PHARMACOLOGICAL REVIEWS
Vol. 18, No. 1, Part I

Printed in U.S.A.

C. FACTORS INFLUENCING ACTIVATION OF PHOSPHOFRUCTOKINASE

TAG E. MANSOUR

Department of Pharmacology, Stanford University School of Medicine, Palo Alto, California

Phosphofructokinase is the enzyme which catalyzes the reaction:

$$\text{Fructose-6-P} + \text{ATP} \xrightarrow{Mg^{++}} \text{Fructose} - 1,6\text{-di-P} + \text{ADP}.$$

The role of phosphofructokinase as a rate-limiting enzyme in the Meyerhoff metabolic scheme for glycolysis was indicated by Cori (2), Engel'hardt and Sakov (3) and Lynen *et al.* (8). In these early reports evidence for enzyme activation was based on a decrease in the substrate and an increase in the product of the reaction. According to this criterion, muscular contraction (2) as well as anoxia (8) caused an increase in the activity of phosphofructokinase. A more direct evidence of phosphofructokinase activation was recently demonstrated in our laboratory in the liver fluke *Fasciola hepatica.* It was found that glycolysis in this parasite is regulated by phosphofructokinase (10). Stimulation of glycolysis by 5-hydroxytryptamine (serotonin) resulted in a marked increase in phosphofructokinase activity (10). Evidence based on our previous finding that serotonin, and not epinephrine (E), activates the synthesis of cyclic 3′,5′-AMP (9, 14) suggested that the effect of serotonin on phosphofructokinase was mediated through cyclic 3′,5′-AMP. It was later reported that cyclic 3′,5′-AMP can activate phosphofructokinase in two different ways (13). First, the cyclic nucleotide can activate phosphofructokinase which was inhibited by ATP. Such activation would appear to be related to an allosteric effect on the enzyme. Second, cyclic 3′,5′-AMP activated a concentrated inactive preparation of phosphofructokinase. Under the latter conditions an effect of the nucleotide on the enzyme during assay was excluded because the final dilution in the assay mixture was below effective concentration of the nucleotide.

The purpose of this paper is to describe some of our recent work on the nature of heart phosphofructokinase and the different mechanisms for its activation and inhibition. An attempt will be made to interpret these results in relationship to the regulation of glycolysis and of glycogenolysis.

ACTIVATION OF MAMMALIAN PHOSPHOFRUCTOKINASE THROUGH ITS KINETIC PROPERTIES

Recent studies from our laboratory and others have shown that phosphofructokinase from mammalian sources (11, 23), yeast (26, 28), and *Escherichia coli* (1) has kinetic properties similar to that from the liver fluke and undergoes allosteric activation by cyclic 3′,5′-AMP as well as other adenylic nucleotides. Studies on a partially purified phosphofructokinase from the guinea pig heart showed that ATP was a potent inhibitor of the enzyme. A similar effect was previously re-

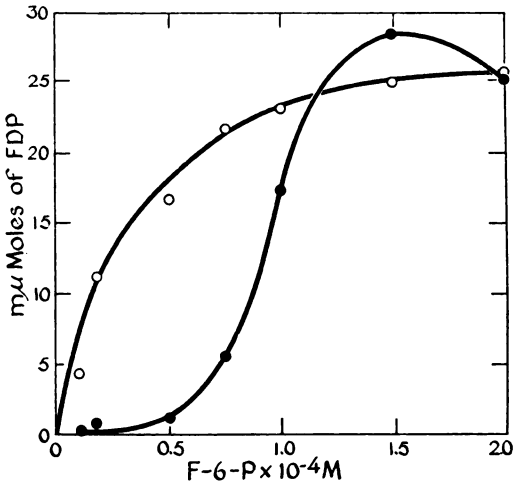

FIG. 1. Plot of initial reaction velocity *versus* fructose-6-P concentration in the absence of cyclic 3′,5 -AMP, ●, and with cyclic 3′,5 -AMP (10^{-4} M), ○. Initial reaction velocities are expressed as millimicromoles of fructose-1,6-di-P (FDP) per 1 ml per 5 min. Assay conditions used are described in the original paper. (From J. biol. Chem. 238: 2285, 1963.)

ported with phosphofructokinase from the skeletal muscle (6). The inhibitory effect of ATP on the heart enzyme was dependent on the presence of suboptimal substrate concentrations of fructose-6-P and a pH 6.9 to 7.5. The ATP-inhibited enzyme can be activated by cyclic 3′,5′-AMP (concentrations as low as 5×10^{-5} M) and to a lesser degree by 5-AMP, ADP, and inorganic phosphate. ATP inhibition is partially competitive with respect to cyclic 3′,5′-AMP (11).

