Monitoring Active Site Alterations upon Mutation of Yeast Pyruvate Kinase Using $^{205}$Tl$^+$ NMR

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The interaction of the monovalent cation with wild type (WT) yeast pyruvate kinase (YPK) and with the T298S, T298C, and T298A mutants was investigated by $^{205}$Tl$^+$ NMR to monitor possible structural alterations at the active site by Thr-298 mutation. TINO$_3$ activates WT YPK with a $k_{cat}$ value similar to that obtained with KCl and an apparent $K_\text{m}$ of 0.96 ± 0.07 mM in the presence of Mn$^{2+}$ and fructose 1,6-bisphosphate. With the three mutants, Tl$^+$ is a better activator than is K$^+$ based on $k_{cat}$ values. Tl$^+$ activation and inhibition of YPK is affected by mutation of the active site Thr-298. The effect of Mn$^{2+}$ on the $1/T_1$ value of $^{205}$Tl$^+$ in the presence of the WT and mutant YPK complexes was determined at 173 MHz (300 MHz, $^1$H) and 346 MHz (600 MHz, $^1$H). For each complex studied, $1/T_1$ $\gg 1/P_{T_1}$ and $1/P_{T_{1p}}$ is frequency-dependent suggesting fast exchange conditions. The values of $1/P_{T_{1p}}$ differ for each mutant. A correlation time of 0.65 ± 0.15 ns was estimated for the Mn$^{2+}$-Thr298$^S$ interaction. The Tl$^+$-$\text{Mn}^{2+}$ distances at the active site of YPK were calculated from the paramagnetic contribution of Mn$^{2+}$ to $1/T_1M$ of YPK-bound $^{205}$Tl$^+$. The calculated Tl$^+$-$\text{Mn}^{2+}$ distance for the Thr-298 mutants is decreased by about 1 Å from 6.0 ± 0.2 Å observed with WT. The results suggest conformational alterations at the active site of YPK where phosphoryl transfer occurs upon mutation of Thr-298. These conformational changes may, in part, explain the alteration in $k_{cat}$ and $k_{cat}/K_m$ observed with the Thr-298 mutants.

Yeast pyruvate kinase (YPK)$^1$ (EC 2.7.1.4.0) is a tetrameric enzyme of identical subunits with a subunit molecular mass of 54.5 kDa (1). PK is a key regulatory enzyme in glycolysis that catalyzes the nearly irreversible reaction of phosphoenolpyruvate (PEP) and ADP to yield pyruvate and ATP. YPK has an absolute requirement for both monovalent and divalent cations, undergoes homotropic activation by PEP and M$^{2+}$, and heterotropic activation by fructose 1,6-bisphosphate (FBP). Potassium is the physiologically important monovalent activator, but several other monovalent cations (Li$^+$, Na$^+$, Rb$^+$, Tl$^+$, Cs$^+$, NH$_4^+$, CH$_3$NH$_2$) can also activate PK (2–4).

The refined x-ray structures of the YPK-K$^+$-$\text{Mn}^{2+}$-phosphoglycolate and YPK-K$^+$-$\text{Mn}^{2+}$-phosphoglycolate-FBP complexes have been solved at 3 Å resolution (5). Phosphoglycolate is a structural analog of the substrate PEP that lacks the C-3 carbon. From the x-ray data, the active site Thr-298 is in the correct orientation to serve as the proton donor to the C-3 of the enolate of pyruvate, the enzyme-bound intermediate (Fig. 1). The role of Thr-298 in the YPK-catalyzed reaction has been addressed by mutation of this residue to serine, alanine (6), and to cysteine. Studies of these mutants suggest that Thr-298 is not the proton donor to the enolate of pyruvate but that enzyme-bound water serves this function. Far-UV CD analysis indicates that none of the mutations cause any significant change in the secondary structure and that wild type and mutant YPK enzymes are folded into a similar, if not identical, structure. Physical and kinetic studies with the Thr-298 mutants of YPK indicate that the single amino acid mutations at the active site can trigger long range effects. These effects were observed at the FBP-binding site, located more than 40 Å away from the active site (5). The Thr-298 residue is about 6.5 Å from the enzyme-bound divalent cation and 8.9 Å from the monovalent cation at the active site (Fig. 1) (5).

All three Thr-298 mutants of YPK, T298S, T298C and T298A, showed altered $k_{cat}$ and $k_{cat}/K_m$ values and altered kinetic cooperativity with PEP relative to wild type PK (6). It is possible that these alterations in kinetic parameters of the YPK Thr-298 mutants may, in part, be explained by structural changes at the active site as a result of the single amino acid mutations. Such alterations may be too subtle to be monitored by methods such as CD and fluorescence spectroscopy, which are sensitive to more gross changes in protein structures.

When performing site-directed mutagenesis studies, the possibility of local structural alterations introduced upon mutation tends to be overlooked in the interpretation and assessment of overall properties and behavior of the resulting protein mutants relative to the wild type protein. The ability to address these questions is normally limited by lack of appropriate experimental tools. Yeast PK has an advantage in that it is amenable to such an analysis. Measurement of the internuclear interactions between the mono- and divalent cations, both located at the active site, provides a specific and sensitive method to monitor such structural alterations in YPK. In vitro, Tl$^+$ has been shown to be a good substitute for K$^+$ in wild type YPK (7).

Thallium is particularly well suited for NMR experiments because of its intrinsic nuclear properties. $^{205}$Tl$^+$ (70.5% natural abundance) is one of the two stable isotopes of thallium with a nuclear spin of $\frac{1}{2}$. The relative receptivity (receptivity is sensitivity multiplied by natural abundance) of $^{205}$Tl$^+$ is 0.1355, in contrast to $^{39}$K$^+$ with a value of 0.000473. $^1$H has an average of 0.65 ± 0.15 ns was estimated for the Mn$^{2+}$-Thr298$^S$ interaction.
Active Site Alterations in Yeast PK

**Experimental Procedures**

*Materials—L-(+)-Lactate dehydrogenase from rabbit muscle was purchased from Roche Applied Science. Wild type, T298S, T298C, and T298A yeast pyruvate kinases were constructed, expressed, and purified as described by Mesecar and Nowak (10). The cyclohexylammonium salts of PEP, ADP, and of FBP, disodium NADH, glycerol, and MES buffer were purchased from Sigma. Thallium nitrate was obtained from Aldrich. Tetramethylammonium hydroxide pentahydrate (TMAOH) and tetramethylammonium nitrate (TMA-NO\textsubscript{3}) were purchased from Acros Organics. Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories. The Bio-Gel® P-6 DG desalting gel was purchased from Bio-Rad.*

