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KINETIC STUDIES ON PHOSPHOFRUCTOKINASE FROM EHRLICH ASCITES TUMOR CELLS

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SUMMARY

1. Kinetic studies are undertaken on the reaction catalyzed by phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) purified from Ehrlich ascites tumor cells.

2. The rate–concentration curves with respect to Fru-6-*P* and ATP show highly cooperative patterns of activation and inhibition, respectively. The inhibition by ATP is reduced by increasing the concentration of Fru-6-*P* or by the addition of the positive effectors such as AMP, P_i, ADP and SO_4^{2-}, while the cooperative binding of Fru-6-*P* and the effectors is exaggarated by increasing the concentration of ATP.

3. The cooperative interactions displayed by this enzyme are completely lost in the presence of a saturating concentration of any of the positive effectors. The resultant hyperbolic saturation curve for Fru-6-*P* or ATP shows the same pattern regardless of the kind of effectors used, except for the case of ADP which, besides functioning as a positive effector, is inhibitory competitively with ATP.

4. These kinetic properties appear to be readily accounted for by the "concerted allosteric transition" by assuming two conformational states, R and T. The R state has an affinity for Fru-6-*P*, ATP and positive effectors while the T state has an affinity only for ATP.

5. This view makes it most plausible to postulate further that the hyperbolic saturation kinetics observed at the saturating concentration of the positive effectors reflect the binding of the substrates to the catalytic sites in the R state. Kinetic patterns under this condition are in accord with the "ping-pong bi-bi" mechanism in which two kind of stable enzyme forms, free and phosphorylated, alternate with each other during the course of the reaction.

6. These kinetic data are readily interpreted in terms of the reaction model of "allosteric ping-pong II" which is one of the reaction models proposed by us (*Biochim. Biophys. Acta*, 276 (1972) 12) from a theoretical viewpoint for the allosteric enzyme exhibiting the ping-pong mechanism. The model suggests that the conversion of free enzyme to another stable enzyme form is an essential step for allosteric transition.

INTRODUCTION

The complex kinetic properties of phosphofructokinase (ATP:D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) are known to be responsible for the control mechanism of the glycolytic pathway in mammalian tissues. Extensive studies have been made of the kinetic behavior of phosphofructokinases from the mammalian tissues, such as skeletal muscle[1], brain[2] and heart[3], which are characterized by a high glycolytic rate. Phosphofructokinase of tumor cells which also possess high glycolytic activity, however, has scarcely been the subject of kinetic studies, presumably because an enzyme preparation freed from other enzymes such as adenylate kinase which might obscure the fine control of phosphofructokinase has not been available. Our recent success in preparing highly purified phosphofructokinase from Ehrlich ascites tumor cells[4] has therefore prompted us to study its kinetic properties in an attempt to analyze the control mechanisms governing the reaction catalyzed by this allosteric enzyme.

The present paper describes several kinetic properties of phosphofructokinase purified from Ehrlich ascites tumor cells; the results showing that the cooperative kinetics could be accounted for in terms of the "concerted allosteric transition"[5]. It was also suggested that the binding of the substrates to catalytic sites takes place by the "ping-pong" mechanism[6]. It will be shown that the combined reaction model of these catalytic and regulatory properties conforms to the reaction model of "allosteric ping-pong II"[7] which is one of the reaction models introduced by us for allosteric enzymes with a ping-pong mechanism.

MATERIALS AND METHODS

Disodium salts of ATP, ADP, Fru-6-*P* and NADH, Tris, imidazole, aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were purchased from Sigma Chemical Company. AMP was from Kowa Chemical Company and bovine serum albumin was the product of Armour Pharmaceutical Company. All other chemicals used were analytical grade reagents from commercial sources.

Ehrlich ascites tumor phosphofructokinase was purified by the procedure described in the previous paper[4]. The purified enzyme stored as $(NH_4)_2SO_4$ precipitate in 0.1 M phosphate buffer, pH 7.5, containing 0.8–saturated $(NH_4)_2SO_4$, 0.1 mM ATP and 1 mM EDTA was dissolved in 0.5% serum albumin and then dialyzed against a large volume of 50 mM Tris–HCl, pH 8.0, containing 0.1 mM ATP and 0.1 mM EDTA before use. Auxiliary assaying enzymes dissolved in 0.5% serum albumin were also dialyzed against large volumes of 50 mM Tris–HCl, pH 8.0, to remove $(NH_4)_2SO_4$.

