Relationship between Fructose 2,6-Bisphosphate Activation and MgATP Inhibition of Rat Liver Phosphofructokinase at High pH. Kinetic Evidence for Individual Binding Sites Linked by Finite Couplings†

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Received July 2, 1986

ABSTRACT: The concentration of fructose 6-phosphate required to produce half-maximal velocity of rat liver phosphofructokinase at pH 9 (K_a) has been measured at 110 different combinations of MgATP and fructose 2,6-bisphosphate (Fru-2,6-BP) concentrations spanning the range 0.1–100 mM and 0.003–100 μM, respectively. The data have been evaluated by nonlinear regression to an equation resulting from a linked-function analysis of an enzyme capable of binding three ligands simultaneously at separate sites. In addition, the data have been fit to equations, derived from the linked-function expression, that would result if various combinations of antagonistic ligands were unable to bind to the enzyme simultaneously, even at high concentration, either because they compete for a single binding site or because they bind exclusively to different conformational forms of the enzyme. The complete linked-function equation is able to predict the K_a for rat liver phosphofructokinase as a function of any Fru-2,6-BP and/or MgATP concentration significantly better than any of the alternatives examined, particularly at high concentrations of one or both modifier ligands. The free energy couplings between all three possible pairs of ligands are of quite moderate magnitude, especially when the multiplicity of binding sites for each ligand that actually exists on the functional enzyme is considered. Therefore, we conclude that any explanation of the action of Fru-2,6-BP and MgATP by a model more elaborate than the simple linked-function case considered herein cannot be simplified by assuming that the properties of rat liver phosphofructokinase result from an equilibrium of limiting conformational states that exhibit exclusive binding properties.

Fructose 2,6-bisphosphate (Fru-2,6-BP)[1] activates rat liver phosphofructokinase (EC 2.7.1.11) by lowering the concentration of Fru-6-P required to produce half-maximal velocity (K_a). In so doing, it acts to mitigate the action of MgATP, which inhibits phosphofructokinase by increasing the apparent K_a for Fru-6-P (Furuya & Uyeda, 1980a,b; Uyeda et al., 1981a,b; Pilkis et al., 1981; Van Schaftingen et al., 1981; Hers & Van Schaftingen, 1982). Although it is qualitatively well established that Fru-2,6-BP is a very potent activator, a comprehensive quantitative evaluation of its action is to date unavailable, largely because its effects are influenced by virtually all other specific ligands of phosphofructokinase, including Fru-6-P and MgATP. This has led to descriptions of Fru-2,6-BP activation under a variety of specific assay conditions, and few attempts have been made to develop a quantitative relationship that would enable one to extrapolate a prediction of its behavior to other conditions. In addition, few models have been proposed to explain the relationship between the action of Fru-2,6-BP and that of MgATP at the molecular level other than to suggest (Uyeda et al., 1981a) that regulation of rat liver phosphofructokinase by Fru-2,6-BP and MgATP might result from an allosteric model similar to that proposed by Monod et al. (1965). In this view, rat liver phosphofructokinase can exist in either an active or an inhibited conformation. Fru-6-P and Fru-2,6-BP bind to, or induce, the active form whereas MgATP binds to, or induces, the inhibited form. We have undertaken the present investigation in an effort to provide a quantitative description of the relationship between Fru-2,6-BP activation and MgATP inhibition of rat liver phosphofructokinase as well as to ascertain whether there is any kinetic evidence supporting the existence of functionally distinct and discrete conformational forms.

A linked-function analysis is an ideally suited approach with which to examine the relationship between Fru-2,6-BP activation and MgATP inhibition because it entails the explicit quantitation of the magnitude of the interactions between multiple ligands bound to a single protein (Reinhart, 1983a, 1985). Such an analysis is particularly useful when related to the energetics of protein structure stabilization and ligand binding (Weber, 1972, 1975) because if two ligands cannot bind to the same form of an enzyme simultaneously, this feature can be recognized by an unusually large apparent coupling free energy between these two ligands (Weber, 1972, 1975; Reinhart, 1983a, 1985). The results obtained in this study convincingly indicate that the interdependence of the MgATP inhibition and Fru-2,6-BP activation of rat liver phosphofructokinase is best described by models that provide for the capability of all three ligands to bind to the enzyme simultaneously in any combination with moderate free energies of interaction between the bound ligands. Specifically, models incorporating binding antagonism between MgATP and Fru-6-P and/or between MgATP and Fru-2,6-BP resulting from the exclusive binding of these ligands to different conformational forms of the enzyme are not able to adequately describe the behavior of rat liver phosphofructokinase, particularly at high concentrations of either effector ligand.

