Conformational Changes in Yeast Pyruvate Kinase Studied by $^{205}Tl^+$ NMR†

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ABSTRACT: The interaction of the monovalent cation with yeast pyruvate kinase (yPK) has been investigated by $^{205}Tl^+$ NMR. $TlNO_3$ activates yPK to 80−90% activity compared to KCl with an apparent K_a of 1.00 ± 0.03 mM in the presence of 4 mM $Mn(NO_3)_2$ as the activating divalent cation. At higher concentrations of Tl^+, enzyme inhibition is observed with an apparent K_I of 180 ± 10 mM. The extent of inhibition is dependent on the nature and concentration of the divalent cation. The effect of Mn^{2+} on the $1/T_1$ and $1/T_2$ values of $^{205}Tl^+$ in the presence of yPK was determined at 173.02 MHz (300 MHz, ^1H) and 346.03 MHz (600 MHz, ^1H). The temperature dependence of the relaxation rates indicates that fast exchange conditions prevail for $^{205}Tl^+$ longitudinal relaxation rates. The correlation time, τ_c, for the Mn^{2+}−$^{205}Tl^+$ interaction was estimated by a frequency dependence of $1/T_{1m}$ for several enzyme complexes, and an average value of τ_c was determined to be 0.91 ns. The distance between Tl^+ and Mn^{2+} at the active site of yPK was calculated from the paramagnetic contribution of Mn^{2+} to the longitudinal ($1/T_{1m}$) relaxation rates of Tl^+ bound to yPK. For the apo yPK complex, the Tl^+ to Mn^{2+} distance is 6.7 ± 0.2 Å. Upon addition of phosphoenolpyruvate (PEP) to form the yPK−Tl−Mn−PEP complex, the inter-cation distance decreases to 6.1 ± 0.3 Å. The addition of the allosteric activator fructose 1,6-bisphosphate (FBP) to form the yPK−Tl^+−Mn^{2+}−PEP−FBP complex gives an intermetal distance of 6.2 ± 0.2 Å. In the yPK−Tl−Mn−FBP complex, a Tl^+−Mn^{2+} distance of 6.0 ± 0.1 Å is observed, indicating that FBP causes a conformational change at the active site in the absence of PEP. Analogous ^{205}Tl NMR experiments with competitive inhibitors of PEP (oxalate, BrPEP) indicate that these ligands do not induce the same conformational changes as do the physiological substrates and activators. Similar experiments with the nonallosteric rabbit muscle PK were also performed and analyzed.

Yeast pyruvate kinase (yPK)[1] is a tetrameric enzyme of identical 54-kDa subunits. PKs from almost all sources have an absolute requirement for both monovalent and divalent metal ions. PK was the first enzyme for which a specific monovalent cation requirement was established (*1*). Potassium is the physiologically important cation, but numerous monovalent cations (Li^+, Na^+, NH_4^+, Rb^+, Tl^+, Cs^+, $CH_3NH_3^+$) can also activate PK. Nearly all of the prior studies investigating the role of the monovalent cation have been performed with the nonallosteric rabbit muscle pyruvate kinase (mPK) (*2*). Nowak (*3, 4*) showed, using difference spectroscopy, that several monovalent cations produce conformational changes within muscle pyruvate kinase. Reuben and Kayne (*5*) provided the first evidence that the monovalent cation binds at the active site. Using $^{205}Tl^+$ NMR, they reported that thallium binds within 8 Å of the enzyme-bound Mn^{2+}. The cation−cation distance was found to be substrate dependent. In the presence of phosphoenolpyruvate (PEP), the two metals moved approximately 3 Å closer. Similar experiments using the weakly activating Li^+ showed that $^7Li^+$ also binds in the vicinity of the enzyme-bound Mn^{2+}. The distance between Li^+ and Mn^{2+} was estimated to be 11 Å (*6*). Reexamination of the mPK−Li^+−Mn^{2+} complex using EPR resulted in refined distance measurements for this complex (*7*). Nowak (*4*) determined the distance between the methyl protons of $CH_3NH_3^+$ and Mn^{2+} to be 6.3 Å in the mPK−Mn−PEP complex. This gives rise to a Mn^{2+}−nitrogen distance for $CH_3NH_3^+$ of 4.65 Å. All investigations with mPK indicate that the monovalent cation binds at the active site and that its location is sensitive to the binding of other ligands.

X-ray crystal structure data has provided conflicting results concerning the monovalent cation site in PK. In the structure of the cat muscle pyruvate kinase (*8*), which shares a 94% sequence identity with mPK, no electron density is observed for the monovalent cation. Its position is inferred from negative electron density in the difference maps. Side chains in the vicinity of the putative monovalent cation are Q328 and E363. Larsen et al. (*9, 10*) solved the crystal structure of rabbit muscle PK in complexes with pyruvate and phospholactate. The K^+ site appears to lie between the

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[1] Abbreviations: BrPEP, 3-bromophosphoenolpyruvate; EPR, electron paramagnetic resonance; FBP, fructose 1,6-bisphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; mPK, rabbit muscle pyruvate kinase; PEP, phosphoenolpyruvate; P-glycolate; phosphoglycolate; TMA-(NO_3), tetramethylammonium nitrate; yPK, yeast pyruvate kinase (*Saccharomyces cerevisiae)*; CPMG, Carr−Purcell modification of the Meiboom−Gill pulsed method for T_2 determinations.

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carbonyl oxygens of T311, O^{γ} of S76, $O^{\delta 1}$ of N74, and $O^{\delta 2}$ of D112. K^+ in this location is 5.7 Å from Mn^{2+}. The K^+ is not observed to interact directly with the bound pyruvate. The allosteric *Escherichia coli* form of PK has recently been crystallized (*11*). No evidence for the K^+ site was found in the crystal structure, although this form of PK requires K^+ for activity. Thus there is considerable ambiguity in the location and function of the monovalent cation in homologous forms of PK.

In contrast to the muscle forms of PK, the enzyme from yeast (yPK) is allosterically regulated by a number of ligands (FBP, H^+, PEP, M^{2+}, M^+, and ATP). yPK is highly homologous with the muscle forms of PK, and the active site residues are 100% conserved among the muscle and yeast enzyme forms. Despite the high active site homology between the muscle and yeast enzymes, there are significant differences in the divalent and monovalent metal activation of these enzymes (*12*−*15*). In addition, previous NMR studies of the monovalent cation with the muscle enzyme were performed at inhibitory concentrations of monovalent cation (*5*) and with weakly activating monovalent cations (*4, 6, 7, 16*). The correlation between the results of these studies and those from studies of a fully active enzyme is tenuous. It was of interest to examine this question more thoroughly and to extend it to the allosteric yeast pyruvate kinase.

