Kinetic Linked-Function Analysis of the Multiligand Interactions on Mg^{2+}-Activated Yeast Pyruvate Kinase†

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ABSTRACT: The multiligand interactions governing the allosteric response of Mg^{2+}-activated yeast pyruvate kinase (YPK) during steady-state turnover were quantitated by kinetic linked-function analysis. The substrate, PEP, the enzyme-bound divalent metal, Mg^{2+}, and the allosteric effector, FBP, positively influence each other's interaction with the enzyme in the presence of saturating concentrations of the second substrate, MgADP. The presence of Mg^{2+} enhances the interaction of PEP and of FBP with YPK by −2.0 and −1.0 kcal/mol, respectively. The simultaneous interaction of PEP, Mg^{2+}, and FBP with YPK is favored by −4.1 kcal/mol over the sum of their independent binding free energies. The coupling free energies measured for Mg^{2+}-activated YPK are weaker than the corresponding coupling free energies measured for Mn^{2+}-activated YPK [Mesecar, A., and Nowak, T. (1997) *Biochemistry 36*, 6792, 6803], but are consistent with results of thermodynamic measurements with the Mg^{2+}−YPK complex [Bollenbach, T. J., and Nowak, T. (2001) *Biochemistry 36*, 13088−13096]. A comparison of ligand binding data measured by kinetic and thermodynamic linked-function analyses reveals that the MgADP complex modulates both the binding of the other three ligands and the two- and three-ligand coupling interactions between the other three ligands. Enzyme-bound Mg^{2+} does not influence the homotropic cooperativity in PEP binding to YPK. It is the MgADP complex that induces homotropic cooperativity in PEP binding. It is the enzyme-bound Mn^{2+} that induces homotropic binding of PEP with Mn^{2+}-activated YPK. These results lend support to the hypothesis that divalent metals modulate the interactions of ligands on YPK and that divalent metals play a role in regulation of the glycolytic pathway.

Yeast pyruvate kinase (YPK)[1] is a key regulatory enzyme of the glycolytic pathway. PK catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate with concomitant phosphorylation of adenosine 5′-diphosphate (ADP) to ATP. YPK requires one monovalent cation and two divalent cations per active site for activity. The physiological monovalent cation is probably K^+, and the physiological divalent cation is probably Mg^{2+} (*1*). One equivalent of divalent metal binds at an enzyme site and helps to orient the substrate phosphoryl groups and to bridge and stabilize the phosphoryl transfer reaction during catalysis. The enzyme-bound divalent metal has also been implicated in modulating the heterotropic interaction between the substrate, PEP, and the allosteric activator fructose 1,6-bisphosphate (FBP) (*2*, *3*). A second divalent metal enters the active site as a chelate to the substrate, ADP (*4*).

The multiligand interactions of PEP, Mg^{2+}, and FBP with YPK were studied by steady-state fluorescence titrations (*19*). The results demonstrate that Mg^{2+}- and Mn^{2+}-activated YPK have significantly different ligand binding properties. PEP binds to the YPK−Mn^{2+} complex with positive homotropic cooperativity. The kinetic interaction of PEP and Mn^{2+} with the enzyme is also positively cooperative (*2*). With Mg^{2+}-activated YPK, the coupled interaction between PEP and Mg^{2+} was weaker and Mg^{2+} did not heterotropically induce homotropic binding of PEP to the enzyme. The velocity response of YPK to variable PEP concentrations in the absence of FBP is sigmoidal, indicating positive kinetic cooperativity. The current kinetic study was undertaken to more clearly define the kinetic allosteric properties of Mg^{2+}-activated YPK. This study also defines the role of the second substrate, MgADP, in modulation of the activity of YPK and compares the kinetic heterotropic effects of Mn^{2+}- and Mg^{2+}-activated YPK.

EXPERIMENTAL PROCEDURES

Materials. L-(+)-Lactate dehydrogenase was purchased from Boehringer-Mannheim. Yeast pyruvate kinase was purified according to the method of Mesecar and Nowak (*2*). PEP, ADP, FBP, and buffers were purchased from Sigma. All other chemicals were reagent grade or better.

Pyruvate Kinase Assay. Pyruvate kinase was assayed according to the methods described by Bücher and Pfleiderer (*5*). The change in absorbance at 340 nm was measured as a function of time using a Gilford 240 spectrophotometer. Typical assays (1 mL) contained 100 mM MES (pH 6.2), 4% glycerol, 200 mM KCl, 15 mM $MgCl_2$, 5 mM PEP, 5 mM ADP, 175 μM NADH, 20 μg of LDH, and 1−1.5 μg of pyruvate kinase. The specific activity of pyruvate kinase

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; FBP, fructose 1,6-bisphosphate; M^{2+}, divalent metal ion; MADP, divalent metal−ADP complex; MES, 2-(*N*-morpholino)ethanesulfonic acid; NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; YPK, yeast pyruvate kinase.

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is expressed as the number of micromoles of NADH oxidized per milliliter per minute per milligram of protein. The concentration of YPK was determined by its absorbance at 280 nm and using an extinction coefficient ϵ_{280} of 0.51 mL/mg (*6*). The specific activity was determined prior to all experiments.

Initial rates of pyruvate formation were measured with PEP as the variable substrate and FBP as the fixed variable substrate at the indicated concentrations. These individual three-dimensional experiments were performed at fixed, variable concentrations of free Mg^{2+} as indicated. The free Mg^{2+} concentration was calculated from the measured concentrations of total Mg^{2+} and total ADP with a K_d of 0.25 mM for the dissociation constant of the MgADP complex (*7*). The total Mg^{2+} concentration was determined by atomic absorption spectroscopy. The concentration of MgADP, the second substrate for the YPK reaction, was held constant at 6.10 mM throughout this study unless otherwise indicated. In all of the kinetic studies that were performed, when appropriate kinetic parameters were to be measured, at least 10, but more often 20−25 data points were obtained. The data were then treated appropriately as described below.

Treatment of Kinetic Data. Initial rates were measured from slopes of the linear portions of progress curves at each concentration of substrate (S). The specific activity at each [S] was plotted versus [S] and fit to either the Michaelis−Menten equation (eq 1) or the Hill equation (eq 2).

$$v = \frac{V_{max}[S]}{K_m + [S]} \tag{1}$$

$$v = \frac{V_{max}[S]^n}{K_m + [S]^n} \tag{2}$$

where v is the initial rate at each [S], V_{max} is the maximal velocity, and K_m is the Michaelis constant, the concentration of substrate producing half-maximal velocity. The Hill coefficient, n_H, has a value of 1.0 in the Michaelis−Menten rate equation.