**Tl\textsuperscript{+} Activation Studies—**YPK was assayed by following the decrease in absorbance at 340 nm due to NADH oxidation using the coupled assay with lactate dehydrogenase (11). The activity assays were performed at 22 °C. The specific activity of YPK is expressed as μmol of NADH oxidized per min/mg of protein. The concentration of YPK was determined by its absorbance at 280 nm. The extinction coefficient used for YPK is ε\textsubscript{280} = 0.51 (mg/ml)\textsuperscript{-1} cm\textsuperscript{-1}. For Tl\textsuperscript{+} activation studies with YPK, the assay conditions were as follows: a 1:ml assay mixture contained 100 mM MES (pH 6.2, adjusted with TMA-OH), 4% glycerol, Tl\textsuperscript{+}NO\textsubscript{3} to 200 mM total salt concentration, 10% D\textsubscript{2}O, 4 mM Mn(NO\textsubscript{3})\textsubscript{2}, 5 mM PEP (cyclohexylammonium salt), 5 mM ADP (cyclohexylammonium salt), and 1 mM FBP (cyclohexylammonium salt), when present. Prior to Tl\textsuperscript{+} activation experiments, YPK was desalted on a Bio-Gel® P-6 DG column with a thin Chelex layer on top that was pre-equilibrated with 100 mM MES, pH 6.2 (adjusted with TMA-OH), 20% glycerol. Care was taken in these studies to eliminate activating monovalent cations such as K\textsuperscript{+} and Na\textsuperscript{+}. Thallous ion was added as TINO\textsubscript{3}, and 10% D\textsubscript{2}O was included in the assay mixture to simulate NMR conditions. The total salt concentration was kept constant at 200 mM by the addition of non-activating TMA-NO\textsubscript{3}.

The initial velocity activation data were fit to either a noncompetitive substrate inhibition model (Equation 1) or to Equation 2, depending on which model best describes the experimental data. K\textsubscript{A} and K\textsubscript{I} values represent the activation constant and inhibition constant for Tl\textsuperscript{+}, respectively.

\[
v_{\text{o}} = V_\text{t}(1 + K_A / A + K_I / I) + V_I (1 + K_A / A + K_I / I) \quad \text{(Eq. 1)}
\]

\[
v_{\text{o}} = V_\text{t}(1 + K_A / A) \quad \text{(Eq. 2)}
\]

**Tl\textsuperscript{205} NMR—**NMR experiments were carried out on Varian 300 MHz (173.02 MHz) and Varian 600 MHz (346.04 MHz) Unity Plus instruments. Tuning of the probes to the thallium resonance was achieved by using a home-built inductor rod. The 90° pulse widths were calibrated prior to each experiment and typically ranged from 8 to 10 μs at 300 MHz (173.02 MHz) and from 36 to 37 μs at 600 MHz (346.04 MHz). D\textsubscript{2}O (10%) was included as an internal field-frequency lock. Samples with YPK were referenced to an external TINO\textsubscript{3} sample that was identical in TINO\textsubscript{3} concentration with the YPK sample and set at 0.0 ppm. At 300 MHz (173.02 MHz) the spectral width was 14,000 Hz, and 16,000 data points were used. The spectral width at 600 MHz (346.04 MHz) was 28,000 Hz, and 34,000 data points were used. The number of acquired transients ranged from 8 to 40. To improve signal to noise, a 2–40-Hz exponential line broadening function was used with the enzyme samples. In most of the cases, the line broadening was 2–9% of the measured line width. All spectra were acquired at 22 ± 0.1 °C.

Prior to the NMR experiments, YPK was desalted on a Bio-Gel® P-6 DG spin column with a thin Chelex layer (~5 mm) on top that was pre-equilibrated with 100 mM MES, pH 6.2 (adjusted with TMA-OH), 20% glycerol. The typical concentration range of desalted YPK was 10–15 mg/ml. Typical NMR samples contained in 0.7 ml: 100 mM MES, pH 6.2, 4% glycerol, 45–140 μM enzyme sites, 15–90 mM TINO\textsubscript{3}, 10% D\textsubscript{2}O, 5 mM PEP, and 1 mM FBP, when present. The total salt concentration of the samples was kept constant at 200 mM by the addition of TMA-NO\textsubscript{3}. Mn\textsuperscript{2+} was titrated directly into the NMR tube as Mn(NO\textsubscript{3})\textsubscript{2}. The added volumes were in the range of 1–15 μl. [Mn\textsuperscript{2+}], [Mn\textsuperscript{2+}], and [Tl\textsuperscript{2+} were corrected for dilution, although these corrections were less than 2% (the denotates total and free, respectively). The external TINO\textsubscript{3} reference contained 100 mM MES, pH 6.2, 4% glycerol, 15–90 mM TINO\textsubscript{3}, and TMA-NO\textsubscript{3} to 200 mM total salt concentration.

Longitudinal relaxation rates of 205Tl (1/T\textsubscript{1}) were determined using the inversion-recovery method (180°-t-90° acquire) (12). Typically 12–15 fits to Equation 3, and the data were fit to Equation 3, in which I is the peak height and A and B are constants. Deviations from a single exponential fit to the T\textsubscript{1} relaxation data were typically 2–6%.

\[
I(t) = A + Be^{-rt} \quad \text{(Eq. 3)}
\]

Transverse relaxation rates (1/T\textsubscript{2}) were determined from the line widths of the resonance signals according to Equation 4, which is based on the assumption of exponential relaxation. In Equation 4, λ\textsubscript{max} is the value for the line width at half-peak height (λ\textsubscript{0}) corrected for the artificial line broadening (LB) according to Equation 5.