When the concentration of fructose-6-P was tested against the velocity of the reaction (concentration of fructose-1,6-di-P produced) a characteristic sigmoidal curve was obtained indicating a second order effect (fig. 1). This indicates that a slight increase in the concentration of fructose-6-P can result in a marked increase in activity of the enzyme. The sigmoidal curve obtained for fructose-6-P is similar to that obtained for hemoglobin in respect to its affinity to oxygen (16). When enzyme assays were carried out in the presence of cyclic 3′,5′-AMP and under the same conditions, a first order kinetics characterized by the hyperbolic curve was observed with fructose-6-P (fig. 1) (11). Thus, heart phosphofructokinase is adapted for regulation by its own substrate.

It was further reported that citrate and other compounds associated with the tricarboxylic acid cycle are strong inhibitors of phosphofructokinase at concentrations found in the tissues (20, 24). Studies on the nature of such inhibition have not yet been fully reported.

A multisite model[1] (11) for phosphofructokinase interaction with its substrates and activators is summarized in figure 2. It is assumed in this model that at inhibitory concentrations of ATP, at least one additional ATP molecule can

[1] The author is indebted to Dr. David S. Hogness for suggesting this model.

be added to the enzyme. The $(ATP)_n$-enzyme complex ($n > 1$) could either have a lesser affinity for the fructose-6-P or a decreased rate of breakdown to products after fructose-6-P is added to the enzyme complex. Either of the possibilities would lead to inhibition of the reaction by ATP. Activation of the ATP-inhibited enzyme by raising the concentration of fructose-6-P is supposed to result from the hexose phosphate displacing the extra ATP molecules on the enzyme. This would lead to the sigmoidal curve shown in figure 1. Activation by cyclic 3′,5′-AMP and related nucleotides can be explained assuming that the activators compete with ATP for the extra site. The assumption must be made that the activator-enzyme complex is fully active with respect to fructose-6-P. Such an explanation is consistent with the observation that ATP inhibition is partially competitive with respect to cyclic 3′,5′-AMP.

On the basis of the kinetic data obtained with the heart phosphofructokinase at concentrations of ATP and fructose-6-P which might well exist in the cell (7×10^{-3} M and 0.8×10^{-4} M respectively) the enzyme should be strongly inhibited by ATP (11). An increase in the intracellular levels of 5-AMP, ADP and inorganic phosphate such as that which occurs during anoxia (7, 8) should result in an increase in phosphofructokinase activity. This might explain the known increase in the activity of phosphofructokinase which results from anoxia (Pasteur effect) (8, 23).

It is also possible that the effect of E in stimulating glycogenolysis during its inotropic action on the heart is achieved by activation of the ATP-inhibited phosphofructokinase (in addition to its known effect on glycogen phosphorylase) (11). According to the model described above this can be achieved by raising the concentration of the substrate, fructose-6-P, or one or more of the activators. E is known to increase the level of fructose-6-P (17) through its effect in activating

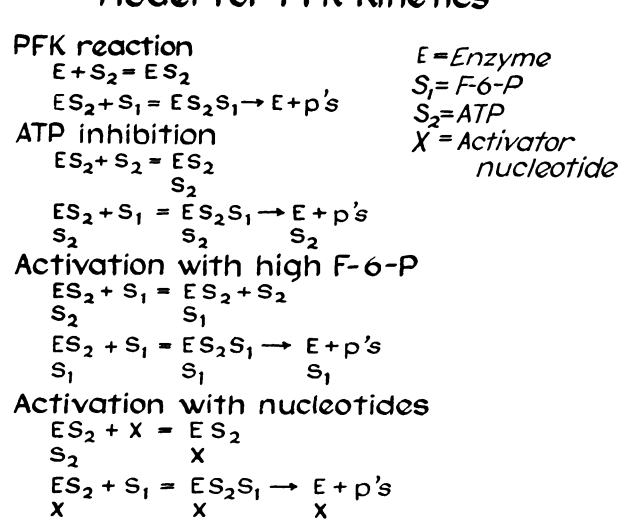

FIG. 2. Model for phosphofructokinase (PFK) kinetics

phosphorylase (25). Furthermore, studies on the heart reported from Sutherland's laboratory have shown an increase in the level of cyclic 3′,5′-AMP in the heart during the onset of the inotropic effect of epinephrine (18). Other activators which might contribute to activation of the ATP-inhibited phosphofructokinase and which are increased in the heart cell as a result of the E-induced increase in the contractility of the heart muscle include ADP, AMP and inorganic phosphate (27). Recently it has been reported from Cori's laboratory that in the frog sartorius muscle the process of phosphofructokinase activation is geared to the contraction process rather than to E (4, 19). It must, therefore, be emphasized here that the ATP-inhibited mammalian phosphofructokinase can be activated not by cyclic 3′,5′-AMP alone, but also by other adenylic nucleotides and inorganic phosphate (11, 23) whose levels are increased under different physiological conditions (*e.g.* contractility, anoxia).