Kinetic Linked-Function Analysis. The multiligand interactions between PEP, Mg^{2+}, and FBP were quantitated by kinetic linked-function analysis. The analysis was described in detail previously (*3*) and is outlined here. The equations describing the multiligand interactions with an enzyme undergoing steady-state turnover were derived by Reinhart (*8*−*10*). The dependence of the apparent K_m of one ligand on the concentration of a second ligand is described by eqs 3−6.

$$K_A = K_A{}^0\left(\frac{K_X{}^0 + [X]}{K_X{}^0 + Q_{AX}[X]}\right) \tag{3}$$

$$K_A = K_A{}^0\left(\frac{K_Y{}^0 + [Y]}{K_Y{}^0 + Q_{AY}[Y]}\right) \tag{4}$$

$$K_X = K_X{}^0\left(\frac{K_Y{}^0 + [Y]}{K_Y{}^0 + Q_{XY}[Y]}\right) \tag{5}$$

$$Q_{AX}' = \frac{(K_Y{}^0 + [Y])(K_Y{}^0 Q_{AX} + Q_{AXY}[Y])}{(K_Y{}^0 + Q_{AY}[Y])(K_Y{}^0 + Q_{XY}[Y])} \tag{6}$$

In eqs 3−6, PEP, FBP, and Mg^{2+} are designated as A, X, and Y, respectively. K_A is the concentration of ligand A giving half-maximal velocity. $K_A = K_A{}^0$ when [X] = [Y] = 0. $K_X{}^0$ is the dissociation constant of ligand X when [A] = [Y] = 0. $K_Y{}^0$ is the dissociation constant of ligand Y when [A] = [X] = 0. Q_{AX} is the coupling parameter for coupling between enzyme-bound A and enzyme-bound X. Q_{AY} is the coupling parameter for coupling between bound A and bound Y. Q_{XY} is the coupling parameter for coupling between bound X and bound Y. Coupling parameters describe the magnitude of the influence that the presence of one ligand on the enzyme has on the affinity of the enzyme for the other ligand. Q is related to the free energy of interaction, or coupling free energy, between two ligands by eq 7.

$$\Delta G_{AX} = -RT \ln Q_{AX} \tag{7}$$

where ΔG_{AX} is the coupling free energy, R is the gas constant, and T is the temperature in kelvin. If $Q_{AX} > 1$, the ΔG_{AX} is negative and the interaction between A and X is termed positively cooperative. If $Q_{AX} < 1$, ΔG_{AX} is positive and the interaction between A and X is termed antagonistic. If $Q_{AX} = 1$, then the two ligands do not influence the binding of each other and their interaction is termed independent.

Steady-State Fluorescence Titrations. Experiments were performed on an SLM-Aminco 8100 spectrofluorimeter thermostated to 25 °C. Fluorescence titrations were performed by monitoring the steady-state fluorescence intensity of the enzyme at 334 nm with an excitation wavelength of 295 nm, both at a bandwidth of 2 nm. Titrations were performed by adding aliquots of a stock FBP solution, usually 1−10 μL, to a 900 μL sample containing 100 mM MES (pH 6.2), 4% glycerol, 200 mM KCl, approximately 0.05 mg/mL YPK, and fixed, variable concentrations of Mg^{2+}, as specified. Percent fluorescence quenching at each addition of ligand was corrected for dilution of the enzyme. All experiments were performed in triplicate and the data averaged. Fluorescence quenching data were fit to eq 8.

$$F = \frac{F_{max}[L]^n}{K_d + [L]^n} \tag{8}$$

which is analogous to the Hill equation. F_{max} represents the maximal percent fluorescence quenching. L represents the titrated ligand. K_d represents the concentration of ligand giving half-maximal quenching. n represents the Hill coefficient.

RESULTS

Steady-State Kinetics. A series of kinetic measurements were performed with Mg^{2+} as the activating divalent cation. The velocity responses to variable concentrations of PEP, free Mg^{2+}, and MgADP were measured with the other ligands kept constant and saturating. These studies were performed in the presence and absence of FBP. The activation response of YPK to FBP was also measured. The results were fit to the appropriate equation, and the constants are summarized in Table 1. V_{max} for Mg^{2+}-activated YPK (257 ± 4 units/mg) is approximately 4-fold higher than for the Mn^{2+}-activated enzyme (56 ± 2 units/mg). The V_{max} for Mg^{2+}-activated YPK is similar in the presence (257 ± 4

Table 1: Steady-State Kinetic Parameters for Mg^{2+}-Activated YPK[a]

ligand	FBP[b]	K_m (mM)	n_H
PEP	without	1.99 ± 0.06	2.41 ± 0.15
PEP	with	0.103 ± 0.004	1.0
Mg^{2+}	without	0.509 ± 0.021	1.42 ± 0.07
Mg^{2+}	with	0.093 ± 0.007	1.0
MgADP	without	0.715 ± 0.020	1.0
MgADP	with	0.667 ± 0.078	1.0
FBP		0.0128 ± 0.0001	1.0

[a] Nonvaried substrates were held saturating: [PEP] = 20 mM, [Mg^{2+}] = 15 mM, and [MgADP] = 6.1 mM. Kinetic constants were calculated from fits of either eq 1 or 2 to initial velocity data as a function of variable ligand concentration. [b] When present as a nonvaried activator, FBP was at a concentration of 1 mM.

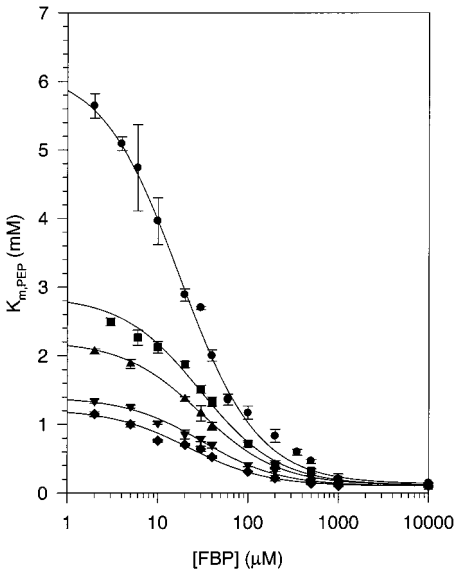

FIGURE 1: Influence of free Mg^{2+} on the FBP dependence of $K_{m,PEP}$. The $K_{m,PEP}$ is plotted as a function of FBP concentration. The concentrations of free Mg^{2+} are 1.09 (●), 2.90 (■), 5.23 (▲), 10.1 (▼), and 20.1 mM (◆). The data at each concentration of Mg^{2+} were fit to eq 3. Curves represent the best fit of eq 3 to the data. Error bars represent the standard error in the fit of the $K_{m,PEP}$ at each FBP concentration.

units/mg) and absence (224 ± 4 units/mg) of FBP. FBP causes a 20-fold decrease in the $K_{m,PEP}$ and a 5-fold decrease in the $K_{m,Mg^{2+}}$. Because FBP has no significant effect on V_{max} but modulates the affinity of the enzyme for substrates, FBP is a "K-type" allosteric effector of YPK. FBP heterotropically abolishes the homotropic cooperativity in the velocity response of YPK as a function of variable PEP or variable Mg^{2+} concentration. At saturating Mg^{2+} and MgADP concentrations and subsaturating PEP concentrations, the velocity response of YPK as a function of FBP concentration is hyperbolic. FBP binds to the YPK−Mg^{2+}−PEP complex with positive cooperativity (*19*). Therefore, MgADP must influence the homotropic cooperativity in FBP binding to YPK.

Multiligand Interactions between PEP, Mg^{2+}, and FBP. The velocity response of YPK as a function of variable PEP concentration was investigated as a function of FBP concentration at fixed, variable concentrations of free Mg^{2+} (Figure 1). The $K_{m,PEP}$ decreases with increasing FBP concentrations until a plateau is reached. This suggests that FBP positively influences the interaction of PEP with YPK. $K_{m,PEP}$ at saturating FBP concentrations is approximately 100 μM at all concentrations of free Mg^{2+}. At subsaturating FBP concentrations, the $K_{m,PEP}$ decreases as the free Mg^{2+} concentration increases. This suggests that under kinetic conditions and in the absence or at subsaturating FBP concentrations, the interaction between free Mg^{2+} and PEP is positively cooperative.