Being an allosteric enzyme, yPK couples the binding of ligands to large conformational changes in the protein structure. Mesecar and Nowak (*13*) observed global conformational changes in yPK that are induced by substrates and by activators that bind to yPK. Binding of Mn^{2+}, PEP, and FBP to yPK caused significant alteration in intrinsic yPK tryptophan fluorescence. We recently observed similar structural changes in yPK upon binding of these ligands using circular dichroism, sedimentation velocity, and sedimentation equilibrium experiments (*17*). These data suggest that binding of FBP results in structural changes at the active site of yPK, which is 40 Å away from the FBP binding site (*18*). There is no direct evidence, however, linking the binding of the allosteric activator, FBP, with specific active-site structural changes. Measurement of the distances between the monovalent and divalent cations provides a specific method to monitor ligand-induced structural changes in yPK and to correlate global changes induced by the allosteric activator, FBP, with specific active site alterations. The monovalent cation to divalent cation distances for a number of ligand complexes of yPK have been determined by ^{205}Tl NMR. In addition, this study was extended to mPK for comparison. Previously determined distances were checked and have been refined.

EXPERIMENTAL PROCEDURES

Materials. Yeast pyruvate kinase (yPK) was purified by the method of Mesecar and Nowak (*13*). yPK was assayed by following the oxidation of NADH at 340 nm using the coupled assay with lactate dehydrogenase (*19*). Enzyme activity typically ranged from 260 to 280 U/mg at 22 °C. For Tl^+ activation studies, yPK was assayed as follows: a 1-mL cuvette contained 100 mM MES (pH = 6.2, adjusted with TMA-OH), 4% glycerol, 200 mM TMA-NO_3, 4 mM $Mn(NO_3)_2$, 5 mM PEP (cyclohexylammonium salt), 5 mM ADP (cyclohexylammonium salt), and 1 mM FBP (cyclohexylammonium salt). Thallous ion was added as $Tl(NO_3)$, and 10% D_2O was included to simulate NMR conditions. All chemicals were reagent grade or better. Thallium nitrate was purchased from Ventron, Inc. D_2O was purchased from Cambridge Isotope Labs. (Cambridge, MA). yPK concentration was determined using an ϵ value of 0.51 $[(mg/mL)^{-1}]$-cm^{-1} at 280 nm (*20*).

$^{205}Tl^+$ NMR. NMR experiments were carried out on Varian 300 MHz (173.02 MHz, ^{205}Tl) and Varian 600 MHz (346.03 MHz, ^{205}Tl) Unity Plus instruments. The probes were tuned to the thallium resonance using a home-built inductor rod. The 90° pulse widths were calibrated prior to each experiment and typically ranged from 9 to 13 μs. Enzyme samples were referenced to an external $TlNO_3$ sample, at 0 ppm. D_2O (10%) was included as a field-frequency lock. A spectral width of 5000 Hz and 5300 data points were used. A 5−10-Hz exponential line-broadening function was used with enzyme samples to improve signal-to-noise. Typically, 80−100 acquisitions were obtained. The sample temperature was maintained to ±0.1 °C for all NMR experiments.

In preparation for NMR experiments, yPK was desalted on a Bio-Rad P6-DG column equilibrated with 100 mM MES, pH = 6.2 (adjusted with TMA-OH), and 20% glycerol and concentrated to at least 10 mg/mL using an Amicon stirred cell concentrator prior to each NMR experiment. Included in the NMR tube was 100 mM MES, pH = 6.2, 4% glycerol, 80−100 μM enzyme sites, 15 mM $TlNO_3$, and 10% D_2O. Ionic strength was kept constant at a salt concentration of 200 mM by the addition of tetramethylammonium nitrate (TMA-NO_3). The added Mn^{2+} was titrated as $Mn(NO_3)_2$. The $Mn(NO_3)_2$ was titrated directly into the NMR tube in microliter amounts. Corrections for volume changes were taken into consideration when determining $[E]_T$, $[Mn]_T$, $[Mn]_f$, and $[Tl^+]_T$, although these corrections were <5%. When added, the concentration of PEP was 5 mM, the concentration of FBP was 1 mM, and the concentration of BrPEP was 1 mM. All experiments with mPK were performed in 50 mM cacodylate or 100 mM HEPES buffer, pH = 7.4.

Longitudinal relaxation rates ($1/T_1$) were determined using the inversion−recovery method (*21*). Transverse relaxation rates ($1/T_2$) were determined by measuring line widths at half peak height and by using the CPMG (*22*) technique. Typically 8−10 τ values were used and the data were fit to a single exponential. Relaxation data were fitted on a Sun Sparc station interfaced with the Varian spectrometers. In most cases T_1 and T_2 values were determined consecutively on the same enzyme sample. T_1 and T_2 values were determined at several (at least 5) concentrations of Mn^{2+}.

K_D Determination. For yPK, the binding constant for Mn^{2+} was primarily determined by fluorescence titrations. Titrations were performed on an SLM-Aminco 8100 spectrofluorimeter. Quenching of the lone tryptophan fluorescence upon Mn^{2+} binding was observed at 340 nm with excitation at 295 nm. Data were fit to either a hyperbolic or a cooperative model of ligand binding depending on the enzyme complex being studied and the appropriate binding model. Mn^{2+} binding to yPK−FBP complexes could not be determined directly by fluorescence since no additional fluorescence quenching of tryptophan is observed upon

ligand binding to enzyme−FBP complexes. The dissociation constant for Mn^{2+} binding to yPK−Tl^+−FBP was determined by the method described below. The reactions in the following scheme describe Mn^{2+} and FBP binding to yPK.

$E + M \leftrightarrow EM$	K_1
$E + A \leftrightarrow EA$	K_2
$EM + A \leftrightarrow EMA$	K_3
$EA + M \leftrightarrow EMA$	K_4

In this scheme, E is yPK, M is Mn^{2+}, and A is FBP, and the dissociation constants for each reaction are given. K_4 was calculated from the measured quantities K_1, K_2, and K_3 using the relationship $K_1K_3 = K_2K_4$. For mPK, binding of Mn^{2+} to the enzyme was determined by loss of EPR signal of free Mn^{2+} upon binding to mPK. All data for mPK were fit to a hyperbolic binding model.