This approach has been utilized recently to characterize the influence of pH on MgATP inhibition (Reinhart, 1985). In that study, we concluded that pH affected the apparent K_a for Fru-6-P in a manner largely independent of the inhibition

†Supported by Grant GM 33216 from the National Institutes of Health.

[1] Abbreviations: Fru-2,6-BP, fructose 2,6-bisphosphate; Fru-1,6-BP, fructose 1,6-bisphosphate; Fru-6-P, fructose 6-phosphate; CHES, 2-(cyclohexylamino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/86/0425-7308$01.50/0

of MgATP, and the apparent pK for this effect was 8.1. We have chosen to perform the present investigation at pH 9 so that a consideration of the binding of protons as a potential fourth ligand (in addition to Fru-6-P, MgATP, and Fru-2,6-BP) could safely be ignored in the analysis. Unlike rabbit muscle phosphofructokinase, the rat liver isozyme continues to exhibit substantial allosteric properties even at high pH (Reinhart & Lardy, 1980; Reinhart, 1985).

Materials and Methods

Phosphofructokinase from the livers of fed, male rats obtained from Holtzman was purified as described previously (Reinhart & Lardy, 1980). Aldolase, triosephosphate isomerase, and glycerol-3-phosphate dehydrogenase were purchased as ammonium sulfate suspensions from Boehringer Mannheim and dialyzed extensively before use.

Fru-2,6-BP was either synthesized from Fru-1,6-BP as described by Van Schaftingen and Hers (1981) as modified by Reinhart (1983b) or purchased as the hexaammonium salt from Sigma. Comparable results were obtained with Fru-2,6-BP obtained from either source. Stock solutions of Fru-2,6-BP were assayed for P_i according to the procedure of Itaya and Ui (1966) and assayed enzymatically for Fru-6-P, both before and after a mild 10-min acid hydrolysis, to confirm the concentration of Fru-2,6-BP. Contamination by Fru-6-P, Fru-1,6-BP, and P_i was negligible. Fru-6-P (potassium salt), ATP (sodium salt), NADH (sodium salt), CHES, and dithiothreitol were also purchased from Sigma. All other chemicals were reagent grade, and deionized, distilled water was used throughout.

Phosphofructokinase activity was assayed at pH 9 in a final volume of 1.0 mL containing 50 mM CHES–KOH, 100 mM KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, 5 μg of triosephosphate isomerase, and the concentrations indicated of Fru-6-P, Fru-2,6-BP, and MgATP. "MgATP" refers to equimolar quantities of $MgCl_2$ and ATP. The high levels of coupling enzymes were used to ensure very low steady-state concentrations of Fru-1,6-BP as discussed previously (Reinhart & Lardy, 1980). Assays were initiated by the addition of 10 μL of an appropriately diluted phosphofructokinase stock solution, yielding a phosphofructokinase subunit concentration of approximately 6×10^{-10} M. Consequently, the lowest concentration of Fru-2,6-BP examined was in 5-fold excess to the binding site concentration. The final results indicate that the dissociation constant for Fru-2,6-BP, even under the most favorable conditions examined, was nearly 30-fold greater than the subunit concentration. Therefore, the assumption that $[\text{Fru-2,6-BP}]_{free} = [\text{Fru-2,6-BP}]_{total}$ was valid for all experiments. Reaction progress curves were recorded on a strip-chart recorder connected to a Gilford Model 252 spectrophotometer. Steady-state rates were determined by measuring the slope of a line drawn tangent to the reaction progress curve after completion of a slow pre-steady-state transient (Reinhart & Lardy, 1980). Substrate concentration at the time of rate determination had decreased by no more than either 5% or 32 μM, whichever was smaller. Consequently, ADP produced by the reaction never exceeded 32 μM, and its influence on the kinetic behavior was considered to be negligible. One unit of activity is equal to the production of 1 μmol of Fru-1,6-BP/min.

At any given concentration of MgATP and Fru 2,6-BP, the concentration of Fru-6-P required to give half-maximal velocity (apparent K_a) was determined by inspection of a plot of activity vs. log [Fru-6-P]. From 8 to 10 assays were performed to determine each apparent K_a, and all determinations were repeated at least once on a separate day.

