THE INHIBITION OF PYRUVATE KINASE BY ATP: A Mg^{++} BUFFER SYSTEM FOR USE IN ENZYME STUDIES

P. D. Boyer

The Molecular Biology Institute and the Department of Chemistry

University of California, Los Angeles, 90024

Received February 4, 1969

SUMMARY

A simple procedure for "buffering" the free Mg^{++} concentration in an enzyme system is described, based on use of moderately dissociated Mg^{++} salts. As an example, glycerol 1-phosphate was used to maintain the free Mg^{++} concentration during measurement of the ATP inhibition of pyruvate kinase. The inhibition was demonstrated to be largely competitive with phosphoenolpyruvate and independent of removal of free Mg^{++} by the ATP.

Recently T. Wood (1968) reported that the ATP inhibition of pyruvate kinase was caused by removal of free Mg^{++} as a result of complexing with ATP. This was in contrast to the earlier report of Reynard, Hass, Jacobson and Boyer (1961) in which the ATP inhibition was demonstrated to be competitive with ADP or with phosphoenolpyruvate (PEP). Clarification of mode of ATP inhibition is of interest because the competition between ATP and PEP has been regarded as giving important evidence for a common binding position on the enzyme for the terminal phosphoryl group of ATP and the phosphoryl group of PEP.

Although Wood (1968) used conditions reportedly close to those of Reynard *et al.* (1961), the experiments of the latter were conducted near 0° whereas Wood used 30°. The inhibition by ATP and its overcoming by ADP or PEP noted by Reynard *et al.* were thus checked at a higher temperature (25°) and were reconfirmed. Solutions of phosphorylated substrates were adjusted to the experimental pH prior to

use (as in the previous experiments of Reynard et al.), and all samples were verified to have the expected pH. Thus the ATP inhibition was not due to inadequate pH control, a possibility suggested by Wood (1968).

These apparent conflicting results as well as other experiments in which control of the level of free Mg^{++} could be of value, prompted design and use of a Mg^{++} buffer system for a further check of the ATP inhibition of pyruvate kinase. An attractive possibility appeared to be use of phosphorylated glycerol or sugars with appropriate affinities for Mg^{++}. For the experiments reported herein, glycerol 1-phosphate was used. The principle is simple and quite analogous to H^{+} ion buffers. The dissociation constant, K_d, for the Mg^{++} salt of glycerol 1-phosphate in 0.1 M KCl is 1.6×10^{-2}M (Schwarzenbach and Anderegg, 1957). Thus in a mixture of the Mg salt and of a completely dissociated salt of glycerol 1-phosphate, the free Mg^{++} will be given by $(Mg^{++}) \approx K_d \frac{(MgA)}{(A^{=})}$, where $A^{=}$ is the glycerol 1-phosphate anion. With concentrations of (MgA) and $(A^{=})$ appreciably greater than the K_d, the (Mg^{++}) will be effectively "buffered". Under such circumstances, substrates or inhibitors that may complex Mg^{++} may be varied at concentrations considerably less than the "buffer" concentration with maintenance of nearly constant free Mg^{++} concentration.

Applicability of such a system was tested with pyruvate kinase by use of 50 mM potassium glycerol 1-phosphate and 30 mM $MgCl_2$. Use of the K^{+} salt of the glycerol phosphate provided the about 0.1 M K^{+} optimal for the pyruvate kinase reaction. In the presence of up to 5 mM ADP plus ATP to complex Mg^{++}, a free Mg^{++} concentration of close to 8 mM would thus be assured. At this Mg^{++} concentration, nearly 99% of the ADP would be present as MgADP and over 99% of the ATP as MgATP. The catalytic activity of the pyruvate kinase was found to be close to the same when 0.1 M KCl and 10 mM $MgCl_2$ replaced the 0.05 M K_2-glycerol phosphate and 30 mM $MgCl_2$, showing that the glycerol phosphate anion had little or no inhibitory effect on the reaction.

The inhibition of the pyruvate kinase reaction by ATP in the presence of the Mg^{++} "buffer" system, and the overcoming of the inhibition by PEP is shown in Fig. 1. This figure shows two important points: first, that ATP gives an inhibition that cannot be caused by its removal of free Mg^{++}, and second, that the inhibition is largely overcome in a competitive manner by increase in the PEP concentration. The results thus amply confirm the earlier findings of Reynard et al. (1961) and the later similar results of Mildvan and Cohn (1966). The small deviation from a common intercept noted in Fig. 1, and confirmed in other separate experiments, suggests that there may be a small non-competitive component to the ATP inhibition under the conditions used.