NMR Data Calculations. The relaxation rates $1/T_1$ and $1/T_2$ of a nucleus bound near a paramagnetic center have been described previously (*23*−*26*), and applications of these methods to study enzyme−metal complex systems have been previously reviewed (*27, 28*). The correlation times (τ_c) governing the Mn−Tl interaction can be determined from the frequency dependence of $1/T_{1m}$ (eq 1) at $\omega_{I,1}$ and $\omega_{I,2}$ and from the ratio of $1/T_{1m}$ and $1/T_{2m}$ determined at a single frequency (eqs 1 and 2). The use of frequency dependence data assumes that τ_c is frequency independent. The use of T_{1m} and T_{2m} ratios requires the assumption that only dipolar contributions affect $1/T_{1m}$ and $1/T_{2m}$ (*28*).

$$\frac{1}{T_{1m}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) \quad (1)$$

$$\frac{1}{T_{2m}} = \frac{1\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) \quad (2)$$

When the correlation time is determined, the correlation function $f(\tau_c)$ can be calculated from eq 3.

$$f(\tau_c) = \frac{3\tau_c}{1+\omega_I^2\tau_c^2} \quad (3)$$

Once τ_c is known, the Tl^+−Mn^{2+} distance r, in angstroms, can be calculated.

$$r = 676\left(\sqrt[6]{f(\tau_c)T_{1m}}\right) \quad (4)$$

As an additional method of determining τ_c for these complexes, the paramagnetic contribution to the relaxation rates of the water protons was determined using the approach of Navon (*29*). The $1/T_{1m}$ and $1/T_{2m}$ values were determined for the water protons in these complexes by the inversion−recovery and CPMG methods, respectively. From these data, τ_c can be calculated using eqs 1 and 2 as with the previous ^{205}Tl data.

RESULTS

Yeast PK. Kinetics and Mn^{2+} Binding. No yPK activity is observed in the absence of an activating monovalent cation where TMA or cyclohexylammonium ions are used as countercations. This agrees with the observations of Boyer (*1*) with muscle PK and of Kayne (*30*) with yPK that an absolute requirement exists for an activating monovalent cation with these enzymes. Thallium can activate yPK to 85% compared to activity in the presence of K^+ (Table 1). At higher Tl^+ concentrations, inhibition is observed (Figure 1). With Mn^{2+} as the divalent cation, Tl^+ has a steady-state activator constant approximately 30-fold less than that for K^+. The activator constant, K_I, and k_{cat} values are dependent upon the divalent cation (Table 1). Large changes in Mn^{2+} binding to yPK occur in the presence of substrate, substrate analogues, and the heterotropic activator (Table 2). Pyruvate kinase from rabbit muscle shows similar activation and inhibition behavior by thallium (*30*). For all NMR experiments with yPK, 15 mM $Tl(NO_3)$ was used. This concentration is 15-fold greater than the K_a for Tl^+ to ensure saturation and 10-fold less than its K_I value (Table 1).

Table 1: Kinetic Constants for Monovalent Cation Activation[a]

complex	k_{cat} (s^{-1})	K_a (mM)	K_I (mM)
yPK−Tl^+−Mn^{2+} [b]	7500 ± 150	1.15 ± 0.06	134 ± 10
yPK−Tl^+−Mn^{2+}−FBP[b]	6850 ± 150	0.96 ± 0.07	181 ± 21
yPK−Tl^+−Mg^{2+} [b]	5850 ± 160	4.70 ± 0.09	52 ± 4.0
yPK−Tl^+−Mg^{2+}−FBP[b]	17400 ± 1550	2.12 ± 0.30	15 ± 2.3
yPK−K^+−Mn^{2+}−FBP[c]	8100 ± 470	35 ± 4	

[a] Assays were performed as described in Experimental Procedures with ionic strength kept at 200 mM with TMA-NO_3. [b] Thallium activation data were fitted to a noncompetitive inhibition model, $v_o = V(A/K_a)/(1 + A/K_a + A/K_I + A^2/K_aK_I)$. [c] K^+ activation data were fitted to $v_o = V/(1 + K_a/A)$.

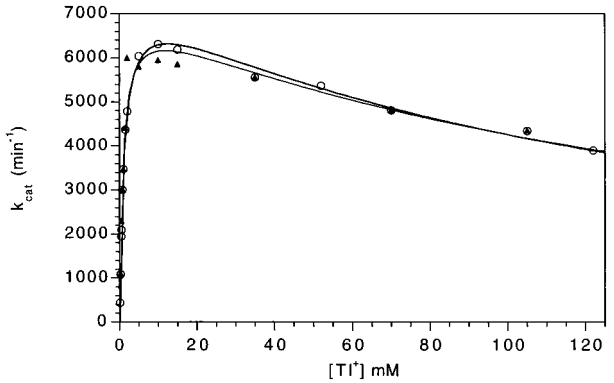

FIGURE 1: Activation of yPK by thallium. The activity of yPK was measured in the presence of 4 mM $Mn(NO_3)_2$, 5 mM PEP, and 5 mM ADP (○) and with the addition of 1 mM FBP (▲) as described in Experimental Procedures. Ionic strength was kept at 200 mM with TMA-NO_3. Data were fitted to a kinetic model for noncompetitive inhibition, and the fits generated the lines through the data.

NMR Experiments with Yeast PK. A solution of 15 mM $Tl(NO_3)$ was used as a reference in the NMR experiments, and its resonance was set to 0.0 ppm. This solution contained the same buffer concentration and ionic strength as those containing enzyme. All pulse calibrations were performed on this solution. The line width of the thallium resonance in solution was 6 Hz at 173.02 MHz. Addition of $Mn(NO_3)_2$ (up to 5 mM) to a solution of $Tl(NO_3)$ in buffer did not affect the T_1 or T_2 relaxation rates. Upon the addition of 100 μM yPK, the thallium resonance was broadened to 30 Hz and shifted downfield by 9 ppm (not shown). In the yPK−PEP complex the thallium resonance broadened to 50 Hz and shifted downfield to 18 ppm. The addition of FBP to this complex caused no further change in the Tl^+ resonance.

