The Proton Transfer Step Catalyzed by Yeast Pyruvate Kinase*

Received for publication, January 9, 2003
Published, JBC Papers in Press, January 31, 2003, DOI 10.1074/jbc.M300257200

Delia Susan-Resiga and Thomas Nowak‡
From the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

The nature of the proton donor to the C-3 of the enolate of pyruvate, the intermediate in the reaction catalyzed by yeast pyruvate kinase, was investigated by site-directed mutagenesis and physical and kinetic analyses. Thr-298 is correctly located to function as the proton donor. T298S and T298A were constructed and purified. Both mutants are catalytically active with a decrease in $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$. $\text{Mn}^{2+}$-activated T298S and T298A do not exhibit homotropic kinetic cooperativity with phosphoenolpyruvate (PEP) in the absence of fructose 1,6-bisphosphate, although PEP binding to enzyme-$\text{Mn}^{2+}$ is cooperative. The pH dependence of $K_m$ for T298A indicates the loss of $K_a$ by 6.4–6.9. Thr-298 affects the ionization ($pK_a = -6.5$) responsible for modulation of $k_{\text{cat}}$. Fluorescence studies show altered dissociation constants of ligands to each enzyme complex upon Thr-298 mutations. The rates of the phosphoryl transfer and proton transfer steps in the pyruvate kinase-catalyzed reaction are altered; pyruvate enolization is affected to a greater extent. Proton inventory studies demonstrate solvent isotope effects on $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$. Fractionation factors are metal-dependent and significantly <1. The data suggest that a water molecule in a water channel is the direct proton donor to enolpyruvate and that Thr-298 affects a late step in catalysis.

Yeast pyruvate kinase (YPK)\(^1\) (EC 2.7.1.4.0) is a key regulatory enzyme in glycolysis that catalyzes the phosphoryl transfer from phosphoenolpyruvate (PEP) to ADP to yield pyruvate and ATP. The reaction requires both monovalent and divalent cations, normally K\(^+\) and Mg\(^{2+}\) or Mn\(^{2+}\). Fructose 1,6-bisphosphate (Fru-6-P\(_2\)) is the heterotropic activator of YPK. The net reaction catalyzed by YPK is the sum of at least two partial reactions. Phosphoryl transfer from PEP to M(II)ADP occurs by an apparent $S_2$ mechanism with inversion of configuration at the phosphoryl group to yield the enolate of pyruvate and M(II)ATP (1). In the second partial reaction, a proton donor at the active site stereospecifically protonates the C-3 of enolpyruvate at the 2-ribose face of the double bond to form ketopyruvate (2–4). The enolate of pyruvate, the common species in both partial reactions, is a tightly bound intermediate in the net reaction catalyzed by PK (5). The two-step character of the net PK reaction is demonstrated by the ability of PK to catalyze the enolization of bound pyruvate without phosphoryl transfer (6–8). Muscle PK requires a group such as inorganic phosphate, methyl phosphonate, or fluorophosphate as a cofactor (6, 7). YPK requires ATP as a cofactor for this activity (8). PK will also catalyze the ketonization of enolpyruvate, generated in situ subsequent to the hydrolysis of PEP catalyzed by alkaline phosphatase (3).