Phosphofructokinase activity was measured in a coupled assay system[8] by following the decrease of absorbance at 340 nm due to the oxidation of NADH with a Gilford model 2400 recording spectrophotometer. The assay solution contained 0.05 mM NADH, Mg^{2+} equal to 3 times the ATP concentration used, 150 mM KCl, the desired concentrations of the substrates and effectors, 1.0 unit of aldolase, 0.8 units of triose phosphate isomerase, 0.4 units of α-glycerophosphate dehydrogenase, 10 μl of the diluted solution of purified phosphofructokinase and 50 mM imidazole–HCl, pH 7.5, in a final volume of 3 ml. The reaction mixture was incubated in a cell

of 1-cm light-path at 26 °C. The reaction was initiated by the addition of the phosphofructokinase in an amount to give a change of absorbance less than 0.05 absorbance units per min.

RESULTS

Kinetic properties

Effects of ATP and Fru-6-P. The inhibition of ascites tumor phosphofructokinase by higher concentrations of ATP at different concentrations of Fru-6-*P* is shown in Fig. 1. The inhibition by ATP tends to be reduced by increasing the concentration of Fru-6-*P*. Replotting of the data of Fig. 1 in the form of log $(V - v/v)$ against log of the inhibition range of ATP concentrations[9] yields straight lines with n values, the index of cooperativity, from 1.0 to 2.2 depending on the concentration of Fru-6-*P* (Fig. 3A). This shows that the cooperative binding of ATP to the inhibitor sites is decreased as the concentration of Fru-6-*P* is increased.

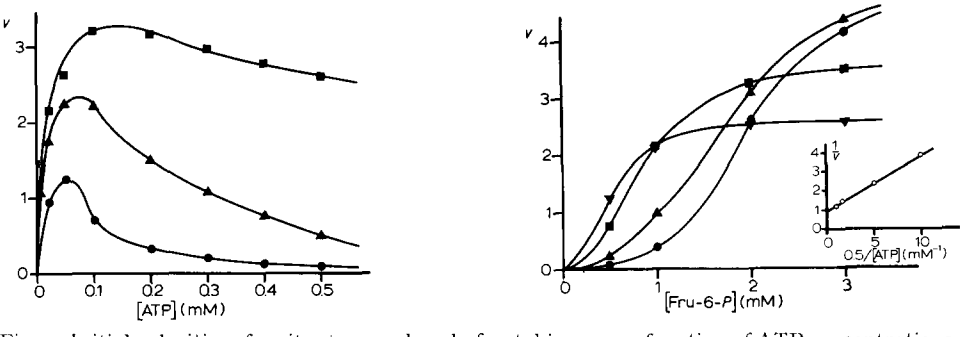

Fig. 1. Initial velocities of ascites tumor phosphofructokinase as a function of ATP concentrations at several fixed concentrations of Fru-6-*P*. ●—●, 0.5 mM Fru-6-*P*; ▲—▲, 1.0 mM Fru-6-*P*; ■—■, 2.0 mM Fru-6-*P*.

Fig. 2. Initial velocities of ascites tumor phosphofructokinase as a function of Fru-6-*P* concentrations at several fixed concentrations of ATP. ▼—▼, 0.05 mM ATP; ■—■, 0.1 mM ATP; ▲—▲, 0.3 mM ATP; ●—●, 0.5 mM ATP. The secondary plot of the reciprocals of the maximum velocities observed against the reciprocals of ATP concentrations is shown in the inset.

Plots of the initial velocities against the concentration of Fru-6-*P* at fixed concentrations of ATP yield a series of sigmoidal curves (Fig. 2). The Hill coefficients with respect to the concentrations of Fru-6-*P* (Fig. 3B) were found to increase upon raising the concentration of ATP, indicating that the cooperative binding of Fru-6-*P* is increased as ATP concentration is increased. The secondary double-reciprocal plot of V obtained at the highest concentration of Fru-6-*P* against ATP gave a straight line as shown in the inset of Fig. 2. This suggests that the binding of ATP to the inhibitor site is completely abolished by Fru-6-*P* at its saturating concentration and that the binding of ATP to any sites other than the inhibitor site is not cooperative under this condition.

Effect of activators. Fig. 4 shows the effects of P_i, AMP, ADP and SO_4^{2-}, positive effectors of ascites tumor phosphofructokinase, on the initial velocities of the enzyme at three fixed concentrations of ATP in the presence of 0.5 mM Fru-6-*P*. It is seen that the saturation curves for these effectors are sigmoidal, especially in the presence