Table 2: Dissociation Constants for Mn^{2+} Binding to Pyruvate Kinase Complexes[a]

enzyme complex	$K_{D,Mn^{2+}}$ (μM)
yPK−Tl^+ [b]	7200
yPK−Tl^+−PEP[b]	10
yPK−Tl^+−PEP−FBP[c]	2
yPK−Tl^+−FBP[d]	586
yPK−Tl^+−BrPEP[b]	7
yPK−Tl^+−BrPEP−FBP[c]	3
yPK−Tl^+ (pH 7.4)[b]	7000
yPK−Tl^+−oxalate[b]	4
mPK−Tl^+(100 mM)[e]	300
mPK−Tl^+(100 mM)−PEP[e]	34
mPK−Tl^+(15 mM)[e]	117
mPK−Tl^+(15 mM)−PEP[e]	27

[a] The dissociation constants for Mn^{2+} binding were determined for various enzyme complexes by [b]fluorescence titrations, [c]kinetic activation constants and direct binding studies, [d]calculation as described in Experimental Procedures, and [e]EPR titration. K_D values determined by fluorescence had standard errors less than 5%, while those determined by EPR were <10%. The concentration values in parentheses for muscle PK are the concentrations of $TlNO_3$ used in the measurement. The total ionic strength was 200 mM, adjusted with TMA-NO_3 when necessary.

Addition of Mn^{2+} to any yPK−Tl^+ complex resulted in an increase in the relaxation rates for thallium, while addition of Mg^{2+} to these same complexes did not cause any change in the T_1 or T_2 relaxation rates or in the chemical shifts of thallium. At 173.02 MHz, $1/T_2$ values determined by the CPMG method were typically 10−15% less than those determined by line-width measurements. At 346.03 MHz, the line-width measurements gave $1/T_2$ values much larger than those obtained using the CPMG method. The values of $1/T_2$ reported in this study were obtained by the CPMG measurements unless otherwise indicated. Deviations from single-exponential fits to the relaxation data were typically less than 5% but ranged up to 25% at higher concentrations of Mn^{2+}.

The temperature dependence of the transverse and longitudinal relaxation rates for enzyme-bound Tl^+ was investigated to determine if rapid exchange conditions exist (*28*). The temperature of the NMR sample was varied from 5 to 40 ± 0.1 °C. An Arrhenius plot of the $1/T_1$ data for each complex studied gave a linear response with a positive slope. The data for several representative yPK complexes are shown in Figure 2. The activation energy determined from Figure 2 for all yPK complexes ranged from −2.7 to −5.3 kcal/mol. The temperature dependence data of $1/T_2$ relaxation rates gave plots that had zero or negative slopes depending on the complex under investigation (not shown), indicating possible intermediate exchange on the time scale of the transverse relaxation rates.

Table 3A contains a summary of the normalized paramagnetic contributions ($1/T_{1m}$ and $1/T_{2m}$) to the $1/T_1$ and $1/T_2$ values determined at 173.02 and at 346.03 MHz for the yPK complexes investigated. In the apo yPK−Tl^+ complex, the $1/T_{1m}$ value is 1500 ± 400 s^{-1} at 173 MHz (Table 3A), an average from 14 different experiments. When yPK binds PEP, this value increases to 2540 s^{-1}. Addition of the allosteric activator FBP to the yPK−Tl^+−PEP complex does not result in a further change in the observed relaxation rates. The yPK−Tl^+−FBP complex gives relaxation results similar to those for the yPK−Tl^+−PEP complex (Table 3A). The substrate analogue BrPEP, a substrate giving V_{max} values 2% that of PEP (*31*) does not elicit the same increase in ^{205}Tl relaxation rates as does PEP. This enhancement only occurs in the presence of BrPEP and FBP (Table 3A). Oxalate, which is a putative intermediate analogue of enolpyruvate, induces a calculated $1/T_{1m}$ value of 380 ± 150 s^{-1}. Addition of FBP to the oxalate complex to form the yPK−Tl−oxalate−FBP complex results in a small increase in $1/T_{1m}$ to 420 ± 35 s^{-1}. The value for $1/T_{1m}$ for the yPK−Tl^+ complex at pH 7.4 increases to 2260 s^{-1}. These data indicate that the Tl−Mn distance varies depending on the bound ligand.

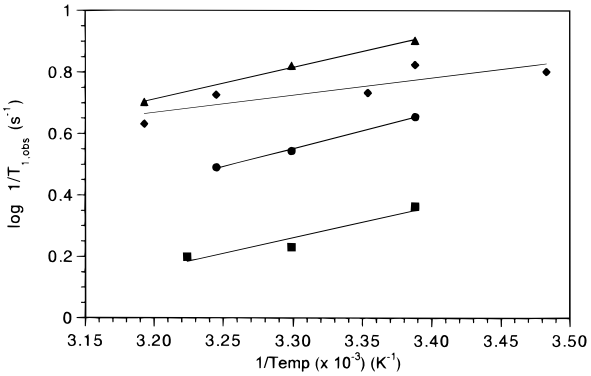

FIGURE 2: Temperature effects on $1/T_1$. The $1/T_1$ values of ^{205}Tl were determined for the (■) yPK(apo, 80 μM sites), (●) yPK(80 μM sites, 5 mM PEP, 18 μM Mn^{2+}), (▲) yPK(80 μM sites, 5 mM PEP, 100 μM Mn^{2+}), and (◆) yPK(80 μM sites, 5 mM PEP, 1 mM FBP, 38 μM Mn^{2+}) complexes. The error in the measurements is less than 10% in all cases. Temperature was maintained to ±0.1 °C using the Varian temperature controller.

NMR Experiments with Rabbit Muscle PK. The results for ^{205}Tl NMR experiments performed with rabbit muscle PK are tabulated in Table 3B. For mPK, the experiments were performed at 15 and at 100 mM Tl^+. These experiments were done for a comparison with previous data reported by Reuben and Kayne (*5*) who used Tl^+ concentrations of 100 mM. Using 15 mM Tl^+, we were unable to reproduce the quantitative relaxation rate values for mPK reported by Reuben and Kayne (*5*). This prompted us to measure Mn^{2+} binding to mPK in the presence of 15 and 100 mM Tl^+ keeping ionic strength constant (Table 2). Muscle PK binds Mn^{2+} three times more tightly at 15 than at 100 mM $TlNO_3$. Not accounting for this variation in Mn^{2+} binding will result in erroneous distance calculations. At 15 mM Tl^+, the binding of PEP results in a 15-fold increase in the measured $1/T_{1m}$ values, while at 100 mM thallium this increase is only 2.5-fold. The newly determined Mn^{2+} binding constants were used to recalculate the $1/T_{1m}$ values determined previously (*5*) at 24.3 MHz. The recalculated $1/T_{1m}$ value of 4300 ± 300, corrected for Mn^{2+} binding, is compared to the nonnormalized value of 2990 ± 300 (Table 3) (*5*).

