Thermodynamic Linked-Function Analysis of Mg^{2+}-Activated Yeast Pyruvate Kinase†

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ABSTRACT: Yeast pyruvate kinase (YPK) is regulated by intermediates of the glycolytic pathway [e.g., phosphoenolpyruvate (PEP), fructose 1,6-bisphosphate (FBP), and citrate] and by the ATP charge of the cell. Recent kinetic and thermodynamic data with Mn^{2+}-activated YPK show that Mn^{2+} mediates the allosteric communication between the substrate, PEP, and the allosteric effector, FBP [Mesecar, A., and Nowak, T. (1997) *Biochemistry 36*, 6792, 6803]. These results indicate that divalent cations modulate multiligand interactions, and hence cooperativity with YPK. The nature of multiligand interactions on YPK was investigated in the presence of the physiological divalent activator Mg^{2+}. The binding interactions of PEP, Mg^{2+}, and FBP were monitored by fluorescence spectroscopy. The binding data were subject to thermodynamic linked-function analysis to determine the magnitudes of the multiligand interactions governing the allosteric activation of YPK. The two ligand coupling free energies between PEP and Mg^{2+}, PEP and FBP, and FBP and Mg^{2+} are 0.88, −0.38, and −0.75 kcal/mol, respectively. The two-ligand coupling free energies between PEP and Mn^{2+} and FBP and Mn^{2+} are more negative than those with Mg^{2+} as the cation. This indicates that the interactions between the divalent cation and PEP with YPK are different for Mg^{2+} and Mn^{2+} and that the interaction is not simply electrostatic in nature, as originally hypothesized. The magnitude of the heterotropic interaction between the metal and FBP is similar with Mg^{2+} and Mn^{2+}. The simultaneous binding of Mg^{2+}, PEP, and FBP to YPK is favored by 3.21 kcal/mol compared to independent binding. This complex is destabilized by 3.30 kcal/mol relative to the analogous YPK−Mn^{2+}−PEP−FDP complex. Interpretation of K_d values when cooperative binding occurs must be done with care as these are not simple thermodynamic constants. These data demonstrate that the divalent metal, which activates phosphoryl transfer in YPK, plays a key role in modulating the various multiligand interactions that define the overall allosteric properties of the enzyme.

The glycolytic enzyme, pyruvate kinase from yeast (YPK),[1] is a key regulatory enzyme. YPK catalyzes the last energy-producing step in glycolysis: the production of ATP from the high-energy phosphate, phosphoenolpyruvate (PEP), and from ADP. The enzyme requires one monovalent cation and two divalent cations per subunit for activation. The physiological monovalent metal is probably K^+ (*1*). The physiological divalent metal is thought to be Mg^{2+}, but Mn^{2+} and Co^{2+} can also activate YPK. While it is generally accepted that Mg^{2+} and Mn^{2+} are interchangeable for the purposes of enzymatic analysis, there are differences in the nature of the Mg^{2+}- and Mn^{2+}-activated PK reactions from different sources. Mn^{2+} is often chosen as an activator because of its paramagnetic properties that are used in biophysical studies.

All pyruvate kinases require two divalent metals per active site for activity. Mg^{2+} elicits the highest activity (k_{cat}) for muscle or yeast PK and is probably the physiological activator. Several other divalent metals activate PK as well (Mg^{2+} > Mn^{2+} > Co^{2+} > Ni^{2+}) (*2*). One equivalent of divalent cation binds to the enzyme at the active site with a stoichiometry of 4 equiv of divalent metal per tetramer in the absence of substrates (*3*−*5*). The second divalent metal enters the active site as a complex with the nucleotide substrate (*6*, *7*). The γ-phosphoryl group of ATP in the product form of the enzyme bridges the two divalent cations at the active site (*8*). The role of the divalent metal is likely to neutralize the net negative charges of the two anionic substrates, thereby facilitating nucleophilic attack during phosphoryl transfer and stabilizing the pentaphosphate transition state.

Several pyruvate kinases from various sources have different biophysical and kinetic properties in the presence of either Mg^{2+} or Mn^{2+}. Pyruvate kinases from the muscle of the sea mollusk *Concholepas concholepas* and sea mussel *Mytilus edulis* display sigmoidal kinetics with the substrate PEP and are allosterically activated by fructose 1,6-bisphosphate (FBP) in the presence of Mg^{2+}. In contrast to the Mg^{2+}-activated enzymes from these organisms, the Mn^{2+}-activated enzymes display hyperbolic kinetics and are insensitive to the presence of FBP (*9*, *10*). Furthermore, it was proposed that the enzyme from *C. concholepas* follows

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

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an ordered sequential mechanism in the presence of Mn^{2+}, and follows a random kinetic mechanism in the presence of Mg^{2+} and FBP (*11*). Evidence which shows that Mn^{2+} acts as an allosteric activator of PEP binding has also been obtained for the mycelium and yeast-like cells of the filamentous fungus *Mucor rouxii* (*12*).

Differences in biophysical properties of PK as a function of divalent metal are not limited to the enzymes from lower organisms. Studies with the human erythrocyte (type R) pyruvate kinase indicate that Mn^{2+} plays a role similar to that of FBP. A single hyperbolic curve is obtained in the presence and absence of FBP with this enzyme in the presence of Mn^{2+} (*13*).

Although the homotropic cooperativity with PEP in yeast PK is similar in the presence of Mg^{2+} and Mn^{2+}, as judged by the magnitude of the Hill coefficient for PEP interaction, there are differences in the kinetic properties. These differences between Mg^{2+}- and Mn^{2+}-activated PK from various sources suggest possible regulatory roles for the divalent metal.

A method has recently been developed in our laboratory for measuring the extent of ligand binding to various yeast PK complexes by fluorescence spectroscopy (*14*). This method was applied to the Mn^{2+}-activated enzyme and was shown to yield quantitative results that were identical to the results of independent methods such as equilibrium dialysis. It was determined that the divalent metal in this system acts as a relay to communicate the signal from the FBP binding site to the PEP binding site that gives rise to the observed regulatory properties of the Mn^{2+}-activated enzyme. It was also shown that it is the enzyme-bound Mn^{2+} that is responsible for inducing the positive homotropic cooperativity in PEP interactions with YPK using both thermodynamic and kinetic methods (*14*, *15*). Thermodynamic linked-function analysis is used to measure the extents of various multiligand interactions on YPK that occur between PEP, Mg^{2+}, and FBP.