**FIG. 1. Active site structure of yeast pyruvate in complex with K\textsuperscript{+}, Mn\textsuperscript{2+}, phosphoglycolate, and FBP (5). K\textsuperscript{+} is shown in gold, Mn\textsuperscript{2+} in blue, and the bound substrate analogue phosphoglycolate (PG) is depicted with the phosphorus atom in purple and oxygen atoms in red. This view of the active site is obtained from the YPK structure published previously (5).**
The relaxation rate of magnetic nuclei of a complexed ligand (1/T_{1p}) was determined at 5–6 concentrations of Mn^{2+}. The paramagnetic contribution to the relaxation rates (1/T_{1p} and 1/T_{2p}) were calculated from the slope of a plot of relaxation rate against concentration of Mn^{2+}. The values of 1/T_{1p} and 1/T_{2p} were normalized by the factor $p = [\text{Mn}^{2+}] / [\text{Mn}^{2+}]_{0}$, as described by Millward and Cohn (13), to generate values of 1/T_{1p}' and 1/T_{2p}'. Under conditions of fast exchange 1/T_{1p}' = 1/T_{1p}.

**K_{p} Determination**—Binding of Mn^{2+} to the YPK-Tl^{+}-PEP-FBP complexes of WT, T298S, and T298A YPK enzymes was determined by loss of EPR signal of free Mn^{2+} upon binding. All data were fit to a hyperbolic binding model. Measurements were performed using a Bruker ER 100E electron spin resonance spectrometer. The experimental conditions used are as follows: frequency 9.86 GHz, modulation amplitude 1.6 × 10 gauss peak-to-peak, time constant 500 ms, field set at 3450 G, sweep time 200–500 s, and variable gain.

For all YPK-Tl^{+}-PEP complexes and for the T298C-Tl^{+}-PEP-FBP complex, the binding constant for Mn^{2+} was determined by tryptophan fluorescence titrations. Measurements were performed on an SLM-Aminco 8100 spectrofluorimeter. Fluorescence titrations were performed by monitoring the change in fluorescence intensity at 334 nm, with excitation at 295 nm upon titration with Mn^{2+}. Data were fit to either a hyperbolic or a cooperative model, depending on which one best describes the experimental data. The enzyme concentration was 0.1-0.2 mg/ml, and the buffer and ligand concentrations were the same as in the NMR experiments. Mn^{2+} binding to the T298C-Tl^{+}-PEP-FBP complex could not be determined directly by fluorescence titration since no additional quenching of tryptophan was observed upon ligand binding to FBP complexes of YPK. Instead the thermodynamic box depicted in Scheme 1 was used to determine the dissociation constant for Mn^{2+}-binding to T298C-Tl^{+}-PEP-FBP. This method requires intrinsic dissociation constants ($K_{p}$), which were calculated from the respective measured dissociation constants ($K_{d}$) and using the model for cooperativity in binding for YPK developed by Bollenbach (14) based on Pauling’s treatment for hemoglobin (15). In Scheme 1, $E'$ is T298C-Tl^{+}-PEP, M is Mn^{2+}, and A is FBP. $K_{e}^{M}$ was calculated from the relationship $K_{e}^{M} = K_{p}^{E'} K_{e}^{E'}$.

**Correlation Time ($\tau_{c}$) and Correlation Function ($f(\tau_{c})$) for Mn^{2+}-Tl^{+} Interaction**—Applications of the longitudinal (1/T_{1}) and transverse (1/T_{2}) relaxation rates of a nucleus bound in the vicinity of a paramagnetic probe to study enzyme-metal complexes have been reviewed previously (13, 16, 17). It has been demonstrated that the longitudinal relaxation rate of magnetic nuclei of a complexed ligand (1/T_{1p}) can be used to calculate the dipolar distance ($r$) between these nuclei (e.g. Tl^{+}) and the paramagnetic metal ion (e.g. Mn^{2+}) (13, 19). In the enzyme complexes that have been studied, the paramagnetic contribution by a metal such as Mn^{2+} to the 1/T_{1} relaxation rates of Tl^{+}, considering only dipolar relaxation, is given by the simplified Solomon-Bloembergen equation (Equation 6),

$$\frac{1}{T_{1\text{rel}}} = \frac{2}{15} \frac{S(S+1)}{g^2 I(1/2)} \left( \frac{3\tau_{c}}{1 + \omega_s^{2}\tau_{c}^2} + \frac{7\tau_{c}}{1 + \omega_s^{2}\tau_{c}^2} \right)$$

where $S$ is the electron spin quantum number; $\gamma$ is the nuclear gyromagnetic ratio; $g$ is the electronic "$g$" factor; $\beta$ is the Bohr magneton; $\tau_{c}$ is the correlation time for the Mn^{2+}-Tl^{+} interaction; and $\omega_s$ and $\omega_e$ are the nuclear and electron resonance frequencies, respectively. In Equation 6 the term due to the electron-nuclear hyperfine interaction has been omitted since it appears to be negligible for most ligands that interact with Mn^{2+}. In macromolecular complexes in which large enhancements of 1/T_{1} are observed, $\omega_e^{2}\tau_{c}^2$ becomes >> 1 and the term $7\tau_{c}/(1 + \omega_e^{2}\tau_{c}^2)$ in Equation 6 is negligible. Hence, the correlation function, $f(\tau_{c})$, for the dipolar term of the Solomon-Bloembergen equation (Equation 6) is simplified to Equation 7,

$$f(\tau_{c}) = \frac{3\tau_{c}}{1 + \omega_s^{2}\tau_{c}^2}$$

The correlation time $\tau_{c}$ was determined from the frequency dependence of 1/T_{1} at $\omega_s$ (173.02 MHz) and $\omega_s$ (346.04 MHz) and by assuming that $\tau_{c}$ is frequency-independent. Knowing $\tau_{c}$, the correlation function $f(\tau_{c})$ can be calculated from Equation 7. Once $f(\tau_{c})$ is determined, the Tl^{+}-Mn^{2+} distance $r$, in Angstroms, can be calculated, according to Equation 8.

$$r = 670(\sqrt{f(\tau_{c})/T_{1\text{rel}}})$$

**RESULTS**

**Tl^{+} Activation and Mn^{2+} Binding**—Tl^{+} activation studies were performed for wild type YPK and the Thr-298 mutants with Mn^{2+} as the divalent activator, and in the absence and in the presence of FBP, respectively (Fig. 2, A–C). Wild type YPK and the Thr-298 mutant enzymes show no measurable YPK activity in the absence of an activating monovalent cation (K^{+} or Tl^{+}) with TMA or cyclohexylammonium ions present as counter cations (<0.1 units/mg, the sensitivity limit of the assay). This is in agreement with the previous observations with muscle PK (20) and with wild type yeast PK (2, 7) that these enzymes have an absolute requirement for an activating monovalent cation.