DISCUSSION

Thallium Activation. yPK is activated by Tl^+ with k_{cat} values similar to that obtained with K^+ and is followed by subsequent inhibition of activity at higher Tl^+ concentrations (Figure 1). It is likely that the inhibition observed is due to Tl^+ binding at the divalent metal site at higher concentrations. This is supported by the measured decrease in affinity of PK for Mn^{2+} at higher Tl^+ concentrations. In our studies,

Table 3: Normalized Longitudinal and Transverse Relaxation Rates

(A) Yeast Pyruvate Kinase Complexes[a]

enzyme complex	$1/T_{1m}$ (s^{-1}) (173 MHz)	$1/T_{1m}$ (s^{-1}) (346 MHz)	$1/T_{2m}$ (s^{-1}) $\times 10^5$ (173 MHz)	$1/T_{2m}$ (s^{-1}) $\times 10^5$ (346 MHz)
yPK−Tl^+	1500 ± 400	1090 ± 200	1.0 ± 0.4	4.4 ± 1.0
yPK−Tl^+−PEP	2540 ±1200	1260 ± 275	3.8 ± 1.5	5.4 ± 1.3
yPK−Tl^+−PEP−FBP	2300 ± 680	N.D.	4.0 ± 1.3	N.D.
yPK−Tl^+−FBP	2920 ± 271	N.D.	2.9 ± 1.1	N.D.
yPK−Tl^+−BrPEP	1080 ± 150	N.D.	1.2 ± 0.5	N.D.
yPK−Tl^+−BrPEP−FBP	2920 ± 780	N.D.	4.3 ± 1.7	N.D.
yPK−Tl^+−oxalate	380 ± 150	N.D.	0.65 ± 0.15[f]	N.D.
yPK−Tl^+−oxalate−FBP	420 ± 35	N.D.	0.62 ± 0.06[f]	N.D.
yPK−Tl^+ (pH 7.4)[b]	2260 ± 16	N.D.	N.D.	N.D.

(B) Muscle Pyruvate Kinase Complexes[c]

enzyme complex	$1/T_{1m}$ (s^{-1}) (24.3 MHz)	$1/T_{1m}$ (s^{-1}) (173 MHz)	$1/T_{1m}$ (s^{-1}) (346 MHz)	$1/T_{2m}$ (s^{-1}) $\times 10^5$ (24.3 MHz)	$1/T_{2m}$ (s^{-1}) $\times 10^5$ (173 MHz)	$1/T_{2m}$ (s^{-1}) $\times 10^5$ (346 MHz)
mPK−Tl^+(100 mM)		2100 ± 201	1560 ± 275		1.3 ± 0.3	1.2 ± 0.4
mPK−Tl^+(100 mM)−PEP		5560 ± 600	4020 ± 380		35.0 ± 0.4	28.0 ± 0.4
mPK−Tl^+(15 mM)		300 ± 50	225 ± 70		N.D.	N.D.
mPK−Tl^+(15 mM)−PEP		4550 ± 900	N.D.		0.5 ± 0.07	N.D.
mPK−Tl^+(100 mM)[d]	2990 ± 300			0.3 ± 0.03		
mPK−Tl^+(100 mM)[e]	4300 ± 300			0.4 ± 0.04		

[a] Normalized longitudinal and transverse relaxation rates were determined for yPK enzyme complexes. Experiments were done at 173.02 and 346.03 MHz as described in Experimental Procedures. [b] Experiment was performed in 100 mM HEPES, pH 7.4. [c] Normalized longitudinal and transverse relaxation rates were determined for mPK enzyme complexes. [d] Values reported by Reuben and Kayne (*5*). These values were measured at 24.3 MHz. [e] Recalculation of relaxation rates reported by Reuben and Kayne (*5*) using measured Mn^{2+} dissociation constants. [f] $1/T_{2m}$ was measured using spectral line widths. N.D., not determined.

15 mM Tl^+ was chosen as the optimal concentration. At 15 mM, good signal-to-noise is obtained in a relatively short amount of time. In addition, 15 mM Tl^+ is saturating (15 times the K_a value) yet well below its K_I value of 130−180 mM. As with other enzymes that require a monovalent cation such as Na^+ or K^+, Tl^+ appears to bind with greater affinity than the physiological activator (*32*). The crystalline ionic radius of thallium is 1.44 Å, compared to 1.37 Å for K^+. Size appears to be an important feature of monovalent cation activation (*16*, *32*). The similarity of the radii of Tl^+ and K^+ also suggests that thallous ion is a good probe for the monovalent cation site.

The dissociation constant of yPK for Mn^{2+} was determined for each enzyme complex studied by NMR (Table 2). The binding data indicate that affinity of Mn^{2+} for various yPK complexes varies widely. It cannot then be assumed that all added Mn^{2+} is bound to yPK. Apo yPK in the presence of $TlNO_3$ binds Mn^{2+} with a K_D of 7200 μM, while the yPK−Tl^+−PEP complex binds Mn^{2+} with 3 orders of magnitude greater affinity. The distribution of Mn^{2+} in each complex investigated must be independently calculated. Clearly, for the apo enzyme, the assumption that all added Mn^{2+} is enzyme bound would greatly underestimate the paramagnetic effect on the $^{205}Tl^+$ relaxation rates, while making this assumption for the yPK−Tl−PEP form would have little effect on the normalized relaxation rates.