Activation of glycogen phosphorylase *b* through a mechanism similar to that described above with phosphofructokinase has been described by Parmeggiani and Morgan (22). Phosphorylase *b* is inhibited by ATP at concentrations which might well exist in the cell. The inhibition can be relieved by AMP, ADP and inorganic phosphate. Recently Krebs *et al.* (5) showed that skeletal muscle phosphorylase *b* kinase is also inhibited by high concentrations of ATP. This evidence, when added to the finding of Parmeggiani and Krebs (21) that during purification of the phosphorylase kinase the phosphofructokinase was found always to accompany the phosphorylase kinase in all the various fractions, suggests that the two enzyme systems are adapted to work in tandem. This is further suggested by the fact that both enzymes are activated together under the same physiologic changes; *e.g.* anoxia or muscular contraction.

STRUCTURAL ACTIVATION OF PHOSPHOFRUCTOKINASE

By structural activation is meant here activation through an effect on a concentrated inactive form of the enzyme. Under these conditions a kinetic effect of the activator during enzyme assay was excluded because of the dilution of the nucleotide to a subeffective concentration.

It was recently reported that partially purified preparations of guinea pig heart phosphofructokinase (11), when incubated at pH values which ranged from 5.8 to 6.5, lost 90 to 95 % of their activity almost instantaneously (12). The acidified inactive enzyme, when tested on sucrose gradients, had a sedimentation coefficient of 8.8 S to 10.4 S. The active enzyme had a sedimentation coefficient which ranged from 15.2 S to 28.8 S. Reactivation of the inactive form of the enzyme to the active form was found to be increased by low concentrations of the following nucleotides and hexose phosphates: cyclic 3′,5′-AMP, ATP, ADP, AMP, fructose-6-P, and fructose-1,6-di-P. Cyclic 3′,5′-AMP and ADP were the most potent nucleotides tested and fructose-1,6-di-P was more potent in reactivating the enzyme than fructose-6-P. It was further demonstrated that combinations of a nucleotide and a hexose phosphate were much more effective in reactivation of phosphofructokinase than either nucleotide or hexose phosphate alone. The best combinations were: both substrates, fructose-6-P and ATP, or both products, fructose-1-6-di-P and ADP.

These results suggest that phosphofructokinase can undergo reversible dissociation from an active to an inactive form. Since such dissociation occurs at a pH which could be that of muscle cell it is possible that there is in the cell an equilibrium between the active and inactive forms of the enzyme. Phosphofructokinase is well adapted for regulation by such a mechanism through the requirement of two agents for maximal reactivation. These include a nucleotide and a hexose phosphate. On the basis of these findings the speculation may be advanced that an increase in the active form of phosphofructokinase may occur during the inotropic effect of E. This would be due to the increase in the levels of fructose-6-P, cyclic 3′,5′-AMP and other nucleotides and inorganic phosphate (see above). Similarly, activation may occur during anoxia of the heart during which an increase in the levels of AMP, ADP, and hexose monophosphate is known to occur (17). As of now it has not been possible to demonstrate such change in extracts from the heart isolated very rapidly during the inotropic effect of E. The fact that several cell components can reactivate heart phosphofructokinase (12) makes it difficult to find the form in which the enzyme is present in the resting cell.

PROPERTIES OF PHOSPHOFRUCTOKINASE FROM SHEEP HEART

Further evidence for the presence of phosphofructokinase in multiple forms can be seen from recent studies on sheep heart phosphofructokinase (15). The enzyme in sheep heart was found to be largely inactive and is present in the insoluble fraction of the cell. When the residue of sheep heart homogenates was incubated with ATP and $MgSO_4$, or with high $MgSO_4$ concentration, high phosphofructokinase activity was observed in the supernatant fluid of the incubate. The process of activation and solubilization was temperature- and time-dependent. The supernatant fluid of the activated incubate had high specific activity and was used as a starting material for further enzyme purification. The enzyme has been purified to a homogenous protein and a crystalline preparation has been isolated (15). Purification of the phosphofructokinase was facilitated after the finding that a combination of fructose-1,6-di-P, an adenylic nucleotide and a protective agent for SH groups can maintain the stability of the enzyme (29).