EXPERIMENTAL PROCEDURES

Materials. Yeast PK was expressed in the PK deficient strain of *Saccharomyces cerevisiae*, pyk 1-5, and purified according to the method of Mesecar and Nowak (*14*). PEP, ADP, FBP, and buffers were purchased from Sigma. All other materials were reagent grade or better.

Steady-State Fluorescence Measurements. Experiments were performed on an SLM-Aminco 8100 spectrofluorimeter thermostated to 25 °C. Emission spectra were recorded from 310 to 400 nm with a bandwidth of 2 nm. Fluorescence titrations were performed by monitoring the steady-state fluorescence intensity of the enzyme at 334 nm (2 nm bandwidth) and an excitation wavelength of 295 nm (2 nm bandwidth). Titrations were performed by adding aliquots of a ligand 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 other ligands as specified. Percent fluorescence quenching was calculated from the following formula

$$Q = (1 - F/F_0) \times 100 \quad (1)$$

where F_0 is the steady-state fluorescence intensity in the absence of ligand and F is the fluorescence intensity in the presence of ligand. Fluorescence quenching at each addition of ligand was corrected for dilution of the enzyme. All experiments were performed in triplicate and the data averaged, unless otherwise indicated. Fluorescence quenching data were fit to either of the following two equations:

$$Q = \frac{Q_{max}[L]}{K_d + [L]} \quad (2)$$

$$Q = \frac{Q_{max}[L]^n}{K_d + [L]^n} \quad (3)$$

which are analogous to the Michaelis−Menten and Hill equations, respectively. L represents the titrated ligand; K_d represents the thermodynamic dissociation constant of the ligand, and n represents the Hill coefficient. When the Hill coefficient is different from 1, it must be remembered that this is a functional dissociation constant and not a true simple thermodynamic constant.

Thermodynamic Treatment of Fluorescence Titrations. The multiple ligand interactions on YPK were quantitated by thermodynamic linked-function analysis. This analysis is based on general principles outlined by Wyman (*16*) and Weber (*17*, *18*) and is described in detail elsewhere (*14*). Briefly, formation of an EMS complex from enzyme, metal, and substrate may follow one of two paths, with either metal binding first followed by substrate, or by substrate binding followed by metal. In either case, the total free energy of formation of the EMS complex (ΔG_T) is described by both unconditional and conditional free energies of binding that represent the binding of a ligand in the absence (ΔG_S) and presence of a second ligand ($\Delta G_{S/M}$), respectively. By the conservation of free energy, the total free energy change on formation of the EMS complex should be path-independent. This gives rise to the following identity:

$$\Delta G_T = \Delta G_S + \Delta G_{M/S} = \Delta G_M + \Delta G_{S/M} \quad (4)$$

In this equation, ΔG_S and ΔG_M are unconditional free energies of binding of substrate and metal, respectively, to the enzyme. The terms $\Delta G_{M/S}$ and $\Delta G_{S/M}$ are conditional free energies of binding for the metal in the presence of substrate and substrate in the presence of metal, respectively. The free energies of binding in each case are related to the dissociation constant of the ligand from the complex by

$$\Delta G = RT \ln(K_d) = -RT \ln(1/K_d) \quad (5)$$

where R is the gas constant, T is the temperature, and K_d is the dissociation constant for ligand binding. The free energy of interaction, or coupling free energy, between ligands represents the difference between the sum of the conditional and unconditional free energy of binding of S and M, and the standard free energy of formation of the EMS complex.

$$\Delta G_{SM} = (\Delta G_S + \Delta G_{M/S}) - (\Delta G_S + \Delta G_M) \quad (6)$$

$$\Delta G_{SM} = (\Delta G_M + \Delta G_{S/M}) - (\Delta G_S + \Delta G_M) \quad (7)$$

or

$$\Delta G_{SM} = \Delta G_{M/S} - \Delta G_M = \Delta G_{S/M} - \Delta G_S \quad (8)$$

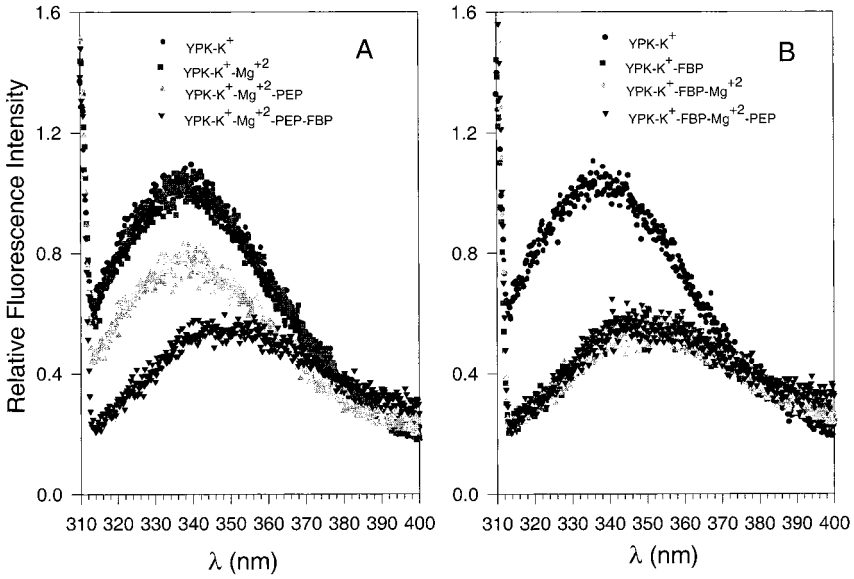

FIGURE 1: Fluorescence emission spectra of various ligated forms of yeast pyruvate kinase. YPK was excited at 295 nm, and emission spectra were monitored from 310 to 400 nm. Fluorescence spectra are as indicated in the inset. Final concentrations for all species were as follows: 60 μg/mL YPK, 200 mM KCl, and 15 mM $MgCl_2$, 5 mM PEP, and 1 mM FBP when present. All ligand concentrations are saturating. Relative fluorescence is calculated relative to an external fluorescent standard and has been corrected for dilution.