Fast Exchange. Fast exchange conditions are suggested for $1/T_1$ from the fact that measured $1/T_{2m}$ values are significantly greater than $1/T_{1m}$ values only if relaxation is due solely to dipolar effects. If significant exchange or scalar effects are manifest in $1/T_2$, then the inequality of $1/T_1$ and $1/T_2$ would not be a reliable indication of the presence of fast exchange conditions. Chemical exchange appears to contribute to T_2 on the basis of negative temperature dependence of $1/T_2$ (vide supra). The off rate (k_{off}) for thallous ion can be estimated to be on the order of 10^5−10^6 s^{-1}, assuming diffusion-limited binding to yPK and using the measured activator constant of 1 mM. This value is of the same magnitude as $1/T_{2m}$ for ^{205}Tl, suggesting that chemical exchange may be contributing to the measured transverse relaxation rates. In addition, scalar superhyperfine coupling has been observed between VO^{2+} (a Mn^{2+} analog) and $^{203,205}Tl^+$ bound at the active site of mPK (*33*). Scalar effects in the Mn−Tl interaction have been previously suggested for mPK (*5*). On the basis of these data, transverse relaxation rates are not solely governed by dipolar interactions and therefore will not be used in the final Tl−Mn distance calculations.

The positive slope of the temperature dependence of the longitudinal relaxation rates as shown in an Arrhenius plot is indicative of the existence of fast exchange conditions (*28*). A positive slope in the temperature dependence of $1/T_1$ was observed for all enzyme complexes investigated (four representative results are shown in Figure 2). The $1/T_{1m}$ values at two magnetic fields can be used to determine the correlation time if τ_c is not frequency dependent. The $1/T_{1m}$ values at three fields were determined for mPK (Figure 3), and the linearity of this plot suggests that τ_c is not frequency dependent and justifies the use of this method for determining the correlation time.

The correlation time for the Tl^+−Mn^{2+} interaction in yPK was determined by two different methods. (1) τ_c was calculated using eq 1 by measuring $1/T_{1m}$ at 173 and 346 MHz. Using this method for the yPK−Tl^+−PEP complex, a τ_c of 0.66 ± 0.38 ns was calculated and a value of 0.35 ± 0.16 ns was calculated for the yPK−Tl^+ complex. (2) The $1/T_{1m}$ and $1/T_{2m}$ values for Mn−H_2O in yPK complexes at a single frequency (300 MHz) were measured, and eqs 1 and 2 were used to calculate τ_c. Approach (2) assumes fast exchange of water and that the same τ_c value that modulates the Mn−Tl^+ interaction also modulates the Mn−H_2O interaction in the same complex. The validity and usefulness

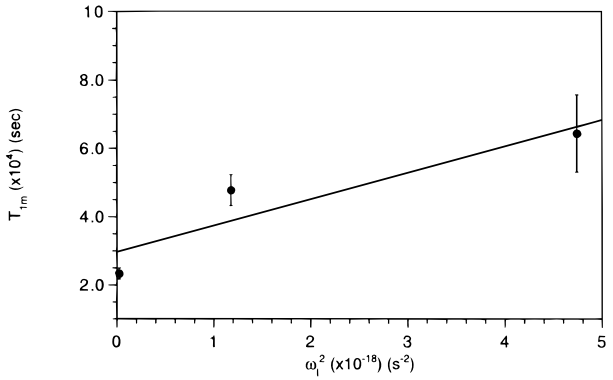

FIGURE 3: Frequency dependence of $1/T_{1m}$ for mPK. The T_{1m} value of ^{205}Tl was determined for apo mPK(100 mM Tl(NO_3), 50 mM cacodylate, pH 7.4) complex at 346.03, 173.02, and 24.3 MHz. Data at 24.3 MHz were taken from Reuben and Kayne (*5*) and were corrected for the K_D for Mn^{2+} binding at 100 mM Tl^+. The solid line is a fit of the data. The τ_c value was determined from the slope to be 0.51 ± 0.27 ns.

of this method for determining τ_c has been described (*29*). Method (2) gives τ_c values of 0.74 ± 0.40, 1.31 ± 0.53, and 1.47 ± 0.33 ns for the yPK−Tl^+−PEP−FBP, yPK−Tl^+−FBP, and yPK−Tl^+−PEP complexes, respectively. Values of τ_c ranging from 0.35 to 1.47 ns result in a calculated distance range differing by less than 0.2 Å. For yPK complexes, the correlation times determined by the T_1 and T_2 ratios for water protons and using the frequency dependence of $1/T_{1m}$ for ^{205}Tl gives an average value for τ_c of 0.91 ± 0.50 ns for all yPK complexes. Although there is some variation in τ_c values estimated for the various yPK−Tl^+−Mn^{2+} complexes studied, these variations are small and are probably due to the experimental errors of each method and the inherent limitations of each technique. It is assumed that the τ_c values for each complex are the same for comparative reasons, and an average value of 0.91 ns will be used. A similar approach was used by Raushel and Villafranca (*16*) in their estimation of correlation times for mPK complexes.

Tl^+−Mn^{2+} Distances for yPK Complexes. With a value for the correlation time, the intermetal distances may be calculated using eq 4. For apo yPK (yPK−Tl^+) a Tl−Mn distance of 6.7 ± 0.1 Å is calculated using a τ_c value of 0.91 ns (Table 4). Binding of PEP by yPK results in an increase in the paramagnetic effects of Mn^{2+} on $^{205}Tl^+$ relaxation and a decrease in the intermetal distance to 6.0 ± 0.3 Å. We observe no differences in relaxation rates between the yPK−Mn−P-glycolate−FBP and yPK−Mn−PEP−FBP complexes (not shown). This distance is in agreement with the Mn−K distance of 5.9 Å measured by X-ray diffraction methods for the yPK−Mn−P-glycolate−FBP complex (*18*). These results are also similar to the value of 5.6 Å obtained from the mPK crystal structure (*10*). The crystal structure of mPK was obtained with the product pyruvate at the active site, which may account for the slight difference.