The following evidence from studies on the purified enzyme indicates the presence of multiple forms for purified sheep heart phosphofructokinase (15).

A. The Schlieren pattern of the enzyme was characteristic of an aggregated protein. The crystalline enzyme showed a pattern which had an asymmetric peak with an S_{20w} of about 38. Enzyme isolated before the process of crystallization which had approximately the same specific activity as that of the crystalline enzyme has a Schlieren pattern which consists of a double peak; a fast moving peak which comprised 80 to 90 % of the area and had an S_{20w} of about 41.0 and a slow moving component with an S_{20w} which ranged from 8 to 11. The sedimentation coefficient of the aggregated enzyme was found to be concentration-dependent when tested at low concentrations on sucrose gradients. The lowest sedimentation coefficient obtained was 15.2 S. These properties suggest a polymer-monomer system in equilibrium. The relationship between the degree of aggregation of the enzyme and its catalytic activity has still to be ascertained. Under the

present assay conditions the enzyme is highly diluted and presumably is in the monomeric form.

B. The aggregated enzyme can also be dissociated in the presence of 2 M NaCl to small enzyme units which have sedimentation coefficient of 16.6 S. This was accompanied by a decrease in enzyme activity by about 50%. A direct effect of NaCl on the enzyme in the assay mixture was ruled out. Thus NaCl causes change in the structure and activity of the enzyme. Removal of NaCl by dialysis resulted in restoration of the original Schlieren pattern and enzyme activity.

CONCLUSIONS

The results so far obtained on crude, partially purified, and purified enzyme indicate that phosphofructokinase is endowed with many properties which make it well adapted for regulation in the cell. Two main possible mechanisms for regulation can be recognized. First, regulation through kinetic properties of the enzyme; second, regulation through structural change of the enzyme. A common effector on these two mechanisms are the adenylic nucleotides and the hexose phosphates. A stimulatory effect by E or other hormones on mammalian heart phosphofructokinase, similar to what has been described before with serotonin on the liver fluke, has not yet been shown. However, a possible indirect effect through the adenylic nucleotides and the hexose phosphates is possible with these two mechanisms.

Acknowledgments. Some of the investigations carried out in the author's laboratory and reported in this paper are supported by research grant A104214 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service; Research Career Development Award GM-K3-3848 from the Division of General Medical Sciences, U. S. Public Health Service; and a grant from the Santa Clara County Heart Association. The author's trip from the United States to Milan was partly supported by a Commonwealth Fund Award.

REFERENCES

1. Atkinson, D. E. and Walton, G. M.: Kinetics of regulatory enzymes. *Escherichia coli* phosphofructokinase. J. biol. Chem. **240**: 757–763, 1965.
2. Cori, C. F.: Phosphorylation of carbohydrates. In: A Symposium on Respiratory Enzymes, pp. 175–189, University of Wisconsin Press, Madison, 1942.
3. Engel'hardt, V. A. and Sakov, N. E.: The mechanism of the Pasteur effect. Biokhimiya **8**: 9–36, 1943.
4. Karpatkin, S., Helmreich, E. and Cori, C. F.: Regulation of glycolysis in muscle. II. Effect of stimulation and epinephrine in isolated frog sartorius muscle. J. biol. Chem. **239**: 3139–3145, 1964.
5. Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L. and Fischer, E. H.: Purification and properties of rabbit skeletal muscle phosphorylase-b kinase. Biochem. **3**: 1022–1033, 1965.
6. Lardy, H. A. and Parks, R. E., Jr.: In: Enzymes: Units of Biological Structure and Function, ed. by O. H. Gaebler, pp. 584–588, Academic Press, Inc., New York, 1956.
7. Lowry, O. H., Passonneau, J. V., Hasselberger, F. X. and Schulz, D. W.: Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. J. biol. Chem. **239**: 18–30, 1964.
8. Lynen, F., Hartmann, G., Netter, K. F. and Schuegraf, A.: In: Ciba Foundation Symposium on Regulation of Cell Metabolism, ed. by G. W. Wolstenholme, pp. 256–273, Little, Brown and Co., Boston, 1959.
9. Mansour, T. E.: Actions of serotonin and epinephrine on intact and broken cell preparations from the liver fluke, *Fasciola hepatica*. Pharmacol. Rev. **11**: 465–466, 1959.
10. Mansour, T. E.: Effect of serotonin on glycolysis in homogenates from the liver fluke *Fasciola hepatica*. J. Pharmacol. **135**: 94–101, 1962.
11. Mansour, T. E.: Studies on heart phosphofructokinase: Purification, inhibition and activation. J. biol. Chem. **238**: 2285–2292, 1963.
12. Mansour, T. E.: Studies on heart phosphofructokinase. Active and inactive forms of the enzyme. J. biol. Chem. **240**: 2165–2172, 1965.