The value ΔG_{SM} can be either positive, negative, or zero. If ΔG_{SM} is negative, then the simultaneous binding of the two ligands is favored over their independent binding, and this interaction is termed synergistic or cooperative. If ΔG_{SM} is positive, then the simultaneous binding of S and M is unfavorable, and their interaction is termed antagonistic. If ΔG_{SM} is zero, then the two ligands bind independent of each other to the enzyme. For these identities to hold, ΔG values must reflect true thermodynamic functions. A third ligand, F, added to this system, introduces one unconditional and three conditional binding free energies, namely, ΔG_F, $\Delta G_{F/S}$, $\Delta G_{F/M}$, and $\Delta G_{F/SM}$.

RESULTS

Yeast pyruvate kinase is a tetramer of identical 54 kDa subunits. Each subunit may be divided into four domains named N, A, B, and C. Overall, the tetramer has D_2 symmetry. The C, or C-terminal, domain is made up of the last 149 amino acids of the YPK monomer and comprises the 1,3 intersubunit contact (*27*). The subunit interactions in the C domain are thought to mediate intersubunit communication in allosteric PKs (*19*). The C domain contains the FBP binding site. YPK contains a single tryptophan residue per subunit (W452). W452 sits atop an α-helix (Cα5), and packs against a loop that connects two β-sheets, Cβ4 and Cβ5. W452 provides an internal fluorescent probe with which to measure the extent of ligand binding at both the active site and the allosteric site on YPK (*14*, *20*).

The fluorescence emission spectra of various Mg^{2+}-activated YPK complexes were determined and are shown in Figure 1. Figure 1A shows the results of emission scans of the YPK−K^+ complex, hereafter termed the apoenzyme, sequentially titrated with Mg^{2+}, PEP, and FBP. The spectrum of apo-YPK has an emission maximum at approximately 334 nm. The addition of kinetically saturating amounts of Mg^{2+} does not cause significant changes in the emission spectrum of W452. Mn^{2+} binding causes a 2 nm blue shift in the fluorescence spectrum, and there is a small but significant fluorescence quenching of 9% (*14*). Addition of PEP to the YPK−Mg^{2+} complex causes 35% quenching and a 2 nm red shift. Saturating the YPK−Mg^{2+}−PEP complex with FBP causes 26% quenching and a large 13 nm red shift. The total fluorescence quenching of 61% is similar to the 60% overall quenching observed at 334 nm for the formation of the YPK−Mn^{2+}−PEP−FBP complex. This suggests that the environments surrounding W452 in the fully ligated YPK complex are similar regardless of the activating divalent cation.

When apo-YPK is saturated with FBP, a 50% quenching occurs at 334 nm with a concomitant 13 nm red shift similar to that observed for saturation with FBP of the YPK−Mg^{2+}−PEP complex (Figure 1B). There are no further fluorescence changes observed at 334 nm on formation of YPK−FBP−Mg^{2+} or YPK−FBP−Mg^{2+}−PEP complexes. This indicates that the structure of the enzyme surrounding W452 is similar in these complexes in the presence of saturating amounts of FBP.

Interaction of PEP with YPK Complexes. Fluorescence titrations of PEP into apo-YPK were performed, and the results are shown in Figure 2A. The data were best fit to eq 2, which describes a rectangular hyperbola. The values for Q_{max} and K_d were 20.1 ± 0.4% and 638 ± 53 μM, respectively. These values are consistent with results obtained previously in this laboratory.

The binding of PEP to YPK was performed in the presence of 15 mM Mg^{2+}. This concentration of Mg^{2+} is kinetically saturating and has been demonstrated to be saturating under the conditions used in these experiments (data not shown). The binding of PEP to the YPK−Mg^{2+} complex is described by a hyperbola, with a Q_{max} of 34.7 ± 0.9% and a K_d of 2810 ± 165 μM (Figure 2B). The YPK−Mg^{2+} complex has properties significantly different from those of the YPK−Mn^{2+} complex. The titration of PEP into the YPK−Mn^{2+} complex is shown in Figure 2C. The data were best fit by eq 3, and the resulting parameters are as follows: Q_{max} = 36.5 ± 0.2%, K_d = 10.5 ± 0.1 μM, and n_H = 2.40 ± 0.07.

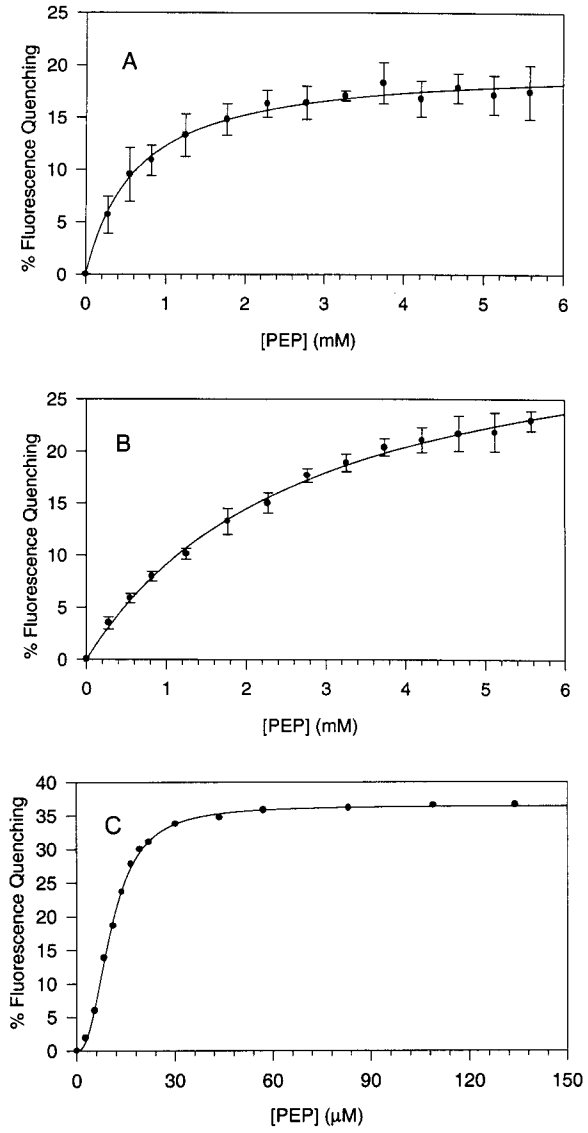

FIGURE 2: Interaction of PEP with YPK complexes. The extent of binding of PEP to apo-YPK, the YPK−Mg^{2+} complex, and the YPK−Mn^{2+} complex was measured by steady-state fluorescence quenching at 334 nm. All complexes contain 200 mM KCl. (A) The interaction of PEP with apo-YPK. The data were best fit to eq 2. (B) The interaction of PEP with the YPK−Mg^{2+} complex where the Mg^{2+} concentration is 15 mM. The data were best fit to eq 2. (C) The interaction of PEP with the YPK−Mn^{2+} complex where the Mn^{2+} concentration is 4 mM. The data were best fit to eq 3. Each data point in panel C represents the average of two determinations. The curves are calculated best fits from either eq 2 or 3 as indicated.