The dependence of the apparent K_a on [MgATP] and/or [Fru 2,6-BP] was evaluated by fitting data to one of the following equations as described in the text:[2]

$$K_a = K_a^0\left[\frac{K_{ix}^0 + [X]}{K_{ix}^0 + Q_{ax}[X]}\right] \quad (1)$$

$$K_a = K_a^0\left[\frac{K_{ix}^0 + [X]}{K_{ix}^0}\right] \quad (2)$$

$$K_a = K_a^0\left[\frac{K_{iy}^0[X] + K_{ix}^0[Y] + Q_{xy}[X][Y] + K_{ix}^0K_{iy}^0}{K_{iy}^0Q_{ax}[X] + K_{ix}^0Q_{ay}[Y] + Q_{axy}[X][Y] + K_{ix}^0K_{iy}^0}\right] \quad (3)$$

$$K_a = K_a^0\left[\frac{K_{iy}^0[X] + K_{ix}^0[Y] + K_{ix}^0K_{iy}^0}{K_{iy}^0Q_{ax}[X] + K_{ix}^0Q_{ay}[Y] + K_{ix}^0K_{iy}^0}\right] \quad (4)$$

$$K_a = K_a^0\left[\frac{K_{iy}^0[X] + K_{ix}^0[Y] + K_{ix}^0K_{iy}^0}{K_{ix}^0Q_{ay}[Y] + K_{ix}^0K_{iy}^0}\right] \quad (5)$$

$$K_a = K_a^0\left[\frac{K_{iy}^0[X] + K_{ix}^0[Y] + Q_{xy}[X][Y] + K_{ix}^0K_{iy}^0}{K_{ix}^0Q_{ay}[Y] + K_{ix}^0K_{iy}^0}\right] \quad (6)$$

The parameters have the following definitions: K_a = concentration of substrate A (in this case Fru-6-P) producing half-maximal velocity, X = allosteric ligand, Y = allosteric ligand, $K_a^0 = K_a$ when [X] = [Y] = 0, K_{ix}^0 = dissociation constant of X when [A] = [Y] = 0, K_{iy}^0 = dissociation constant of Y when [A] = [X] = 0, Q_{ax} = coupling parameter between bound A and bound X, Q_{ay} = coupling parameter between bound A and bound Y, Q_{xy} = coupling parameter between bound X and bound Y, and Q_{axy} = coupling parameter between bound A and bound X and bound Y. The coupling parameters essentially describe the influence that the binding of one of the indicated ligands has on the binding of the other ligand [see Reinhart (1983a) for details]. They are each related to the corresponding free energy of interaction (coupling free energy) between the indicated ligands (for example, ΔG_{ax} for ligands A and X) according to the expression:

$$\Delta G_{ax} = -RT \ln Q_{ax} \quad (7)$$

where R equals the gas constant and T equals the absolute temperature (Reinhart, 1983a). If $Q_{ax} = 1$, then A and X bind independently; if $Q_{ax} < 1$, A and X each antagonize the binding of the other; if $Q_{ax} > 1$, A and X encourage each other's binding. Unless otherwise noted, for this investigation we have let X = MgATP and Y = Fru-2,6-BP.

Nonlinear regression analysis was performed with a Hewlett Packard 9826A desktop computer using programs, written in HP Enhanced BASIC, that followed the general approach described by Cleland (1967). The parameter values were initially estimated as described previously (Reinhart, 1985). A constant relative error in the apparent K_a determinations was assumed (see below).

Results

The nature and interdependence of the activation by Fru-2,6-BP and the inhibition by MgATP of rat liver phospho-

[2] The notation used in the following equations has been previously described (Reinhart, 1983a).

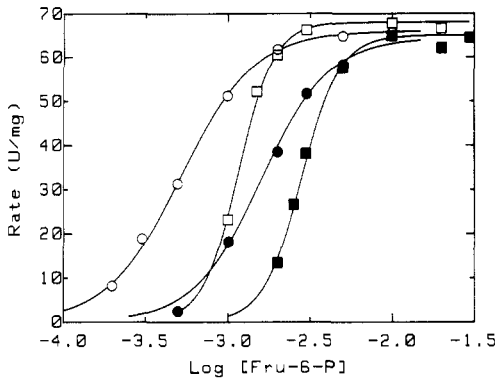

FIGURE 1: Typical steady-state velocity profiles as a function of log [Fru-6-P] at various concentrations of MgATP and Fru-2,6-BP. [Fru-6-P] expressed in molar units. [MgATP] = 3 mM (○, □) or 12 mM (●, ■). [Fru-2,6-BP] = 30 nM (○, ●) or 0 (□, ■).