In the presence of the heterotropic activator FBP, Tl^{+} can activate wild type YPK to 85% the activity in the presence of K^{+} (Table I). With T298S, T298C, and T298A, Tl^{+} is a 1.2–1.8-fold better activator than is K^{+} based on the measured $k_{cat}$ values. With Mn^{2+} as the divalent activator, Tl^{+} has a steady-state activator constant ($K_{a}$) that is ~35-fold smaller than the value for K^{+} with wild type and with T298A YPK. The $K_{a}$ for Tl^{+} compared with K^{+} is ~11-fold and 17-fold smaller with T298S and T298C YPK, respectively. At higher Tl^{+} concentrations, inhibition is observed with wild type YPK (data not shown) and with the mutants T298S (Fig. 2A) and T298C (Fig. 2B). With both mutants, the inhibition constant for Tl^{+} ($K_{i}$) is increased 3–4-fold relative to the value with wild type YPK. These values are summarized in Table I, along with the results of activation studies with K^{+}. T298A shows no inhibition by Tl^{+} in the concentration range studied (Fig. 2C).

Activation by monovalent cations shows no kinetic cooperativity in the absence or presence of FBP. In the absence of FBP, Tl^{+} activates wild type YPK and the mutants T298S and T298C with $k_{cat}$ values similar to the values measured in the presence of FBP (Table I). With T298A, Tl^{+} activation in the presence of FBP gives a 32% increase in $k_{cat}$. The values measured for the Tl^{+} activation constant, $K_{a}$, and the inhibition constant, $K_{i}$, with wild type and T298S YPK, and for $K_{i}$ with T298A YPK are similar in the absence and in the presence of FBP. With T298C, the presence of FBP decreases the apparent activation constant for Tl^{+} ($K_{a}$) and increases the inhibition constant for Tl^{+} ($K_{i}$) both by a factor of 3. No inhibition of T298A is observed with Tl^{+} in the absence or presence of FBP (Fig. 2C).

The mutation of Thr-298 to serine, cysteine, and to alanine results in decreased $k_{cat}$ values upon activation by Tl^{+} and by K^{+}. Increased values for the kinetic constants $K_{a}(Tl^{+})$, $K_{a}(K^{+})$, and $K_{i}(Tl^{+})$ relative to those of wild type YPK are also measured (Table I).

The following Tl(NO$_3$)$_2$ concentrations were used for the NMR experiments with YPK based on the data in Table I: 15 mM for wild type, 70 and 90 mM for T298S, 60 and 70 mM for T298C,
used in the NMR experiments to 60 mM, which is only 3-fold greater than $K_0$ and 2.5-fold less than $K_f$.

**Mn$^{2+}$ Binding Studies**—To determine the contribution of Mn$^{2+}$ to the $^{205}$Tl$^{+}$ relaxation rates of enzyme-bound thallium, the amount of Mn$^{2+}$ bound to YPK must be known. The dissociation constants for Mn$^{2+}$ binding to the complexes of wild type YPK and the Thr-298 mutant enzymes were measured and are summarized in Table II. The dissociation constants for Mn$^{2+}$ binding to the YPK-Tl$^{+}$-PEP complexes measured with wild type and with T298S and T298C mutants are similar. Mn$^{2+}$-binding to the T298A-Tl$^{+}$-PEP complex is 20 times weaker than Mn$^{2+}$ binding to the similar wild type complex. Table II shows that a 5-fold increase of Tl$^{+}$ concentration in the T298S-Tl$^{+}$-PEP complex or a 6-fold increase of Tl$^{+}$ concentration in the WT-Tl$^{+}$-PEP-FBP complex results in no significant effect on $K_{D,Mn}$. This suggests that Tl$^{+}$ does not significantly compete for the Mn$^{2+}$-binding site. The presence of the heterotrophic activator FBP tightens the binding of Mn$^{2+}$ to each of the enzyme complexes. The measured $K_{D,Mn}$ values for the YPK-Tl$^{+}$-PEP-FBP complexes are similar for wild type and mutant enzymes. The $K_D$ for Mn$^{2+}$ binding to the T298C-Tl$^{+}$ (60 mM)-PEP-FBP complex ($K'_d$) was calculated from the relationship $K_d = K'_d/K_n/K_2$, as described under “Experimental Procedures.” The values for $K'_1$, $K'_2$, and $K'_3$ are 39, 986, and 57.5 μM, respectively.

**$^{205}$Tl$^{+}$ NMR Studies**—Depending on the YPK-Tl$^{+}$ complex studied, solutions of 15, 60, 70, and 90 mM Tl(NO$_3$)$_2$ were used as reference in the NMR experiments. In each case, the thallium resonance was set to 0.0 ppm. The reference solution contained the same concentrations of Tl(NO$_3$)$_2$, buffer, and total salt as the samples containing enzyme. All 90° pulse width calibrations were performed on the respective reference solutions. The measured line width of thallium in solution was 4 at 173 Hz and 5 at 346 Hz. Addition of Mn(NO$_3$)$_2$, up to 1 mM, to a solution of 70 mM Tl(NO$_3$)$_2$ in buffer had no effect on the $T_1$ or $T_2$ values (the highest concentration of total Mn(NO$_3$)$_2$ used in the NMR experiments was 0.7 mM). This is in agreement with the previous observation of Loria and Nowak (7) where the addition of up to 5 mM Mn(NO$_3$)$_2$ to a solution of 15 mM Tl(NO$_3$)$_2$ in buffer caused no $T_1$ or $T_2$ effects. Fig. 3, A–D, shows the comparative NMR spectra of Tl(NO$_3$)$_2$ in solution and in the presence of wild type YPK and the Thr-298 mutants as their enzyme + PEP and enzyme + PEP + FBP complexes, at 173 MHz. At saturating levels of PEP, the thallium resonance is broadened to 56 Hz in the presence of 78 μM wild type YPK sites (Fig. 3A–2), to 43 Hz in the presence of 80 μM T298S sites (Fig. 3B–2), to 56 Hz in the presence of 134 μM T298C sites (Fig. 3C–2), and to 41 Hz in the presence of 101 μM T298A sites (Fig. 3D–2). The respective downfield chemical shifts for the thallium resonance in the YPK-Tl$^{+}$-PEP complexes were 16.5 ppm (wild type), 14.3 ppm (T298S), 14.8 ppm (T298C), and 14.5 ppm (T298A). Each enzyme elicits different downfield shifts even when normalized for the concentration of enzyme. This suggests that enzyme-bound Tl$^{+}$ “sees” different environments in wild type YPK and in each of the Thr-298 mutants. The YPK-Tl$^{+}$-PEP-FBP complex of each enzyme species, there was a slight change in the line broadening and in the chemical shift of the thallium resonance relative to values from the YPK-Tl$^{+}$-PEP complex (Fig. 3, A–3 to D–3). The addition of Mn$^{2+}$ to each of the YPK-Tl$^{+}$ (±PEP, ±FBP) complexes results in an increase in the $1/T_1$ and $1/T_2$ values for thallium, and the increase is proportional to the Mn$^{2+}$ concentration (data not shown). For each complex, there is a slight upfield chemical shift (<0.5 ppm) of the thallium resonance upon addition of Mn$^{2+}$ (data not shown). It was demonstrated previously (7) that the addition of Mg$^{2+}$ to Tl$^{+}$ complexes of wild type YPK

