# A KINETIC MODEL FOR PHOSPHOFRUCTOKINASE BASED ON THE PARADOXICAL ACTION OF EFFECTORS

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SUMMARY. Effectors of muscle phosphofructokinase show opposing action on the activity of the enzyme depending upon the concentration of phosphoryl donor employed in the assay. Established inhibitors, such as citrate, activate at low ATP or ITP concentrations while known activators, such as AMP, ADP, and cyclic AMP inhibit at low ATP or ITP concentrations. Inorganic phosphate, on the other hand, activates at all substrate concentrations. The paradoxical effects at low substrate concentrations are dependent upon the order of addition of reaction components. A model is proposed to explain these and other regulatory phenomena of phosphofructokinase.

The activity of phosphofructokinase* (PFK) is influenced by a great variety of regulatory effectors. The enzyme is inhibited by ATP which acts synergistically with creatine-P, citrate, 3-P-glycerate, and P-enolpyruvate (1), and activation is achieved with adenosine di- and monophosphates and inorganic phosphate. Recently we have observed that the inhibitors, creatine-P, citrate, 3-P-glycerate, and P-enolpyruvate, increase the activity of muscle PFK at low subsaturating concentrations of nucleoside triphosphate substrate (1). In the present study we show that the established activators are capable of inhibiting the enzyme at subsaturating ATP or ITP concentrations.

MATERIALS AND METHODS. Muscle PFK was prepared from frozen rabbit skeletal muscle as previously described (2). The crystals were collected by centrifugation and dissolved in a buffer (pH 7.0) consisting of 25 mM Na glycero-P, 25 mM glycylglycine, 1 mM EDTA, 0.1 mM ATP, and 0.1 mM dithiothreitol and the solution was dialyzed against the same buffer for two to three hours at 4°.

Aldolase, glycero-P dehydrogenase, and triose-P isomerase were purchased from Sigma Chemical Company. Preceeding their use in assays, these enzymes were dialyzed to remove ammonium sulfate. Kinetic analyses were performed at 30° and at pH 7.0 in 3 ml of a medium containing 50 mM N-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid, 0.1 mM dithiothreitol, 0.15 M KCl, 1 mM EDTA, 0.6 unit of aldolase, 0.3 unit of triose-P isomerase, 0.3 unit of glycero-P dehydrogenase and 0.2 mM NADH. The concentrations of ATP or ITP, and fructose-6-P were as indicated in the Results. $MgCl_2$ was added in an amount

*Abbreviation used: Phosphofructokinase, PFK

5 mM in excess of the concentration of nucleoside triphosphate. PFK was diluted to the desired concentration in 25 mM Na-glycero-P, 25 mM glycyl-glycine, 1 mM EDTA, 0.1 dithiothreitol, 0.1 mM ATP, all at pH 7.0 and room temperature, and was added to the incubation in a volume of 50 µl. Except where indicated, reactions were started by the addition of fructose-6-P and the rate was determined three to four minutes after the final addition.

RESULTS. Inhibitors as Activators. In 1968, Randle et al., (3) reported that citrate increased the affinity of PFK for ATP at the catalytic site, a result consistent with earlier equilibrium binding studies (4). Recently (1) we have extended this observation to show that citrate, creatine-P, 3-P-glycerate, 2,3-$P_2$-glycerate, and P-enolpyruvate all increase the affinity of the enzyme for ATP at the catalytic site. The activation was even more profound

Table 1: Effect of Known Inhibitors of Phosphofructokinase at High and Low Phosphoryl Donor Concentrations[a]

| Addition (mM) | Phosphoryl Donor ATP (mM) | ITP (mM) | Relative Activity |
|---|---|---|---|
| None | 1 | | 100 |
| Citrate (0.06) | 1 | | 15 |
| 3-P-Glycerate (0.075) | 1 | | 31 |
| P-Enolpyruvate (0.5) | 1 | | 11 |
| 2,3-$P_2$-Glycerate (0.5) | 1 | | 6 |
| None | 0.1 | | 45 |
| Citrate (0.05) | 0.1 | | 54 |
| 3-P-Glycerate (0.10) | 0.1 | | 58 |
| P-Enolpyruvate (0.5) | 0.1 | | 58 |
| 2,3-$P_2$-Glycerate (0.5) | 0.1 | | 59 |
| Creatine-P (3.5) | 0.1 | | 60 |
| None | | 1 | 50 |
| Citrate (0.15) | | 1 | 94 |
| 3-P-Glycerate (0.1) | | 1 | 98 |
| P-Enolpyruvate (0.5) | | 1 | 82 |
| 2,3-$P_2$-Glycerate (0.5) | | 1 | 85 |
| Creatine-P (3.5) | | 1 | 98 |