Mn^{2+} heterotropically induces homotropic binding of PEP to YPK, and is a heterotropic affector. The binding of PEP to the YPK−Mg^{2+} complex is weaker than to apo-YPK by a factor of ~4. Binding of PEP to the YPK−Mg^{2+} complex is also significantly weaker than binding to the YPK−Mn^{2+} complex. The level of binding of PEP to the YPK−FBP complex was not measured since no additional fluorescence change occurs on addition of PEP to this complex (Figure 1). Other methods of measuring the extent of PEP binding to PK or related enzymes have been shown to be unreliable for technical reasons (*14*). Kinetic data in the presence of FBP show that FBP heterotropically abolishes homotropic PEP interactions with YPK (*32*).

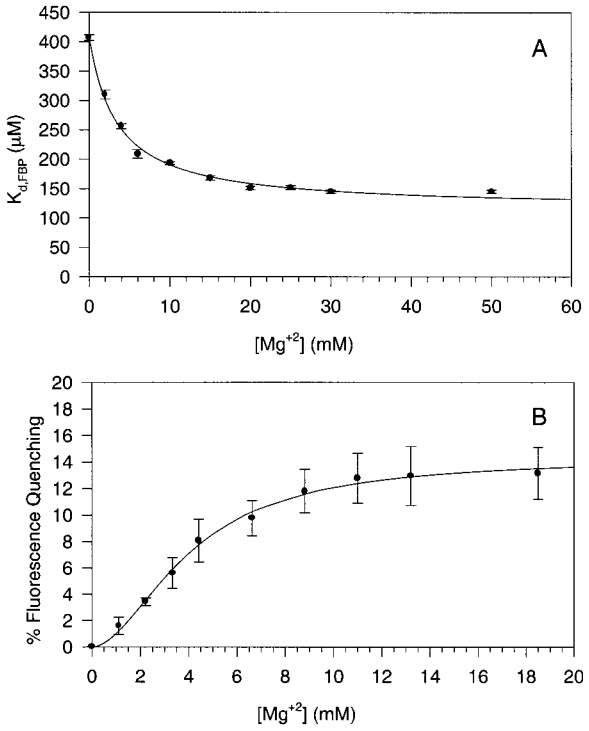

FIGURE 3: Interaction of Mg^{2+} with YPK complexes. All complexes contain 200 mM KCl. (A) The interaction of Mg^{2+} with YPK. The extent of binding of Mg^{2+} was measured by observing the change in $K_{d,FBP}$ as a function of Mg^{2+} concentration. The $K_{d,FBP}$ was measured by steady-state fluorescence titrations as described in the text. The data were fit to eq 9. Error bars represent the standard error in the fit of $K_{d,FBP}$ at each Mg^{2+} concentration. (B) The interaction of Mg^{2+} with the YPK−PEP complex where the PEP concentration is 5 mM. The data were best fit to eq 3. The curves represent fits to the data.

Interaction of Mg^{2+} with YPK Complexes. Mg^{2+} binding to YPK complexes was investigated by steady-state fluorescence titrations. No significant change in the fluorescence emission spectrum is observed on addition of Mg^{2+} to apo-YPK (Figure 1). This would suggest that the binding of Mg^{2+} to YPK causes only local structural perturbations at the active site, and not at the allosteric site, where W452 is located. This is not the case, however, since Mg^{2+} has a significant effect on FBP binding. Because the change in fluorescence of YPK upon Mg^{2+} binding is very small (Figure 1), the affinity of apo-YPK for free Mg^{2+} was measured indirectly by monitoring the change in $K_{d,FBP}$ as a function of Mg^{2+} concentration (Figure 3A). The data in Figure 3A were fit to eq 9.

$$K_{FDP} = K_{FDP}{}^{0}\left(\frac{K_{Mg}{}^{0} + [Mg^{2+}]}{K_{Mg}{}^{0} + Q_{Mg-FDP}[Mg^{2+}]}\right) \qquad (9)$$

Equation 9 describes the apparent K_{FBP} in terms of three parameters. $K_{FBP}{}^{0}$ is the affinity of the apoenzyme for FBP. $K_{Mg}{}^{0}$ is the affinity of the apoenzyme for Mg^{2+}. Q_{Mg-FBP}, which is a thermodynamic coupling parameter, is the $K_{FBP}{}^{0}/K_{FBP}{}^{\infty}$ ratio. $K_{FBP}{}^{\infty}$ is the affinity of YPK for FBP in the presence of a saturating Mg^{2+} concentration. Q_{Mg-FBP} is related to the free energy of the interaction of FBP and Mg^{2+} by eq 5 (*21*). The resulting best fit parameters of the data in Figure 3A to eq 9 are as follows: $K_{FBP}{}^{0} = 410 \pm 8$ μM, $K_{Mg}{}^{0} = 12.0 \pm 1.8$ mM, and $Q_{Mg-FBP} = 3.53 \pm 0.19$.

