Influence of pH on the Regulatory Kinetics of Rat Liver Phosphofructokinase: A Thermodynamic Linked-Function Analysis†

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ABSTRACT: The relationship between pH and the MgATP inhibition of rat liver phosphofructokinase has been quantitatively evaluated by utilization of a thermodynamic linked-function approach. This approach obviates the need to presuppose discrete inhibited and active states of the enzyme. The behavior of the apparent Michaelis constant for fructose 6-phosphate (Fru-6-P) over a 1000-fold concentration range of MgATP conforms to the behavior predicted by the linked-function theory in that, at high concentrations of MgATP, saturation of the inhibitory effect is achieved, a result not predicted by a mutually exclusive two-state model. This behavior is described by the relationship $K_a = K_a^0[(K_{ix}^0 + [X])/(K_{ix}^0 + Q[X])]$, where K_a is the apparent Michaelis constant for Fru-6-P, K_a^0 is the Michaelis constant for Fru-6-P in the absence of MgATP, K_{ix}^0 is the dissociation constant of MgATP in the absence of Fru-6-P, and Q is the coupling term that quantitatively describes the finite degree of antagonism between MgATP and Fru-6-P. The free energy of interaction between MgATP and Fru-6-P, obtained from Q, is 1.9 kcal/mol at 25 °C. K_a^0 and K_{ix}^0 are 0.17 and 0.3 mM, respectively. The influence of pH on these three parameters was then systematically investigated, and only K_a^0 increased substantially with decreasing pH. Consequently, it is concluded that decreasing the pH does not increase the apparent K_a for Fru-6-P by augmenting the binding or inhibition by MgATP to a significant extent but rather by directly affecting the intrinsic affinity of the enzyme for Fru-6-P. The pK for this effect is 8.1.

Phosphofructokinase (EC 2.7.1.11) has long been recognized to be a fascinatingly complex regulatory enzyme. It is subject to allosteric regulation by a wide variety of physiologically relevant metabolites. So numerous and interdependent are the various effectors that attempts in the past to characterize the influence of even a single ligand have usually produced a phenomenological description whose quantitative characteristics are only valid within a highly restrictive set of experimental conditions.

Quantitative models of a more general nature describing the allosteric behavior of phosphofructokinase that have been developed previously (Frieden et al., 1976; Goldhammer & Hammes, 1978; Pettigrew & Frieden, 1979a,b) have utilized variations of the model proposed by Monod et al. (1965) in which the enzyme is assumed to exist in one of two conformational states. Goldhammer & Hammes (1978) invoked active and inactive states that selectively bound Fru-6-P[1] at the active site and MgATP at an inhibitory site, respectively. Frieden and co-workers (Frieden et al., 1976; Pettigrew & Frieden, 1979a,b) modified the model by proposing that the enzyme conformations were also deprotonated and protonated, respectively.

We prefer to view the actions of the various substrate and regulatory ligands from a thermodynamic linked-function perspective, an approach first elucidated by Wyman (1967) and more recently related to the energetics of protein structure and ligand binding by Weber (1972, 1975). This approach has several advantages, not the least of which is an avoidance of a restrictive a priori mechanistic assumption regarding enzyme conformational states. Rather, attention is focused on parameters that can quantitatively describe the affinity of an allosteric ligand for the enzyme and its effectiveness as an inhibitor (or activator) once bound. For ligands that act primarily by modifying the apparent affinity or Michaelis constant of the substrate, efficacy is quantitatively described by the unique free energy of interaction (free-energy coupling) between the allosteric ligand and substrate (Weber, 1972, 1975; Reinhart, 1983).

In this study we have analyzed the MgATP inhibition of rat liver phosphofructokinase in such a fashion. It is demonstrated that the inhibition characteristics of MgATP can be adequately described without resort to postulated intrinsic inhibited and active states or conformations of the enzyme that display differing affinities for the various ligands. The analysis is then extended to consider the three ligand case of Fru-6-P, MgATP, and H^+, which entails a determination of the free-energy couplings between all of the various combinations of these ligands as well as their individual dissociation constants. A complete description of the interdependence of MgATP and pH is thereby obtained and as such serves as a model for examining the interdependence of other ligands in the future.

Materials and Methods

Phosphofructokinase was purified from fresh rat livers as previously described (Reinhart & Lardy, 1980a) and stored in a buffer consisting of 20 mM potassium phosphate, 1 mM Fru-6-P, 10 mM dithiothreitol, 3 mM $MgCl_2$, 100 μM EDTA, and 20% (v/v) glycerol, pH 7.6. Typically, kinetic assays were initiated by adding 10 μL of phosphofructokinase, appropriately diluted in this buffer, to an assay mixture measuring 1.0 mL. The assay mixture contained 50 mM buffer, 100 mM

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[1] Abbreviations: Fru-6-P, fructose 6-phosphate; Fru-1,6-BP, fructose 1,6-bisphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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KCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mM NADH, 250 μg of aldolase, 50 μg of glycerol-3-phosphate dehydrogenase, 5 μg of triosephosphate isomerase, plus varying concentrations of Fru-6-P and MgATP. The following buffers were used according to the pH desired: glycylglycine-HCl (pH 9.0 and 8.5), HEPES–KOH (pH 8.0 and 7.5), MOPS–KOH (pH 7.0 and 6.5), and MES–KOH (pH 6.0 and 5.5). Stock solutions of Fru-6-P (dipotassium salt) and equimolar $MgCl_2$–ATP (disodium salt) were adjusted to the pH of the experiment with either KOH or HCl. Steady-state rates were measured after the completion of any slow pre-steady-state transients of the type described previously (Reinhart & Lardy, 1980a). Rates are expressed as units per milligram of protein where a unit is defined as equal to the production of 1 μmol of Fru-1,6-BP/min at 25 °C. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Data were fit to equations described in the text by nonlinear least-squares regression analysis following the approach outlined by Cleland (1967). Computer programs were written in Hewlett-Packard Enhanced Basic 2.0 and run on a Hewlett-Packard 9826A desktop computer.

