{K_a}{\rightleftharpoons} E' + H^+ \qquad (1)$$

in which the enzyme isomerization step is slower than the ionization step; k_1 and k_{-1} are rate constants for the isomerization, and K_a is a mixed acid dissociation constant. If one assumes that the hydrogen ion concentration is buffered during the temperature jump perturbation, the expression for $1/\tau$ has the following form (French and Hammes, 1965):

$$1/\tau = k_1 + k_{-1}[a_H/(a_H + K_a)] \qquad (2)$$

where the constants are defined above and a_H is the hydrogen ion activity.

Our data do not include the relaxation times over the entire region of pH dependency for two reasons. First, the relaxation process becomes too rapid to be distinguished from the proton transfer process at the lower pH values. Second, the signal-to-noise ratio decreases rapidly below pH 6.3. We had, therefore, to be sure that processes other than isomerization coupled to a rapid proton transfer could not be responsible for our results.

The series of tests involving the indicator (see above) precluded the possibility that the observed effects were indicator–enzyme artifacts. In addition, two mechanisms, other than isomerization, were examined in detail and found not to be consistent with the hexokinase data. These are (1) a slow proton transfer, and (2) self-association of the enzyme. A proton transfer reaction was eliminated on two bases. First,

TABLE I: Rate Constants and pK for the Hexokinase Systems.[a,d]

	P_{II}	P_I	
	Curve A	Prep 1: Curve C	Prep 2: Curve B
k_1 ($\times 10^{-3}$ s^{-1})	6	3	7
$(k_{-1}/K_a)^b$ ($\times 10^{-11}$ M^{-1} s^{-1})	2.0	0.5	0.8
k_{-1} ($\times 10^{-5}$ s^{-1})	1.6	≤ 1.7	1.3
pK_a	6.1[c]	≤ 5.5[c]	5.8[c]

[a] At 15 °C and I = 0.1 M KNO_3. [b] Limiting slope of curves in Figure 3b, estimated error: +50%, −30%. [c] Mixed pK. [d] Uncertainties in k_{-1} and K_a are at least as large as the uncertainty in their ratio.

graphs of $1/\tau$ vs. a_H for two of the sets of data (Figure 3b) clearly show nonlinearity at high values of a_H. This is inconsistent with a proton transfer reaction (even under buffered conditions such that the enzyme concentration does not appear in the functional dependence). The curvature observed, however, is precisely that predicted by eq 2 for a slow isomerization coupled to a rapid ionization. Second, proton transfer rate constants calculated from the limiting (low a_H) slopes in Figure 3b are at least an order of magnitude larger (see Table I) than the theoretically allowed limit for a diffusion-controlled combination of a proton with a spatially-shielded macromolecule.

The data are easily shown to be incompatible with a self-association mechanism coupled to a proton transfer. While such a mechanism can yield the proper pH dependence (that of eq 2 at fixed enzyme concentration), the pH-dependent terms of the relaxation time must be directly proportional to the enzyme concentration, even under buffered conditions. The concentration range employed was easily sufficient to detect any such dependence. No enzyme concentration dependence is, in fact, observed (see Figure 2).

Curves B and C of Figure 3b correspond to two different preparations of P_I: curve A, to a single preparation of P_{II}. For two systems—P_I, curve B and P_{II}, curve A—the pH values were sufficiently close to the pK to allow determination of all the constants in eq 2. For the other system, only k_1 and the ratio k_{-1}/K_a could be determined with some certainty from the low a_H regions of Figure 3b. These constants are given in Table I.

The difference between the two hexokinase P_I preparations may be due to the presence of slightly modified or proteolyzed forms, or to slight variations in the yeast strain. Numerous slightly modified forms of hexokinase from yeast have been identified by slight differences in their chromatography patterns (Rustum et al., 1971; Gazith et al., 1968; Schulze and Colowick, 1969). Similarly, there might be kinetic differences between preparations which are so subtle that neither chromatography nor acrylamide gel electrophoresis can detect differences in the enzyme forms.