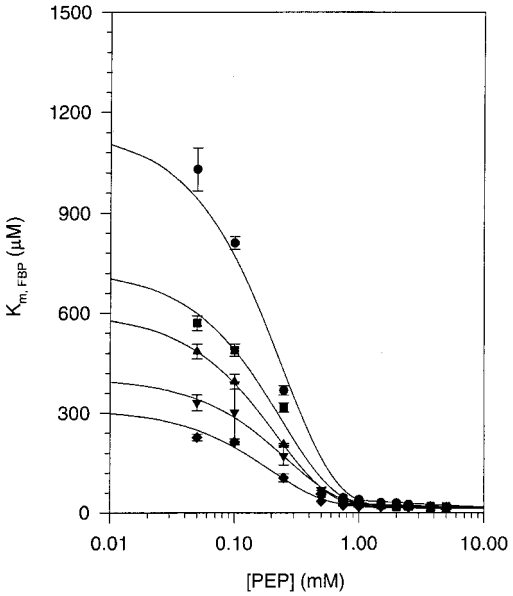

FIGURE 2: Influence of free Mg^{2+} on the PEP dependence of $K_{m,FBP}$. The activator constant, $K_{m,FBP}$, is plotted as a function of PEP concentration. The concentrations of free Mg^{2+} are 1.09 (●), 2.90 (■), 5.23 (▲), 10.1 (▼), and 20.1 mM (◆). Error bars represent the standard error in the fit of the $K_{m,FBP}$ at each concentration of PEP. Curves represent the trend in the data.

Figure 2 shows the trend in the $K_{m,FBP}$ values as a function of PEP concentration and fixed, variable concentrations of free Mg^{2+}. $K_{m,FBP}$ is used here to denote the kinetically determined concentration of FBP producing a half-maximal change in velocity. This is a kinetically determined thermodynamic constant. The $K_{m,FBP}$ decreases with increasing PEP concentration until a plateau is reached. The PEP positively influences the binding of FBP to YPK at all concentrations of free Mg^{2+}. The data from Figures 1 and 2 show the same qualitative results as predicted by the principles of thermodynamic linkage (*11*).

At saturating PEP concentrations, the $K_{m,FBP}$ approaches a value of approximately 12 μM and is unaffected by the concentration of free Mg^{2+}. At subsaturating PEP concentrations, $K_{m,FBP}$ decreases with increasing Mg^{2+} concentrations. Therefore, free Mg^{2+} positively influences the binding of FBP to the enzyme. From Figures 1 and 2, it can be seen that the three ligands (PEP, Mg^{2+}, and FBP) positively influence the interaction of each other with YPK under kinetic conditions.

The magnitude of the heterotropic interactions between PEP and FBP can be described by eq 3. Equation 3 describes the apparent $K_{m,PEP}$ in terms of three parameters: $K_{PEP}{}^{0'}$, which represents the apparent steady-state dissociation constant for the interaction of PEP with the YPK−MgADP complex; $K_{FBP}{}^{0'}$, which represents the apparent dissociation constant of FBP from the YPK−MgADP complex; and $Q_{PEP-FBP}{}'$, which is an apparent coupling parameter for PEP and FBP and is related to the coupling free energy, $\Delta G_{PEP-FBP}$, by eq 7. These parameters are apparent since they

Table 2: Steady-State Fluorescence Titration of FBP into the YPK−Mg^{2+}−MgADP Complex at Fixed, Variable Free Mg^{2+} Concentrations

$[Mg^{2+}]_f$[a] (mM)	$K_{d,FBP}$ (μM)[b]	n_H[b]
1.09	1070 ± 9	3.65 ± 0.10
2.90	742 ± 7	3.17 ± 0.09
5.23	597 ± 5	3.19 ± 0.09
10.1	406 ± 3	2.87 ± 0.05
20.1	319 ± 5	2.86 ± 0.10

[a] [MgADP] was held constant at 6.1 mM. The concentration of free Mg^{2+} was calculated from total [Mg^{2+}] and [ADP] using a K_d of 0.25 mM. [b] K_d and n_H were obtained from a fit of fluorescence quenching data to eq 8.

Table 3: Steady-State Kinetic Parameters Describing the Interaction between PEP and FBP at Fixed, Variable Mg^{2+} Concentrations[a]

$[Mg^{2+}]_f$ (mM)	$K_{PEP}^{0'}$ (mM)	$K_{FBP}^{0'}$ (μM)	$Q_{PEP-FBP}'$
1.09	6.20 ± 0.23	814 ± 79	47.2 ± 2.3
2.90	2.87 ± 0.07	746 ± 434	25.3 ± 1.3
5.23	2.22 ± 0.04	589 ± 186	19.6 ± 5.2
10.1	1.41 ± 0.06	403 ± 75	13.8 ± 1.1
20.1	1.22 ± 0.05	322 ± 53	14.1 ± 1.4

[a] $K_{PEP}^{0'}$, $K_{FBP}^{0'}$, and $Q_{PEP-FBP}'$ were obtained from fits of the data in Figure 1 to eq 3 at each free Mg^{2+} concentration. The prime (′) indicates that the values for these parameters are apparent values.

are measured as a function of free Mg^{2+} concentration. Since these are derived from kinetic studies, MgADP must also be present and is kept at a constant and saturating concentration.

Michaelis constants derived from the rate equation describing the mechanism depicted in Figure 1 are not, in general, equal to thermodynamic dissociation constants. The use of the data in Figure 2 in describing the kinetic interaction between PEP and FBP must be justified. If both PEP and FBP are in rapid equilibrium during steady-state turnover, the $K_{m,PEP}$ and $K_{m,FBP}$ can be treated as thermodynamic terms. This would simplify the rate equation for the kinetic scheme outlined in Figure 1 to one that is first-order in both PEP and FBP concentrations. The association between PEP and YPK appears not to occur by a rapid equilibrium mechanism (*12*). A less restrictive assumption is that only the allosteric ligand is in rapid equilibrium with the enzyme during steady-state turnover. Reinhart has shown that the rate equation obtained under these conditions is also first-order in both A concentration and X concentration (*8*). It follows that $Q_{AX} = K_X^0/K_X^\infty = K_A^0/K_A^\infty$. Therefore, the ratios of $K_{m,PEP}$ in the absence and presence of saturating FBP concentrations should give the true coupling parameter.

The $K_{m,FBP}$ approaches a value of 12.8 μM as both the PEP and Mg^{2+} concentrations become saturating. This is similar to the $K_{d,FBP}$ measured by fluorescence (7.1 μM). As the concentration of substrate approaches 0, ligand X is important in the binding equilibrium. Therefore, $K_{FBP}^{0'}$ should approach $K_{d,FBP}$ as the PEP concentration approaches 0. This was verified by measuring the dissociation constant for the interaction between FBP and the YPK−Mg^{2+}−MgADP complex by fluorescence titrations at 1.09, 2.90, 5.23, 10.1, and 20.1 mM free Mg^{2+}. These results are summarized in Table 2. The kinetic $K_{m,FBP}$ in Figure 2 approaches the $K_{d,FBP}$ in Table 2 at each of the concentrations of free Mg^{2+} that was analyzed. Therefore, FBP is in thermodynamic equilibrium with YPK during steady-state turnover, and the ratio of the apparent $K_{m,PEP}$ in the absence and presence of saturating FBP yields the thermodynamic parameter $Q_{PEP-FBP}$ by fitting the data in Figure 1 to eq 3.

The fitted parameters are listed in Table 3. Fits to eq 3 yielded good estimates of $K_{PEP}^{0'}$ at all concentrations of free Mg^{2+} compared to the measured values of $K_{m,PEP}$ without FBP. The kinetically determined values for $K_{FBP}^{0'}$ are also in excellent agreement with the apparent $K_{d,FBP}$ determined by fluorescence measurements (Table 2). In general, eq 3 describes the $K_{m,PEP}$ versus FBP concentration data quite well.