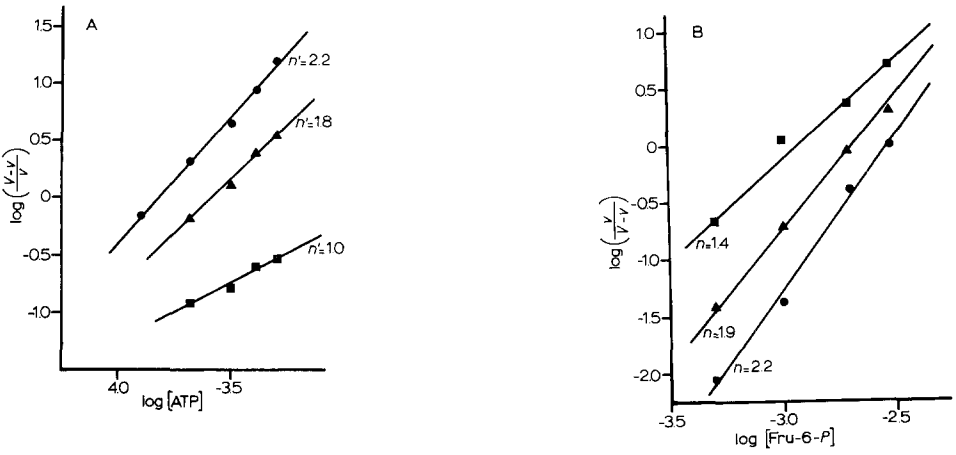

Fig. 3. The Hill plots (A) with respect to the inhibitory concentrations of ATP at several fixed concentrations of Fru-6-*P*, and (B) with respect to Fru-6-*P* concentrations at several fixed concentrations of ATP. (A) ●—●, 0.5 mM Fru-6-*P*; ▲—▲, 1.0 mM Fru-6-*P*; ■—■, 2.0 mM Fru-6-*P*. (B) ■—■, 0.1 mM ATP; ▲—▲, 0.3 mM ATP; ●—●, 0.5 mM ATP.

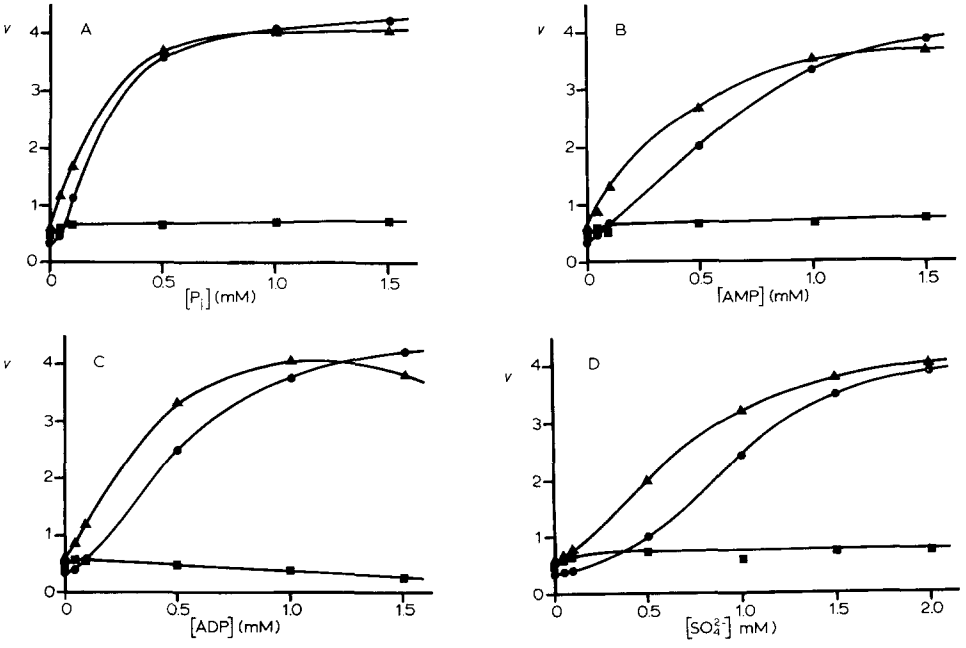

Fig. 4. Initial velocities of phosphofructokinase as a function of concentrations of P_i, AMP, ADP and $SO_4^{=}$ at several fixed concentrations of ATP. Fru-6-*P* concentration, 0.5 mM. ■—■, 0.01 mM ATP; ▲—▲, 0.1 mM ATP; ●—●, 0.3 mM ATP.

of higher concentrations of ATP. As a result, the inhibition induced by higher concentrations of ATP is strikingly reduced as the concentrations of these effectors increase. In Figs 5A and 5B, the rate-concentration curves for Fru-6-*P* and ATP are compared in the presence and absence of the saturating concentrations of these effectors. It is

noteworthy that, at saturating concentrations of these effectors, the kinetics with respect to ATP and Fru-6-*P* were no longer cooperative; the reaction rate approximated a normal Michaelis function of the Fru-6-*P* or ATP concentration. The maximum velocity obtained under this condition was found to be the same regardless of the kind of the effector used. Furthermore, a combination of any of these effectors at its saturating concentration caused no additive effect (Fig. 6). It can be said from Figs 4 to 6 that essentially the same kinetic pattern is obtained by most of these effectors; an exception is ADP which is not only stimulatory like other effectors but also inhibitory as shown in Figs 4C and 5B. The failure of ADP to exhibit its inhibitory action in Figs 5A and 6 suggests that the inhibition induced by ADP results from a competitive interaction with ATP, since the concentration of ATP used for these experiments were higher than the concentrations at which the inhibition by ADP was observed in Figs 4C and 5B. This will be confirmed in the following section.