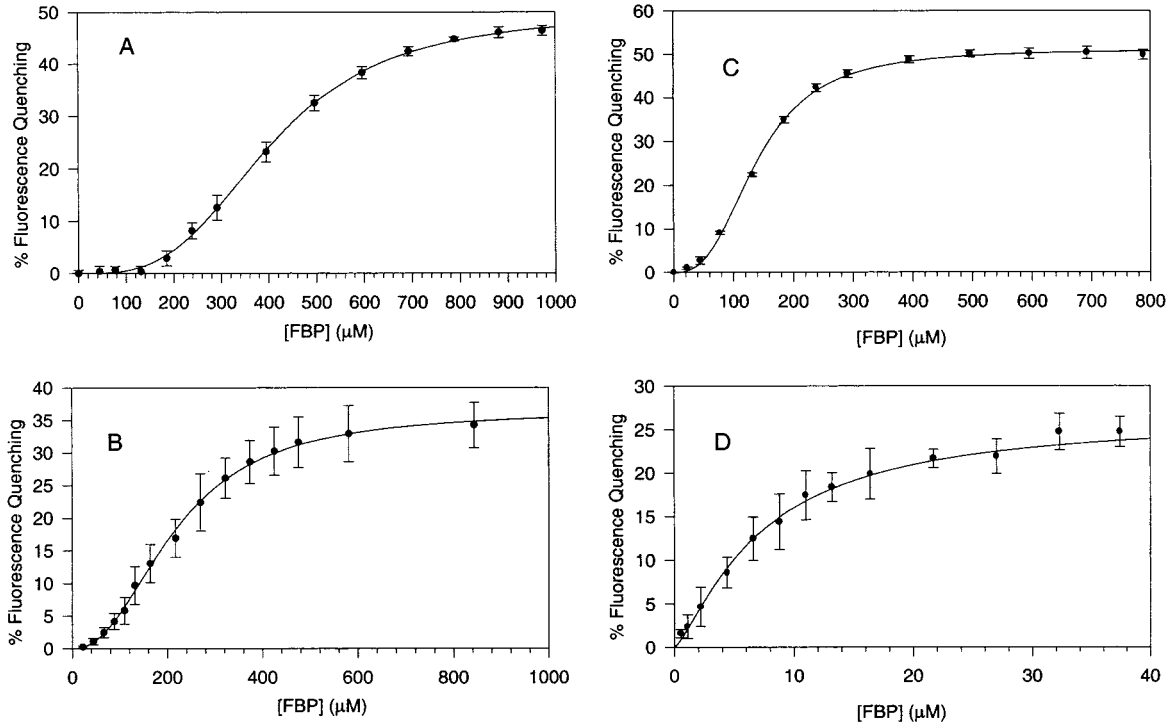

FIGURE 4: Interaction of FBP with YPK complexes. The extent of binding of FBP to apo-YPK, the YPK−Mg^{2+} complex, the YPK−PEP complex, and the YPK−Mg^{2+}−PEP complex was measured by steady-state fluorescence quenching at 334 nm. All complexes contain 200 mM KCl. (A) The interaction of FBP with apo-YPK. The data were best fit to eq 3. (B) The interaction of FBP with the YPK−Mg^{2+} complex where the Mg^{2+} concentration is 15 mM. The data were best fit to eq 3. (C) The interaction of FBP with the YPK−PEP complex where the PEP concentration is 5 mM. The data were best fit to eq 3. (D) The interaction of FBP with the YPK−Mg^{2+}−PEP complex where the Mg^{2+} concentration is 15 mM and the PEP concentration 5 mM. The data were best fit to eq 3. The curves are calculated fits to the data.

The fluorescence titration of Mg^{2+} into the YPK−PEP complex is shown in Figure 3B. The data are best described by eq 3, and the resulting Q_{max}, K_d, and n_H values are 14.3 ± 0.4%, 4040 ± 210 μM, and 1.85 ± 0.14, respectively. Thus, Mg^{2+} binds to the YPK−PEP complex with positive cooperativity. In the case of a tetrameric enzyme, the observed K_d for a ligand binding to four sites with positive cooperativity is a complex function of both the intrinsic K_d values and the strength of the homotropic interactions between individual binding sites. In fitting the data to eq 3, we report the K_d as the concentration of ligand that elicits half-maximal response. The overall percent fluorescence changes are 22.2 ± 0.3 and 14.3 ± 0.4% for titration of Mn^{2+} and Mg^{2+}, respectively, into the YPK−PEP complex. This indicates that the conformations of the YPK−Mn^{2+}−PEP and YPK−Mg^{2+}−PEP complexes are different. The percent fluorescence change is insignificant upon saturation of apo-YPK with Mg^{2+}, whereas saturation of the enzyme with Mn^{2+} causes a maximal fluorescence quenching of 9.3 ± 0.5%. This indicates that the structures of metal-saturated YPK in the presence Mg^{2+} or Mn^{2+} are also significantly different.

Interaction of FBP with YPK Complexes. The interaction of FBP with various YPK complexes was investigated by steady-state fluorescence titrations. Figure 4A shows the percent fluorescence quenching as a function of FBP concentration for titration of FBP into apo-YPK. The data were best fit by eq 3, and the resulting parameters are as follows: Q_{max} = 49.6 ± 0.6%, K_d = 407 ± 5 μM, and n_H = 3.27 ± 0.10. The Hill coefficient indicates that FBP binds to apo-YPK with positive cooperativity.

In the presence of 5 mM PEP, the cooperativity of FBP binding changes significantly, with an n_H of 2.27 ± 0.09 upon fitting of the data to eq 3 (Figure 4B). The K_d for FBP binding to this complex decreases to 217 ± 5 μM.

The binding of FBP to the binary YPK−Mg^{2+} complex shows a decrease in the degree of homotropic cooperativity in FBP binding. The resulting n_H after fitting to eq 3 is 3.09 ± 0.16. Similar quantitative results were observed with FBP binding to the YPK−Mn^{2+} complex (*14*). The presence of Mg^{2+} positively influences FBP binding to YPK, with a resulting K_d of 145 ± 3 μM.

Binding of FBP to the ternary YPK−Mg^{2+}−PEP complex results in significant changes in both the homotropic cooperativity and the affinity of the enzyme for FBP. The percent fluorescence quenching versus FBP concentration data are shown in Figure 4D. The data are best described by eq 3, and the resulting values for Q_{max}, K_d, and n_H are 26.6 ± 1.1%, 7.27 ± 0.57 μM, and 1.30 ± 0.08, respectively. These data are similar to those for FBP binding to the analogous YPK−Mn^{2+}−PEP complex (K_d = 3.13 ± 0.14 μM and n_H = 1.30 ± 0.07). The kinetically determined parameters are as follows $K_{m,FBP}$ = 12.8 ± 0.63 μM and $n_{H,FBP}$ = 1.0 for FBP activation in the presence of Mg^{2+} (*32*). Thus, in the fully ligated kinetic complex, the homotropic interaction of FBP is heterotropically abolished. The Mg^{2+}- and Mn^{2+}-activated enzymes differ in their response to FBP. In the presence of Mn^{2+}, FBP interacts with the kinetically competent complex with an n_H of 1.4 ± 0.4 (*15*). Table 1 summarizes the binding data for Mg^{2+}, PEP, and FBP. The apparent binding free energy, ΔG, was calculated from the K_d values using eq 5. Values for ΔG are summarized in Table 1.