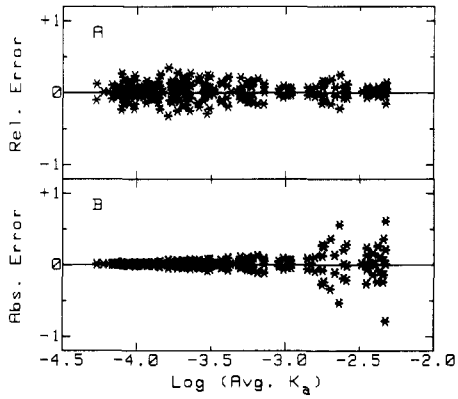

FIGURE 2: Scatter plot of relative (A) or absolute (B) error in replicate values for each apparent Michaelis constant determined plotted as a function of the log of the average value for each replicate set. Absolute error was calculated according to absolute error = K_a − av K_a. Relative error was calculated according to relative error = (K_a − av K_a)/av K_a.

fructokinase are illustrated in Figure 1. Both modifiers affect activity almost exclusively by changing the concentration of Fru-6-P required to produce half-maximal activity, which we define to be the apparent K_a. In the absence of Fru-2,6-BP, increasing the concentration of MgATP from 3 to 12 mM increases the K_a by a factor of 2.4, whereas a comparable change of MgATP concentration produces an increase in the K_a of 3.1 when the concentration of Fru-2,6-BP is 30 nM. Concurrently, 30 nM Fru-2,6-BP lowers the K_a 2.2-fold when the concentration of MgATP is 3 mM but produces a decrease of only 1.8-fold when the MgATP concentration is 12 mM.

Experiments such as these were performed for 110 different combinations of MgATP and Fru-2,6-BP concentrations. The concentration of MgATP ranged from 0.1 to 100 mM and that of Fru-2,6-BP from 3 nM to 100 μM (plus 0). These data were fit to various equations, described below, in an effort to provide a comprehensive description of the interrelationship between MgATP inhibition and Fru-2,6-BP activation. In so doing, different results are obtained depending upon the choice of weighting factors used in the calculations. Previously, we have assumed that the relative error of the apparent K_a's was more constant than the absolute error (Reinhart, 1985). We have been able to explicitly test this assumption since virtually all of the experiments were performed in duplicate, with some done in triplicate or quadruplicate. Figure 2 shows a plot of the spread of the replicates on a relative and absolute basis as a function of the average value of the K_a for that set. It can be seen that the absolute error increases dramatically at high values of K_a but that the relative error is nearly constant

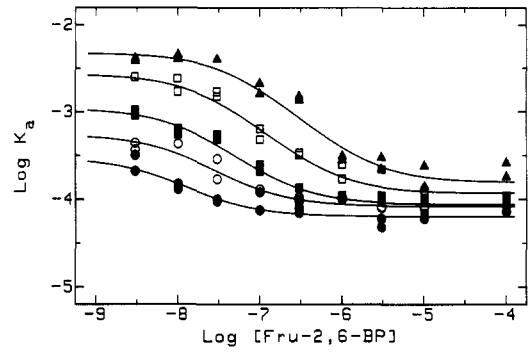

FIGURE 3: Influence of [Fru-2,6-BP] on the apparent Michaelis constant for Fru-6-P at various concentrations of MgATP. [Fru-2,6-BP] expressed in molar units. [MgATP] = 0.19 (●), 0.75 (○), 3.0 (■), 12 (□), and 48 mM (▲). Curves represent the best fit of the data at each concentration of MgATP to eq 1.

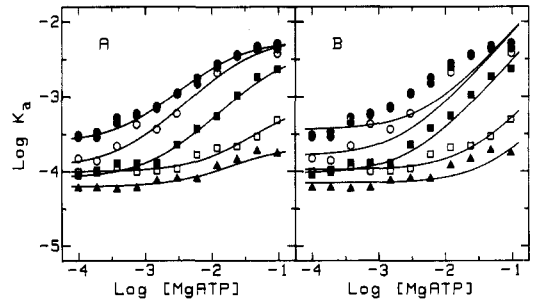

FIGURE 4: Influence of [MgATP] on the apparent Michaelis constant for Fru-6-P at various concentrations of Fru-2,6-BP. [MgATP] expressed in molar units. [Fru-2,6-BP] = 0 (●), 0.010 (○), 0.10 (■), 1.0 (□), and 10 μM (▲). Curves in panel A represent the best fit of the data at each concentration of Fru-2,6-BP to eq 1. Curves in panel B represent the best fit of the same data to eq 2.

throughout the range of values obtained for K_a in this study, thus justifying the use of a weighting factor $(1/K_a)^2$ during the nonlinear regression calculations (Cleland, 1967).