Initial crystallographic data from cat muscle PK (9) indicated that Lys-269 (Lys-240 in the YPK numbering sequence) serves as the putative proton donor because it was positioned close to the methyl carbon of the bound pyruvate. The e-amino group of Lys-269 as the possible candidate for the acid/base catalyst in PK was supported by the measurement of multiple protons that are incorporated into pyruvate with rabbit muscle pyruvate kinase (10). Subsequent refined x-ray crystal structures of the yeast enzyme with bound phosphoglycolate (11) and of the rabbit muscle enzyme complexed with pyruvate (12) demonstrated that Lys-240 is at the 2-re face of the double bond of the enolato and is in apparent contact with the phosphoryl group of PEP. The recent studies of the K240M mutant of YPK support the role of this lysine in facilitating phosphoryl transfer but not enolpyruvate protonation (13). The location of the methyl group of pyruvate is oriented such that Thr-298 (Thr-327 in the muscle PK numbering sequence) is in the correct position to protonate the enolate intermediate at C-3 (12). Thr-298 of YPK relative to bound phosphoglycolate is in the same location (11). Rose et al. (14) have suggested that the proton donor in pyruvate kinase is a high $K_a$ monoprotic acid that rapidly exchanges protons with solvent in the unliganded enzyme. The secondary alcohol of Thr-298 satisfies these requirements and that of the stereochemistry. On the other hand, pH rate profiles with rabbit muscle PK reveal an ionization with a $K_a = 8.3$. This ionization has been interpreted as the $K_a$ of the acid-base catalyst (15). The strict conservation of both Lys-240 and Thr-298 in all PKs that have been sequenced and their critical location within the active site of PK suggest that the side chains of both residues play important roles in catalysis. Studies of YPK by Bollenbach et al. (13) demonstrate that the $K_a$ of 8.8 is lost on mutation of Lys-240 to methionine. Furthermore, the results of the kinetic characterization of K240M suggest that Lys-240 is not the direct proton donor to the enolpyruvate intermediate in YPK. In its protonated form, Lys-240 is important in stabilization of the pentavalent transition state of the phosphoryl group undergoing transfer.

The present study focuses on the role of Thr-298 in catalysis of the pyruvate kinase reaction. This question was addressed by altering Thr-298 by site-directed mutagenesis and by physical and kinetic analyses of the wild type and the resulting mutant enzymes. The results indicate that it is water at the active site that serves as the proton donor.

\* This work was supported in part by Research Grant DK 17049 from the National Institutes of Health (to T.N.) and by the University of Notre Dame. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556-5670. Tel.: 574-631-5859; Fax: 574-631-3567; E-mail: Nowak.1@ND.edu.

\(1\) The abbreviations used are: YPK, yeast pyruvate kinase; PK, pyruvate kinase; Fru-6-P\(_2\), fructose 1,6-bisphosphate; MII, divalent metal cation; MESS, 2-(N-morpholino)ethanesulfonic acid; PEP, phosphoenolpyruvate; SIE, solvent isotope effect; TAPS, 3-[N-tris(hydroxymethyl)methylamino]-propanesulfonic acid.

\(2\) The present study focuses on the role of Thr-298 in catalysis of the pyruvate kinase reaction. This question was addressed by altering Thr-298 by site-directed mutagenesis and by physical and kinetic analyses of the wild type and the resulting mutant enzymes. The results indicate that it is water at the active site that serves as the proton donor.
Role of Thr-298 in Pyruvate Kinase Catalysis

EXPERIMENTAL PROCEDURES

Materials—t-(L)-Lactate dehydrogenase from rabbit muscle was purchased from Roche Molecular Biochemicals. Wild type, T298S, and T298A yeast pyruvate kinases were purified as described by Mesecar and Nowak (16). PEP, ADP, Fru-6-P, disodium NADH, glycerol, Dowex 1 chloride (400-mesh), and buffers were purchased from Sigma. Deutero oxide (99.9%) was obtained from Cambridge Isotope Laboratories.

Site-directed Mutagenesis and Cell Growth— Mutagenesis reactions were performed in the pSelectTM-1 vector according to the Promega Altered Sites II manual. The mutagenic oligonucleotides used for constructing the specific point mutants are as follows: T298S, CCAAGATCCACAGATCTGG; T298A, CCAAGATCCACAGGCTGGACAGTAAACTGG; and T298V, CCAACATTTGGACAGCACAGATAACAGATAACTGG; and T298V, CCAACATTTGGACAGCACAGATAA-CTGG. The underlined sequences correspond to the mutated nucleotides. A 1402-bp XbaIEcoRI fragment of the YPK gene was subcloned into the XbaIEcoRI sites of the pSelectTM-1 vector (18). For each of the Thr-298 mutants, the 751-bp fragment of DNA containing the desired mutation in the YPK gene was excised from the pSelect vector by EcoRI/BamHI/IEcoRI digestion, followed by isolation on a 1% agarose gel. The 751-bp fragments were cloned into the EcoRI/BamHI site of the yeast shuttle vector, pYPK101, that contains the entire PK gene. In order to verify the presence of the desired mutations, the mutated genes were sequenced (DNA Sequence Facility, Iowa State University, Ames, IA). The mutant pYPK101 constructs were transformed into the pyruvate kinase-deficient yeast strain, pkyl-5 (19), using a lithium acetate procedure (20).