Attempts at fitting the data in Figure 2 to eq 3 failed at all concentrations of free Mg^{2+} because the algorithms would not converge to a well-defined minimum. This resulted in significant errors in the fitted parameters. Equation 3 does not adequately describe the data for $K_{m,FBP}$ versus PEP concentration.

Interaction of PEP and Mg^{2+} in the Absence of FBP. The $K_{m,PEP}$ was measured as a function of free Mg^{2+} concentration in the absence of FBP to estimate K_{PEP}^0 (the affinity of YPK for PEP when [FBP] = [Mg^{2+}] = 0) and K_{Mg}^0 (the affinity of YPK for Mg^{2+} when [FBP] = [PEP] = 0), as well as to quantitate Q_{PEP-Mg}. The results of these experiments are shown in Figure 3A. The data are described well by eq 4. The resulting best fit value for K_{PEP}^0 is 30.6 ± 0.78 mM. This value is significantly higher than the $K_{d,PEP}$ of 638 ± 53 μM obtained in the absence of FBP by fluorescence titrations. The difference in K_{PEP}^0 and $K_{d,PEP}$ exists because K_{PEP}^0 describes the kinetic interaction of PEP with the YPK−MgADP complex, and $K_{d,PEP}$ describes the binding between PEP and apo-YPK. The kinetic assays were performed in the presence of saturating MgADP concentrations. At low free Mg^{2+} concentrations, the interaction between MgADP and PEP is antagonistic. This was demonstrated by measuring the $K_{m,PEP}$ as a function of fixed, variable concentrations of MgADP at 97.5 μM free Mg^{2+}. In these experiments, the concentrations of total Mg^{2+} and ADP were varied such that the concentration of free Mg^{2+} was constant. The results of this analysis are listed in Table 4.

At low free Mg^{2+} concentrations, the $K_{m,PEP}$ increases with increasing MgADP concentrations, suggesting that the relationship of PEP and MgADP to YPK is antagonistic. This may account for the increase in $K_{m,PEP}$ at low concentrations of free Mg^{2+}. The interaction between PEP and MgADP at saturating free Mg^{2+} concentrations is cooperative. Therefore, free Mg^{2+} must mediate the interaction between the two substrates. Furthermore, a comparison of thermodynamic dissociation constants (K_d) and the kinetic constant, K_m, is not valid unless the substrate in question is in rapid equilibrium with the enzyme during steady-state turnover (vide infra). The K_{Mg}^0 is 6.82 ± 1.0 mM which is lower than the $K_{d,Mg^{2+}}$ measured by steady-state fluorescence measurements (11.9 ± 1.8 mM). Q_{PEP-Mg}, the thermodynamic coupling parameter between PEP and free Mg^{2+}, is 31.1 ± 2.8 and corresponds to a coupling free energy of −2.04 ± 0.09 kcal/mol.

Influence of Free Mg^{2+} on $K_{FBP}^{0'}$. The dissociation constant for the interaction of FBP with the YPK−Mg^{2+}−MgADP complex was estimated from the fits of the data in Figure 1 to eq 3. The resulting $K_{FBP}^{0'}$ was plotted as a function of free Mg^{2+} concentration (Figure 3B). The data indicate that the free Mg^{2+} concentration positively influences the binding of FBP to the enzyme. The kinetically determined $K_{FBP}^{0'}$ at

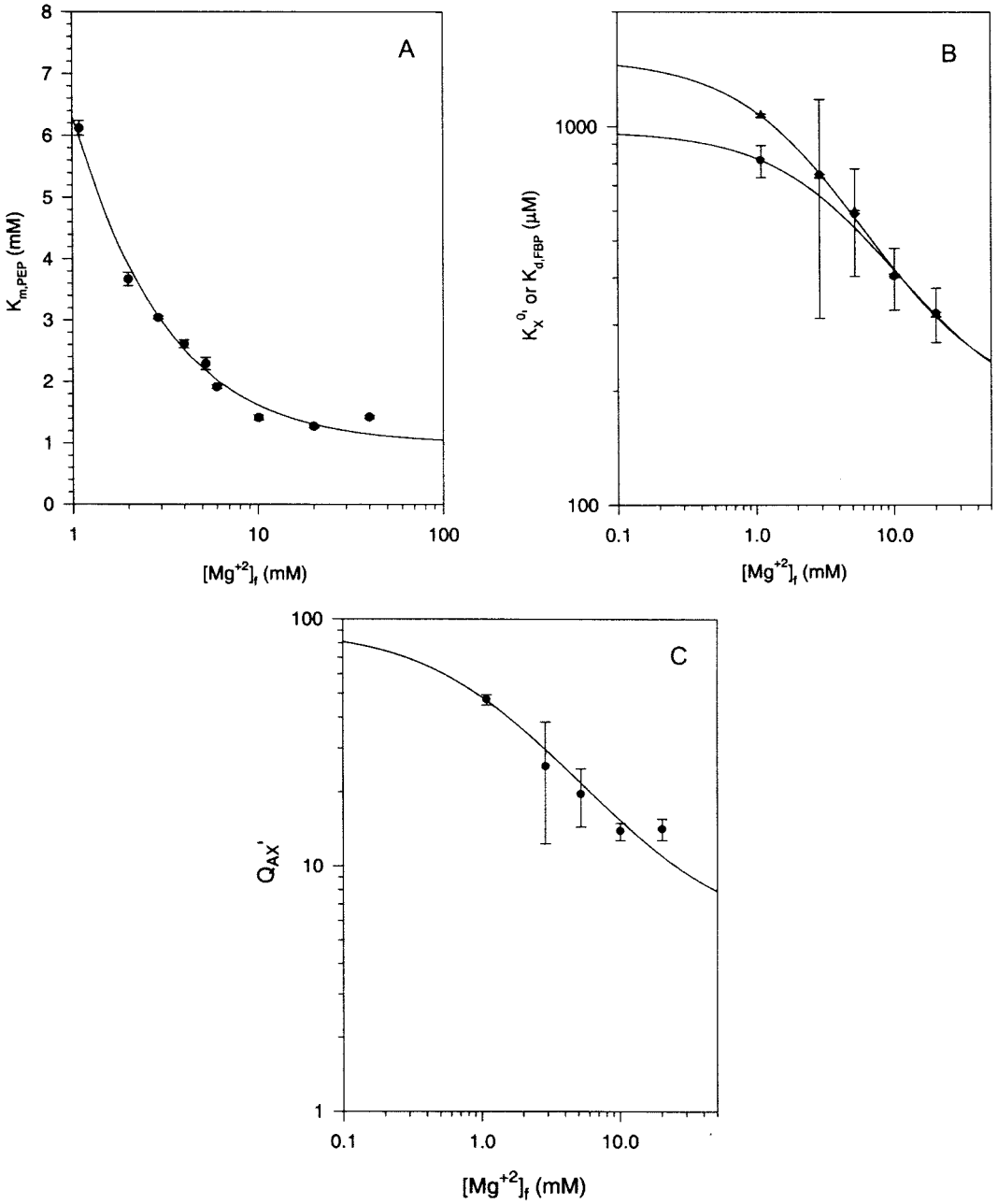

FIGURE 3: Influence of free Mg^{2+} on $K_A{}^{0'}$, $K_X{}^{0'}$, and Q_{AX}. (A) The curve represents the best fit of eq 4 to the data. Error bars represent the standard error in the fit of the $K_{m,PEP}$ at each concentration of free Mg^{2+}. (B) Influence of free Mg^{2+} on $K_X{}^{0'}$ and $K_{d,FBP}$. $K_X{}^{0'}$ (●) and $K_{d,FBP}$ (▲) are plotted as a function of free Mg^{2+} concentration. Both data sets were fit to eq 5. Curves represent the best fit of the data to eq 8. Error bars represent the standard error in the fit of the $K_X{}^{0'}$ or $K_{d,FBP}$ at each concentration of free Mg^{2+}. (C) Influence of free Mg^{2+} on Q_{AX}'. The data were fit to eq 6. The curve represents the best fit of the data to eq 6 where the following parameters were fixed: $K_X{}^0 = 25.1$ mM, $Q_{AY} = 31.1$, and $Q_{XY} = 5.64$. Error bars represent the standard error in the estimate of Q_{AX}' by eq 4 at each concentration of free Mg^{2+}.