Apparent Michaelis constants for Fru-6-P[2] were determined from the x-axis intercept of the Hill plot resulting from a least-squares linear regression of data plotted as log $[v/(V-v)]$ vs. log [Fru-6-P] where V is maximal velocity estimated from a direct plot of v vs. log [Fru-6-P] and v is reaction rate. Only rates between 10 and 90% of V were used in the calculation.

Biochemicals and buffers were purchased from Sigma Chemical Co. Coupling enzymes were purchased as ammonium sulfate suspensions from Boehringer Mannheim and dialyzed extensively before use to remove the ammonium sulfate. All other reagents were of the highest purity available, and deionized, distilled water was used throughout.

Theory

The rate equation[3] describing the influence of a single allosteric ligand on the initial velocity, v, of a single substrate enzyme is given by eq 1 provided that either the substrate is in rapid equilibrium (Botts & Morales, 1953; Frieden, 1964) or the allosteric ligand achieves a true (not necessarily rapid) binding equilibrium in the steady-state (Reinhart, 1983):

$$v/E_T = \frac{V^0K^0_{ix}[A] + V^0QW[A][X]}{K^0_{ix}[A] + K^0_a[X] + Q[A][X] + K^0_aK^0_{ix}} \quad (1)$$

where [A] = substrate concentration, [X] = allosteric ligand concentration, E_T = total enzyme concentration, V^0 = maximal velocity when [X] = 0, K^0_a = Michaelis constant when [X] = 0, K^0_{ix} = dissociation constant of X when [A] = 0, Q = ratio of Michaelis constants when [X] = 0 to [X] = ∞, and W = ratio of maximal velocity when [X] = ∞ to [X] = 0. If X is a "K-type" inhibitor, it is more convenient to express its action with the equation describing its influence on the apparent Michaelis constant for A, K_a. This expression follows from eq 1 when $W = 1$ and $v/E_T = V^0/2$, although it is also generally true for any value of W. Solving for [A], which is equal to K_a, yields

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

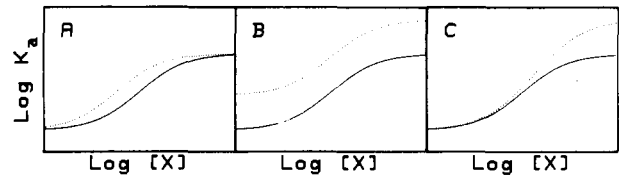

FIGURE 1: Differentiating the three mechanisms by which a second allosteric ligand, Y, can appear to increase the apparent K_a for the substrate A, at any given concentration of the first allosteric ligand X. The solid curve in each figure was calculated from eq 2, which describes the expected influence of X on the apparent K_a when X antagonizes the binding of A with a finite coupling free energy. Arbitrary values of K^0_a, K^0_{ix}, and Q were assumed. If Y acts by decreasing the apparent dissociation constant of X, K^0_{ix}, the resulting change in the curve is indicated by the dotted line in panel A. If the effect of Y is independent of X, the apparent Michaelis constant for A in the absence of X, K^0_a, will change and the curve will shift as indicated by the dotted line in panel B. The dotted curve in panel C will result if Y increases the antagonism between A and X, an effect reflected by a decreased value of Q.

Thus, we see that three parameters determine the apparent Michaelis constant: K^0_a, which reflects the steady-state affinity of the free enzyme for the substrate; K^0_{ix}, which reflects the affinity of the free enzyme for the allosteric ligand; Q, which reflects the unique free energy of interaction between substrate and allosteric ligand according to the relationship (Reinhart, 1983)

$$\Delta G_{ax} = -RT \ln Q \quad (3)$$

where T is the temperature in kelvin and R is the gas constant. In essence, Q is a measure of the effectiveness of X at inhibiting the binding of A and vice versa.

Equation 2 predicts that a plot of log K_a vs. log [X] will yield a hyperbolic curve similar to a binding profile[4] for X (see Figure 1). A second ligand, Y, that empirically changes the apparent K_a of the substrate when added to such a system can achieve its effect by apparently altering any or all of the parameters K^0_a, K^0_{ix}, or Q. Each of these three effects can qualitatively be discerned by examining the effects that Y has on the plot of log K_a vs. log [X] as shown in Figure 1.