![Graph](https://example.com/graph.png)

**Fig. 2. Activation of YPK by thallium.** A, T298S; B, T298C; and C, T298A. The activity of YPK with thallium was measured in the presence of 4 mM Mn(NO$_3$)$_2$, 5 mM ADP, and 5 mM PEP in the absence (○) and in the presence of 1 mM FBP (▪). Ionic strength was kept constant at 200 mM with TMA-NO$_3$. The points represent the average of two sets of experiments, and the error bars represent the S.D. For T298S and T298C, data were fit to a kinetic model for noncompetitive substrate inhibition (Equation 1), and for T298A data were fit to Equation 2. The fits generated the curves through the data.

and 70 and 90 mM for T298A. With the exception of T298C in the absence of FBP, these concentrations were 10–15-fold greater than the $K_n$ for Tl$^{+}$ to ensure saturation and 7–10-fold less than its $K_f$ value (with wild type and T298S) to minimize the effects from inhibition. In the case of T298C in the absence of FBP, the large $K_n$ for Tl$^{+}$ limited the Tl(NO$_3$)$_2$ concentration...
has no effect on the $1/T_1$ and $1/T_2$ values nor on the chemical shift for thallium. This suggests that the observed effect of the divalent metal on the Tl$^+$ relaxation rates is due to the paramagnetic properties of Mn$^{2+}$.

The chemical shifts and paramagnetic effects on relaxation at 346 MHz were similar to those measured at 173 MHz for each of the YPK-Tl$^+$ complexes of wild type YPK and of the T298S mutant.

A summary of the normalized paramagnetic contributions ($1/pT_{1p}$ and $1/pT_{2p}$) to the $1/T_1$ and $1/T_2$ values measured at 173 and at 346 MHz is presented in Table III. For all YPK-Tl$^+$ complexes studied, $1/pT_{2p} \gg 1/pT_{1p}$. As argued previously (21), $1/pT_{1p}$ approximates $1/T_{1M}$ (see below), the relaxation rate of the nucleus bound to the macromolecule. The data are listed as such in Table III. In the WT-Tl$^+$ (15 mM)-PEP complex at 173 MHz, the $1/T_{1M}$ value is $2350 \pm 300$ s$^{-1}$ (Table III), in good agreement with the value of $2540 \pm 1200$ s$^{-1}$ obtained by Loria and Nowak (7) under the same conditions. The addition of the allosteric activator FBP to this complex does not result in a further change in $1/T_{1M}$, as also observed previously (7). An increase in the Tl$^+$ concentration by a factor of 6 in the WT-Tl$^+$-PEP-FBP complex results in a 4-fold increase of $1/T_{1M}$. With the three Thr-298 mutants, the $1/T_{1M}$ values measured at 173 MHz for the enzyme-Tl$^+$-PEP complexes are different from the respective $1/T_{1M}$ values determined for the wild type YPK complex and from each other (Table III). The addition of FBP to the enzyme-Tl$^+$-PEP complex of the Thr-298 mutants results in a small but significant change in $1/T_{1M}$. In the enzyme-Tl$^+$-PEP-FBP complex of the Thr-298 mutants, the values for $1/T_{1M}$ differ among these mutants and are 2.5–4-fold larger than the $1/T_{1M}$ value in the wild type-Tl$^+$ (15 mM)-PEP-FBP complex.

The values for $1/T_{1M}$ are normalized for 100% saturation (activation) with Tl$^+$, $1/T_{1M,100}$ (see below). These values are also summarized in Table III. The $1/pT_{2p}$ values measured at 173 MHz for the Tl$^+$ complexes of wild type and of Thr-298 mutants are on the order of $10^5–10^6$ s$^{-1}$. These values are 2–3 orders of magnitude larger than the $1/T_{1M}$ values for these complexes (Table III).

The measurement of $1/T_{1M}$ values at 346 MHz for wild type YPK and the T298S mutant enzyme-Tl$^+$-PEP and enzyme-Tl$^+$-PEP-FBP complexes indicates that there is a frequency dependence for $1/T_{1M}$, based on Equation 6 and that the measured values reflect relaxation (Table III). There is no frequency dependence for the $1/pT_{2p}$ values for these complexes (Table III).

**Calculation of $1/T_{1M}$ Normalized for 100% Saturation with Tl$^+$**—Each of the enzyme complexes studied has a different activator constant ($K_a$) and inhibitor constant ($K_i$) for Tl$^+$, thus requiring different concentrations of Tl$^+$ to optimize complex formation (Table I). For a more accurate comparison of the $1/T_1$ relaxation rates measured for the YPK-Tl$^+$ complexes at different concentrations of Tl$^+$, hence at different saturation levels with Tl$^+$, the relaxation rates were normalized to 100% saturation ($1/T_{1M,100}$). For each enzyme-Tl$^+$ complex, the value for $1/T_{1M,100}$ was calculated as $1/T_{1M,100} = (1/T_{1M}(\%A)) \times 100$, where $1/T_{1M}$ is the calculated and normalized (by $1/p$) longitudinal relaxation rate of $^{205}$Tl$^+$ in the enzyme-Tl$^+$ complexes studied, and $\%A$ is the percent saturation (activation) with Tl$^+$. The percent saturation (activation) with Tl$^+$ was calculated from Equation 2, as $(n/V) \times 100 = ([Tl^+]/(K_a+[Tl^+])) \times 100$, assuming no inhibitory effect at higher Tl$^+$ concentrations (Table III).