If one examines the influence that Fru-2,6-BP has on the apparent K_a for Fru-6-P, results such as those shown in Figure 3 are obtained. Increasing the Fru-2,6-BP concentration decreases the apparent K_a until a plateau is reached such that a further increase in Fru-2,6-BP concentration produces no further effect. This behavior is characteristic of a model in which both ligands are able to bind to the enzyme simultaneously (Reinhart, 1983a), a feature not surprising when considering allosteric activation. Equation 1 describes the expected relationship between the apparent K_a and an allosteric ligand, X, when both ligands bind simultaneously provided either that the rapid equilibrium assumption is valid for substrate A (Botts & Morales, 1953; Frieden, 1964) or that X achieves a true binding equilibrium with the enzyme in the steady state (Reinhart, 1983a, 1985). The curves in Figure 3 represent the best fit of the data at each MgATP concentration to eq 1 (with X = Fru-2,6-BP), and it is apparent the data are well described by this relationship. Increasing the concentration of MgATP tends to shift the curves up and to the right and increase the distance between the plateaus approached at low and high concentrations of Fru-2,6-BP. Such changes indicate that MgATP influences all three parameters that describe the mode of action of Fru-2,6-BP, i.e., K_a^0, K_{ix}^0, and Q_{ax} (Reinhart, 1985).

Similarly, the inhibition of rat liver phosphofructokinase by MgATP at various concentrations of Fru-2,6-BP is characterized by data of the type shown in Figure 4. Increasing the MgATP concentration causes the apparent K_a for Fru-6-P to increase. The dependence of the apparent K_a on varying

Table I: Summary of Parameters Resulting from Fitting Apparent Michaelis Constant Data Obtained at Various Concentrations of MgATP and Fru-2,6-BP to Equations 3–6[a]

	equation			
	3	4	5	6
K_a^0 (mM)	0.173 ± 0.013	0.163 ± 0.017	0.183 ± 0.014	0.202 ± 0.015
K_{ix}^0 (mM)	0.399 ± 0.048	0.368 ± 0.065	2.24 ± 0.22	2.43 ± 0.23
K_{iy}^0 (μM)	0.032 ± 0.005	0.028 ± 0.007	0.160 ± 0.029	0.143 ± 0.025
Q_{ax}	0.031 ± 0.007	0.031 ± 0.008		
ΔG_{ax} (kcal/mol)	+2.05 ± 0.09	+2.06 ± 0.11		
Q_{ay}	2.38 ± 0.49	2.08 ± 0.48	2.43 ± 0.59	2.87 ± 0.69
ΔG_{ay} (kcal/mol)	−0.51 ± 0.09	−0.43 ± 0.10	−0.53 ± 0.11	−0.62 ± 0.10
Q_{xy}	0.012 ± 0.004			0.050 ± 0.017
ΔG_{xy} (kcal/mol)	+2.62 ± 0.13			+1.78 ± 0.15
Q_{axy}	0.004 ± 0.002			
ΔG_{axy} (kcal/mol)	+3.27 ± 0.24			
variance ($\times 10^4$)	8.6	10.9	29.9	27.2

[a] A = Fru-6-P, X = MgATP, and Y = Fru-2,6-BP. Coupling free energies were calculated according to eq 7 with T = 25 °C.

concentrations of MgATP was analyzed by fitting the data either to eq 1 or to eq 2 (with X = MgATP).

As indicated above, eq 1 results from a linked-function analysis of allosteric behavior where it is assumed that substrate and allosteric ligand bind to separate sites and that their bindings are not mutually exclusive (Reinhart, 1983a, 1985) but rather linked with coupling free energies of finite magnitude. The curves in Figure 4A describe the best fit of these data to eq 1.

A model describing allosteric inhibition by the binding of antagonistic ligands, such as Fru-6-P and MgATP in this case, to distinct enzyme "forms" or conformations such that both ligands cannot bind to the same enzyme form (Monod et al., 1965) predicts kinetic behavior indistinguishable from a competitive mechanism with respect to the nature of the inhibitory ligand's effect on K_a. This relationship is described by eq 2. Note that eq 2 can be derived from eq 1 by letting Q_{ax} = 0, implying an infinite coupling free energy between the two antagonistic ligands. When the data of Figure 4 are analyzed by nonlinear regression to eq 2, the curves presented in Figure 4B result.

A simple qualitative comparison of Figure 4A,B suggests that in most cases eq 1 provides a much better description of the inhibition by MgATP of rat liver phosphofructokinase, a conclusion supported by a statistical comparison of the error associated with the respective fits (data not shown). Moreover, increasing the concentration of Fru-2,6-BP apparently decreases K_a^0, increases K_{ix}^0, and reduces the magnitude of the apparent interaction between MgATP and Fru-6-P.

Equation 3 represents an extension of the linked-function approach just described to the case of two allosteric ligands, X and Y, each binding to separate sites on an enzyme and linked with finite couplings (Reinhart, 1983a, 1985). As such, it can serve as the basis for evaluating the combined effects of MgATP and Fru-2,6-BP simultaneously. Note that a total of four interaction parameters appear in this equation, one for each combination of ligands that might exist on an enzyme molecule simultaneously.