Yeast pyk1-5 containing the wild type pYPK101 was grown on rich media containing the following per liter: 10 g of yeast extract, 20 g of bactopeptone, and 2% glucose. Transformed pyk1-5 containing the pYPK101 with the T298S and T298A mutations were grown on glucose minimal medium; 2% glycerol and 2% ethanol; pH 5.0; and Nowak (16). PEP, ADP, Fru-6-P2, disodium NADH, glycerol, Dowex 1 chloride (400-mesh), and buffers were purchased from Sigma. Deutero oxide (99.9%) was obtained from Cambridge Isotope Laboratories.

Fluorescence Measurements—The dissociation constants of the ligands to various enzyme complexes were measured by steady-state fluorescence. Fluorescence quenching of the intrinsic single tryptophan residue of YPK upon ligand binding was monitored on an SLM model MC200 fluorometer thermostated at 24 ± 1 °C. The excitation monochro- mator was a model MC400, and the emission monochromator was a model MC200. Fluorescence titrations were performed by monitoring the change in fluorescence intensity at 334 nm upon excitation at 295 nm. Titrations were performed by sequentially adding 1–10-μl aliquots of a concentrated ligand solution to 900 μl of a mixture containing 100 mM MES (pH 6.2), 5% glycerol, 200 mM KCl, 0.05–0.07 mM wild type YPK or Thr-298 mutants, and other ligands as specified. The percent fluorescence quenching, Q, was calculated using the relationship: Q = (F₀ - F) × 100, where F₀ and F are the fluorescence intensities of YPK in the presence and absence of a ligand, respectively. F was corrected for dilution effects. Each fluorescence titration data set fit to Equations 6 and 7, where [L] represents the variable ligand concentration; Kₒ is the dissociation constant for ligand L to the respective enzyme complex; Qmax is the percent maximal quenching; and nL is the Hill coefficient. Equations 6 and 7 are analogous to the Michaelis-Menten and the Hill equations, respectively.

Rate of Phosphoryl Transfer—The rate of phosphoryl transfer catalyzed by YPK was measured using the glycolate kinase activity, as described by Bollenbach et al. (13). The reaction mixture contained in 1 ml, 100 mM HEPES (pH 7.5), 4% glycerol, 200 mM KCl, 2 mM Fru-6-P₂, 50 mM glycylate, 10 mM MnCl₂, or 15 mM MgCl₂, 0.2–3 mM ATP, and YPK (100–200 μM) wild type and T298S, or 200–300 μM of T298A). The glycylate kinase reaction was followed as the decrease of ATP peaks over time using high pressure liquid chromatography separation. High pressure liquid chromatography analysis was performed on a Beckman 421 liquid chromatograph equipped with a Beckman 334 Gradient System and a Rainin 218TPT5-4C18 column (5 μm, 4.6 × 250 mm) with a flow rate of 500 μl/min. Detection was at 260 nm. Integration and plotting of chromatograms were performed on a Spectra Physics SP4290 integrator. Initial velocities of phosphoryl transfer were calculated from measurements of ATP disappearance with time. The kₜₐ for phosphoryl transfer catalyzed by YPK (vₒ) was determined from initial velocities versus [ATP] and fitting the data to Equation 1. A nonenzymatic blank was added to each sample.
was run at each ATP concentration. There was no detectable decrease in ATP concentration over the course of the experiment in the absence of enzyme.

**Rate of Pyruvate Elongation**—The elongation of pyruvate was measured as the time-dependent exchange of tritium from $^{3}{\text{H}}$pyruvate into water, as described previously (13). The activity of $^{3}{\text{H}}$pyruvate was 75,170 dpm/μmol pyruvate in the experiments with wild type YPK and T298S or 72,900 dpm/μmol pyruvate in the experiments with T298A. The PK-catalyzed rate of pyruvate elongation, $v_{p}$, is defined as $\mu$ mol of protons exchanged into water/min/mg protein.