In any case these data appear to be accounted for by postulating that all of these effectors but ADP do not interact with the catalytic sites and that they, including ADP, exert their influence on the reaction rate by abolishing the allosteric properties of phosphofructokinase.

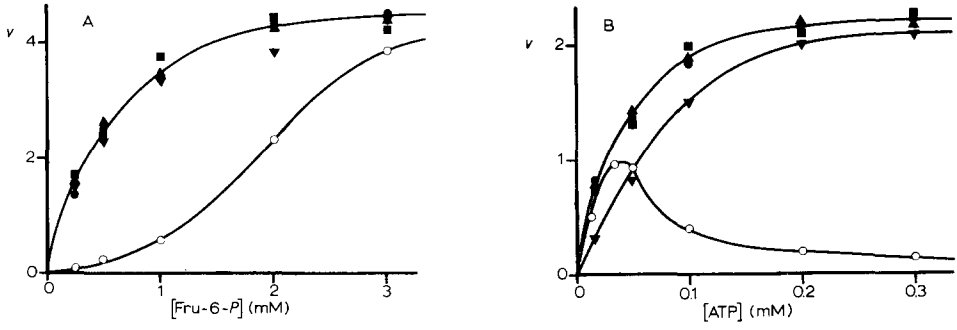

Fig. 5. Effects of a saturating concentration of P_i, AMP, ADP and SO_4^{2-} on the kinetic patterns with respect to Fru-6-*P* and ATP. (A) ATP concentration, 0.3 mM. (B) Fru-6-*P* concentration, 0.5 mM. ○—○, control; ▲—▲, 1.5 mM P_i; ■—■, 2.0 mM AMP; ●—●, 2.5 mM SO_4^{2-}; ▼—▼, 2.0 mM ADP.

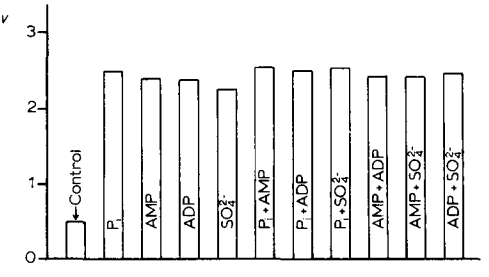

Fig. 6. Effects of combination of a saturating concentration of P_i, AMP, ADP and SO_4^{2-} on an initial velocity. ATP concentration, 0.3 mM. Fru-6-*P* concentration, 0.5 mM. The concentration of each activator is the same as indicated in the legend to Fig. 5.

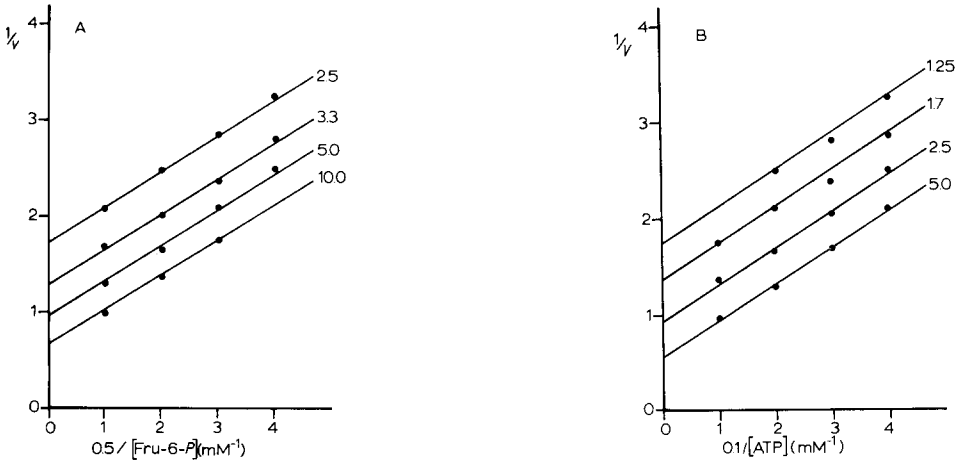

Fig. 7. Initial velocities as a function of Fru-6-*P* and ATP concentrations in the presence of a saturating concentration of AMP. AMP concentration, 2.0 mM. Numbers indicated below each lines are 10^5 times ATP concentrations (A), and 10^4 times Fru-6-*P* concentrations (B).

