A Slow Transient Kinetic Process of Yeast Hexokinase

By J. P. SHILL and K. E. NEFT

Department of Biochemistry, Case Western Reserve University,
Cleveland, Ohio 44106, U.S.A.

(Received 29 March 1971)

During a study of some kinetic properties of yeast hexokinase (EC 2.7.1.1) we have observed transient changes in reaction progress curves, i.e. transients, going from an initial high activity to a lower steady-state activity. From steady-state kinetics Kosow & Rose (1970) have suggested the possibility of negative co-operativity in yeast hexokinase (P_{II}), an enzyme that appears to be identical with yeast hexokinase B. In view of the increasing prevalence of transient kinetics and the possible significance of such behaviour in co-operativity, we now report experiments documenting a slow change in hexokinase activity during assay and suggest a unique mechanism to correlate transients and co-operativity.

Materials and methods. Yeast hexokinases A and B were purified from Red Star baker's yeast by a modification of the procedure of Lazarus, Ramel, Rustum & Barnard (1966). Hexokinases A and B were greater than 95% pure as indicated by disc polyacrylamide-gel electrophoresis and polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Pringle, 1970), in which the enzyme appeared as a polypeptide chain of molecular weight 50 000. Specific activities were 217 and 842 $\mu\text{mol/min per mg}$ respectively. The results reported in this paper were obtained primarily with hexokinase B possessing a specific activity of 300 $\mu\text{mol/min per mg}$. All reagents used were of the highest possible commercial grade.

Results. At pH 6.5 the observed velocity of the hexokinase reaction measured as the rate of proton formation continuously decreases during the first few minutes to a slower steady-state value (Fig. 1 inset). This transient change in the reaction progress was analysed by plotting the logarithm of the instantaneous velocity (the logarithm of the slope of the progress curve at any time) as a function of time. From such graphs we have calculated $t_{\frac{1}{2}}$, the apparent half-time for the first-order decay of the initially observed velocity to the steady-state value, ϕ_1 , the activity of the enzyme at zero time, and ϕ_2 , the activity in the steady state. The burst height, representing product formed in excess of that which would have been formed by the steady state alone, and ϕ_1 and ϕ_2 are linearly dependent on protein concentration over a range of 0.75–

6.0 $\mu\text{g/ml}$. Over the same range of protein concentration $t_{\frac{1}{2}}$ is constant.

ϕ_1 and ϕ_2 have a similar dependence on pH, both increasing as the pH increases from 5.5 to 8.0, where hexokinase is normally assayed. The pH-dependence of $t_{\frac{1}{2}}$ for the transition from the initial to the steady-state velocity is shown in Fig. 1. The transient is most readily observable in our assay systems at about pH 6.5, because of the magnitude of $t_{\frac{1}{2}}$ and the relative magnitudes of ϕ_1 and ϕ_2 .

The transition process is not caused by product inhibition, since the addition of products to the assay, either alone or in combinations, does not eliminate the curvature of the recorder tracing. Both substrates are required for this transition, since it cannot be eliminated by preincubation with either MgATP^{2-} or glucose, nor by initiating the reaction with either substrate rather than with enzyme. Substitution of either magnesium acetate or magnesium sulphate for magnesium chloride did not eliminate the transient. Preincubation with relatively high concentrations of both substrates produces an essentially linear progress curve on subsequent assay. However, the transient can be regained from enzyme in the steady state by removal of substrates. Modification of the enzyme by methylation (Lazarus, Derechin & Barnard, 1968), by limited proteolysis with trypsin or by denaturation and renaturation from 5 M-guanidinium chloride or 8 M-urea does not eliminate the transient.

A possibility existed that the hexokinase transient was an artifact of the pH-stat, or that the H^+ formation was not specific for the hexokinase reaction but was in part due to some other reaction such as an adenosine triphosphatase reaction. These possibilities were examined by using the glucose 6-phosphate dehydrogenase coupled enzyme assay (Rose & Warms, 1967) with either tri(hydroxyethyl)ammonium chloride or the pH-stat to maintain the pH at 6.5. In either case the progress curves obtained were similar to Fig. 1 (inset). Further, the appearance of the transient on the pH-stat is dependent on the source of the enzyme preparation. Hexokinase A shows less of a transient than that shown here for hexokinase B, and commercial yeast hexokinases (General Biochemicals and Boehringer Mannheim) show even