**Solvent Isotope Effect Studies**—All buffers, divalent and monovalent cations, and substrates were exchanged in 99.9% D$_2$O by dissolving solutes 3 times in D$_2$O followed by lyophilization. In each case the final sample was re-dissolved in D$_2$O to give the desired solute concentrations. The pD and pH values of the assay buffer were adjusted to 6.2 using KOD and KOH, respectively. The assay buffer was adjusted to pH 6.2 according to the relationship pD = (pH)$_{water}$ + 0.4 (22). NADH was dissolved in the assay buffer, prepared as above, immediately prior to use. Assays (1 ml) were prepared and covered with parafilm prior to measurement. Assays were performed in duplicate. Solvent isotope effects (SIE) on $k_{cat}$, $v_{cat}$, and on $k_{cat}/K_{m,PEP}$, $v_{cat}/K_{m,PEP}$, were determined from fitting initial velocity versus [PEP] data in H$_2$O and in D$_2$O to Equation 1.

**Proton Inventory Studies**—The stock solutions were prepared as described above. The final deuteron content of the deuterated stock solutions was estimated to be 97%. Wild type YPK and Thr-298 mutants were assayed in a series of isotopically mixed water ([$^{1}\text{H}]$H$_2$O and [2H]H$_2$O) of deuterium molar fraction $n$. In each case, initial velocities were measured at saturating PEP concentration (5 mM). Assays were performed in triplicate. Data were fit to the Gross-Butler equation either in the linear form (Equation 8) or in the nonlinear form (Equation 9) (23). Equation 8 describes the case where a single proton in the transition state of the PK-catalyzed reaction contributes to the observed isotope effect. Equation 9 represents the situation of a single proton in the transition state and one in the reactant state, respectively. Alternatively, one proton may have different contributions in the reactant state than in the transition state.

$$V_{n}/V_{0} = 1 + n(\phi^{T-1})$$  \hspace{1cm}  (Eq. 8)

$$V_{n}/V_{0} = (1 + n(\phi^{T-1}))(1 + n(\phi^{R-1}))$$  \hspace{1cm}  (Eq. 9)

In these equations, $\phi^{T}$ and $\phi^{R}$ are the fractionation factors for the proton in the transition state and reactant state, respectively, and $V_{n}$ and $V_{0}$ represent the maximal velocities observed in a mixture of isotopic water of $n$ mole fraction of D$_2$O and in H$_2$O, where $n = 0$, respectively (23, 24).

**RESULTS**

**Cell Growth and Purification of T298S and T298A**

T298S, T298A, and T298V were constructed and expressed using the same procedure as for wild type YPK. The pyruvate kinase-deficient Saccharomyces cerevisiae strain, pyk1-5, containing the pPYK101 plasmid with either the T298S or the T298A mutation was grown on media containing glucose as the sole carbon source (glucose minimal media). These cells were successfully grown on glycerol-ethanol media. The T298V pPYK101 was unable to grow on media containing glucose to pyruvate. The T298S and T298A mutants were purified using the same procedure as for wild type YPK. The pyruvate kinase-deficient yeast do not have the ability to catabolize glucose to pyruvate. The T298S and T298A mutants were purified to greater than 95% homogeneity based on SDS-PAGE, with yields of 35 and 30 mg/liter of culture, respectively. The T298V mutant was not purified.

**Biophysical Characterization of T298S and T298A**

The secondary structures of the apo T298S and T298A mutants were monitored by far UV-CD and compared with wild type YPK (data not shown). Both mutants showed a slight and consistent difference in the magnitude of the molar ellipticity relative to wild type enzyme. The general shape of the far UV spectra of the Thr-298 mutants and wild type YPK was the same over the full spectral range (200–280 nm). These results indicate that neither mutation caused any significant changes in the secondary structure and that the wild type and mutant YPKs were folded into a similar if not identical structure.

The intrinsic tryptophan fluorescence emission spectra of apo T298S and apo T298A mutants were recorded between 310 and 400 nm and compared with wild type YPK. The single tryptophan, Trp-452, was selectively excited at 295 nm so that observed changes in the intrinsic fluorescence could be correlated with conformational changes at a specific localized region in YPK. The apo forms of T298S and T298A YPK have a tryptophan emission maximum at ~334 nm, similar to that for apo wild type YPK (16).