Kinetics with respect to the binding of Fru-6-P and ATP to the respective catalytic sites. If the above postulate is correct, it should follow that the change of the reaction rate as a function of ATP or Fru-6-*P* in the presence of a saturating concentration of AMP, P_i or SO_4^{2-} reflects only the interaction of the substrates with the catalytic sites. The double reciprocal plots with respect to Fru-6-*P* and ATP under this condition gave two sets of essentially parallel lines as shown in Figs 7A and 7B, respectively. The results seem to suggest that the two substrates, Fru-6-*P* and ATP, are not present simultaneously on the enzyme, since the lines do not converge at a common

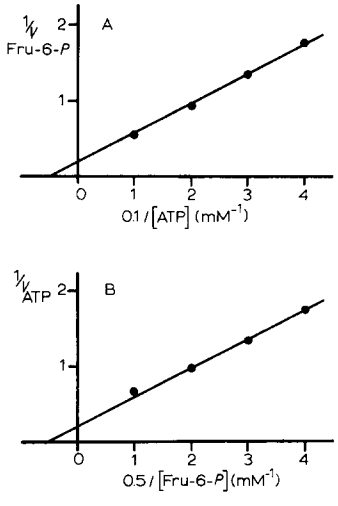

Fig. 8. Double reciprocal plots of the V obtained from the normalized kinetic patterns in the presence of a saturating concentration of AMP against ATP and Fru-6-*P* concentrations. The V was obtained from the intercepts on the abscissae of Figs 7A and 7B. The experimental conditions are the same as indicated in the legend to Fig. 7.

point. Instead, this type of data is consistent with a "ping-pong" mechanism[6] and seems to exclude the mechanism involving random or ordered addition of substrates. Double reciprocal plots of V obtained in Figs 7A and 7B against ATP and Fru-6-*P*, respectively gave straight lines as shown in Figs 8A and 8B. The negative reciprocals of the point at which this line cuts the horizontal axis for each case represents the apparent K_m for ATP or Fru-6-*P* with respect to its binding to the catalytic site. The K_m value thus obtained is 0.2 mM for ATP and 1.0 mM for Fru-6-*P*.

The apparent competition of ADP with ATP for the catalytic site, as discussed in the foregoing section based on the data in Figs 4C and 5B, was further studied in the presence of a saturating concentration of AMP. The results are presented in Figs 9A and 9B in the form of double reciprocal plot. It is seen that the inhibition by ADP is competitive with respect to ATP and non-competitive with respect to Fru-6-*P*.

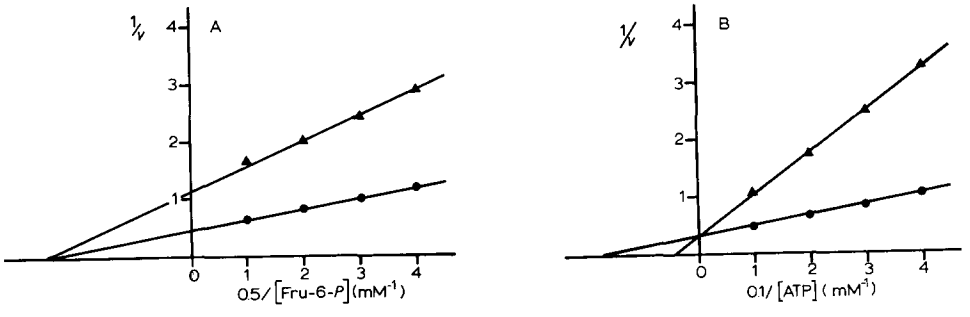

Fig. 9. Inhibitory effect of ADP on the initial velocity in the presence of a saturating concentration of AMP. AMP concentration, 2.0 mM. (A) ATP concentration, 0.05 mM. (B) Fru-6-*P* concentration, 0.5 mM. ●—●, control; ▲—▲, 0.5 mM ADP.

A plausible model serving to explain the kinetic data

The theoretical basis currently available for cooperative kinetics is the allosteric transition theory which has been successfully applied to many regulatory enzymes. In the case of ascites tumor phosphofructokinase, the cooperative kinetics presented above could be accounted for in terms of the concerted allosteric transition theory introduced by Monod *et al.*[5]. According to this theory, the cooperative kinetic data obtained can be explained qualitatively in the following way. (a) Ascites tumor phosphofructokinase is a perfect K system; *i.e.* the kinetic parameter V is not modified by allosteric effectors. (b) Fru-6-*P* has an affinity for the R state, one of two possible conformational states, but its affinity for the T state is negligible, whereas ATP has affinities for both the R and T states. R is catalytically active while T is substantially inactive. (c) Allosteric effectors such as P_i, AMP, ADP and SO_4^{2-} occupy their binding sites in the R state but have no appreciable affinity for the T state.