13. MANSOUR, T. E. AND MANSOUR, J. M.: Effects of serotonin (5-hydroxytryptamine) and adenosine 3′,5′-phosphate on phosphofructokinase from the liver fluke *Fasciola hepatica*. J. biol. Chem. **237**: 629–634, 1962.
14. MANSOUR, T. E., SUTHERLAND, E. W., RALL, T. W. AND BUEDING, E.: The effect of serotonin (5-hydroxytryptamine) on the formation of adenosine-3′,5′-phosphate by tissue particles from the liver fluke, *Fasciola hepatica*. J. biol. Chem. **235**: 466–470, 1960.
15. MANSOUR, T. E., WAKID, N. W. AND SPROUSE, H. M.: Purification, crystallization and properties of activated sheep heart phosphofructokinase. Biochem. Biophys. Res. Comm. **19**: 721–727, 1965.
16. MANWELL, C.: Comparative physiology: Blood pigments. Ann. Rev. Physiol. **22**: 191–244, 1960.
17. NEWSHOLME, E. A. AND RANDLE, P. J.: Regulation of glucose uptake by muscle. Biochem. J. **80**: 655–662, 1961.
18. ØYE, I., BUTCHER, R. W., MORGAN, H. E. AND SUTHERLAND, E. W.: Epinephrine and cyclic 3′,5′-AMP levels in working rat hearts. Fed. Proc. **23**: 562, 1964.
19. ÖZAND, P. AND NARAHARA, H. T.: Regulation of glycolysis in muscle. III. Influence of insulin, epinephrine, and contraction on phosphofructokinase activity in frog skeletal muscle. J. biol. Chem. **239**: 3146–3152, 1964.
20. PARMEGGIANI, A. AND BOWMAN, R. H.: Regulation of phosphofructokinase activity by citrate in normal and diabetic muscle. Biochem. Biophys. Res. Comm. **12**: 268–273, 1963.
21. PARMEGGIANI, A. AND KREBS, E. G.: Crystallization of rabbit muscle phosphofructokinase. Biochem. Biophys. Res. Comm. **19**: 84–94, 1965.
22. PARMEGGIANI, A. AND MORGAN, H. E.: Effect of adenine nucleotides and inorganic phosphate on muscle phosphorylase activity. Biochem. Biophys. Res. Comm. **9**: 252–256, 1962.
23. PASSONNEAU, J. V. AND LOWRY, O. H.: Phosphofructokinase and the Pasteur effect. Biochem. Biophys. Res. Comm. **7**: 10–15, 1962.
24. PASSONNEAU, J. V. AND LOWRY, O. H.: Phosphofructokinase and the control of the citric acid cycle. Biochem. Biophys. Res. Comm. **13**: 372–379, 1963.
25. RALL, T. W. AND SUTHERLAND, E. W.: The regulatory role of adenosine-3′,5′-phosphate. Cold Spring Harbor Symposia on Quantitative Biology, XXVI, pp. 347–354, Long Island Biological Association, Cold Spring Harbor, Long Island, New York, 1961.
26. RAMAIAH A., HATHAWAY, J. A. AND ATKINSON, D. E.: Adenylate as a metabolic regulator. Effect on yeast phosphofructokinase kinetics. J. biol. Chem. **239**: 3619–3622, 1964.
27. REGEN, D. M., YOUNG, D. A. B., DAVIS, W. W., JOCK, JR., J. AND PARK, C. R.: Adjustment of glycolysis to energy utilization in the perfused rat heart. The effect of changes in the ionic composition of the medium on phosphofructokinase activity. J. biol. Chem. **239**: 381–384, 1964.
28. VINUELA, E., SALAS, M. L. AND SOLS, A.: End-product inhibition of yeast phosphofructokinase by ATP. Biochem. Biophys. Res. Comm. **12**: 140–145, 1963.
29. WAKID, N. AND MANSOUR, T. E.: Factors influencing the stability of heart phosphofructokinase. Molecular Pharmacol. **1**: 53–59, 1965.