[a]Assays performed at pH 7.0 and 30° as described in Materials and Methods with additions shown. Reactions were started by the addition of 1 mM fructose-6-P.

when the phosphoryl donor was ITP, a substrate that binds with lower affinity than MgATP to the catalytic site and very poorly to the inhibitory site. Additional experiments demonstrating both the inhibiting and activating properties of citrate and related metabolites are described in Table 1.

Activators as Inhibitors. ADP, AMP, and cyclic AMP are known as activators of PFK, or more specifically, as deinhibitors. An inhibitory action of AMP on the activity of PFK is described in Fig. 1. In addition to the profound activation seen under conditions where ATP was inhibiting, inhibition was observed at low concentrations of ATP. Table 2 describes similar actions of cyclic AMP and ADP at low and high concentrations of ATP and ITP. The inhibitory effect of the adenine nucleotides was particularly potent with ITP as the phosphoryl donor. In contrast to the action of adenine nucleotides, a dual role was not observed with another activator, inorganic phosphate, which increased the activity of PFK at all concentrations of both ATP and ITP (Table 2).

Importance of the order of addition of reaction components. We have previously noted (1) differences in the sensitivity to inhibition by citrate depending upon the order of addition of assay components. The enzyme was less sensitive to citrate inhibition if citrate was added after the reaction was started by fructose-6-P. Activation by citrate and inhibition by AMP at low ATP concentrations also were not observed if the reaction had previously been

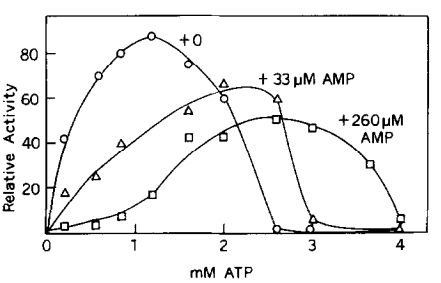


Figure 1. Effect of AMP at varying ATP concentrations. Conditions of assay described in Materials and Methods using 1 mM fructose-6-P and the indicated concentrations of AMP and ATP.

started by fructose-6-P. The effects are interpreted as reflecting slow conformational changes between two or more forms of the enzyme.

DISCUSSION. Figure 2 shows a model for the regulation of PFK which consists of two, active conformational states (I and II) and an inactive dimeric form (III) of the enzyme. Effectors in heavy type indicate tight binding by that conformation whereas those written in lighttype indicate weaker binding. Evidence for and consequences of such a model are as follows: (a) Equilibrium binding studies (1,4,5) have indicated the presence of single sites per protomer for fructose-6-P, fructose-1,6-$P_2$, a site that binds competitively cyclic AMP, AMP, ADP, and, very poorly, ATP, and a site that binds compettively citrate, 3-P-glycerate, and P-enolpyruvate. Two binding sites for

Table 2: Effect of Known Activators of Phosphofructokinase at High and Low Phosphoryl Donor Concentrations[a]

| Additions (mM) | Phosphoryl Donor ATP (mM) | ITP (mM) | Relative Activity |
|---|---|---|---|
| None | 1 | | 100 |
| None | 2.6 | | < 1 |
| Cyclic AMP (0.07) | 2.6 | | 78 |
| AMP (0.26) | 2.6 | | 50 |
| ADP (0.20) | 2.6 | | 60 |
| Pi (4) | 1 | | 50 |
| None | 0.1 | | 45 |
| Cyclic AMP (0.07) | 0.1 | | < 3 |
| AMP (0.20) | 0.1 | | < 3 |
| ADP (0.20) | 0.1 | | < 3 |
| Pi (4) | 0.1 | | 54 |
| None | | 1 | 50 |
| Cyclic AMP (0.07) | | 1 | 9 |
| AMP (0.20) | | 1 | 18 |
| ADP (0.20) | | 1 | 5 |
| Pi (4) | | 1 | 74 |

[a]Conditions described in footnote to Table 1.