Fig. 1. pH-dependence of the half-time of the conversion of the initial into the steady-state velocity of hexokinase. $t_{1/2}$ was calculated as the time for the velocity to fall halfway from ϕ_1 to ϕ_2 from a semilogarithmic plot of the progress curve typical of the inset. Experimental conditions are the same as that shown for the inset except that enzyme concentration varied tenfold across the pH range. The four points at pH 6.5 were obtained over an eightfold range of enzyme concentration with no trend. In the inset, progress of the hexokinase B reaction is indicated by the volume of CO_2 -free 1.2 mM-NaOH added to the reaction under N_2 to maintain constant pH. The rate of the reaction was automatically recorded as a function of time in a pH-stat with a Radiometer TT11-SBR2-ABUI apparatus. Assay was initiated by the addition of $1 \mu\text{g}$ of hexokinase B to a total volume of 1 ml of 20 mM-glucose, 2 mM-ATP (sodium salt) and 10 mM- MgCl_2 at pH 6.5 at 25°C .

less than hexokinase A when assayed under the conditions of Fig. 1 (inset). Lastly, the transition process was not greatly affected by the purification, since a transient in the progress curve is present in all steps from crude extract of yeast cells to bands eluted from disc gel electrophoresis. It is also present in the supernatant of crude extract (French press) of *Saccharomyces cerevisiae* (Harden and Young strain) freshly grown by the method of Young, Tolbert & Utter (1969).

Discussion. The results presented here indicate that yeast hexokinase B undergoes a slow change in activity under assay conditions. Such changes do not appear to be an artifact of the assay method,

but rather appear to be dependent on some reversible change induced by the presence of both substrates, MgATP^{2-} and glucose. A conformational change in the enzyme from an initial form to a second, less active, form would be consistent with the results. The requirements for this conversion appear similar to the change of hexokinase B into hexokinase C, reported by Rustum, Massaro & Barnard (1970), which requires both substrates but occurs at higher pH values and is detected by a change in the chromatographic properties.

The significance of the transient of hexokinase may be related to recent reports of allosteric enzymes that possess positively co-operative kinetics and demonstrate a lag type of transient. These enzymes are threonine deaminase (Hatfield, Ray & Umbarger, 1970a,b), glutamine phosphoribosyl pyrophosphate amidotransferase (Rowe, Coleman & Wyngaarden, 1970), homoserine dehydrogenase I (Barber & Bright, 1968) and *Escherichia coli* D-lactate dehydrogenase (Tarmy & Kaplan, 1968). We now find that hexokinase, an enzyme with apparent negative co-operativity as reported by Kosow & Rose (1970) and confirmed in our laboratory, has a burst type of transient. The coincidence of co-operativity and transients in these enzymes suggests that a mechanistic relationship may exist. The substrate-induced conformation-isomerization model proposed by Rabin (1967) may be extended to include instances in which the conformational change occurs slowly enough to be readily observable. We have derived a more general form of the conformation-isomerization model and conclude that the concurrence of positive or negative co-operativity with lag or burst transients can be adequately explained by an appropriate choice of rate constants (G. R. Ainslie, J. P. Shill & K. E. Neet, unpublished work). Such a process may be operating in yeast hexokinase.

This work was supported by U.S. Public Health Service Research Grant no. AM-12881. J.P.S. is aided by Pre-doctoral Research Scholarship Grant no. PRE-24 from the American Cancer Society. K.E.N. is a Faculty Research Awardee of the American Cancer Society (PRA-55).

- Barber, E. D. & Bright, H. (1968). *Proc. natn. Acad. Sci. U.S.A.* **60**, 1363.
- Hatfield, G. W., Ray, W. J. & Umbarger, H. E. (1970a). *J. biol. Chem.* **245**, 1742.
- Hatfield, G. W., Ray, W. J. & Umbarger, H. E. (1970b). *J. biol. Chem.* **245**, 1748.
- Kosow, D. P. & Rose, I. A. (1970). *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 399.
- Lazarus, N. R., Derechin, M. & Barnard, E. A. (1968). *Biochemistry, Easton*, **7**, 2390.
- Lazarus, N. R., Ramel, A. H., Rustum, Y. M. & Barnard, E. A. (1966). *Biochemistry, Easton*, **5**, 4003.

- Pringle, J. R. (1970). *Biochem. biophys. Res. Commun.* **39**, 46.
- Rabin, B. R. (1967). *Biochem. J.* **102**, 22c.
- Rose, I. A. & Warms, J. V. B. (1967). *J. biol. Chem.* **242**, 1635.
- Rowe, P. B., Coleman, M. D. & Wyngaarden, J. B. (1970). *Biochemistry, Easton*, **9**, 1498.
- Rustum, Y. M., Massaro, E. J. & Barnard, E. A. (1970). *Fedn Proc. Fedn Am. Soc. exp. Biol.* **29**, 334.
- Tarmy, E. M. & Kaplan, N. O. (1968). *J. biol. Chem.* **243**, 2587.
- Young, M. R., Tolbert, B. & Utter, M. F. (1969). In *Methods in Enzymology*, vol. 13, p. 250. Ed. by Lowenstein, J. M. New York: Academic Press Inc.