From eq 3, one can also readily derive the equations that describe the behavior of several plausible models in which one or two of these three ligands bind excluisvely to specific enzyme forms. If Fru-2,6-BP activates rat liver phosphofructokinase by directly competing with MgATP for binding to a single site, for example, the expected behavior of the apparent K_a for Fru-6-P is given by eq 4. Since neither modifying ligand can bind in the presence of the other according to this model, the coupling free energy between them must be infinite, and Q_{xy} = 0 as given by eq 7. By the same reasoning, Q_{axy} = 0 because all three ligands do not bind simultaneously in this model. Equation 4 directly results, therefore, from eq 3 by making these substitutions. The same equation would result if Fru-2,6-BP and MgATP exclusively bound to two distinct conformational forms of the enzyme, each of which was able to bind Fru-6-P with a different affinity.

The regulatory properties of phosphofructokinase with respect to a wide variety of effector ligands are often interpreted by assuming that the enzyme can exist in either an "active" or an "inactive" form (Uyeda et al., 1981a; Frieden et al., 1976; Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979a,b; Roberts & Kellett, 1979, 1980a,b). To conform to this model, Fru-6-P and Fru-2,6-BP would be expected to bind to the active form, and MgATP would bind to the inactive form. Consequently, not only would Q_{xy} and Q_{axy} equal 0 but also Q_{ax} would equal 0 since Fru-6-P and MgATP could not bind to the same enzyme molecule concurrently. Equation 5, resulting from substituting these values into eq 3, therefore, describes this general mechanism.

Equation 6 represents a model in which Fru-6-P and MgATP bind exclusively to different enzyme forms, but Fru-2,6-BP can bind to either. In such a case, Q_{ax} = 0 and Q_{axy} = 0, but Q_{xy} would not. Again, eq 6 can be readily derived from eq 3.

The results of fitting the entire data set of 221 apparent K_a values to eq 3–6 are presented in Table I. As indicated in Table I, the overall variance[3] of the fit to eq 3 is 27% lower than it is for the fit to eq 4 and is over 3-fold lower than the variance for the fit to either of the two remaining models.

We can evaluate the significance of these differences by considering the ratio of variances to be proportional to the reduced χ^2 statistic. Because the data set is so large (the number of degrees of freedom is greater than 200 in all cases), the significance of the improvement of the fits to eq 3 and 4 compared to eq 5 and 6 is overwhelming (Bevington, 1969). This observation is not surprising in light of the data shown in Figure 4 since both eq 5 and 6 incorporate the assumption of an infinite coupling free energy between Fru-6-P and MgATP. In addition, there is a less than 1% probability that

[3] The variance of each fit was calculated in the following manner (Bevington, 1969):

$$\text{variance} = \frac{[1/(N-P)]\sum_{i=1}^{N}(K_{calcd} - K_i)^2 W_i}{(1/N)\sum_{i=1}^{N} W_i}$$

where K_i = the measured apparent K_a for Fru-6-P, K_{calcd} = the apparent K_a calculated according to the indicated equation, N = the number of data points (=221), P = the number of parameters (=7 for eq 3, =5 for eq 4, etc.), and $W_i = (1/K_i)^2$.

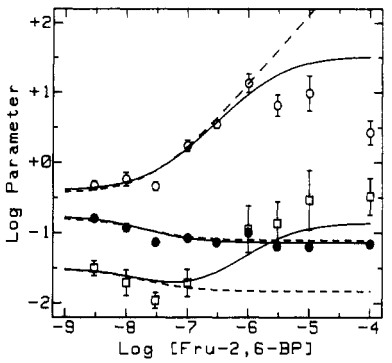

FIGURE 5: Comparison of the ability of eq 3 and 4 to describe the influence of [Fru-2,6-BP] on the parameters describing the allosteric inhibition by MgATP. The parameters K_a^0 (●), K_{ix}^0 (○), and Q_{ax} (□) were determined by fitting data of the type presented in Figure 4 to eq 1. The solid curves represent the predicted values for these parameters based upon the overall fit to eq 3. The dashed curves are the parameter values predicted by the overall fit to eq 4. Curves were calculated by using eq 8–10 and the parameter values given in Table I. [Fru-2,6-BP] is expressed in molar units. K_a^0 and K_{ix}^0 are expressed in millimolar units.

the difference in variance between the fits to eq 3 and 4 is due to statistical fluctuation, indicating that eq 3 is a significantly more appropriate model for the data. This conclusion is reinforced by the fact that the larger variance associated with eq 4 is attributable, at least in part, to a nonrandom error that is correlated with the independent variables, particularly [Fru-2,6-BP]. This correlation can be readily seen in Figure 5.