An explicit quantitative description of these effects can be obtained by considering the rate equation applicable to a system of one substrate and two allosteric ligands, each binding to separate sites (Reinhart, 1983):

$$\begin{aligned} v/E_T = (&V^0K^0_{ix}K^0_{iy}[A] + V^0K^0_{iy}Q_{ax}W_x[A][X] + \\ &V^0K^0_{ix}Q_{ay}W_y[A][Y] + V^0Q_{axy}W_{xy}[A][X][Y])/ \\ (&K^0_{ix}K^0_{iy}[A] + K^0_aK^0_{iy}[X] + K^0_aK^0_{ix}[Y] + K^0_{iy}Q_{ax}[A][X] + \\ &K^0_{ix}Q_{ay}[A][Y] + K^0_aQ_{xy}[X][Y] + Q_{axy}[A][X][Y] + \\ &K^0_aK^0_{ix}K^0_{iy}) \end{aligned} \quad (4)$$

where K^0_{iy} = dissociation constant of Y from free enzyme, Q_{ax} = coupling term between A and X when [Y] = 0, Q_{ay} = coupling term between A and Y when [X] = 0, Q_{axy} = cou-

[2] The use of the term "Michaelis constant" in this context is meant only to convey the concentration of Fru-6-P that gives half-maximal velocity under steady-state conditions. Since the [Fru-6-P]–velocity profiles are highly cooperative, other implications usually associated with this phrase are inappropriate.

[3] The notation used in the following equations has been defined previously (Reinhart, 1983). Essentially, it conforms to the notation of Cleland (1963) with the addition of a superscript indicating the concentration of the other ligands not pertaining to the dissociation or Michaelis constant in question.

[4] One should note that the inflection point of this curve does not represent $K^{0\prime}_{ix}$, the apparent dissociation constant for X from free enzyme, but rather $K^{0\prime}_{ix}/Q^{1/2}$, because the antagonism between A and X is mutual.

pling term between all three ligands [the coupling free energy associated with this term ($\Delta G_{axy} = -RT \ln Q_{axy}$) is equal to the difference between the free energy of binding all three ligands sequentially and the sum of the free energies of binding each ligand individually in the absence of the other two; see Reinhart (1983) for details], W_x = relative change in maximal velocity induced by saturating X alone, W_y = relative change in maximal velocity induced by saturating Y alone, and W_{xy} = relative change in maximal velocity induced by saturating with both X and Y.

The expression for the apparent Michaelis constant as a function of both [X] and [Y] can be derived from eq 4 in a manner similar to that used in deriving eq 2 from eq 1:

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

Let us define the following terms:

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

$$K_{ix}^{0\prime} = K_{ix}^0 \left[\frac{K_{iy}^0 + [Y]}{K_{iy}^0 + Q_{xy}[Y]} \right] \quad (7)$$

$$Q' = \frac{(K_{iy}^0 + [Y])(K_{iy}^0 Q_{ax} + Q_{axy}[Y])}{(K_{iy}^0 + Q_{ay}[Y])(K_{iy}^0 + Q_{xy}[Y])} \quad (8)$$

Equation 5 can now be rewritten in the same form as eq 2:

$$K_a = K_a^{0\prime} \left[\frac{K_{ix}^{0\prime} + [X]}{K_{ix}^{0\prime} + Q'[X]} \right] \quad (9)$$

Therefore eq 6–8 describe the expected influence that a second ligand Y can have on the apparent constants $K_a^{0\prime}$, $K_{ix}^{0\prime}$, and Q'.

Results

Qualitatively, the analysis just set fourth should be applicable to the study of rat liver phosphofructokinase because it has been observed that allosteric modifiers predominantly affect the apparent K_a for Fru-6-P (Reinhart & Lardy, 1980a). Most have little, if any, effect on either maximal velocity or the behavior of MgATP as a substrate (Bloxham & Lardy, 1973; Reinhart & Lardy, 1980a). Fundamental to the regulatory properties of this enzyme is the allosteric inhibition by MgATP. It should be possible to obtain a description of this inhibition in terms of the three parameters set fourth in eq 2, namely, an apparent "true" K_a for Fru-6-P in the absence of any inhibition by MgATP, an apparent dissociation constant of MgATP from free enzyme, and the free energy of interaction between Fru-6-P and MgATP. Once obtained, the mechanistic impact of a second allosteric ligand, in this case H^+, can be evaluated by determining its influence on these three parameters. Although it might be expected that the final quantitative relationship between these parameters will ultimately prove to be more complex than that given by eq 9 (see Discussion), this equation, as well as eq 5, should provide a good starting point for the following analysis.

The effect on the activity of rat liver phosphofructokinase that MgATP has when varied over a very wide concentration range is shown as a function of log [Fru-6-P] in Figure 2. These data were obtained at pH 7.5 with total MgATP concentration varied from 0.1 to 100 mM. Two effects are apparent at high concentrations of MgATP. The first is a decrease in the apparent maximal velocity. The second is a lessening of the relative influence that MgATP has on the concentration of Fru-6-P producing half-maximal velocity.