Table 1: Summary of Ligand Dissociation Constants for Pyruvate Kinase Complex Formation

enzyme complex	ligand	K_d (μM)	n_H	ΔG (kcal/mol)
YPK−K^+	PEP	638 ± 53	1	−4.36 ± 0.08
YPK−K^+−Mg^{2+}	PEP	2810 ± 160	1	−3.48 ± 0.06
YPK−K^+	Mg^{2+}	11900 ± 1800	nd[a]	−2.62 ± 0.15
YPK−K^+−PEP	Mg^{2+}	4040 ± 210	1.85 ± 0.15	−3.26 ± 0.05
YPK−K^+	FBP	407 ± 5	3.27 ± 0.10	−4.62 ± 0.01
YPK−K^+−PEP	FBP	217 ± 6	2.27 ± 0.09	−5.00 ± 0.03
YPK−K^+−Mg^{2+}	FBP	145 ± 3	3.09 ± 0.16	−5.23 ± 0.02
YPK−K^+−PEP−Mg^{2+}	FBP	7.3 ± 0.6	1.30 ± 0.08	−7.01 ± 0.08

[a] Not determined.

Table 2: Two- and Three-Ligand Coupling Free Energies for Mg^{2+}- and Mn^{2+}-Activated YPK

	Mg^{2+}	Mn^{2+} [a]
ΔG_{M-PEP}[c] (kcal/mol)	−0.64 ± 0.19	−3.88 ± 0.08
ΔG_{M-PEP}[d] (kcal/mol)	0.88 ± 0.10	−2.47 ± 0.10
ΔG_{M-FBP} (kcal/mol)	−0.75 ± 0.03	−1.09 ± 0.02
$\Delta G_{PEP-FBP}$ (kcal/mol)	−0.38 ± 0.04	−0.22 ± 0.03
$\Delta G_{PEP-FBP/M}$ (kcal/mol)	−1.78 ± 0.08	−1.63 ± 0.03[b]
$\Delta G_{M-PEP-FBP}$ (kcal/mol)	−3.21 ± 0.03	−6.60 ± 0.09

[a] From ref *14*. [b] From ref *31*. [c] Calculated using eq 6 (PEP binding first). [d] Calculated using eq 7 (Mg^{2+} binding first).

DISCUSSION

Yeast PK contains a single tryptophan residue per subunit that was used as an intrinsic fluorescent probe to measure the extent of ligand binding. Because these binding experiments were performed in the absence of any M^{2+}−ADP complex, YPK is not undergoing steady-state turnover. The thermodynamic analysis, therefore, is straightforward, and the binding free energies are calculated directly from ligand binding constants and not from kinetic constants (see ref *32*). The binding constants are thermodynamic parameters and are related directly to the binding free energy using eq 5. In biological systems, virtually all binding constants are "apparent" since they are linked to some additional variable. When conditions are defined under which these constants are measured, they are important in the elucidation of mechanisms and in the quantitation of binding phenomena. Careful measurements of the extent of ligand binding to a protein in the presence and absence of a second ligand can identify synergistic or antagonistic ligand binding and the extent of these effects by a calculation of $\Delta(\Delta G)$ values for binding. Temperature, pH, and ionic strength effects on ΔG of binding of a specific ligand can also prove to be revealing.

When cooperativity in binding is observed, apparent binding is quantitated using the K_d value calculated using eq 3. From this treatment, the ligand concentration that elicits half-maximal binding is obtained. It is important to note that a true interpretation of K_d values obtained from cooperative binding is unclear at this time. Such data can be fit to a model with microscopic binding constants to a particular site on the protein and with coupling factors for binding. Calculations of apparent binding free energies are also made from the K_d values from eq 3 using eq 5. The specific meaning of such values is unclear. These $\Delta G'$ values can be used for comparison and for calculations of apparent coupling. For instance, do the conditional ΔG values increase or decrease in the presence of a second ligand, and by how much? Such information is important in the study of ligand−protein interactions and in the influence of ligand−ligand effects on binding phenomena. Comparisons and interpretations of apparent binding constants calculated from eqs 2 and 3 are not direct and must be done with great care (vide infra).

In this study, the biophysical properties of Mg^{2+}-activated yeast PK were investigated. The physiological divalent metal activator of PK is generally considered to be Mg^{2+}; however, Mn^{2+} and Co^{2+} can substitute and are catalytically competent activators (*22*). In previous studies with this enzyme, Mn^{2+} was the divalent cation of choice for several pragmatic reasons. The most important of these is that Mn^{2+} is paramagnetic, and therefore may serve as a probe in measuring the extent of ligand binding and ligand interactions by magnetic resonance techniques (*15*, *23*). The general kinetic properties of Mg^{2+}- and Mn^{2+}-activated yeast pyruvate kinase are similar. The velocity response as a function of PEP concentration for both Mg^{2+}- and Mn^{2+}-activated enzymes is sigmoidal in the absence of the allosteric affector, FBP. The velocity responses as a function of Mg^{2+} and Mn^{2+} concentrations in the absence of FBP are also sigmoidal. Saturating concentrations of FBP cause the velocity response of YPK as a function of PEP and of the divalent metal to become hyperbolic; FBP heterotropically abolishes homotropic cooperativity in PEP and in divalent metal interactions with YPK. Since FBP affects the apparent affinity of YPK for PEP and for Mg^{2+} and does not significantly alter V_{max}, FBP is a K-type allosteric effector of YPK in the presence of either divalent metal (*32*).

Mg^{2+} binding to apo-YPK does not cause a significant fluorescence spectral change because the structural change in the environment surrounding W452 is probably negligible. Mg^{2+} does bind to the free enzyme because the presence of a kinetically saturating Mg^{2+} concentration alters the K_d for subsequent PEP and FBP binding. Because the binding of PEP, Mg^{2+}, and FBP can each be measured independent of the other ligands, a random mechanism is proposed, consistent with previous kinetic data (*24*).