The logic to normalize the $1/T_{1M}$ values for 100% saturation with Tl$^+$ is described. The activation of the YPK complexes by Tl$^+$ has an inhibitory component at higher Tl$^+$ concentrations (see Fig. 2). The measured $1/T_1$ values for $^{205}$Tl$^+$ in several of
these complexes consequently have an inhibitory component. Since the mechanism of inhibition is unclear, we minimized the effect of the inhibitory component on the $1/T_1$ values of $Tl/HOPO_1$ for the YPK-$Tl/HOPO_1$ complexes studied. Presumably $Tl/HOPO_1$ binds at two sites on the enzyme, an activator site and an inhibitor site, and these sites are independent. Each of these sites has a contribution to the overall observed $1/T_1M$. This model is described by Equation 9.

$$\frac{1}{T_1M_{obs}} = \frac{1}{T_1M_A} \frac{\% A}{[E]} + \frac{1}{T_1M_I} \frac{\% I}{[E]}$$

(Eq. 9)

In Equation 9, $\% A$ is the percent activation (saturation) by $Tl^+$ calculated as described above, $\% I$ is the percent inhibition by $Tl^+$ defined by $\frac{[E-Tl]}{[E]} = \left(\frac{[Tl^+]}{K_I}\right)$ assuming that $K_I$ is a dissociation constant, and $[E]$ is the total concentration of enzyme sites. ($1/T_1M_A$ and $1/T_1M_I$) are the individual contributions to $(1/T_1M)_{obs}$ of $Tl^+$ bound at the activator site and at the inhibitor site in YPK, respectively. Based on the model for Equation 9, $(1/T_1M_A)$ and $(1/T_1M_I)$ are independent of the $Tl/HOPO_1$ concentration and should be unique for each system. To test this model three equations are required. This implies that the relaxation rates $(1/T_1M)$ need to be measured at three different $Tl/HOPO_1$ concentrations and at the same frequency. Two $Tl^+$ concentrations will generate two equations of the type described by Equation 9 that are necessary to calculate the values for $(1/T_1M_A)$ and $(1/T_1M_I)$. A third $Tl^+$ concentration is required to compute the overall $(1/T_1M)_{obs}$ by using the calculated values for $(1/T_1M_A)$ and $(1/T_1M_I)$ from Equation 9. If, for the third $Tl^+$ concentration, the values for the calculated $(1/T_1M)_{calc}$ and for the measured $(1/T_1M)_{obs}$ are comparable, then the assumption of independent sites for activation and for inhibition by $Tl^+$ in YPK is consist-
Normalized longitudinal and transverse relaxation rates were determined for YPK complexes of wild type and Thr-298 mutants. Experiments were performed at 173 MHz and 346 MHz as described under “Experimental Procedures.”

| Enzyme complex | % saturationa | 1/T_{1M} (s⁻¹) (173 MHz) | 1/T_{1M} (s⁻¹) (346 MHz) | 1/T_{1M} (s⁻¹) (346 MHz) | 1/pT_{1p} (s⁻¹) (173 MHz) | 1/pT_{1p} (s⁻¹) (346 MHz) |
|----------------|---------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
| WT-T1(15 mM)-PEP | 87            | 2350 ± 300               | 2700 ± 341               | 1050 ± 157               | (4.2 ± 1.5) × 10⁸        | (4.3 ± 0.7) × 10⁸        |
| WT-T1(15 mM)-PEP-FBP | 94           | 2300 ± 800               | 2450 ± 851               | 1340 ± 83                | (3.7 ± 0.4) × 10⁸        | (5.5 ± 1.7) × 10⁸        |
| WT-T1(90 mM)-PEP-FBP | 99           | 8840 ± 1370              | 8930 ± 1380              | ND                       | ND                       | ND                       |
| T298S-T1(15 mM)-PEP | 71            | ND                       | ND                       | 2093 ± 108               | ND                       | ND                       |
| T298S-T1(35 mM)-PEP | 85            | ND                       | ND                       | 3800 ± 213               | ND                       | ND                       |
| T298S-T1(70 mM)-PEP | 92            | 10900 ± 782              | 11850 ± 850              | 5210 ± 260               | (16.8 ± 0.7) × 10⁸       | (19.4 ± 1.8) × 10⁸       |
| T298S-T1(90 mM)-PEP-FBP | 95          | 9500 ± 650               | 10000 ± 684              | 5390 ± 930               | (33.3 ± 2.7) × 10⁸       | (30.7 ± 7.8) × 10⁸       |
| T298C-T1(60 mM)-PEP | 73            | 3645 ± 512               | 4990 ± 700               | ND                       | (4.7 ± 0.7) × 10⁸        | ND                       |
| T298C-T1(70 mM)-PEP-FBP | 90           | 6100 ± 567               | 6780 ± 630               | ND                       | (14.6 ± 2.5) × 10⁸       | ND                       |
| T298A-T1(70 mM)-PEP | 93            | 8780 ± 1960              | 9440 ± 2100              | ND                       | (13.2 ± 6.0) × 10⁸       | ND                       |
| T298A-T1(90 mM)-PEP-FBP | 94          | 6800 ± 1200              | 7230 ± 1280              | ND                       | (18.2 ± 9.0) × 10⁸       | ND                       |

a Percentage saturation with Tl⁺ is calculated from Equation 2 as (νp/V) × 100 = ([Tl⁺]/[K⁺ + [Tl⁺]]) × 100.

b 1/T_{1M} normalized for 100% saturation with Tl⁺.

c 1/pT_{1p} = 1/(T_{1M} + τ_{m}), where p = [Mn_{bound}]²/[Tl_{total}]; it shows that in this case τ_{m} > T_{2M} therefore 1/pT_{1p} ≠ 1/T_{2M} (see “Discussion”).

d ND, not determined.

**DISCUSSION**

Recent studies (6) with YPK were performed to determine the functional group at the active site that is responsible for the enzyme-catalyzed protonation of the enolate of pyruvate. In the yeast enzyme, the 100% conserved Thr-298 is in the optimal location to perform this function (5). Several mutations at Thr-298 were constructed, and the mutant proteins were isolated and characterized. Each mutant enzyme has different kinetic characteristics. The results led to the conclusion that a molecule of bound water at the active site serves as the proton donor (6). In an effort to characterize further the effect of these mutations at the active site, the interaction between the enzyme-bound divalent activator (Mn²⁺) and the monovalent activator (Tl⁺) was investigated in each of the mutants by appropriate NMR studies.