The allosteric activator FBP does not induce any further change in the Tl−Mn distance upon binding to the yPK−Tl−PEP complex (Table 4). Binding of FBP to the apo complex induces a conformational change that brings the monovalent and divalent cations to within 6.0 Å of one another (Table 4). This is the first direct evidence linking FBP-induced conformational changes with specific active site

Table 4: Correlation Times and Calculated Tl^+−Mn^{2+} Distances in Pyruvate Kinase Complexes[a]

enzyme complex	τ_c ($\times 10^{-9}$ s)[b]	$f(\tau_c)$ ($\times 10^{-9}$ s)[b]	r (Å)[c]
yPK−Tl^+	0.91	1.38	6.7 ± 0.2
yPK−Tl^+−PEP	0.91	1.38	6.1 ± 0.3
yPK−Tl^+−PEP−FBP	0.91	1.38	6.2 ± 0.2
yPK−Tl^+−FBP	0.91	1.38	6.0 ± 0.1
yPK−Tl^+−BrPEP	0.91	1.38	7.0 ± 0.1
yPK−Tl^+−BrPEP−FBP	0.91	1.38	6.0 ± 0.3
yPK−Tl^+−oxalate	0.91	1.38	7.8 ± 0.3
yPK−Tl^+−oxalate-FBP	0.91	1.38	7.7 ± 0.1
yPK−Tl^+ (pH 7.4)	0.91	1.38	6.2 ± 0.1
mPK−Tl^+(15 mM)	0.38	0.98	8.2 ± 0.1
mPK−Tl^+(15mM)−PEP	0.38	0.98	5.2 ± 0.2
mPK−Tl^+(100 mM)	0.38	0.98	5.9 ± 0.1
mPK−Tl^+(100 mM)−PEP	0.38	0.98	5.0 ± 0.1

[a] The values for τ_c, $f(\tau_c)$, and r were determined for yPK and mPK complexes. [b] The values for τ_c and $f(\tau_c)$ were determined from an average τ_c as described in the text. [c] The distance was determined using the $f(\tau_c)$ value in the second column and T_{1m} relaxation rates at 173 MHz.

changes. FBP binding to yPK also causes large quenching of tryptophan fluorescence (*13*), indicative of a global FBP-induced structural change. These results indicate that, in addition to global changes in yPK structure upon binding of PEP and FBP, there are specific structural changes at the active site of yPK in the formation of the catalytic complex. One result of FBP binding is to bring the monovalent and divalent cations closer, a remarkable feat considering the FBP site and the active site are 40 Å apart (*18*).

BrPEP is a slow substrate with yPK and a competitive inhibitor vs PEP. BrPEP, unlike PEP, does not cause a decrease in the Tl^+−Mn^{2+} distance compared to the apo form. This result was surprising because BrPEP binds to yPK with the same affinity that PEP has for yPK. BrPEP binding causes Trp quenching in yPK upon binding, identical to the quenching induced by PEP binding (*31*). Binding of FBP to the yPK−Tl−BrPEP complex does result in an intermetal separation of 6.0 Å, identical to the structure measured for the enzyme−Tl−Mn−PEP−FBP complex. A possible explanation of this phenomenon is that, with PEP as the substrate, FBP behaves as a K-type activator (*34*); FBP lowers the K_m of yPK for PEP while not affecting V_m and alters the interaction of PEP with yPK from sigmoidal (cooperative) in the absence of FBP to hyperbolic (noncooperative) in its presence. When BrPEP is the substrate, no cooperative kinetic behavior is observed in the absence of FBP. When FBP is present, the K_m for BrPEP is not changed but the V_m more than doubles in value in the absence of FBP (*31*). These data suggest that the physiological substrate PEP can induce the conformational changes necessary in yPK to achieve maximal activity (one result of this conformational change is the reduction in the distance between monovalent and divalent cations). The substrate BrPEP cannot induce this same change in yPK in the absence of FBP. The ^{205}Tl resonance in the PEP complex is 4 ppm downfield of the ^{205}Tl resonance in the BrPEP complex, indicating slightly different environments for the thallous ion in both these complexes or somewhat different exchange rates for the thallous ion between solution and its enzyme-bound state.

Oxalate has been suggested as an analogue of the enolate intermediate of the PK reaction (*35, 36*). For this reason, the Tl^+−Mn^{2+} distance for the yPK oxalate complex was

investigated, as it may provide a snapshot of the yPK active site during a transient phase of catalysis. Assuming a correlation time of 0.91 ns, the Tl^+ to Mn^{2+} distance in the yPK−Tl^+−Mn^{2+}−oxalate complex was calculated to be 7.8 ± 0.2 Å. This distance is significantly greater than that measured for the yPK−Mn and yPK−Mn−PEP complexes. In addition, the chemical shift for $^{205}Tl^+$ in the yPK oxalate complex is 6 ppm downfield of thallous ion in aqueous solution. This is compared to a 18 ppm downfield shift for thallium in the yPK−PEP complex indicating differing chemical environments for $^{205}Tl^+$ in the oxalate and PEP enzyme complexes. It is tempting to speculate, on the basis of this distance change, that after transfer of the phosphoryl group of PEP to ADP, the two metals move apart prior to enolization. Alternatively, oxalate may simply serve as a tight-binding ligand to the bound Mn^{2+} and may not be a viable analogue of enolpyruvate in this complex. In contrast to effects seen with BrPEP, FBP is unable to cause additional changes in the intermetal distance upon binding to the yPK−Tl−oxalate complex (Table 4). This suggests that oxalate does not behave like BrPEP or PEP. The yPK conformation induced by oxalate appears to be immune to the effects of FBP.

The effects of pH on the intermetal distance were investigated. The Tl−Mn distance in yPK was measured at pH 7.4. The Tl−Mn distance for yPK at pH 7.4 is 6.2 Å, 0.5 Å less than that determined for the yPK−Tl^+ complex at pH 6.2. The difference in distances at the two pH values is not entirely surprising given the ionic nature of the enzyme-metal interactions.

Rabbit Muscle PK. In the process of studying the yPK complexes, mPK was also investigated as a control to replicate previous data (*5*) and to compare effects with homologous isozymes. Using 15 mM $Tl(NO_3)$, we were unable to reproduce the results obtained by Reuben and Kayne (*5*) who performed such studies at 100 mM Tl^+. As described, these differences were due to the inhibition of Mn^{2+} binding at higher concentrations of thallium. $1/T_{1m}$ and $1/T_{2m}$ values were normalized to take into account the amount of enzyme-bound Mn^{2+}. The correlation time was determined from the frequency dependence of $1/T_{1m}$ for the various complexes and from the value determined from Figure 3. The linearity of the data in Figure 3 indicates that τ_c is not frequency dependent; therefore, as was done with yPK (vide supra), the individual correlation time values were averaged to give a τ_c of 0.38 ± 0.23 ns. This value is in agreement with values of 0.7, 1.7, and 2.6 ns for the Mn^{2+}−Li^+ interaction in mPK (*7*) and a value of 3.4 ns determined for the Mn^{2+} interaction with the two isotopes of Li^+, NH_4^+, and Rb^+ in mPK (*16*). Values of τ_c ranging from 0.38 to 3.4 ns give corresponding distances that differ by only 0.3 Å.