Thermodynamic linked-function analysis was used to quantitate the heterotropic multiligand interactions that occur between PEP, Mg^{2+}, and FBP with YPK in the absence of the second substrate, MgADP. This approach has been successful in the study of the analogous interactions with Mn^{2+}-activated YPK. With the Mn^{2+}-activated enzyme, linked-function analysis revealed that the allosteric communication between the FBP binding site and the PEP binding site was mediated by the divalent metal ion (*14*). This led to development of the differential ligation model, or extended sequential model, for describing the multiligand interactions on YPK (*14*).

The standard free energies of formation of various YPK complexes were calculated using eq 5 and are summarized in Table 1. These standard free energy changes were used to calculate the two- and three-ligand coupling free energies that are listed in Table 2.

Two significantly different two ligand coupling free energies for coupling between Mg^{2+} and PEP are calculated, depending on the model of whether Mg^{2+} or PEP binds first to the apoenzyme. If we assume that PEP binds first and use eq 6, ΔG_{Mg-PEP} equals −0.64 ± 0.19 kcal/mol. Thermodynamically, a negative ΔG_{M-PEP} indicates that simultaneous binding of Mg^{2+} and PEP to YPK to form the ternary

YPK−Mg^{2+}−PEP complex is synergistically favorable over formation of either the YPK−Mg^{2+} or YPK−PEP complex. A relatively strong cooperative interaction between PEP and Mn^{2+} was identified for both yeast PK [−3.88 ± 0.08 kcal/mol (*14*)] and muscle PK [−3.13 ± 0.08 kcal/mol (*25*)]. This large ΔG_{M-PEP} is explained in part by the formation of a strong inner sphere coordination complex between the phosphate group of PEP and the divalent metal. Formation of an inner sphere complex between the phosphoryl group of PEP and enzyme-bound Mn^{2+} has been demonstrated by both X-ray crystallography (*26*−*28*) and spectroscopic methods (*29*, *30*). Although there is a significant amount of crystal structure and spectroscopic data for PK in the presence of Mn^{2+}, there are no data describing the interaction of the substrate phosphoryl group with enzyme-bound Mg^{2+}. The ΔG_{M-PEP} with Mg^{2+} is significantly smaller than the ΔG_{M-PEP} in the presence of Mn^{2+}. This may be due to the fact that binding of Mn^{2+} to the YPK−PEP complex is significantly more favorable than binding of Mg^{2+} to the YPK−PEP complex, while the free energy of formation of the YPK−M^{2+} complex is similar with both metals.

Using eq 7, and therefore using the assumption that Mg^{2+} binds first to apo-YPK, the calculated ΔG_{M-PEP} equals 0.88 ± 0.10 kcal/mol. The positive ΔG_{M-PEP} suggests that the interaction between PEP and Mg^{2+} binding to YPK is antagonistic when Mg^{2+} binds first. Thermodynamically, this means that simultaneous binding of Mg^{2+} and PEP to form the YPK−Mg^{2+}−PEP complex is unfavorable compared to formation of either of the two binary complexes, namely, YPK−PEP or YPK−Mg^{2+}. This is surprising since the interaction between the divalent metal and PEP is at least partially determined by electrostatic interactions. One potential explanation is that the high concentration of $MgCl_2$ required to saturate the enzyme would introduce a significant ionic strength effect on the binding of PEP. This was tested by measuring the dissociation constant for the interaction between PEP and the apoenzyme in the presence of 245 mM KCl. The ionic strength of a solution of 245 mM KCl is the same as the ionic strength in the presence of 200 mM KCl and 15 mM $MgCl_2$. The K_d for PEP was found to be 770 ± 56 μM. Therefore, the K_d measured in the presence of 15 mM Mg^{2+} must have a small ionic strength component, but this does not account for the 4-fold decrease in the affinity of PEP binding to the YPK−Mg^{2+} complex versus binding to the apoenzyme. An alternative explanation is that small differences in the active site conformation induced by the binding of Mg^{2+} could antagonize the subsequent binding of PEP. For example, the binding of Mn^{2+} to the rabbit muscle PK apoenzyme has a dissociation constant of 55 ± 5 μM (*25*). The binding of Mn^{2+} to apo-YPK has a dissociation constant of 7160 ± 930 μM (*7*). The active site residues for 27 species of PK, including the rabbit muscle and yeast enzymes, are 100% conserved (*31*). Therefore, there must be a significant conformational contribution to the relative affinities of binding of the metal to each of these enzymes. Differences in the binding affinities of YPK for PEP in the presence and absence of Mg^{2+} may be modified by such changes.

By conservation of free energy, the total free energy of formation of the YPK−Mg^{2+}−PEP complex should be the same regardless of the order of addition of ligands. The total free energy of formation of the YPK−Mg^{2+}−PEP complex is −6.10 ± 0.16 and −7.62 ± 0.09 kcal/mol for Mg^{2+} binding first and PEP binding first to apo-YPK, respectively. These values differ by 1.5 kcal/mol. The difference in the ΔG_{Mg-PEP} calculated from eqs 6 and 7 suggests that the YPK−Mg^{2+}−PEP complexes that formed depend on the order of addition of ligands. This phenomenon might be explained if pre-equilibration of the enzyme in the presence of saturating concentrations of a given ligand will generate different forms of the enzyme, each of which may stabilize subsequent ligand binding to a different extent. Therefore, the coupling free energies measured for each path may not be identical with those of YPK.[2] Since the free energy of formation of a particular complex should be path-independent, the total free energy of formation of the YPK−Mg^{2+}−PEP complex should be the same, regardless of the order of addition of substrates. The total percent fluorescence change for formation of the YPK−Mg^{2+}−PEP complex is the same, regardless of the order of addition of ligands. Therefore, the structure surrounding W452 is the same whether Mg^{2+} or PEP binds first. These discrepancies in coupling free energies are probably due to the use of apparent free energy changes in these calculations. Cooperativity in Mg^{2+} binding to the YPK−PEP complex occurs, and the K_d value calculated from the sigmodal data was used for these analyses. Therefore, this apparent anomaly in thermodynamic linkage does not arise from a breakdown of thermodynamic principles but from the presence of complex thermodynamic systems that are presently unresolvable. Similar data have been obtained with Mn^{2+}-activated YPK, where the coupling free energies that were measured differ by approximately 1.3 kcal/mol, depending of the order of addition of Mn^{2+} and PEP (*14*).