Table 4: Interaction of PEP with MgADP at 97.5 μM Free Mg^{2+} [a]

[MgADP] (mM)	$K_{m,PEP}$ (mM)	$n_{H,PEP}$	V_{max} (units/mg)
0.698	10.7 ± 0.8	2.98 ± 0.48	0.524 ± 0.030
1.40	13.2 ± 0.2	3.01 ± 0.10	3.27 ± 0.06
2.75	17.2 ± 0.7	2.61 ± 0.14	11.5 ± 0.5
4.21	19.1 ± 0.6	2.56 ± 0.09	21.0 ± 0.7

[a] K_m, n_H, and V_{max} were obtained from fits of initial velocity vs PEP concentration to eq 2.

each free Mg^{2+} concentration was compared to the thermodynamically measured values to test the validity of the estimates of $K_{FBP}{}^{0'}$ from eq 3. The thermodynamically determined values are superimposed on the kinetically determined values in Figure 3B. Both the kinetic and thermodynamic plots were fit to eq 5, which describes the dissociation constant of FBP in terms of the dissociation constant for the interaction of free Mg^{2+} with the YPK−MgADP complex ($K_{Mg}{}^0$) and the magnitude of the coupling interaction between free Mg^{2+} and FBP. The results are listed in Table 5.

Agreement between the kinetically and thermodynamically determined values for $K_{FBP}{}^0$, $K_{Mg}{}^0$, and Q_{Mg-FBP} is satisfactory. The values probably differ because the $K_{FBP}{}^0$ determined kinetically at 1.09 mM free Mg^{2+} is an underestimate of the thermodynamically determined value, which would cause the best fit of eq 9 to deviate slightly from the thermodynamic plot (Figure 3B).

Table 5: Comparison of the Kinetically and Thermodynamically Determined Values of K_{FBP}^0, K_{Mg}^0, and Q_{Mg-FBP}

method	K_{FBP}^0 (μM)	K_{Mg}^0 (mM)	Q_{Mg-FBP}
fluorescence[a]	1506 ± 95	17.9 ± 5.7	8.15 ± 1.2
kinetics[b]	973 ± 65	25.2 ± 1.7	5.64 ± 1.6

[a] Thermodynamic K_{FBP}^0 values were measured by fluorescence titrations of FBP into the YPK−Mg^{2+}−MgADP complex. [b] Kinetic K_{FBP}^0 values were obtained from fits of the $K_{m,PEP}$ vs FBP concentration data in Figure 1 to eq 3.

Influence of Free Mg^{2+} on $Q_{PEP-FBP}'$. The apparent heterotropic coupling parameter between PEP and FBP is plotted as a function of free Mg^{2+} concentration in Figure 3C. The data were fit to eq 6, which describes the apparent coupling parameter as a function of K_{Mg}^0, $Q_{PEP-FBP}$, Q_{PEP-Mg}, Q_{Mg-FBP}, and $Q_{PEP-Mg-FBP}$. The algorithm repeatedly missed convergence when all five parameters were left as floating point parameters, even when the previously determined values for several coupling parameters were used as starting estimates. Since the two parameters of interest were $Q_{PEP-FBP}$ and $Q_{PEP-Mg-FBP}$, and since the other parameters have been measured, these three parameters were fixed as follows: $K_{Mg}^0 = 25.1$ mM, $Q_{PEP-Mg} = 31.1$, and $Q_{Mg-FBP} = 5.64$. Fixing these values in eq 6 allowed convergence and a determination of the remaining two parameters. The two-ligand coupling parameter describing the interaction between PEP and FBP in the absence of Mg^{2+} ($Q_{PEP-FBP} = 88.7 \pm 12$) and the three-ligand coupling parameter ($Q_{PEP-Mg-FBP} = 974 \pm 124$) gave the best global fits for the data.

Homotropic Cooperativity of PEP Binding to Mg^{2+}-Activated YPK. The various homotropic interactions on YPK were measured. As shown in Table 1, the velocity response of YPK as a function of PEP and Mg^{2+} concentrations in the absence of FBP occurs with positive cooperativity. This was investigated in more detail, and the results are shown in Figures 4−6. Figure 4 shows the response of $n_{H,PEP}$ as a function of FBP concentration and of free Mg^{2+} concentration. Increasing the concentration of FBP decreases the degree of positive cooperativity as indicated by the decrease in $n_{H,PEP}$ with FBP concentrations. At saturating concentrations of FBP, $n_{H,PEP}$ approaches a value of 1.0 at all concentrations of free Mg^{2+}. In the absence of FBP, the values of $n_{H,PEP}$ converge to a value of approximately 2.8, which does not vary as a function of the concentration of free Mg^{2+}. The $n_{H,FBP}$ was measured as a function of PEP concentration at fixed, variable concentrations of free Mg^{2+} (not shown). At saturating concentrations of PEP and Mg^{2+}, the data tend toward a value of 1, indicating that PEP is able to heterotropically abolish homotropic cooperativity in FBP binding.

Figure 5 shows the influence of free Mg^{2+} on the $n_{H,PEP}$ in the absence of FBP. In the range of 1.09−40.1 mM free Mg^{2+}, there is no apparent trend in the $n_{H,PEP}$, suggesting that Mg^{2+} does not modulate the homotropic binding of PEP. This is consistent with the results of PEP binding studies by fluorescence titrations. With Mn^{2+}, the homotropic binding of PEP is modulated by the divalent metal in the absence of FBP (*2*, *3*). The reciprocal experiment of Figure 5 is shown in Figure 6. Here, the $n_{H,Mg^{2+}}$ is measured as a function of PEP concentration from 1.09 to 20.1 mM PEP. The homotropic binding of Mg^{2+} is modulated by PEP. PEP also modulates the homotropic binding of Mn^{2+} (*2, 3).* Direct

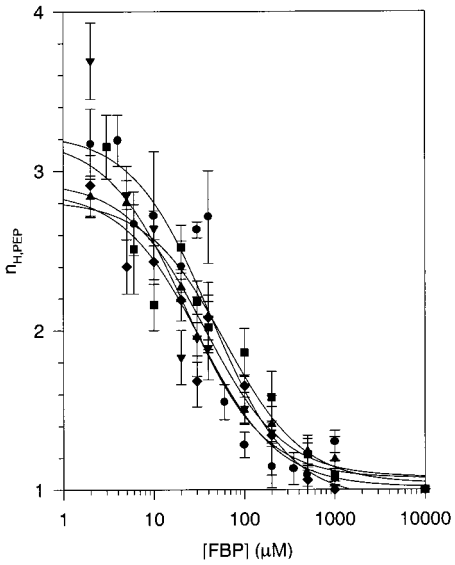

FIGURE 4: Influence of the concentration of free Mg^{2+} on the FBP dependence of $n_{H,PEP}$. The Hill coefficient for PEP, $n_{H,PEP}$, is plotted as a function of FBP concentration. $n_{H,PEP}$ was determined from fits of the velocity response of the enzyme as a function of PEP concentration at fixed, variable concentrations of FBP and of free Mg^{2+} and fit to eq 3. Free Mg^{2+} concentrations are 1.09 (●), 2.90 (■), 5.23 (▲), 10.1 (▼), and 20.1 mM (◆). Error bars represent the standard error in the fit of the $n_{H,PEP}$ values at each concentration of FBP. Curves represent the trend in the data.