The correlation times for the mPK complex determined by ratios of $1/T_1$ and $1/T_2$ result in calculated τ_c values that range from 9 to 15 ns (Table 4). These values are larger than anticipated and larger than those measured for other mPK−Mn complexes (*4*, *7*, *16*). As was done with yPK, transverse relaxation rates were not used for the determination of the correlation time.

The calculated distances for mPK complexes are listed in Table 4. At 15 mM Tl^+ the intercation distance is 8.2 Å for apo mPK, while at 100 mM Tl^+ the calculated distance is 5.9 Å. The difference may be due to Tl^+ binding at other sites on mPK, and thus the calculated distance may be an average value. In the presence of PEP, the Mn−Tl distance is 5.2 Å for the mPK−Tl (15 mM)−PEP complex and 5.0 Å for the same complex with 100 mM $TlNO_3$. The Mn−Tl distances at 15 and 100 mM Tl^+ are similar in the presence of PEP. This is probably due to PEP inhibition of thallium binding at the "other site", which is consistent with the binding data in Table 2.

CONCLUSIONS

These experiments reaffirm a crucial role for the monovalent cation in supporting PK activity. The two metals move closer to one another in yPK upon formation of the active enzyme form. X-ray crystal data of the yPK−K−Mn−P-glycolate−FBP complex show the monovalent cation within 2.8 Å of the phosphoryl oxygen of P-glycolate. Thallium NMR studies were performed on the yPK−Tl−Mn−P-glycolate−FBP complex (not shown), and the relaxation results are identical to those from the yPK−Tl−Mn−PEP−FBP complex, indicating similar active site environments in both complexes. It has been suggested by Zhang (*37*) that the monovalent cations activate fructose-1,6-bisphosphatase by aiding the Arg276 residue to deshield the negative charge on the 1-phosphoryl group of the substrate, thereby facilitating nucleophilic attack at the 1 position. The monovalent cation in PK may orient an enzymic group necessary for substrate binding or catalysis. Alternatively, the monovalent cation may serve to orient the phosphate for attack by the incoming nucleophile. Certainly specific changes are occurring at the active site upon ligand binding.

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REFERENCES

1. Boyer, P. D., Lardy, H. A., and Phillips, P. H. (1942) *J. Biol. Chem. 146*, 673−682.
2. Nowak, T., and Suelter, C. H. (1981) *Mol. Cell. Biochem. 35*, 65−75.
3. Nowak, T. (1973) *J. Biol. Chem. 248*, 7191−7196.
4. Nowak, T. (1976) *J. Biol. Chem. 251*, 73−78.
5. Reuben, J., and Kayne, F. J. (1971) *J. Biol. Chem. 246*, 6227−6234.
6. Hutton, W. C., Stephens, E. M., and Grisham, C. M. (1977) *Arch. Biochem. Biophys. 184*, 166−171.
7. Ash, D. E., Kayne, F. J., and Reed, G. H. (1978) *Arch. Biochem. Biophys. 190*, 571−577.
8. Muirhead, H., Clayden, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schiltz, E., and Schmitt, W. (1986) *EMBO J. 5*, 475−481.
9. Larsen, T. M., Benning, M. M., Wesenberg, G. E., Rayment, I., and Reed, G. H. (1997) *Arch. Biochem. Biophys. 345*, 199−206.
10. Larsen, T. M., Laughlin, L. T., Holden, H. M., Rayment, I., and Reed, G. H. (1994) *Biochemistry 33*, 6301−6309.
11. Mattevi, A., Valentini, G., Rizzi, M., Speranza, M. L., Bolognesi, M., and Coda, A. (1995) *Curr. Biol. Struct. 3*, 729−741.
12. Mesecar, A. M., and Nowak, T. (1997) *Biochemistry 36*, 6792−6802.
13. Mesecar, A. M., and Nowat, T. (1997) *Biochemistry 36*, 6803−6813.

14. Nowak, T., and Lee, M. J. (1977) *Biochemistry 16*, 1343−1350.
15. Baek, Y. H., and Nowak, T. (1982) *Arch. Biochem. Biophys. 217*, 491−497.
16. Raushel, F. M., and Villafranca, J. J. (1980) *Biochemistry 19*, 5481−5485.
17. Loria, J. P., and Nowak, T. (1997) (submitted for publication).
18. Jurica, M. S., Mesecar, A. M., Heath, P. J., Shi, W., Nowak, T., and Stoddard, B. J. (1997) *Structure 6*, 195−210.
19. Bucher, T., and Pfleiderer, G. (1955) *Methods Enzymol. 1*, 435−440.
20. Yun, S.-L., Aust, A. E., and Suelter, C. H. (1976) *J. Biol. Chem. 251*, 124−128.
21. Allerhand, A., Doddrell, D., and Komoroski, R. (1971) *J. Chem. Phys. 55*, 189−197.
22. Meiboom, S., and Gill, D. (1958) *Rev. Sci. Instrum. 29*, 688−691.
23. Bloembergen, N. (1957) *J. Chem. Phys. 27*, 572−573.
24. Luz, Z., and Meiboom, S. (1964) *J. Chem. Phys. 40*, 2686−2692.
25. Swift, T. J., and Connick, R. E. (1962) *J. Chem. Phys. 37*, 307−320.
26. Solomon, I. (1955) *Phys. Rev. 99*, 559−565.
27. Nowak, T. (1981) in *Spectroscopy in Biochemistry*, p 109, CRC Press, Boca Raton, FL.
28. Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, Clarendon, Oxford.
29. Navon, G. (1970) *Chem. Phys. Lett. 7*, 390−394.
30. Kayne, F. J. (1971) *Arch. Biochem. Biophys. 143*, 232−239.
31. Loria, J. P. (1997) Ph.D. Thesis, University of Notre Dame.
32. Markham, G. D. (1986) *J. Biol. Chem. 261*, 1507−1509.
33. Lord, K. A., and Reed, G. H. (1987) *Inorg. Chem. 26*, 1464−1466.
34. Neet, K. E. (1980) *Methods Enzymol. 64*, 139−226.
35. Reed, G. H., and Morgan, S. D. (1974) *Biochemistry 13*, 3537−3541.
36. Poyner, R., Reed, G. H., and Scholes, C. P. (1993) *Biochemistry 32*, 7799−7810.
37. Zhang, R., Villeret, V., Lipscomb, W. N., and Fromm, H. J. (1996) *Biochemistry 35*, 3038−3043.

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