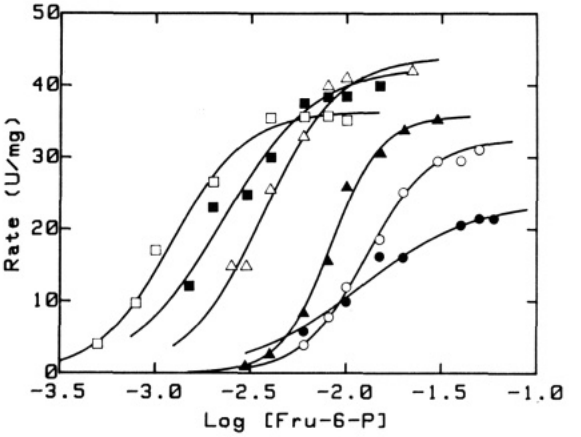

FIGURE 2: Influence of MgATP on the acitivity of rat liver phosphofructokinase expressed as a function of log [Fru-6-P] at pH 7.5. Curves were determined from the Hill plots described under Materials and Methods. MgATP concentrations are 0.2 (□), 0.5 (■), 2.0 (△), 10 (▲), 35 (○), and 100 mM (●). For clarity, the results obtained for only six concentrations of MgATP are shown whereas typically 10–12 concentrations of MgATP in the range of 0.1–100 mM are examined.

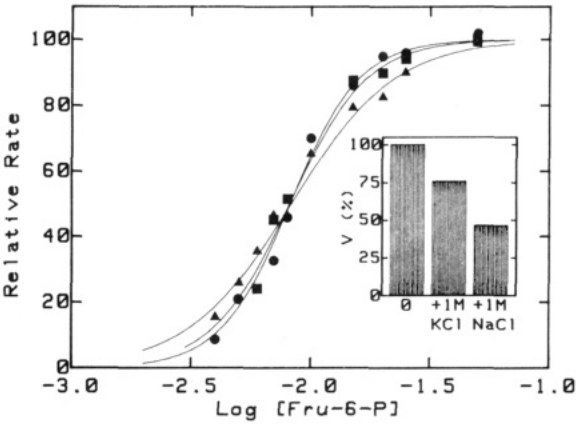

FIGURE 3: Influence of high salt concentration on the apparent K_a for Fru-6-P. Conditions of each assay were as described under Materials and Methods with [MgATP] = 1 mM and pH 7.5. Salt concentrations were 0 (●), 1.0 M KCl (■), or 1.0 M NaCl (▲). These concentrations were in addition to the 100 mM KCl, 50 mM HEPES–KOH, and other ions normally present in each assay. pH was rechecked and readjusted as necessary after the addition of the high salt. The apparent maximal velocity for each curve was normalized to 100. The inset shows the relative effects of 1 M additional KCl or NaCl on maximal velocity.

The possibility that these two effects are caused by the ionic strength or cation concentration increases that accompany the high MgATP concentrations is addressed in Figure 3. The Fru-6-P saturation profile was measured at 1 mM MgATP in the presence of either 1 M NaCl or 1 M KCl at pH 7.5. Although high salt concentration has a substantial effect on maximal velocity, the apparent Michaelis constant is not significantly affected.

Data similar to that presented in Figure 2 were obtained over a pH range of 5.5–9.0. The dependence of the apparent Michaelis constant for Fru-6-P on the MgATP concentration for each pH tested is summarized in Figure 4. The curves drawn in Figure 4 represent the expected behavior when data for each pH were fit to eq 9, which apparently provides a good

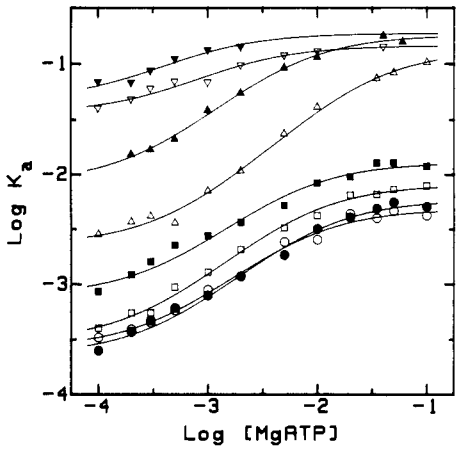

FIGURE 4: Influence of pH on the MgATP dependence of the apparent K_a for Fru-6-P. Points represent the concentration of Fru-6-P yielding half-maximal velocity as determined from Hill plots as described under Materials and Methods. Curves represent the best fit of the data for each pH to eq 9 given in the text. pH values are 9.0 (○), 8.5 (●), 8.0 (□), 7.5 (■), 7.0 (△), 6.5 (▲), 6.0 (▽), and 5.5 (▼). Compare the pattern of these curves to the alternatives illustrated in Figure 1 and note the similarity to Figure 1b.

description of the influence of MgATP on K_a. The fitting was performed by assuming a constant relative error in each K_a determination. This assumption introduces a weighting factor of $(1/K_a)^2$ into the nonlinear regression procedure (Cleland, 1967). Preliminary estimates for the parameters $K_{ix}^{0\prime}$ and Q' were obtained by performing a least-squares regression to the following linear form of eq 9:

$$\frac{1}{r-1} = \frac{1}{[X]}\left(\frac{K_{ix}^{0\prime}}{1-Q'}\right) + \frac{Q'}{1-Q'} \qquad (10)$$

where $r = K_a/K_a^{0\prime}$ (Reinhart, 1983). If M is the slope and B is the intercept of a plot of $1/(r-1)$ vs. $1/[X]$ where X = MgATP, it follows from eq 10 that $K_{ix}^{0\prime} = M/(B+1)$ and $Q' = B/(B+1)$. The preliminary estimate of $K_a^{0\prime}$ was taken to be the value that provided the best line for the data plotted according to eq 10. In performing this linear regression, a constant relative error in r was also assumed.