In Figure 5, we summarize the overall fits of eq 3 and 4 by presenting the apparent parameters describing the MgATP inhibition, K_a^0, $K_{ix}^0{}'$, and Q_{ax}' (obtained by fitting the data to eq 1 as described for Figure 4A), as a function of Fru-2,6-BP concentration. The variation of these parameters predicted by eq 3 and 4 was calculated by using the following expressions (Reinhart, 1985) and is presented in these figures by the solid and dashed curves, respectively:

$$K_a^{0\prime} = K_a^0\left[\frac{K_{iy}^0 + [Y]}{K_{iy}^0 + Q_{ay}[Y]}\right] \quad (8)$$

$$K_{ix}^{0\prime} = K_{ix}^0\left[\frac{K_{iy}^0 + [Y]}{K_{iy}^0 + Q_{xy}[Y]}\right] \quad (9)$$

$$Q_{ax}' = \frac{(K_{iy}^0 + [Y])(K_{iy}^0 Q_{ax} + Q_{axy}[Y])}{(K_{iy}^0 + Q_{ay}[Y])(K_{iy}^0 + Q_{xy}[Y])} \quad (10)$$

Y represents Fru-2,6-BP, and the parameter values are those listed in Table I. While both equations (3 and 4) do a good job of describing the data at low concentrations of Fru-2,6-BP, eq 4 fails to adequately predict the behavior observed at high concentrations of Fru-2,6-BP. Specifically, the apparent dissociation constant for MgATP, $K_{ix}^0{}'$, does not continue to increase but rather clearly approaches a plateau at high concentrations of Fru-2,6-BP. Also, the apparent inhibition effectiveness of MgATP, after initially increasing, ultimately decreases at high concentrations of Fru-2,6-BP (as indicated by the increase in the value of Q_{ax}'). Both of these features of the data are more correctly accommodated by eq 3.

Discussion

The most notable difference between eq 3 and eq 4–6 is that eq 3 is the only one that provides for full and finite linkage between all three ligands of rat liver phosphofructokinase examined in this investigation, namely, Fru-6-P, Fru-2,6-BP, and MgATP. Each of the other equations entails an assumption that one ligand cannot bind simultaneously with one or more of the other ligands. Such a situation can arise either when two different ligands bind to the same binding site or when two ligands bind exclusively to each of two different conformational forms of the enzyme, respectively. In either case, certain features of the enzyme's behavior are predicted to be unsaturable. For example, if Fru-6-P and MgATP cannot bind to the same enzyme form, the apparent K_a for Fru-6-P should continually increase as MgATP concentration increases (Figure 4). If Fru-2,6-BP and MgATP were truly competitive, the apparent dissociation constant of MgATP, $K_{ix}^0{}'$, should increase without limit as Fru-2,6-BP concentration increases (Figure 5). However, in each case saturation has been observed. Even the simplest application of finite linkage represented by eq 3, therefore, is able to describe these features better than the corresponding equations incorporating the competitive relationships inherent in models where opposing ligands bind exclusively to different enzyme forms.

One might argue that total exclusivity is only an unreasonable limiting case for mechanisms involving functionally distinct conformational states. Ligands might reasonably be expected to have a small, nonzero, affinity for the "wrong form". However, as Tanford (1981) has pointed out, though these mechanisms might be inherently plausible, ligand binding data alone will not provide any information pertaining to the existence of specific conformational isomers one way or the other. It would seem to be preferable, therefore, to analyze the data in ways that are not dependent on a priori assumptions that will not improve the description of observable behavior. This philosophy is reflected in the common procedures used when deriving conventional kinetic rate equations, for example. The various isomerizations that might, indeed must, take place upon the conversion of the ternary complex of enzyme plus substrates to form the ternary complex of enzyme plus products have no influence on the form of the resultant initial velocity rate equations (Cleland, 1963) and so are ignored when deriving the rate expressions. So it is with allosteric enzymes. The possible existence of interconvertible enzyme conformational states at equilibrium, not accompanied by *mandatory* changes in the state of ligation, will not affect the ultimate form of the rate equation. Therefore, until data (other than ligand binding or steady-state rate data) are obtained that directly imply the existence of such forms, they are best left out of the analysis.