**Steady-state Kinetics**

Steady-state kinetic measurements were performed for the wild type YPK and for the mutant enzymes under identical conditions. An example of the steady-state kinetic rate profiles for T298S at variable PEP concentrations with Mg$^{2+}$ and with Mn$^{2+}$ as divalent activator in the absence or presence of Fru-6-P$_2$, is shown in Fig. 1. Similar profiles were obtained with T298A (data not shown). The kinetic responses of wild type and the Thr-298 mutants with either Mg$^{2+}$ or Mn$^{2+}$ as the activator and in the absence or presence of Fru-6-P$_2$ were fit to Equations 1 and 2 as appropriate. The best fits from the appropriate equations were used accordingly. A summary of the calculated steady-state parameters is presented in Table I.

The mutant enzymes showed differential allosteric responses, depending upon the divalent metal activator (Mg$^{2+}$ or Mn$^{2+}$). The T298S mutation showed alterations primarily in $k_{cat}$. The $k_{cat}/K_{m,PEP}$ values for wild type and T298S were similar. The divalent metal specificity with T298S was the same as with wild type. Based on $k_{cat}$ values, Mg$^{2+}$ > Mn$^{2+}$. The T298S mutation had a minor effect on $K_{m,PEP}$ only in the presence of Mn$^{2+}$. The Mn$^{2+}$-activated T298S did not display kinetic cooperativity with PEP in the absence of Fru-6-P$_2$, with $n_H = 1$ compared with $n_H = 2.5$ for wild type YPK. T298S activated by Mg$^{2+}$ showed cooperative behavior ($n_H = 1.9$) similar to that of wild type enzyme ($n_H = 2.8$).

The turnover rates for T298A decreased by a factor of 24 and
Role of Thr-298 in Pyruvate Kinase Catalysis

Steady-state kinetics were measured as described under “Experimental Procedures,” with either PEP or divalent metal as the variable substrate. Measurements were performed in the absence or presence of saturating concentrations of Fru-6-P₂ and with either wild type YPK, T298S, or T298A mutants as indicated.

| Divalent activator | FBP | YPK | kₐₛ | Kₐₛ,PEP | kₐₛ/Kₐₛ,PEP | Kₐₛ,app, M | nᵢₜ,PEP | nᵢₜ,LM |
|--------------------|-----|-----|------|----------|-------------|------------|----------|--------|
| Mn²⁺               |     |     | s⁻¹  | µM       | m⁻¹ s⁻¹     | µM         |          |        |
| – Wild type        | 58.4 ± 0.9 | 45 ± 1 | (1.3 ± 0.1) × 10⁶ | 2.5 ± 0.1 |
| – T298S            | 35.3 ± 0.6 | 26 ± 1 | (1.4 ± 0.1) × 10⁶ | 1         |
| + Wild type        | 3.3 ± 0.1 | 40 ± 1 | (0.82 ± 0.06) × 10⁵ | 1         |
| + Wildtype⁹        | 66.0 ± 1.8 | 21 ± 3 | (3.1 ± 0.6) × 10⁶ | 1         |
| + T298S            | 36.0 ± 0.8 | 16 ± 2 | (2.3 ± 0.3) × 10⁶ | 1         |
| + T298A            | 4.0 ± 0.1 | 14 ± 2 | (2.9 ± 0.5) × 10⁶ | 0.7 ± 0.1 |
| Mg²⁺               |     |     | s⁻¹  | µM       | m⁻¹ s⁻¹     | µM         |          |        |
| – Wild type        | 232 ± 5 | 1180 ± 222 | (2.9 ± 0.5) × 10⁵ | 2.8 ± 0.1 |
| – T298S            | 64.4 ± 1.6 | 835 ± 36 | (0.77 ± 0.06) × 10⁵ | 1.9 ± 0.2 |
| + T298A            | 9.4 ± 0.3 | 6956 ± 230 | (0.14 ± 0.01) × 10⁴ | 2.1 ± 0.1 |
| + Wild type        | 226 ± 11 | 310 ± 5 | (7.5 ± 0.5) × 10⁵ | 1.2 ± 0.1 |
| + T298S            | 77.5 ± 0.2 | 304 ± 13 | (2.6 ± 0.1) × 10⁵ | 1.5 ± 0.2 |
| + T298A            | 20.9 ± 0.3 | 680 ± 25 | (3.2 ± 0.1) × 10⁵ | 1.6 ± 0.1 |

¹ Taken from Ref. 13.
² Total MgCl₂ concentration was 22 mM.