It should be noted that the enzyme is "desensitized" by the addition of the saturating concentration of the effectors such as AMP, P_i, ADP and SO_4^{2-} in a sense that the regulatory properties of the enzyme are completely lost because of the predominant occurrence of the R state over the T state in the presence of these effectors. As a result, the kinetic pattern in the presence of these effectors at their saturating concentrations must reflect the manner in which the substrates, Fru-6-*P*

and ATP bind to the catalytic sites in the R state. Fig. 7 shows such patterns which suggest that the binding of Fru-6-*P* and ATP to the respective catalytic sites in the R state occurs by the mechanism nick-named by Cleland[6] "ping-pong" type. Thus, the reaction sequence conforming to the observed kinetic pattern may be characterized by the alternate occurrence of two stable enzyme forms, the free enzyme and the phosphorylated one; ATP is bound to the former while Fru-6-*P* to the latter to produce ADP and Fru-1,6-P_2, respectively.

Thus the kinetic properies of ascites tumor phosphofructokinase will be interpreted in terms of "allosteric ping-pong" models proposed by us[7] for the allosteric enzymes with ping-pong mechanism. It should be remembered that the catalytically active conformational state of the enzyme is stabilized by Fru-6-*P* as revealed by Figs 1 and 2. In contrast, the rate-concentration curves for ATP appear to approximate the Michaelis-Menten kinetics at its non-inhibitory concentrations as shown in Fig. 1. Therefore, the experimental data are compatible with the reaction model in which the phosphorylated enzyme form which directly binds Fru-6-*P* does, but the free enzyme form which directly binds ATP does not undergo allosteric transition. This reaction model belongs to the "allosteric ping-pong" model of "Type II"[7]. Thus, the reaction model of the enzyme is formulated as follows:

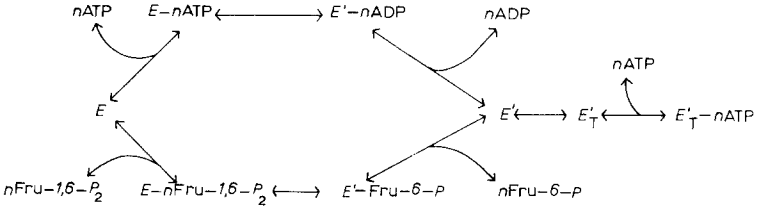

Diagram 1

where E, E' and E'_T represent the free enzyme, the phosphorylated enzyme of R state and the phosphorylated enzyme of T state respectively, and n number of subunits of the enzyme. The initial rate equation in the absence of the products is then derived according to Sumi and Ui[7] as follows:

$$\frac{v}{n} = \frac{k_1 k_2 [\text{ATP}]\,[\text{Fru-6-}P]\,e_0}{k_1 K_F [\text{ATP}] \left[\left\{ 1 + \frac{[\text{Fru-6-}P]}{K_F} \right\} + L \frac{\left\{ 1 + \frac{[\text{ATP}]}{K_{TA}} \right\}^n}{\left\{ 1 + \frac{[\text{Fru-6-}P]}{K_F} \right\}^{n-1}} \right] + k_2 K_A [\text{Fru-6-}P] \left\{ 1 + \frac{[\text{ATP}]}{K_A} \right\}} \quad (1)$$

where K_A, K_F and K_{TA} represent the intrinsic dissociation constants of E–ATP, E'–Fru-6-P and E'_T–ATP, respectively, k_1 and k_2 the rate constants involved in the rate limiting steps for dephosphorylation of ATP and phosphorylation of Fru-6-P on the enzyme protein, respectively, L the allosteric constant and e_0 the total concentration of the enzyme. If the allosteric effectors such as AMP, P_i, ADP and SO_4^{2-} are taken into account, Eqn 1 should be further transformed into Eqn 2

$$\frac{v}{n} = \frac{k_1 k_2 [\text{ATP}]\,[\text{Fru-6-}P]e_0}{k_1 K_F [\text{ATP}]\left[\left\{1+\frac{[\text{Fru-6-}P]}{K_F}\right\} + L\frac{\left\{1+\frac{[\text{ATP}]}{K_{TA}}\right\}^n}{\left\{1+\frac{[\text{Fru-6-}P]}{K_F}\right\}^{n-1}\left\{1+\frac{[M]}{K_M}\right\}^n}\right] + k_2 K_A [\text{Fru-6-}P]\left\{1+\frac{[\text{ATP}]}{K_A}\right\}} \quad (2)$$

where $[M]$ represents the concentration of effector and K_M the intrinsic dissociation constant of the enzyme–modifier complex. This equation is based on an assumption that these effectors are directly bound to E'; such a binding is essentially required for their role as positive allosteric effectors, but the binding to E, if it occurs, has no influence on the equation.