**Thallium Activation**—Wild type YPK is activated by Tl⁺ with Mn²⁺ as divalent activator and in the presence of FBP with a k_{cat} value similar to that measured with K⁺ (Table I). With the three Thr-298 mutants, Tl⁺ is a better activator than K⁺ based on k_{cat} values. It has been suggested by Loria and Nowak (7) that the monovalent cation in PK may orient either an enzymic group for substrate binding and/or catalysis or the phosphate group for attack by the incoming nucleophile. It is possible that the greater k_{cat} value with Tl⁺ than with K⁺ for the Thr-298 mutants is due to a more favorable accommodation of Tl⁺ at the active site with respect to the group whose orientation is aided by the monovalent cation. Tl⁺ binds with higher affinity than the physiological activator K⁺ to wild type YPK and Thr-298 mutants (Table I), as observed with other enzymes that require a monovalent cation such as Na⁺ or K⁺ (22). The ionic radii of thallium and potassium are 1.44 and 1.37 Å, respectively. These results and previous studies by Raushel and Vilafranca (23), Markham (22), and Loria and Nowak (7) suggest that size has an important role in monovalent cation activation of enzymes that require such a cofactor for activity. Binding of Tl⁺ to YPK is affected by mutation of Thr-298 in the active site. The apparent K_{m} values for Tl⁺ with the Thr-298 mutant enzymes are five to eight times greater than the values measured with wild type YPK. Mn²⁺-activated T298C in the absence of FBP is an exception; the value for K_{m} for Tl⁺ is 20-fold larger than the value determined with wild type YPK. At higher Tl⁺ concentrations, wild type YPK, T298S, and T298C show inhibition of activity (Fig. 2, A and B). No inhibition by Tl⁺ is observed with T298A (Fig. 2C). Perhaps with

![Fig. 4. Dependence of (%A/[E]ₚ) on 1/T{1M} defined in Equation 9, on the concentration of Tl⁺ for the T298S-T1-PEP complex at 346 MHz. The term (%A/[E]ₚ)(1/T{1M}) was derived as described under "Results." The curve through the points represents the fit of the data to Equation 2. The best fit parameters are K_{m} = 13 ± 3 mM, the activation constant for Tl⁺, and 1/T{1M}_{obs} = 5000 ± 300 s⁻¹, the value for 1/T{1M} at saturating Tl⁺ concentration at the activator site.](image-url)
The concentration of PEP (5 mM) was saturating under kinetic conditions for wild type YPK and the Thr-298 mutants. The $K_{m,PEP}$ value was measured by steady-state kinetics for all enzymes studied in the presence of saturating Tl$^{+}$ concentrations (15 mM for wild type, 90 mM for T298S and T298A, and 60 mM for T289C), at 200 mM total salt concentration, and in the absence or in the presence of FBP (data not shown). In all cases, the values for $K_{m,PEP}$ were in the micromolar range (10–500 μM) similar to the values measured in the presence of 200 mM K$^{+}$. These results demonstrate that altered $K_{c}$ and $K_{r}$ values for Tl$^{+}$ are not due to subsaturating concentrations of PEP with the Thr-298 mutants.

The dissociation constants for Mn$^{2+}$ binding to the complexes of wild type YPK and of the Thr-298 mutants were determined (Table II). Mn$^{2+}$ binds to the enzyme-Tl$^{+}$-PEP complex for the wild type, T298S, and T298C enzymes with the same affinity. The T298A mutation weakens the binding of Mn$^{2+}$ to the enzyme-Tl$^{+}$-PEP complex by about an order of magnitude. With the T298S-Tl$^{+}$-PEP complex, a 5-fold increase of Tl$^{+}$ concentration has no significant effect on $K_{D,Mn^{2+}}$. Table III shows an increase in the observed 1/$T_{1M}$ with increasing Tl$^{+}$ concentration in the T298S-Tl$^{+}$-PEP complex. If Tl$^{+}$ were binding at the Mn$^{2+}$ site at higher concentrations, a decrease of the paramagnetic effect of Mn$^{2+}$ on $^{205}$Tl$^{+}$ relaxation would be observed due to Mn$^{2+}$ displacement by Tl$^{+}$. The increase in 1/$T_{1M}$ at higher concentrations of Tl$^{+}$ may result from greater occupancy at the inhibitory site of Tl$^{+}$. Similar results were obtained with rabbit muscle PK in the enzyme-Tl$^{+}$-PEP complex at 15 mM Tl$^{+}$ and 100 mM Tl$^{+}$ (7). These results indicate that Tl$^{+}$ inhibition is not due to displacement of Mn$^{2+}$ and that the inhibitory site is near or at the active site of YPK. The presence of the heterotrophic activator FBP tightens the binding of Mn$^{2+}$ to YPK, and the measured $K_{D,Mn^{2+}}$ values are similar for the wild type and mutant complexes (Table II). With WT-Tl$^{+}$-PEP-FBP, a 6-fold increase of Tl$^{+}$ concentration does not affect the $K_{D,Mn^{2+}}$ value. The explanation for this observation may be the same as for the T298S-Tl$^{+}$-PEP complex (see above).

**Longitudinal and Transverse Relaxation Rates**—The NMR spectra for $^{205}$Tl$^{+}$ were measured in the presence of wild type YPK and the three mutants. The $^{205}$Tl$^{+}$ resonance in the PEP complex of wild type YPK is 2 ppm downfield of the $^{205}$Tl$^{+}$ resonance in the PEP complex of the Thr-298 mutants (Fig. 2, A-2 to D-2). These data indicate different environments for the thallous ion in each of these complexes. Nuclear relaxation effects of bound Mn$^{2+}$ on the $^{205}$Tl$^{+}$ resonance were used to further investigate the nature of the environmental differences.

Table III shows that for each complex studied, the normalized values of 1/$T_{2p}$ are significantly larger than the normalized values of 1/$T_{1p}$. The measured and normalized value of 1/$T_{1p}$ is frequency-dependent (Table III). Both observations suggest that the relaxation phenomenon and non chemical exchange is measured for 1/$T_{1p}$.