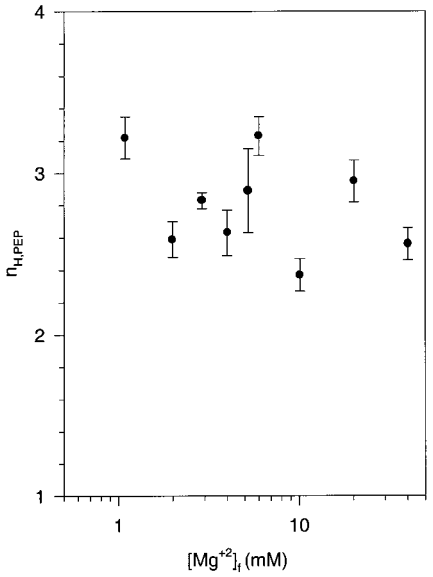

FIGURE 5: Influence of free Mg^{2+} on the $n_{H,PEP}$ without FBP. $n_{H,PEP}$ was obtained from the velocity response of YPK as a function of PEP concentration at fixed, variable concentrations of free Mg^{2+} and fit to eq 2. Error bars represent the standard error in the fit of the $n_{H,PEP}$ values at each concentration of Mg^{2+}.

measurements of Mg^{2+} binding to the YPK apoenzyme were not possible since Mg^{2+} does not induce a significant fluorescence change in the enzyme. Apparent binding is sufficiently weak that other equilibrium methods were not feasible. No estimate of the $n_{H,Mg^{2+}}$ for binding to the apoenzyme was made. The binding of Mn^{2+} to the apoenzyme is hyperbolic (*2*). Binding of Mg^{2+} to the YPK−PEP complex is sigmoidal (*19*).

MgADP Effects on Cooperativity in YPK. PEP shows hyperbolic binding to apo-YPK with an apparent dissociation constant of 638 ± 53 μM. Mn^{2+} heterotropically induces

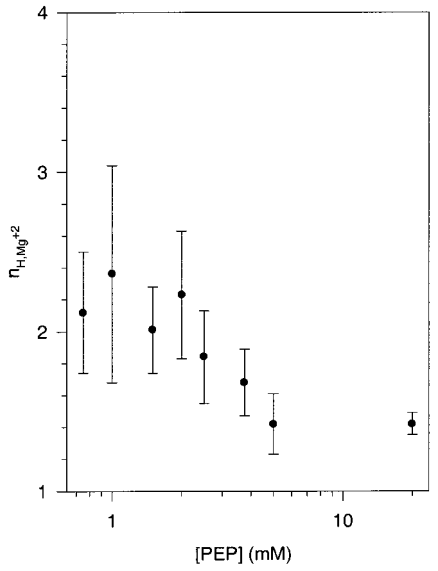

FIGURE 6: Influence of PEP on the $n_{H,Mg^{2+}}$ without FBP. $n_{H,Mg^{2+}}$ was obtained from the velocity response of YPK as a function of Mg^{2+} concentration at fixed, variable concentrations of PEP and fit to eq 2. Error bars represent the standard error in the fit of the $n_{H,Mg^{2+}}$ values at each concentration of PEP.

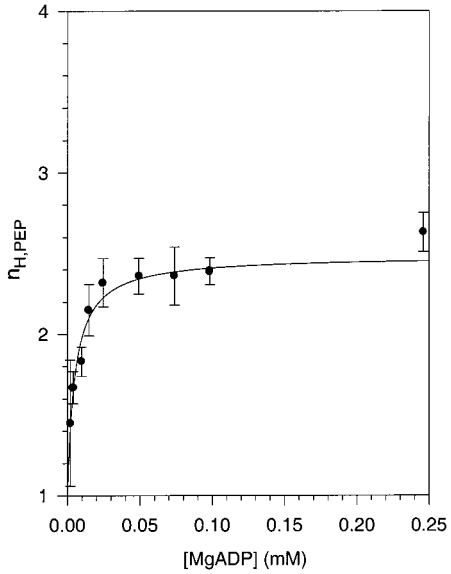

FIGURE 7: Influence of MgADP on the $n_{H,PEP}$ without FBP and with 15 mM free Mg^{2+}. The value for $n_{H,PEP}$ was obtained from the velocity response of YPK as a function of PEP concentration at fixed, variable concentrations of MgADP, and the results were fit to eq 2. Error bars represent the standard error in the fit of the $n_{H,PEP}$ values at each concentration of MgADP. The curve represents the fit of the data to a hyperbola with a finite intercept on the ordinate.

homotropic cooperativity in PEP binding ($K_d = 10.5 \pm 0.1$ μM and $n_H = 2.40 \pm 0.07$). PEP binding to the YPK−Mg^{2+} complex fits best to a hyperbolic response. Therefore, Mg^{2+} does not induce homotropic binding of PEP to YPK. Kinetically, there is a sigmoidal velocity response as a function of PEP concentration in the absence of FBP but in the presence of either Mn^{2+} or Mg^{2+}. The difference between the kinetic experiments and the thermodynamic binding experiments is that the M^{2+}−ADP complex is excluded from the thermodynamic experiments. The presence of free nucleotide does not heterotropically induce homotropic cooperativity in PEP binding (*3*). It is possible that the metal−nucleotide complex, MgADP, the catalytically important form of the substrate, plays a role in modulating the homotropic binding of PEP. This was investigated by steady-state kinetic experiments, with MgADP as the fixed, variable substrate in the absence of FBP (Figure 7). Figure 7 shows the values of $n_{H,PEP}$ as a function of MgADP concentration, and the results are best fit by a rectangular hyperbola with a non-zero intercept. The resulting curve intersects the ordinate at 1.01 ± 0.08. This value for $n_{H,PEP}$ is the same as the thermodynamic value for the Hill coefficient obtained by the fluorescence titration of PEP into the YPK−Mg^{2+} complex or into the apoenzyme in the absence of MgADP and FBP. The value for $n_{H,PEP}$ increases in a hyperbolic manner and plateaus at a fit value of 2.48 ± 0.09. The apparent K_d for MgADP, obtained from the midpoint in this curve, is 2.5 ± 0.5 μM.

DISCUSSION

Mg^{2+}-activated yeast pyruvate kinase behaves as a K-type allosteric system, indicating that a ligand binding to the enzyme affects the affinities of the enzyme for other ligands, without significantly altering V_{max}. Mg^{2+}-activated pyruvate kinases from several sources are also K-type allosteric systems (*13*−*16*). Significant differences have been observed in the activation of pyruvate kinase by Mg^{2+} and Mn^{2+} from various sources. The activator Mn^{2+} mimics the activation by FBP and abolishes the homotropic cooperativity in the velocity response of the pyruvate kinases from *Concholepas concholepas* and *Mytilus edulis* L. (*13*, *14*). These enzymes are completely insensitive to FBP in the presence of Mn^{2+}. Binding experiments with Mn^{2+}-activated yeast pyruvate kinase showed that Mn^{2+} heterotropically induces homotropic cooperativity in PEP binding (*2*). Subsequent binding studies with Mg^{2+}-activated yeast PK revealed that Mg^{2+} does not induce homotropic binding of PEP (*19*). The velocity response of Mg^{2+}-activated YPK as a function of PEP concentration is sigmoidal. The current study was undertaken to gain a more detailed understanding of the kinetic activation of YPK in the presence of Mg^{2+}.