The initial velocity equation (Eqn 2), as a mathematical representation of the proposed model, could account for most of the kinetic properties exhibited by Ehrlich ascites tumor phosphofructokinase at least qualitatively; *e.g.* the cooperative inhibition by ATP which is released by increasing the concentration of Fru-6-*P* or the modifiers, and the cooperation with respect to Fru-6-*P* and the modifiers exaggerated by raising the concentration of ATP. Furthermore, if we let $[M]$ in Eqn 2 approach infinity, it is transformed to the equation of the following type:

$$V/v = 1 + K_\alpha/[\text{Fru-6-}P] + K_\beta/[\text{ATP}]$$

which should represent the kinetic pattern in the presence of the allosteric effector at its saturating concentration. This is clearly in accord with the experimental results since the slope of the plot of $1/v$ against $1/[\text{Fru-6-}P]$ or $1/[\text{ATP}]$ according to this equation is not affected by the co-substrate. K_α and K_β in this equation correspond to the apparent K_m of Fru-6-*P* and ATP respectively which were determined from the plot of Fig. 8. It is seen that K_α is actually $(k_1/(k_1 + k_2))K_F$ while K_β is $(k_2/(k_1 + k_2))K_A$ in Eqn 2. Thus, $k_1 K_F k_2 K_A$ is 5. Likewise, the slope of the Hill plots in Fig. 3 suggests that the enzyme is the oligomer consisting of 3 or more subunits.

DISCUSSION

Two types of cooperative bindings of the substrates, cooperation with respect to Fru-6-*P* and cooperation with respect to ATP as a negative effector, are the kinetic feature widely exhibited by phosphofructokinases from mammalian[1,3,9–12] and other sources[13–17]. In this respect, phosphofructokinase from Ehrlich ascites tumor cells is no exception; sigmoidal patterns were obtained with Fru-6-*P* (Figs 2 and 3B) as well as with ATP at its inhibitory concentrations (Figs 1 and 3A). This kinetic feature could be interpreted in terms of a model for allosteric interactions. Among several allosteric models now available we adopted a model based on the "concerted transition theory" by Monod *et al.*[5] because rather simpler assumptions are sufficient for the explanation of the present kinetic data by this model.

The stimulatory effect of P_i[2,3,11,18–22], AMP[2,11,16–18,20–24] and ADP[2,3,21–24] has been described on phosphofructokinases from various sources, some of which also responded to SO_4^{2-} [19,21,22,25] in increasing the catalytic activity. The present results show that, despite a considerable distinction in chemical structure, SO_4^{2-} shares the

common mechanism of activation with other phosphorylated effectors in ascites tumor phosphofructokinase; these effectors all showed the same saturation kinetic patterns (Fig. 4) and gave, at their saturating concentrations, the same normalized hyperbolic kinetic pattern with respect to Fru-6-*P* or ATP (Fig. 5) in a "non-additive manner" (Fig. 6). These results do not necessarily imply that these effectors occupy the same binding site on the enzyme protein, because, according to the concerted transition model adopted here, the allosteric ligands exert a "heterotropic effect" only by displacement of the equilibrium between the R and T state of the enzyme protein. At all events, the fact that all of these effectors, despite having no common structural unit, share the same kinetic mechanism appear to make it reasonable to adopt the concerted allosteric transition model in interpreting the present kinetic data. Likewise, it has been reported that the kinetic properties of *Escherichia coli*[24], rat muscle[26] and yeast[27] phosphofructokinases can be fit with this Monod's model using only two conformational states, R and T.

The velocity of several phosphofructokinases from other sources such as sheep brain[2] *etc.* has been reported to obey the normal Michaelis–Menten kinetics at lower concentrations of ATP, despite the strong cooperation at its higher concentrations. Such a normalized kinetic pattern obtained in the presence of non-inhibitory concentrations of ATP has been used for an estimation of the non-regulatory kinetic parameters. In the case of phosphofructokinase from ascites tumor cells, however, the rate-Fru-6-*P* concentration curve was sigmoidal even at the lowest concentration of ATP (Fig. 2). This may be readily accounted for in the present model by postulating that the allosteric constant, L, is such as to maintain an appreciable fraction of the enzyme protein in the T state in the absence of any allosteric ligands. Since, according to the reaction model discussed above, ascites tumor phosphofructokinase exists exclusively in the R state in the presence of the positive effectors at the saturating concentration, it can be reasonably assumed that the normal Michaelis–Menten kinetics observed in the presence of 5 mM AMP (Fig. 7) may serve in this case to reveal the manner in which the substrates occupy their respective catalytic sites on the R state. Under this condition, double reciprocal plots of the initial velocity against Fru-6-*P* or ATP at a series of fixed concentrations of ATP or Fru-6-*P*, respectively yielded a set of parallel lines as shown in Fig. 7. The same pattern has been described for phosphofructokinases from rabbit muscle[28,29], yeast[14], calf liver[30], *Dictyostelium discoideum*[31], human muscle[12] and erythrocytes[12] in the presence of the phosphate donor at its non-inhibitory concentration.