The logarithm of the values obtained for $K_a^{0\prime}$, $K_{ix}^{0\prime}$, and Q' from the direct nonlinear regression analysis are plotted as a function of pH in Figure 5. The error bars represent plus or minus the standard error for each determination. It is immediately apparent that of the three parameters only $K_a^{0\prime}$ varies substantially and systematically with pH. Moreover, the slope of the log–log presentation of this variation is approximately equal to 1, suggesting that a single protonation is involved.

All of the data from Figure 4 were then fit to eq 5, which describes the expected behavior of the apparent K_a as a function of two allosteric ligands X and Y. In this case, X = MgATP and Y = H^+. Preliminary estimates of the parameters were made by inspection of Figure 5 as follows: $K_a^0 = K_a^{0\prime}$ when $H^+ = 0$ (i.e., the high-pH limiting value) = 0.2 mM; $K_{ix}^0 = K_{ix}^{0\prime}$ at the high-pH limit = 0.3 mM; $K_{iy}^0 = 10^{-\log(pH)}$ of the transition in $K_a^{0\prime} = 1 \times 10^{-8}$; $Q_{ax} = Q'$ at the high-pH limit = 0.04; Q_{ay} = the ratio of $K_a^{0\prime}$ at the high-pH limit to $K_a^{0\prime}$ at the low-pH limit = 0.004; Q_{xy} = ratio of $K_{ix}^{0\prime}$ at the high-pH limit to $K_{ix}^{0\prime}$ at the low-pH limit = 1; $Q_{axy} = Q_{ay}Q_{xy}$(Q' at the low-pH limit) = 0.001 (this relationship can be obtained from eq 8 by letting [Y] = ∞). By use of these preliminary estimates, the nonlinear regression analysis converges[5] and

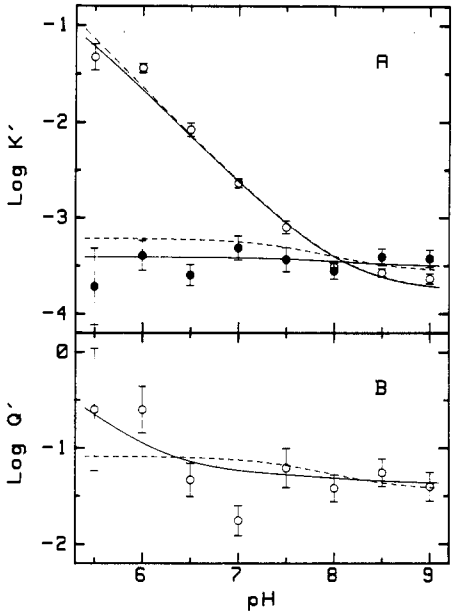

FIGURE 5: Influence of pH on the parameters describing the inhibition of rat liver phosphofructokinase by MgATP. (A) Logarithm of the apparent Michaelis constant for Fru-6-P in the absence of MgATP, $K_a^{0\prime}$ (○); logarithm of the apparent dissociation constant of MgATP in the absence of Fru-6-P, $K_{ix}^{0\prime}$ (●). (B) Logarithm of the apparent coupling term between MgATP and Fru-6-P, Q' (○). Values were obtained by fitting the data presented in Figure 4 for each pH to eq 9 as described in the text. Error bars indicate plus or minus standard error for each determination. Significance of the solid and dashed curves is described in the text.

Table I: Parameters Describing the Combined Influence of MgATP and H^+ on the Apparent Michaelis Constant for Fru-6-P[a]

	model I	model II
K_a^0 (mM)	0.171 ± 0.040	0.172 ± 0.044
K_{ix}^0 (mM)	0.31 ± 0.10	0.27 ± 0.10
K_{iy}^0 (M)	(7.6 ± 2.5) × 10^{-9}	(7.6 ± 2.3) × 10^{-9}
pK_{iy}^0	8.12 ± 0.14	8.12 ± 0.13
Q_{ax}	0.042 ± 0.023	0.036 ± 0.021
ΔG_{ax} (kcal/mol)	1.87 ± 0.31	1.96 ± 0.34
Q_{ay}	(4.2 ± 5.6) × 10^{-4}	
ΔG_{ay} (kcal/mol)	4.61 ± 0.79	
Q_{xy}	0.80 ± 0.44	0.44 ± 0.25
ΔG_{xy} (kcal/mol)	0.13 ± 0.32	0.48 ± 0.34
Q_{axy}	(4.0 ± 2.7) × 10^{-4}	
ΔG_{axy} (kcal/mol)	4.63 ± 0.40	

[a] Values were obtained by fitting data presented in Figure 4 to either eq 5 (model I) or eq 17 (model II). Parameter definitions are given in the text with A = Fru-6-P, X = MgATP, and Y = H^+. Coupling free energies were calculated according to eq 3 with T = 25 °C.

yields the values and standard errors for these parameters listed in Table I under model I. The behavior of the apparent constants $K_a^{0\prime}$, $K_{ix}^{0\prime}$, and Q' as a function of pH that is predicted by these values according to eq 6–8 is shown by the solid curves in Figure 5.

[5] The search strategy of Cleland (1967) has been modified in the fitting of this equation. Because of the large number of parameters, small errors in the preliminary estimates can cause the search to "blow up" and miss convergence. We have limited the change in each subsequent parameter estimation to 10%. This slows the progression to convergence considerably but allows for greater tolerance in preliminary estimates. For the example cited in the text, convergence was obtained after 22 iterations. The regression analysis was considered to have converged when a new estimate for each parameter changed its value by no more than 0.01%.