MgATP have been described (1,4,6), and one of these, the catalytic site, also binds MgITP. This latter site is shown on the model as MgITP site to distinguish it from the MgATP inhibitory site. (b) The two different conformations, I and II, have been proposed previously (7) on the basis of the reactivity of a single thiol group per protomer with several reagents. AMP, fructose-6-P, and fructose-1,6-$P_2$ increase the reactivity of the thiol group whereas MgATP and citrate block thiol reactivity (6,7). (c) Dissociation to the dimer (III) is favored by low temperatures, low pH, and dilute enzyme concentrations (8,9). Lad et al. (10) have shown that citrate stabilizes an inactive dimer while fructose-6-P and fructose-1,6-$P_2$ stabilize a tetramer. Binding of citrate to a tetrameric form, II, however, has been demonstrated (1). (d) Interconversions among the forms are not rapid. Frieden originally described slow conformational changes associated with changes in activity of PFK (11). The enhanced sensitivity to citrate inhibition that results when PFK is preincubated with citrate (1) can be explained by the slow conversion of the enzyme to conformation III. (e) The model accounts for the well-described inhibitory actions of MgATP, citrate, 3-P-glycerate, and P-enolpyruvate by their binding to state II and thereby decreasing the affinity of the enzyme for fructose-6-P. It also accounts for activation by AMP and

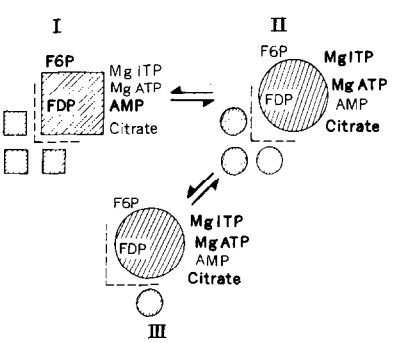


Figure 2. A Kinetic Model for PFK. F6P = site for binding of fructose-6-P; FDP = fructose-1,6-$P_2$ binding site; citrate = binding site for citrate, 3-P-glycerate, and P-enolpyruvate; AMP = binding site for AMP, ADP, and cyclic AMP; MgITP = catalytic site; MgATP = inhibitory site.

fructose-1,6-$P_2$ which favor state I. Previous studies of PFK have evaluated effector actions under conditions where ATP concentrations were high relative to the dissociation constant for MgATP at the catalytic site of either state I or state II. In the present study, fructose-6-P was present at high concentrations and ATP levels were low. Under these conditions the rate was limited by the relative affinities of MgATP for conformations I or II. AMP favored state I and led to inhibition and citrate favored state II and produced activation. Thus, the activity may be controlled by either the relative saturation of the fructose-6-P site, the saturation of the MgATP catalytic site, or by the fraction of the enzyme in the dissociated form, conformation III.

Two important regulators, inorganic phosphate and creatine phosphate, have not been included in Fig. 2. Binding studies (1) of fructose-6-P showed that inorganic phosphate enhanced the binding of that effector but other studies (7) showed that inorganic phosphate could not by itself stabilize conformation I. Creatine-P does not compete for the citrate site but does enhance MgATP binding (1) and thus apparently binds to conformation II.

Figure 2 is undoubtedly a simplification of the real situation and conformations or "sub-conformations" other than those shown may exist. In earlier studies, for example, we have shown that both fructose-6-P and fructose-1,6-$P_2$ stabilize conformation I (7), but, in addition, fructose-6-P (and AMP) protect one pair of more slowly reacting thiol groups whereas fructose-1,6-$P_2$ protects a different pair of thiol groups (12). This suggests that conformation I has at least two "sub-conformations". The scheme of Figure 2, however, does present a means of logically viewing many of the properties of PFK and provides a basis for the design of other investigations into the extremely complex regulatory behavior of the enzyme.

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