If only dipolar relaxation is considered, 1/$T_{2M}$ is given by the simplified Solomon-Bloembergen equation (Equation 11).

$$\frac{1}{T_{2M}} = \frac{9}{15} S(S+1)\pi_{g}[\frac{\rho}{p} + \frac{3}{4} ig + \frac{3}{4} g_{I} \frac{1}{1 + 0.2 g_{s}^{2} g_{I}^{2}} + \frac{12}{1 + 0.2 g_{s}^{2} g_{I}^{2}}]$$

(Eqn 11)

The more general form of Equation 11 has an additional scalar term for the relaxation rate (19). If scalar effects contribute to relaxation, then 1/$T_{2M}$ is even greater. In macromolecular complexes in which large enhancements are observed, $\rho_{g}g_{s}^{2}g_{I}^{2} >> 1$ and the correlation function of the dipolar term in Equation 11 is simplified to $4g_{s} + 3g_{I} + 0.3g_{s}^{2}g_{I}^{2}$. 1/$pT_{2p}$ could be frequency independent if (a) $g_{s}$ dominates the relaxation rate 1/$pT_{2p}$ in Equation 10 or (b) if in the simplified form of Equation 11, the frequency independent term is much larger than the frequency dependent term, $4g_{s} + 3g_{I} + 0.3g_{s}^{2}g_{I}^{2}$. If $a$ is the case then 1/$pT_{2p} = 1/T_{1M}$, the rate of chemical exchange. The temperature dependence data of 1/$T_{2p}$ for enzyme-bound Tl$^{+}$ in complexes of wild type YPK gave Arrhenius plots (1/$T_{2p}$ versus 1/temperature) that had zero or negative slopes, depending on the complex (7). The frequency-independent value calculated for $g_{s} = 0.65 \times 10^{-5}$ s (see below). This value for 1/$T_{2M}$ was calculated for the WT-Tl$^{+}$-PEP complex at both frequencies from the simplified form of Equation 11 assuming only dipolar effects and was compared with the measured 1/$pT_{2p}$ values listed in Table III. This calculation eliminates argument $b$, suggests scalar contributions to 1/$T_{2M}$, and further indicates that chemical exchange contributes to 1/$T_{2p}$. Scalar superhyperfine coupling has been reported between VO$^{2+}$ (a Mn$^{2+}$...
analogy and $^{205,203}$Tl$^+$ bound at the active site of muscle PK (24). Reuben and Kayne (9) have also suggested scalar effects in the Mn$^{2+}$-Tl$^+$ interaction with muscle PK. The analysis indicates that transverse relaxation rates are not solely governed by dipolar interactions and therefore will not be used to calculate the Tl$^+$-Mn$^{2+}$ distance in the complexes of wild type YPK and the Thr-298 mutants that were studied.

The values of $1/p_{T_{1p}}$ appear to be in fast exchange (Table III). From the Swift-Connick relationship for $1/p_{T_{1p}}$ and $1/p_{T_{2p}}$ (Equation 10), $1/p_{T_{1p}} = 1/T_{1M}$. The existence of fast exchange conditions for $1/T_{1p}$ is also supported by the positive slopes in the Arrhenius plots for longitudinal relaxation rates of enzyme-bound Tl$^+$ in complexes of wild type YPK, as reported by Loria and Nowak (7), and by the frequency dependence of $1/p_{T_{1p}}$ (Table III).

Studies with muscle PK (7) indicate that $\tau_r$ is not a function of frequency over the range of the magnetic field studied. Assuming similar properties of $\tau_r$ with YPK, the correlation time for the Tl$^+$-Mn$^{2+}$ interaction in YPK was calculated using Equation 6 for values of $1/T_{1M}$ at 173 and 346 MHz. By using this method for the WT-Tl$^+$ (15 mm)-PEP, WT-Tl$^+$ (15 mm)-PEP-FBP, T298S-Tl$^+$ (70 mm)-PEP, and T298S-Tl$^+$ (90 mm)-PEP-FBP complexes, the following values for $\tau_r$ were calculated: $0.77 \pm 0.52$, $0.52 \pm 0.46$, $0.70 \pm 0.31$, and $0.54 \pm 0.36$ ns, respectively. Although there is some variation in the $\tau_r$ values estimated, these variations are probably due to experimental errors and limitations of the method. Values of $\tau_r$ ranging from 0.52 to 0.77 ns result in a calculated distance range differing by less than 0.14 Å. For simplicity, it is assumed that the $\tau_r$ values for each complex of wild type YPK and Thr-298 mutants are the same, and an average value of 0.65 ± 0.35 ns will be used for further calculations. A similar approach has been previously used for estimation of correlation times for muscle PK complexes (7, 23) and yeast PK complexes (7). These results indicate that the variations in $1/T_{1M}$ between wild type YPK and the various Thr-298 mutants are due to variations in the dipolar distances and not in $\tau_r$ values.

**Tl$^+$-Mn$^{2+}$ Distances in YPK Complexes**—The Tl$^+$-Mn$^{2+}$ distances in the enzyme-Tl$^+$-Mn$^{2+}$ complexes of wild type YPK and Thr-298 mutants were calculated using Equation 8, in which the values for $T_{1M}$ were normalized for saturation ($T_{1M,100}$) and are listed in Table III. The calculated Tl$^+$-Mn$^{2+}$ distances in complexes of wild type and the Thr-298 mutants of YPK are summarized in Table IV. For the WT-Tl$^+$ (15 mm)-PEP complex the calculated inter-metal distance is 6.0 ± 0.1 Å (Table IV). This value is in excellent agreement with the value of 6.1 ± 0.3 Å determined by Loria and Nowak (7) and with the K$^+$-Mn$^{2+}$ distance of 5.8 Å measured by x-ray diffraction methods for the YPK-Mn$^{2+}$-phosphoglycolate complex (5).
that mutation of the catalytically important Thr-298 residue induces a closer proximity of the monovalent and divalent cations that are located at the site of phosphoryl transfer (Fig. 1). Since both cations interact with the phosphate group of PEP (see Ref. 5), the alterations measured by $^{203}$Tl$^+$ NMR may reflect a conformational change of PEP at the active site. The substrate-induced conformational changes in YPK measured by $^{203}$Tl$^+$ NMR may, in part, explain the alteration in $k_{cat}$ and $k_{cat}/K_m$PEP observed with T298S, T298C, and T298A (6). These results emphasize that interpretation and assessment of overall properties and behavior of mutant enzymes relative to the wild type protein must be done with caution. Amino acid mutation in a protein may introduce subtle structural alterations at the site of mutation or at remote sites. These structural changes may be, in part, responsible for the altered catalytic properties of the mutant enzymes. The unique properties of YPK have allowed for such detailed analyses.

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