Discussion

There is obvious concern over the interpretation of effects of high MgATP concentration because of the high ionic strength and cation concentrations that accompany these experiments. The data in Figure 3 suggest that ionic strength or cation effects do not influence significantly the parameters most pertinent to this discussion, namely, the apparent K_a for Fru-6-P. The same is not true, however, for the maximal velocity or degree of cooperativity. A complete kinetic description of phosphofructokinase needs to include these parameters, and it will be a more difficult task to separate the influence specifically due to MgATP from those associated with general salt effects.

One must also consider the degree of complexation that ATP may assume with the various cations in solution, particularly at the high end of the concentration range of MgATP and Fru-6-P studies. It has previously been observed that MgATP is preferred over free ATP as the inhibitory ligand (Reinhart & Lardy, 1980a). Pettigrew & Frieden (1979a) have calculated the distribution of the various complex forms of ATP under conditions virtually identical with those used in this study at low concentration. They found that, with total [ATP] < 1 mM, total [$MgCl_2$] = 5 mM, and ionic strength = 0.13, 90% of the ATP existed as $MgATP^{2-}$. In the experimental design described herein, total ATP and total $MgCl_2$ are increased above 1 mM in unison, with a 5 mM $MgCl_2$ concentration continually maintained in excess. Increasing ionic strength up to 0.42 has not been found to change the complexation equilibrium constant significantly (Phillips et al., 1966). Mass action would cause, if anything, an even greater relative proportion of the total ATP to exist as MgATP. Consequently, there would be at most a 10% error involved in assuming that total ATP concentration is equal to the actual concentration of the inhibiting species, and any systematic error associated with this assumption would work against seeing the limit to the inhibition by ATP evident in Figure 4.

No attempt has been made to correct for the complexation of Fru-6-P with Mg^{2+}, which occurs with a K_d = 25.7 mM (Schwarzenback & Anderegg, 1957), primarily because it is not known to what extent this complexation alters the behavior of Fru-6-P as a substrate. If one did assume that MgFru-6-P is not a substrate for phosphofructokinase, however, the concentration of MgFru-6-P relative to total Fru-6-P concentration varies from 16 to 4% as the total Fru-6-P concentration varies from 0.1 to 100 mM. Therefore, this complexation would also not be the cause of the plateaus seen in Figure 4 at high MgATP concentration, and the error introduced in the apparent K_a's would be no greater than 10%.

In considering the energetics of small molecular weight ligand binding to proteins, Weber (1972, 1975) concluded that the absolute value of the free energy of interaction between two ligands binding to separate binding sites should be no more than approximately 2 kcal/mol for ligands with individual dissociation constants in the range of 10^{-2}–10^{-6} M. This conclusion arises from a recognition that binding energies, as well as the stabilization energies of protein tertiary structure, are obtained from many interactions with individual energetic magnitudes no greater than 1 kcal/mol. In every case where coupling free energies have been measured, this expectation has been confirmed (Weber, 1975). Consequently, the mutual binding of both ligands at high concentration is expected to occur. In other words, to use liver phosphofructokinase as an example, there is a limit to the expected magnitude of the antagonism between the binding of MgATP and Fru-6-P. Saturation of the MgATP binding site can be achieved such that further increases in MgATP concentration should have no further effect on the binding of Fru-6-P. This is exactly the type of behavior observed in the data presented in Figures 2 and 4 and contrasts with the behavior predicted by models in which MgATP and Fru-6-P exclusively to different enzyme forms (Frieden et al., 1976; Goldhammer & Hammes, 1978; Pettigre & Frieden, 1979a,b). For this latter case, one would expect no limit to the competitive nature of antagonism between MgATP and Fru-6-P at high concentration (Reinhart, 1983). Moreover, the magnitude of the coupling free energy between Fru-6-P and MgATP, given in Table I as ΔG_{ax}, is 1.9 kcal/mol, exactly within the magnitude range predicted by Weber.

At first glance it seems somewhat fortuitous that a hyperbolic equation such as eq 9 adequately describes data from an enzyme so notorious for its cooperative behavior. Upon closer examination, however, this result can be rationalized. Although phosphofructokinase is well-known to be very cooperative in its response to Fru-6-P, the applicability of eq 9 as utilized in this study is dependent upon the nature of the saturation profile of MgATP since [MgATP], and not [Fru-6-P], is the independent variable.

Ignoring possible effects on aggregation state, there are three mechanisms that can contribute to cooperativity in the saturation profile of a given ligand: homotropic, subsaturating heterotropic, and heterotropically induced homotropic interactions (Reinhart, 1983). Homotropic effects arise from nonzero coupling free energies between the various binding sites for the same ligand and should therefore be manifest in the saturation profile in the absence of all other ligands. Heterotropically induced homotropic interactions occur when the binding of another ligand causes there to be a finite coupling free energy between the binding sites of the same ligand and should therefore be apparent at saturating concentrations of the other ligands. In either case, however, the cooperativity would not be a function of the linkage between heterologous sites and therefore not be influenced by the inherent cooperativity displayed by the other ligand. Consequently, the fact that the binding of Fru-6-P shows an apparent high degree of positive cooperativity does in no way require that the binding of MgATP be similarly cooperative with respect to these mechanisms. In light of this distinction, it is significant that Goldhammer & Hammes (1978) and Pettigrew & Frieden (1979a) have found that the binding of MgATP by muscle phosphofructokinase in the absence of other ligands is noncooperative. Though somewhat at variance with our intuitive expectations regarding the complexity of phosphofructokinase, such an observation need not be surprising theoretically.