Effect of Ligands on the Isomerization. We have determined the effect of substrates upon the isomerization process. None of the substrates investigated perturbed the rate of the isomerization, but the amplitude of the isomerization process was qualitatively observed to be affected by a variety of ligands. The results of these experiments are summarized in Table II. The amplitude of the isomerization effect decreased in the presence of glucose; the same loss of amplitude occurs at lower glucose concentrations when the pH is decreased. In the presence of $MgCl_2$ and glucose, the relaxation amplitude increased. The relaxation amplitude decreased with addition of MgADP and increased in the presence of mannose. A decrease in amplitude was observed upon addition of $Cr(NH_3)_2ATP$; the addition of 1 mM glucose to the solution from the previous experiment partially restored (55% of original) the amplitude of the relaxation effect.

Addition of glucose to hexokinase P_{II} did not cause dissociation of the dimer, as indicated by sedimentation velocity studies at an enzyme concentration of 96 μM in 0.1 M KNO_3 and 50 mM glucose at pH 7.6.

Effect of Urea. The effect of the denaturant urea upon the enzyme isomerization process was determined at pH 8.2. A solution containing 200 μM hexokinase P_I showed a large, well-defined relaxation process which completely disappeared upon addition of urea (final concentrations, 6.7 M urea, 140 μM hexokinase). In a second experiment, hexokinase P_I was dialyzed into 8 M urea; no relaxation effect was observed (200 μM hexokinase, pH 8.2). These results indicate that the enzyme isomerization process is dependent upon the tertiary and/or quaternary structure of the enzyme.

Discussion

pH Dependence. Differences observed for the isomerization process between isozymes or between different preparations of the same isozyme involve primarily a shift in the estimated pK of the ionizing group and/or a change in the magnitude of k_1. If all forms of hexokinase are considered together for comparison purposes, several general observations may be made: (1) the $1/\tau$ values vary by a factor of ten or more over the pH range studied. Other enzymes for which a pH-dependent isomerization process has been published (French and Hammes, 1965; French et al., 1974; Giannini et al., 1975) have not exhibited nearly so wide a range of $1/\tau$ values. (2) The value of k_{-1} for the isomerization process is similar to that

TABLE II: Effect of Various Ligands on the Amplitude of the Hexokinase Relaxation Effect.[a]

Enzyme (Concn)	pH	Ligand (concn)	Amplitude[b]
P_I (100 μM)	7.1	Glucose (3 mM)	0.5
P_I (270 μM)	6.8	MgADP (160 μM)	0.7
P_{II} (290 μM)	7.2	Mannose (1.2 mM)	1.3
P_{II} (200 μM)	6.5	$Cr(NH_3)_2ATP$ (10 μM)	1.0
	6.5	$Cr(NH_3)_2ATP$ (500 μM)	0.3
	6.5	$Cr(NH_3)_2ATP$ (500 μM) + 1 mM glucose	0.55
P_I (215 μM)	6.7	Mg^{2+} (4.7 mM)	1.05
	6.7	Mg^{2+} (4.7 mM) + 2.4 mM glucose	1.21

[a] None of the ligands changed the value of τ. [b] Relative to the system in which the ligand was absent.

observed for other enzymes (French and Hammes, 1965; French et al., 1974; Giannini et al., 1975). (3) The estimated p*K* for hexokinase is comparable to p*K* values determined for the fastest isomerizations of other enzymes (French and Hammes, 1965; French et al., 1974, Giannini et al., 1975). In general, when ionization of a specific amino acid residue has been suggested to account for the relaxation behavior of these enzymes, a histidine residue was implicated (French and Hammes, 1965, Giannini et al., 1975).

Implications for the Enzyme Mechanism. The isomerization process of hexokinase P_I or P_{II} is at least 50 times faster than a single catalytic cycle at the same temperature and pH in the presence of 0.1 M KNO_3. For example, at pH 8 the isomerization of P_I occurs at a rate of about $10^4\ s^{-1}$, whereas our measured turnover number for the enzyme under the same conditions is $40\ s^{-1}$. With these relative rates, such a single step is rapid enough to occur during each catalytic cycle and, hence, may be a mandatory step in the mechanism. The isomerization can therefore occur during catalysis, but is not, itself, a rate-limiting step.

The maximum p*K* of 6.1 reported here for the isomerization coupled enzyme ionization is very different from the p*K* of 7.3 reported (Kosow and Rose, 1971; Shill and Neet, 1975) for enzyme activity. Thus, the ionization reported here is probably a separate process from the protonation which causes a decrease in hexokinase activity at low pH. However, at least two other processes of hexokinase with p*K* values in the region of 5.5–6 have been reported: (1) the pH dependence of the rate of the transient in enzyme activity (Shill and Neet, 1975), and (2) the pH dependence of the rate of hexokinase inactivation induced by xylose and MgATP (Menzes and Pudles, 1976).