Parallel double reciprocal plots are usually considered to be a kinetic evidence for the "ping-pong" mechanism in which the first product is released from the enzyme before the second substrate combines. In no case has any mechanism other than the one involving the ping-pong type attachment of substrates been suggested for the enzymic reaction which shows the kinetics characterized by parallelism in double-reciprocal plots. Thus, it appears that the most plausible reaction mechanism for Ehrlich ascites tumor phosphofructokinase with respect to the binding of ATP and Fru-6-*P* to their catalytic sites on the R state may be the one which involves the "ping-pong" mechanism.

Thus, ascites tumor phosphofructokinase has provided the example of the kinetic mechanism which conforms to the reaction models of "allosteric ping-pong" type introduced by us[7] from a theoretical point of view for the allosteric enzymes function-

ing with ping-pong bi-bi mechanism. As described in Results, all the experimental data could be interpreted in terms of "Type II" of the proposed models in which the conversion of free enzyme to another stable enzyme form, probably phosphorylated one, is an essential requirement for allosteric transition to occur.

It is worthy to note that, in the present model, the binding of ATP to its catalytic sites never exerts any influence on the equilibrium between the R and T states. An experimental support for this striking characteristic of the model may be found in Fig. 1 where the rate-concentration curve at the non-inhibitory level of ATP appears not sigmoidal but rather hyperbolic. Generally speaking, the rate-substrate concentration curve in the presence of the non-inhibitory concentration of ATP could be sigmoidal or hyperbolic depending on the equilibrium position between the R and T state in the absence of ligand. The curve will be hyperbolic if the equilibrium is shifted far towards the R state even in the absence of the substrates, as has been observed for phosphofructokinase from several sources[2,12,14]. In this type of allosteric enzyme, the cooperativity of Fru-6-*P* emerges only if the T state has been favored by binding ATP. In the case of ascites tumor enzyme, however, the saturation kinetics with respect to Fru-6-*P* in the lowest concentration of ATP employed proved sigmoidal (Fig. 2). This suggests that the ratio of T_0 to R_0 is kept at a significantly high level before the addition of any allosteric ligand, as discussed above. It is very reasonable to anticipate that under this condition the rate–concentration curve for ATP at its non-inhibitory level must be also sigmoidal, if the occupation of the catalytic sites by ATP can cause a shift of the R–T equilibrium as a result of stabilizing the R state. This was not the case as observed in Fig. 1; the initial velocity plotted as a function of ATP at its non-inhibitory concentrations appeared hyperbolic rather than sigmoidal in contrast to the curve with Fru-6-*P*. Thus, the experimental results are likely to lend support to a view that the binding of ATP to the catalytic site is distinct from the binding of Fru-6-*P* in its incapability of stabilizing the R state. This is comformable to the reaction model proposed here.

The failure of ATP to show an allosteric interaction has been reported for phosphofructokinases from *E. coli*[24] and *Clostridium pasteurianum*[32], although both enzymes nevertheless exhibited a strong cooperation with respect to Fru-6-*P*. In the latter case, the ping-pong mechanism was likewise suggested for the binding of the substrates to the catalytic sites. In the case of phosphofructokinase from *E. coli*, the kinetic data were accounted for in terms of the concerted allosteric transition with an assumption that ATP has the same affinity for the R state and T state. On the basis of the foregoing discussion, however, a lack of the cooperation with respect to the binding of substrate to the enzyme undergoing the concerted allosteric transition might be alternatively explained by assuming that its binding to the R state fails to affect the equilibrium between the R and T states by a mechanism still unknown but somewhat similar to the present one involving "ping-pong" type addition of substrates. Then, it is very tempting to speculate that the failure of ATP to stabilize the R state as suggested by the present model, though it appears very strange, is not unique to the ascites tumor phosphofructokinase but rather common to the enzymes from several sources.

Though more direct evidence is required for the proposed model to be accepted conclusively, the present model, together with the theory extended for building the

model, may offer new basic concepts which could be taken into consideration in the analysis of complex kinetic properties of phosphofructokinase from many sources.

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