ΔG_{M-FBP}, the free energy for coupling between the divalent metal and FBP without PEP, equals −0.75 ± 0.03 and −1.09 ± 0.02 kcal/mol with Mg^{2+} and with Mn^{2+}, respectively. The absolute magnitude of this two-ligand interaction is smaller with Mg^{2+} than with Mn^{2+}. The coupling between the divalent metal binding site and the FBP binding site is communicated by structural changes in the polypeptide rather than by a direct electrostatic interaction, since the FBP and divalent metal binding sites are separated by approximately 40 Å (*27*). Mg^{2+} clearly does not induce the same structural change at the FBP binding site as does Mn^{2+}. Given that formation of the binary YPK−M^{2+} complex with both metals occurs with a similar free energy change, the total free energy of formation of the YPK−M^{2+}−FBP complexes and the coupling free energy ΔG_{M-FBP} should be metal-independent if the structures induced by each metal are the same. Results presented here show that the structures induced by each metal are not identical.

The coupling free energy between PEP and FBP binding to the enzyme is weak (−0.38 ± 0.04 kcal/mol). Kinetic data suggest that the coupling between these two ligands is large, since FBP causes a 20-fold decrease in the apparent affinity of the enzyme for PEP (*32*). These data, however, were obtained in the presence of a saturating divalent metal.

[2] One reviewer suggested that the thermodynamic discrepancy can occur if there is a kinetic well or that a kinetic component may be present and we are not correcting for this. While such an explanation is possible, there are no data that suggest a slow process is occurring on the time scale of these experiments. The most logical explanation for the failure of this linkage is the utilization of both apparent and actual thermodynamic constants for such calculations.

Previous kinetic and thermodynamic studies with the Mn^{2+}-activated enzyme have shown that it is the interaction between PEP and metal, and FBP and metal, that communicates the coupling between PEP and FBP sites (*14*, *15*). The coupling free energy between the PEP and FBP sites in the presence of saturating divalent metal is -1.78 ± 0.08 and -1.63 ± 0.03 kcal/mol for the Mg^{2+}- and Mn^{2+}-activated YPK, respectively. Thus, it appears that Mg^{2+} plays the same role as Mn^{2+} in communicating the heterotropic interaction between PEP and FBP with YPK, and that both metals mediate this communication to the same extent.

The proposed extended sequential model of cooperativity is based on thermodynamic linked-function analysis of Mn^{2+}-activated YPK (*14*). A preferred ordered kinetic mechanism was inferred with the $Mn^{2+}-$YPK complex, where either PEP or FBP bound the enzyme first, followed by Mn^{2+}. On the basis of the results obtained with Mg^{2+}-activated YPK, the order of binding of PEP, Mg^{2+}, and FBP is probably the same. It is unlikely that Mg^{2+} binds the enzyme before PEP, since the resulting ternary complex would be significantly less stable than either the YPK−PEP or YPK−Mg^{2+} binary complex. The ternary complex would only form at saturating concentrations of both PEP and Mg^{2+}. In the absence of MgADP, the addition of ligands is probably PEP followed by Mg^{2+}, which increases the affinity of the enzyme for FBP. Conversely, the binding of FBP could increase the affinity of YPK for Mg^{2+}which, in turn, would increase the affinity of the enzyme for PEP.

The three-ligand coupling free energy, $\Delta G_{PEP-Mg-FBP}$, equals -3.21 ± 0.03 kcal/mol. This value is significantly weaker than the three-ligand coupling free energy with Mn^{2+}-activated YPK [$\Delta G_{PEP-Mn-FBP} = -6.60 \pm 0.09$ kcal/mol (*14*)]. The formation of the quaternary YPK−PEP−Mg^{2+}−FBP complex is favorable by -3.21 kcal/mol over the formation of other binary or ternary complexes with Mg^{2+}, and is 3.30 kcal/mol less favorable than the formation of the analogous quaternary YPK−PEP−Mn^{2+}−FBP complex. Formation of the Mg^{2+}-activated quaternary complex is significantly less favorable because ΔG_{PEP-Mg} is significantly less negative than ΔG_{PEP-Mn}.

An interesting facet of the PEP−Mg^{2+} interaction is that Mg^{2+} does not heterotropically induce homotropic cooperativity in PEP binding. Binding of PEP to the YPK−Mn^{2+} complex occurs with a Hill coefficient of approximately 2.4. The studies of binding of PEP to the YPK−Mg^{2+} complex yield a K_d of 2.8 mM and an n_H of 1.0. Kinetically, the velocity response of Mg^{2+}-activated YPK as a function of PEP concentration under similar conditions is sigmoidal, with a Hill coefficient of approximately 2.6. Since the binding experiments were carried out in the absence of ADP, the nucleotide (MgADP) must assist in the modulation of the homotropic binding of PEP to YPK. Free ADP antagonizes the binding of PEP to YPK, but does not induce homotropic cooperativity in PEP binding (*15*). It is therefore hypothesized that the nucleotide-bound divalent metal plays a role in the cooperativity of PEP binding. Kinetic measurements of the $n_{H,PEP}$ as a function of MgADP concentration show that the degree of homotropic cooperativity in the PEP interaction is modulated by the MgADP complex (*32*). Extrapolation of the kinetic data at 0 mM MgADP gives an $n_{H,PEP}$ of 1.0. Therefore, the kinetically measured Hill coefficient approaches the thermodynamically measured value in the absence of steady-state turnover.

The multiligand interactions between YPK and PEP, Mg^{2+}, and FBP were quantitated by thermodynamic linked-function analysis and compared to the analogous interactions with Mn^{2+}-activated YPK. Both ΔG_{M-PEP} and ΔG_{M-FBP} were significantly weaker in the presence of Mg^{2+} than in the presence of Mn^{2+}. Both Mg^{2+} and Mn^{2+} serve to mediate the allosteric interaction between the PEP binding site and the FBP binding site. The magnitude of the PEP−FBP interaction in the presence of either divalent metal is the same. The three-ligand coupling free energy for the Mg^{2+}-activated enzyme was significantly lower than that for the Mn^{2+}-activated enzyme, but the coupling results in a significantly negative value. The use of apparent K_d values for ligand binding when cooperativity in binding occurs must be recognized as apparent quantitative data. The results of these studies reinforce the key role played by the enzyme-bound cation in the ligand interactions and cooperative behavior of YPK. Physiologically, the cell (yeast, erythrocytes, etc.) probably requires a less highly coupled PK system with Mg^{2+}-activated PK for efficient control than the more highly coupled system that would occur with Mn^{2+}-activated PK.

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