On the other hand, subsaturating heterotropic cooperativity is caused by a nonzero coupling interaction between different ligands at more than one binding site for each when the nonvaried ligand concentration is not saturating (or zero) and must be reciprocally apparent in the binding profiles of either ligand. Since the active phosphofructokinase species under normal assay conditions is predominantly a tetramer (Reinhart & Lardy, 1980b) consisting of four binding sites each for Fru-6-P and MgATP, this mode of cooperativity is quite likely. Weber (1972, 1975) has shown that this type of cooperativity must always appear positive regardless of whether the free energy of interaction between the different ligands is positive or negative. Indeed, most forms of phosphofructokinase do display an increase in Hill coefficient of the Fru-6-P saturation profile at substrating concentrations of MgATP. Such effects must equally pertain to the saturation profile of MgATP. However, subsaturating heterotropic cooperativity should also

be symmetrical about the half-saturation point of a binding profile and consequently not affect the form of eq 9 as shown in the following:

To simplify the algebra, let us consider an enzyme with two binding sites each for A and X such as might occur in a dimer. Moreover, let Q be the coupling parameter between A and each X. Ignoring modifier-induced effects on maximal velocity, the initial velocity expression should be analogous to the saturation profile described by Weber (1975):

$$v/E_T = \frac{V^0(K_{a2}[A] + [A]^2)}{K_{a1}K_{a2} + 2K_{a2}[A] + [A]^2} \quad (11)$$

where

$$K_{a1} = K_a^0\left[\frac{K_{ix}^0 + [X]}{K_{ix}^0 + Q[X]}\right]^2 \quad (12)$$

$$K_{a2} = K_a^0\left[\frac{K_{ix}^0 + Q[X]}{K_{ix}^0 + Q^2[X]}\right]^2 \quad (13)$$

The concentration of A that gives half-maximal velocity, which we have operationally defined as the apparent Michaelis constant, K_a, can be derived from eq 11 to be equal to

$$K_a = (K_{a1}K_{a2})^{1/2} \quad (14)$$

Substituting eq 12 and 13 into eq 14 yields

$$K_a = K_a^0\left[\frac{K_{ix}^0 + [X]}{K_{ix}^0 + Q^2[X]}\right] \quad (15)$$

Since eq 15 has the same form as eq 9, we can conclude that the existence of subsaturating heterotropic cooperativity would only influence our interpretation of the magnitude of Q but otherwise not affect the applicability of eq 9 to describe the behavior of the apparent Michaelis constant for Fru-6-P as a function of MgATP concentration.

The value of Q obtained by a fit to eq 9 therefore represents an upper limit to actual coupling between two heterologous sites. If each MgATP only interacts with one Fru-6-P, then its coupling is 1.9 kcal/mol. If each MgATP interacts equally with all four Fru-6-P ligands, the individual couplings would be equal to $-RT \ln [(0.042)^{0.25}] = 0.47$ kcal/mol. Reality probably lies somewhere in between, and 1.9 kcal/mol represents the sum of all of the coupling interactions between a single MgATP and the various Fru-6-P ligands (and correspondingly between a single Fru-6-P and the several MgATP ligands) per functional oligomer.

In a system of three ligands, in this case H^+, MgATP, and Fru-6-P, four different coupling free energies exist that correspond to the four Q terms in eq 4. Three of these terms represent interactions between each possible pairing of ligands, and as such, they are subject to the same expectation with regard to magnitude as discussed above.

It is notable, therefore, that the coupling free energy between H^+ and Fru-6-P ($\Delta G_{ay} = 4.6$ kcal/mol; see Table I) is significantly greater than 2 kcal/mol. This large value might result from either multisite interactions or mutual exclusivity between Fru-6-P and H^+. If each protonation equally influenced all four Fru-6-P binding sites, the individual coupling free energies would equal (4.6)(0.25) = 1.15 kcal/mol. Alternatively, H^+ and Fru-6-P might bind at the same site in an exclusive manner in which case an infinite coupling energy ($Q_{ay} = 0$) would be predicted. Since the standard error in Q_{ay} is greater than its value, this possibility should be considered further.

Any model that provides for $Q_{ay} = 0$ should also anticipate that $Q_{axy} = 0$ because of the identity (Reinhart, 1983):

$$\Delta G_{axy} = \Delta G_{ax} + \Delta G_{ay} + \Delta G_{xy/a}$$

or equivalently

$$Q_{axy} = Q_{ax}Q_{ay}Q_{xy/a} \quad (16)$$

where $\Delta G_{xy/a}$ and $Q_{xy/a}$ represent the coupling terms between ligands X and Y in the saturating presence of A. Consequently, the equation describing the behavior of the apparent Michaelis constant for A when A and Y are mutually exclusive can be obtained from eq 5 by letting $Q_{ay} = Q_{axy} = 0$, resulting in

$$K_a = K_a^0\left[\frac{K_{iy}^0[X] + K_{ix}^0[Y] + Q_{xy}[X][Y] + K_{ix}^0K_{iy}^0}{K_{iy}^0Q_{ay}[X] + K_{ix}^0K_{iy}^0}\right] \quad (17)$$

When all of the data in Figure 4 are fit to eq 17, the resulting parameters are those listed in Table I under model II. The behavior of $K_a^{0\prime}$, $K_{ix}^{0\prime}$, and Q' predicted by those parameters is shown by dashed lines in Figure 5.