The rate of the isomerization process is unchanged by the addition of substrates, which indicates that the isomerization process is not directly coupled to binding of substrates of the enzyme. However, the changes in amplitude observed may indicate that interactions of enzyme and substrate cause a shift in the ratio of concentrations of the enzyme species which undergo the isomerization reaction. A decrease in amplitude would occur if binding substrate prevented the enzyme from participating in the isomerization process; amplitude decreases were observed with glucose, MgADP, and inhibitory concentrations (500 μM) of $Cr(NH_3)_2ATP$. Amplitude increases may be due to a shift of equilibria towards hexokinase forms (E and E′) undergoing the isomerization; mannose or glucose in the presence of $Cr(NH_3)_2ATP$ (500 μM) cause an increase in amplitude. Thus, the amplitude of the isomerization process is sensitive to species which bind in the sugar and nucleotide *active* sites on hexokinase (Table II); this suggests that the isomerization process is affected in some way by events at the active site of the enzyme. On the other hand, concentrations of $Cr(NH_3)_2ATP$ which activate the enzyme (10 μM) (Peters and Neet, 1976) did not affect the amplitude of the isomerization; the isomerization may, therefore, be insensitive to events at the *activator* site on hexokinase.

Conclusions. The isomerization and ionization described here establish kinetically at least three interconvertible forms of hexokinase not previously recognized (EH, EH′, E′). Isomerization between EH and EH′ forms is rapid relative to catalytic activity; the p*K* for the ionization (EH′ to E′) is lower (5.5–6) than observed ionizations affecting enzymatic activity. Data have been presented which suggest that the isomerization process is indirectly affected by events at the active site of hexokinases P_I and P_{II}. The equilibria between these forms of hexokinase may be important in a consideration of the overall mechanism and regulation of the enzyme.

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References

Anderson, W. F., Fletterick, R. J., and Steitz, T. A. (1974), *J. Mol. Biol. 86,* 261.

Anderson, W. F., and Steitz, T. A. (1975), *J. Mol. Biol. 92,* 279.

Danenberg, K., and Cleland, W. W. (1975), *Biochemistry 14,* 28.

Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

DePamphilis, M. L., and Cleland, W. W. (1973), *Biochemistry 12,* 3714.

Fletterick, R. J., Bates, D. J., and Steitz, T. A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 38.

French, T. C., and Hammes, G. G. (1965), *J. Am. Chem. Soc. 87,* 4669.

French, T. C., Yu, N-T., and Auld, D. S. (1974), *Biochemistry 13,* 3877.

Gazith, J., Schulze, I. T., Gooding, R. H., Womack, F. C., and Colowick, S. P. (1968), *Ann. N.Y. Acad. Sci. 151,* 307.

Giannini, I., Baroncelli, V., Boccalon, G., and Fasella, P. (1975), *FEBS Lett. 54,* 307.

Hammes, G. G., and Kochavi, D. (1962), *J. Am. Chem. Soc. 84,* 2069, 2073, 2076.

Kosow, D. P., and Rose, I. A. (1971), *J. Biol. Chem. 246,* 2618.

Noat, G., and Ricard, J. (1968), *Eur. J. Biochem. 5,* 71.

Noat, G., Ricard, J., Borel, M., and Got, C. (1968), *Eur. J. Biochem. 5,* 55.

Menzes, L. C., and Pudles, J. (1976), *Eur. J. Biochem. 65,* 41.

Peters, B. A., and Neet, K. E. (1976), *J. Biol. Chem.* (in press).

Purich, D. L., Fromm, H. J., and Rudolph, F. B. (1973), *Adv. Enzymol. 39,* 249.

Rustum, T. M., Ramel, A. H., and Barnard, E. A. (1971), *Prep. Biochem. 1,* 309.

Schulze, I. T., and Colowick, S. P. (1969), *J. Biol. Chem. 244,* 2306.

Schulze, I. T., Gazith, J., and Gooding, R. H. (1966), *Methods Enzymol. 9,* 376.

Shill, J. P., and Neet, K. E. (1975), *J. Biol. Chem. 250,* 2259.

Womack, F. C., Welch, M. K., Nielsen, J., and Colowick, S. P. (1973), *Arch. Biochem. Biophys. 158,* 451.