The rate equation and kinetic constants describing turnover and ligand binding to an enzyme under steady-state conditions have been derived (*8*). The general rate equation can be simplified to one that is first-order in both substrate and effector concentration by assuming that the allosteric effector is engaged in a binding equilibrium with the enzyme during steady-state turnover, a condition that is less restrictive than the assumption of rapid equilibrium binding of the substrates. This allows use of the kinetic parameter, K_m, in the calculation of linked interactions of ligands on the enzyme. This type of analysis was used to describe the multiligand interactions on Mg^{2+}-activated YPK. Table 6 summarizes the kinetic and thermodynamic data obtained for the three-ligand heterotropic interactions that occur with Mg^{2+}-activated YPK. The kinetic coupling parameters and their corresponding coupling free energies are conditional, since they represent multiligand interactions in the presence of saturating MgADP concentrations.

The interaction between PEP and free Mg^{2+} in the absence of FBP is cooperative. The coupling free energy, $\Delta G_{PEP-Mg/MgADP}$, between PEP and free Mg^{2+} is -2.04 ± 0.09 kcal/mol. The difference between the conditional and

Table 6: Summary of Kinetic and Thermodynamic Parameters for Mg^{2+}-Activated YPK

parameter[a]	kinetics	thermodynamics[b]
K_{PEP}^0 (mM)	30.6 ± 0.8	0.638 ± 0.053
K_{Mg}^0 (mM)	25.2 ± 1.7	11.9 ± 1.8
K_{FBP}^0 (mM)	0.973 ± 0.065	0.407 ± 0.005
$Q_{PEP-FBP}$	88.7 ± 12.0	1.89 ± 0.2
$\Delta G_{PEP-FBP}$ (kcal/mol)	−2.66 ± 0.13	−0.38 ± 0.04
Q_{PEP-Mg}	31.1 ± 2.8	−
ΔG_{PEP-Mg} (kcal/mol)	−2.04 ± 0.09	−
Q_{Mg-FBP}	5.64 ± 1.60	3.55 ± 0.10
ΔG_{Mg-FBP} (kcal/mol)	−1.02 ± 0.28	−0.75 ± 0.03
$Q_{PEP-Mg-FBP}$	974 ± 124	306 ± 2
$\Delta G_{PEP-Mg-FBP}$ (kcal/mol)	−4.07 ± 0.13	−3.39 ± 0.03

[a] The kinetically determined coupling parameters and coupling free energies are conditional, and therefore should contain the subscript /MgADP. This is omitted for clarity. [b] Thermodynamic coupling free energies were determined by steady-state fluorescence titrations (*19*).

unconditional two-ligand coupling free energy between PEP and Mg^{2+} suggests that MgADP facilitates formation of the YPK−Mg^{2+}−PEP complex.

The $K_{m,FBP}$ at low concentrations of PEP decreases as a function of free Mg^{2+} concentration, suggesting that the interaction between the two ligands (FBP and Mg^{2+}) is cooperative. The $K_{FBP}^{0'}$ was measured by direct binding assays and by fitting the data in Figure 1 to eq 3. The resulting conditional $\Delta G_{Mg-FBP/MgADP}$ is −1.02 ± 0.28 kcal/mol. The ΔG_{Mg-FBP} measured by steady-state fluorescence titrations is −0.75 ± 0.03 kcal/mol. The presence of MgADP increases the cooperativity in the Mg^{2+}−FBP interaction in the absence of PEP by approximately −0.25 kcal/mol.

The coupling parameter between PEP and FBP at $[Mg^{2+}]_f = 0$, $Q_{PEP-FBP/MgADP}$, has a value of 88.7 ± 12.0 which corresponds to a conditional coupling free energy of −2.66 ± 0.13 kcal/mol. The unconditional $\Delta G_{PEP-FBP}$ is −0.38 ± 0.04 kcal/mol. The substrate MgADP also increases the heterotropic cooperativity between the PEP and FBP binding sites in the absence of free Mg^{2+}. This is in contrast to coupling in the presence of Mn^{2+} since the coupling parameter between PEP and FBP in the presence of saturating MnADP is 1, meaning that there is no communication between the PEP and FBP binding sites in the absence of free Mn^{2+} ($\Delta G_{PEP-FBP/MnADP} = 0$) (*3*). A $Q_{PEP-FBP/MnADP}$ of 1 would reduce eq 6 to the following form:

$$Q_{AX}' = \frac{(K_Y^0 + [Y])(K_Y^0 + Q_{AXY}[Y])}{(K_Y^0 + Q_{AY}[Y])(K_Y^0 + Q_{XY}[Y])} \qquad (9)$$

Attempts at fitting the data in Figure 3C to eq 9 failed as the algorithm would not converge. Therefore, the conditional coupling free energy with Mg^{2+}, $\Delta G_{PEP-FBP/MgADP}$, is finite, whereas the conditional coupling free energy with Mn^{2+}, $\Delta G_{PEP-FBP/MnADP}$, is 0.

The three-ligand coupling parameter describing the interaction between PEP, Mg^{2+}, and FBP in the presence of saturating MgADP concentrations is 974 ± 124, giving a conditional three-ligand coupling free energy, $\Delta G_{PEP-Mg-FBP/MgADP}$, of −4.08 ± 0.13 kcal/mol. This is larger than the unconditional three-ligand coupling free energy determined by fluorescence titrations (−3.21 ± 0.03 kcal/mol). Therefore, MgADP increases the cooperativity in formation of the quaternary YPK−PEP−Mg^{2+}−FBP complex.

Kinetic linked-function analysis has been successful in determining the extents of the heterotropic interactions between PEP, Mg^{2+}, and FBP in the presence of saturating MgADP concentrations. It would be useful to add a fourth dimension of complexity to this set of experiments and include specific interactions between each of these ligands and MgADP. A comparison of K_m and K_A values in the presence and absence of MgADP is not valid, in general, since the Michaelis constant for a particular ligand is not always equal to its corresponding dissociation constant. The comparison is valid in the case of FBP, since we have determined that the $K_{m,FBP}$ is equal to its thermodynamic dissociation constant over the entire range of free Mg^{2+} and PEP concentrations that was analyzed. This allows the calculation of the extent of the heterotropic interaction between MgADP and FBP when [PEP] = 0 in the absence and presence of saturating free Mg^{2+} concentrations. The K_d for FBP dissociation from apo-YPK is 407 ± 5.2 μM (*19*). In the presence of saturating MgADP concentrations and when $[PEP] = [Mg^{2+}]_f = 0$, the $K_{m,FBP}$ = 973 ± 65 μM. Since the affinity of YPK for FBP is weaker in the presence of saturating MgADP concentrations, the interaction between MgADP and FBP is antagonistic. This is consistent with the hypothesis that MgADP is able to induce a conformational change in the enzyme that modulates the binding of subsequent ligands. This was not observed with Mn^{2+}-activated YPK (*3*).