By inspection of Figure 5, we see that both models I and II fit the available data well, predicting significantly different behavior only at low pH where accurate data are difficult to obtain. Because the overall standard errors of fit in the two cases are not appreciably different, the additional two parameters appearing in model I cannot be statistically justified by the available and model II should be favored.

Both models are consistent, however, in ascribing the most substantial effect of pH to an influence on $K_a^{0\prime}$, the apparent K_a for Fru-6-P in the absence of MgATP. The pH effect is not mediated through the MgATP inhibition. In fact, if anything, the apparent dissociation constant for MgATP, $K_{ix}^{0\prime}$, increases slightly at low pH, and the effectiveness of MgATP, as measured by Q', decreases.

These conclusions stand in contrast to the conclusions reached on the basis of binding data (Pettigrew & Frieden, 1979a) and intrinsic fluorescence quenching studies (Pettigrew & Frieden, 1979a; Kitajima et al., 1983) performed with phosphofructokinase isolated from rabbit muscle. In those studies, lowering the pH was found to decrease the apparent dissociation constant for MgATP. This descrepancy probably reflects a very real difference in the molecular mechanism of the allosteric regulation of these two isozymes, which is consistent with earlier observations that, unlike the muscle enzyme, liver phosphofructokinase continues to exhibit significant cooperativity and MgATP inhibition even at high pH (Reinhart & Lardy, 1980a).

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Tryptophan Residues of Creatine Kinase: A Fluorescence Study†

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ABSTRACT: Spectroscopic studies of rabbit skeletal muscle creatine kinase (CPK) and its complexes with adenosine phosphates have long suggested the occurrence of a tryptophan residue at or near the coenzyme binding sites [Kägi, J. H. R., Li, T.-K., & Vallee, B. L. (1971) *Biochemistry 10*, 1007-1015; Price, N. C. (1972) *FEBS Lett. 24*, 21-23]. This conjecture was further supported by nuclear Overhauser effect (NOE) ^{1}H NMR studies indicating through-space interactions between protons of the adenine ring of bound ADP and one or more aromatic side chains of the proteins [Vašák, M., Nagayama, K., Wüthrich, K., Mertens, M. L., & Kägi, J. H. R. (1979) *Biochemistry 18*, 5050-5055]. Further evidence for a tryptophan residue in the environment of the active site has now been obtained by fluorescence-quenching studies using iodide and acrylamide as external quenchers. Thus, while by the addition of iodide the tryptophan fluorescence of unliganded CPK is reduced to about 75% of the unquenched control, no such effect is manifested upon addition of this quencher to the CPK·ADP and CPK·ATP complexes. Similarly, the relative effectiveness of quenching of the CPK–coenzyme complexes by acrylamide is only about 60% of that measured in the unliganded enzyme. Both these data and the spectral characteristics of the quenched fluorescence suggest that coenzyme binding perturbs a tryptophan residue that is close to the active site and that is partially exposed to the solvent. The differential effectiveness of external quenchers on unliganded and liganded CPK allows the determination of the ligand binding equilibria by fluorescence-quenchability titration. The values obtained for complexes of CPK with ATP, ADP, and AMP are in good agreement with those obtained from other measurements.

Although the spatial structure of creatine kinase (CPK)[1] is still unknown, much information has been collected over the past 25 years on the organization of the binding sites of its substrates ADP/ATP and creatine phosphate/creatine. By chemical and physical studies carried out in the presence and absence of coenzymes and substrates, a number of amino acid residues have been identified at or near the active center of CPK. Thus, the reaction with certain thiol reagents resulted in derivatization of a single active site cysteine (Mahowald et al., 1962) whose proximity to the substrate binding site was demonstrated by magnetic resonance techniques using a covalently attached spin-label (Taylor et al., 1971; McLaughlin et al., 1976). From analogous chemical modification studies, the presence of lysine (Kassab et al., 1968), arginine (Borders & Riordan, 1975), and histidine (Pradel & Kassab, 1968) at the active site was also inferred.

There is also some evidence for the occurrence of aromatic amino acids, especially tryptophan, at the active center. Thus, difference absorption spectra reflecting perturbation of tryptophanyl residues of the protein were observed on binding of ADP or ATP to CPK (Noda, 1963; Roustan et al., 1968). This interaction was proposed to be the basis of the large extrinsic Cotton effect of the CPK–coenzyme complexes (Kägi et al., 1971). The presence of a tryptophan near the coenzyme binding site was also suggested by the observation that a part of the tryptophan fluorescence of CPK is quenched on coenzyme binding (Price, 1972; Mertens, 1978) and is consistent with ^{1}H NMR data, which, on the basis of measurements of "truncated driven nuclear Overhauser effects", indicated that the adenine ring of the coenzymes is close to the location of

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[1] Abbreviations: CPK, creatine kinase (adenosine-5′-triphosphate: creatine *N*-phosphotransferase, EC 2.7.3.2); Gdn·HCl, guanidine hydrochloride.

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