The parameters determined by the fit of these data to eq 3 confirm that Fru-2,6-BP can bind with very high affinity to free enzyme (dissociation constant = K_{iy}^0 = 32 nM; see Table I). Moreover, the small negative coupling free energy between Fru-6-P and Fru-2,6-BP indicates that at saturating Fru-6-P concentrations the dissociation constant for Fru-2,6-BP will actually drop to K_{iy}^0/Q_{ay} = 32/2.38 = 13 nM. The binding of Fru-2,6-BP is also strongly antagonized, however, by the binding of MgATP. Saturation by MgATP raises the dissociation constant for Fru-2,6-BP to K_{iy}^0/Q_{xy} = 0.032/0.012 = 2.7 μM. As expected, the dissociation constant with both Fru-6-P and MgATP saturating assumes a value intermediate between these extremes and is given by K_{iy}^0 (Q_{ax}/Q_{axy}) = 0.25 μM. These effects are, of course, reciprocal in nature, and the influences of the binding of Fru-2,6-BP and MgATP on the K_a for Fru-6-P, the most informative parameter for directly assessing the net impact of these allosteric ligands on the activity of phosphofructokinase, can be calculated by direct substitution into eq 3.

An energetic interpretation of ligand–ligand interactions on proteins leads to an expectation that the free energy of interaction between any two ligands able to bind simultaneously to a protein will usually not exceed roughly 2 kcal/mol in absolute value (Weber, 1972, 1975). It is noteworthy, therefore, that all of the two ligand coupling free energies determined by the fit to eq 3 fall well within this range with the possible exception of ΔG_{xy}, the coupling free energy between Fru-2,6-BP and MgATP, which is equal to +2.6 kcal/mol (Table I). Although a value of this magnitude cannot be considered to seriously violate this general rule of thumb, it is important to recognize that the coupling free energies listed in Table I represent the sum of all of the interacting sites between the ligands indicated (Reinhart, 1985) and hence evaluating them on this basis subjects the finite coupling hypothesis to its most stringent test. Ogilvie (1985), for example, has recently observed that two activating nucleotide sites appear to be coupled, with equal magnitudes, to each ATP inhibitory site in rabbit muscle phosphofructokinase. If the Fru-2,6-BP sites behave similarly, then the coupling between each Fru-2,6-BP and MgATP ligand would be +1.3 kcal/mol, a value fully in keeping with Weber's expectation.

We do not suggest that eq 3 represents the final picture of the kinetic behavior of rat liver phosphofructokinase with respect to these three ligands. In fact, it is somewhat remarkable that a relationship derived from the simplest implementation of linked-function analysis provides as good a description as it does of the behavior of such a notoriously complex enzyme. Specifically, certain possible ramifications of the actual oligomeric nature of rat liver phosphofrucotkinase are not incorporated into eq 3. For example, the interaction of Fru-6-P with rat liver phosphofructokinase is known to be highly cooperative (Reinhart & Lardy, 1980), suggesting an appreciable degree of interaction between the several Fru-6-P binding sites on the functional enzyme. Equation 3 has essentially avoided this complication by factoring out Fru-6-P concentration as a variable and considering the apparent half-maximal concentratin of Fru-6-P to be the dependent variable. Consequently, a complete description of activity as a function of Fru-6-P concentration will also need to incorporate information pertaining to the magnitude of this cooperativity and the influence that Fru-2,6-BP and MgATP might have on it. We are now in a better position to determine the relative contribution that homotropic and heterotropic mechanisms might make to the degree of this cooperativity, however, since quantifying the finite heterotropic couplings is a necessary prerequisite for determining the magnitude of subsaturating heterotropic and heterotropically induced homotropic cooperativity (Weber, 1972, 1975; Reinhart, 1983a). Similarly, MgATP and Fru-2,6-BP may display cooperative behavior, although homotropic interactions between the several binding sites of each of these ligands are not required by the existence of the Fru-6-P cooperativity (Reinhart, 1985) and have not been observed in binding experiments with MgATP and rabbit muscle phosphofructokinase (Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979a). Finally, each bound Fru-6-P might, on average, be strongly coupled to more than one bound MgATP and/or Fru-2,6-BP molecule as discussed above. All of these possibilities will undoubtedly lead to a more complex relationship between these three ligands than that described by eq 3. However, when attempting to develop and test more elaborate models to improve upon the description provided by eq 3, we must know if we are justified in simplifying the analysis by invoking forms with exclusive binding properties. Clearly, these data suggest that we are not and that the more appropriate premise for such an elaboration will be a model where all three ligands have their own saturable binding domains mutually linked with finite coupling free energies of moderate magnitude.

ACKNOWLEDGMENTS

We thank Professor Sherril Christian for helpful discussions regarding the statistical interpretation of the data.

Registry No. Fru-2,6-BP, 77164-51-3; Fru-6-P, 643-13-0; MgATP, 1476-84-2; phosphofructokinase, 9001-80-3.

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