A similar comparison of the K_{PEP}^0 ($K_{m,PEP}$ when $[Mg^{2+}]_f = 0$ and [FBP] = 0) with $K_{d,PEP}$ cannot be made. The K_{PEP}^0 is 30.6 mM, which is significantly higher than the $K_{d,PEP}$ for the interaction between PEP and apo-YPK (638 μM). There are differences between these two values. First, the K_{PEP}^0 is the kinetically determined dissociation constant of PEP from the YPK−MgADP complex. $K_{d,PEP}$ describes the dissociation of PEP from apo-YPK. There is a significant antagonistic interaction between PEP and MgADP as the concentration of free Mg^{2+} approaches zero (Table 4). Second, $K_{m,PEP}$ is a kinetic constant that is generally not equivalent to its corresponding thermodynamic dissociation constant. Several pieces of evidence suggest that PEP is a "sticky" substrate with pyruvate kinase (*12*, *18*). The rate constant for turnover is significantly higher than the off rate for dissociation of PEP from the catalytic complex. With muscle PK, PEP reacts to give pyruvate approximately 5 times faster than it dissociates from the enzyme−substrates complex (*12*). A sticky substrate would have a K_m that is higher than its corresponding K_d because the rate expression for K_m would contain an additional kinetic term(s) in the numerator. As the concentration of free Mg^{2+} approaches zero, and as k_{cat} approaches zero, the $K_{m,PEP}$ (or K_{PEP}^0) should approach the thermodynamic value $K_{d,PEP}$. If K_{PEP}^0 can be assumed to be a dissociation constant, the coupling parameter between PEP and MgADP would be equal to 0.638/30.6 (0.0208), giving a coupling free energy of +2.29 kcal/mol. This is an upper-limit estimate of the coupling free energy since the K_{PEP}^0 is probably higher than the K_d describing the interaction between PEP and the YPK−MgADP complex. This represents a significant antagonistic relationship. The direct measurement of the $K_{d,PEP}$ from this complex by fluorescence is not possible. The formation of a YPK−Mg−MgADP complex where [MgADP] = 6.1 mM and $[Mg^{2+}]_f = 0$ would require an extremely high concentration of ADP.

Table 7: A Comparison of Kinetically Determined Two- and Three-Ligand Conditional Coupling Free Energies for Mg^{2+}- and Mn^{2+}-Activated YPK

	Mg^{2+}	Mn^{2+} [a]
$\Delta G_{M-PEP/MADP}$ (kcal/mol)	-2.04 ± 0.09	-2.75 ± 0.14
$\Delta G_{M-FBP/MADP}$ (kcal/mol)	-1.02 ± 0.28	-1.55 ± 1.03
$\Delta G_{PEP-FBP/MADP}$ (kcal/mol)	-2.66 ± 0.13	0
$\Delta G_{PEP-M-FBP/MADP}$ (kcal/mol)	-4.07 ± 0.13	-4.32 ± 0.40

[a] Coupling free energies for Mn^{2+}-activated YPK were obtained from ref *3*.

The free Mg^{2+} concentration would have to be zero in this complex to prevent turnover of the enzyme. Measurement of this dissociation constant at positive concentrations of free metal is not feasible since the enzyme would be turning over at a significant rate.

A comparison of the kinetically determined conditional two- and three-ligand coupling free energies for Mg^{2+}- and Mn^{2+}-activated YPK is presented in Table 7. The Mg^{2+}-activated YPK system is significantly less cooperative than the Mn^{2+}-activated YPK system. The conditional two-ligand coupling free energy between PEP and the divalent metal in the absence of FBP is weaker for Mg^{2+}-activated YPK (-2.04 ± 0.09 kcal/mol) than for Mn^{2+}-activated YPK (-2.75 ± 0.14 kcal/mol). Similarly, ΔG_{Mg-FBP} (-1.02 ± 0.28 kcal/mol) is significantly weaker than ΔG_{Mn-FBP} (-1.55 ± 1.03 kcal/mol). These trends are similar to those observed by steady-state fluorescence titrations and thermodynamic linked-function analysis (*19*). The conditional three-ligand coupling free energy, however, is similar in the presence of Mg^{2+} ($\Delta G_{PEP-Mg-FBP/MgADP} = -4.07 \pm 0.13$ kcal/mol) and Mn^{2+} ($\Delta G_{PEP-Mn-FBP/MnADP} = -4.32 \pm 0.07$ kcal/mol).

A comparison of the binding and kinetic data for Mg^{2+}- and Mn^{2+}-activated YPK in the absence of FBP demonstrates that there are significant differences in the influence of divalent metal on the homotropic interactions of PEP binding sites on YPK. Mn^{2+} heterotropically induces homotropic cooperativity in PEP binding, and the rate response of Mn^{2+}-activated YPK as a function of PEP concentration is sigmoidal. The velocity response of Mg^{2+}-activated YPK as a function of PEP concentration is also sigmoidal, indicating that there is homotropic cooperativity in the kinetic response of YPK as a function of PEP concentration. The binding of PEP to the YPK−Mg^{2+} complex, however, is hyperbolic (*19*). The sigmoidal velocity response with Mn^{2+}-activated YPK is simple to explain, since the binding of PEP to the YPK−Mn^{2+} complex is sigmoidal. The correlation between the binding data and kinetic response for Mg^{2+}-activated YPK is not as clear on initial inspection.

There are two explanations to account for the observed response of the enzyme toward PEP. The sigmoidal kinetic response may be a purely kinetic phenomenon, and may not be the result of a homotropic binding interaction between PEP sites on the tetramer. This would be observed if there was a cooperative response in the intrinsic turnover rate at each active site. For example, the intrinsic rate of turnover for the ES_2 complex (the enzyme−substrates complex with two substrate binding sites occupied) could be greater than 2 times the intrinsic turnover rate of the ES complex (the enzyme−substrates complex with one substrate binding site occupied). This would result in sigmoidal kinetics even if the binding of S were hyperbolic. Alternatively, the level of binding of PEP to the YPK−Mg^{2+}−MgADP complex could be measured by steady-state fluorescence titrations provided the rate of turnover could be limited such that the disappearance of added ligand would be negligible over the course of the experiment. This could be used to determine whether the binding of PEP to form the kinetically competent complex is hyperbolic or is induced to be sigmoidal. This experiment was attempted by slowing the reaction rate and measuring the binding of the pseudosubstrate (*Z*)-3-fluoro-PEP to the YPK−Mg^{2+}−MgADP complex. The binding of (*Z*)-3-fluoro-PEP to this complex is sigmoidal (not shown). Therefore, the second explanation for the observed response of the enzyme to PEP is that the sigmoidal kinetic response appears to be due to the induction of homotropic cooperativity in PEP binding by MgADP.

The binding isotherm describing the interaction between PEP and the YPK−ADP complex is hyperbolic (*3*). A possible explanation for the difference in behavior in PEP binding and kinetics is that both the enzyme-bound divalent metal and the nucleotide-bound divalent metal modulate the homotropic binding of PEP. This was tested kinetically by varying the concentration of MgADP at saturating free Mg^{2+} concentrations and measuring the Hill coefficient for the velocity response of YPK as a function of PEP concentration. As the MgADP concentration is increased, $n_{H,PEP}$ increases in a saturatable manner and plateaus at a value of approximately 2.6. A fit of these data to a hyperbola passes through an n_H of 1.0 at [MgADP] = 0. This is the same as the thermodynamic value obtained by fluorescence titrations when [MgADP] = 0 (*19*). In contrast, varying the free Mg^{2+} concentration from 1.09 to 40.1 mM had no effect on the Hill coefficient for PEP. Thus, it appears that with Mg^{2+}-activated YPK, the nucleotide-bound divalent metal plays a role in controlling the cooperative binding of PEP. With Mn^{2+}, it is both the enzyme-bound divalent metal and nucleotide-bound divalent metal that modulate cooperativity in PEP binding (*19*). This would explain the difference in the Hill coefficients obtained in the absence and presence of MnADP by fluorescence titrations and kinetics, respectively (*2*, *3*).

In summary, there are significant differences in the kinetic allosteric properties of Mg^{2+}-activated YPK and Mn^{2+}-activated YPK. The most significant difference is the role that the metal−nucleotide complex plays in the activation of the enzyme. MgADP modulates both the cooperativity in the formation of the kinetic enzyme−ligand complex and the cooperativity in the activation of the enzyme by PEP. The role of the enzyme-bound divalent metal is to modulate the communication between the PEP and FBP binding sites.

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