The apparent K_i for ATP from the results given in Fig. 1, calculated

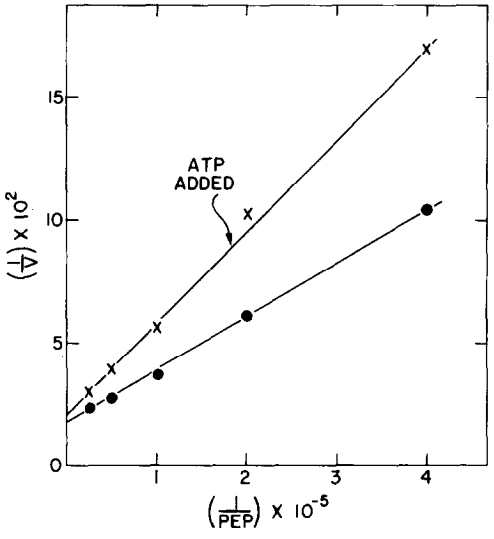

Fig. 1. The competitive inhibition of pyruvate kinase by ATP with a Mg "buffer" system present. 1/v is the reciprocal of the observed velocity expressed as μmolar per minute; 1/(PEP) is the reciprocal of the PEP molarity ATP when added was 3 mM.

A 3 ml. reaction mixture contained 10 mM Tris-Cl, 2.1 mM ADP, 50 mM dipotassium glycerol 1-phosphate, 30 mM $MgCl_2$, 0.2 mM EDTA and 0.13 mM DPNH at a final pH of 7.8 and temperature of 25°. Velocity was measured by decrease in the linear portion of the A_{340} versus time recording following addition of 25 μl of a solution containing 0.16 mM glutathione, 16 μM EDTA, and, per ml., 0.64 mg of bovine serum albumin, 0.32 mg. of lactate dehydrogenase and 27 μg of pyruvate kinase.

by the equation of Reynard et al. (1961), is 0.14 mM. This agrees satisfactorily with the previous value of 0.12 mM reported by Reynard et al. and 0.33 mM reported by Mildvan and Cohn (1966) under slightly different experimental conditions. Wood (1968), as an argument against a competitive effect of ATP, cited the apparent discrepancy between these values and values of K_i of 3.5 mM and 1.0 mM from results of Tanaka et al. (1967) and Lowry and Passoneau (1964), respectively. However, as pointed out by A. S. Mildvan (personal communication) these high values are for concentrations of ATP for half-inhibition under specified conditions. When the K_i's are calculated by the equation of Reynard et al. (1961), they become 0.14 mM and 0.23 mM, respectively. This calculation considers only the competitive component of inhibition with brain pyruvate kinase; the data of Lowry and Passoneau suggest a "mixed" type of inhibition. In addition, behavior of the brain enzyme may not be analogous to that of the muscle enzyme.

An adequate explanation for the results reported by Wood (1968) is not apparent. Under some conditions ATP can inhibit pyruvate kinase by removal of Mg^{++} but this does not justify ascribing the inhibitions noted with excess Mg^{++} present to Mg^{++} removal. It might be pertinent that the failure to note inhibition by ATP with 15 mM $MgCl_2$ added (see Fig. 1 of Wood, 1968) was with limiting ADP (0.25 mM) present. Unless adequate correction was made for the presence of ADP in ATP (e.g., about 2 to 3% in recent commercial preparations tested), increase in the ADP concentration upon addition of 5 mM ATP could largely mask the ATP inhibitory effect.

ACKNOWLEDGMENTS

This research was supported in part by grant GM-11094 of the Institute of General Medical Sciences, U. S. Public Health Service. Mrs. Susan Galyean gave able technical assistance.

REFERENCES

Lowry, O. H., and Passoneau, J. V. (1964), J. Biol. Chem., 239, 31.
Mildvan, A. S., and Cohn, M. (1966), J. Biol., 241, 1178.
Reynard, A. M., Hass, L. F., Jacobson, D. D., and Boyer, P. D. (1961), J. Biol. Chem., 236, 2277.
Schwarzenbach, G., and Anderegg, G. (1957), Helv. chim. Acta, 40, 1229.
Tanaka, T., Harano, Y., Sue, F., and Morimura, H. (1967), J. of Biochem., (Tokyo), 62, 71.
Wood, T., Biochem. Biophys. Res. Comm. (1968), 31, 779