11 compared with wild type YPK when activated by Mg²⁺ in the absence and in the presence of Fru-6-P₂, respectively. Fru-6-P₂ increased the kₐₛ of the reaction with T298A and decreased Kₐₛ,PEP with Mn²⁺ as the activator. The decrease in kₐₛ for T298A relative to wild type YPK in the absence or presence of Fru-6-P₂ was ~18-fold with Mn²⁺ as the activator. The divalent metal specificity with T298A based on kₐₛ was the same as with wild type and with T298S. The steady-state interaction between PEP and YPK, as measured by Kₐₛ and kₐₛ/Kₐₛ values, has been affected significantly by the T298A mutation. The thermodynamic interaction of PEP with the enzyme was unaffected (see below). In the absence of Fru-6-P₂, the Mn²⁺-activated T298S and T298A enzymes did not exhibit positive cooperativity with PEP. T298A displayed slight negative cooperativity for FPEP in the presence of Fru-6-P₂ (nᵢₜ = 0.7 compared with nᵢₜ = 1 in wild type). T298A activated by Mg²⁺ had kinetic behavior similar to that observed for wild type enzyme and T298S YPK activated by Mg²⁺.

The Kₐₛ,Mₐ₄ for Mn²⁺ and for Mg²⁺ (Table I) is an apparent value based on the total metal concentration and was not corrected for the divalent metal bound by ADP in solution. The concentration of ADP is constant and saturating in all of the kinetic studies. Mutation of Thr-298 to serine had no effect on Kₐₛ,Mₐ₄ and resulted in a 3-fold decrease of Kₐₛ,Mₐ₄, compared with wild type YPK. Mutation of Thr-298 to alanine had no effect on the Kₐₛ,Mₐ₄ values.

Effects of pH on Catalysis by Wild Type YPK and Thr-298 Mutants

Any relevance of Thr-298 to an ionization that affects catalysis was addressed by measuring pH rate profiles. The effect of pH on the Vₘₐₓ,ₜₐₜ for the Mn²⁺- and the Mg²⁺-activated wild type, T298S, and T298A enzymes in the presence of Fru-6-P₂ was measured over the pH range of 4.5–9.1. The pH rate profiles for the enzymes activated by Fru-6-P₂ and with Mn²⁺ and with Mg²⁺ are shown in Fig. 2, A and B, respectively. A summary of the pKₐ values obtained from the fit of the data in Fig. 2 to Equations 3–5, as appropriate, is presented in Table II. Interpretation of the pKₐ values obtained from kinetic studies should be done with care. They may not be true thermodynamic values reflecting the microscopic pKₐ of a specific ionizable group (25). The Kₐₛ,PEP values measured for the Mn²⁺-activated wild type, T298S, and T298A mutants in the presence of Fru-6-P₂ were invariant over this pH range allowing the calculation of pKₐₐ values for “E freely.”

The pH data for the Fru-6-P₂- and Mn²⁺-activated wild type YPK in Fig. 2, A and B, were fit to Equation 3, and describe three ionizations for catalysis in the ES complex. A single deprotonation with a pKₐ of ~5.5 and a single protonation of a group with a pKₐ of ~8.5 were required for catalytic activity. Deprotonation of a group in the pH range of 6–8 altered the rate of reaction (Table II) (13). Studies (13) with K240M YPK indicate that the pKₐ of 8.8 was lost upon mutation of Lys-240. Lys-240 plays a putative role in phosphorly transfer in the mechanism of YPK catalysis.

The data for the pH effect on Vₘₐₓ,ₜₐₜ for the Mn²⁺- and Fru-6-P₂-activated T298S (Fig. 2A) were fit to Equation 3, and the resulting pKₐ values are very similar to the values obtained for wild type YPK (Table II). The data for the effect of pH on Vₘₐₓ,ₜₐₜ for the Mg²⁺- and Fru-6-P₂-activated T298S (Fig. 2B) were fit to Equation 4. This equation describes only two ionizations, Kₐₐ and Kₐₐ, in the ES complex and gives a better statistical fit than Equation 3. The calculated pKₐ values are similar to the values for pKₐ and pKₐ, obtained with the wild type YPK. The value for pKₐ appears to be greater than 9.1 and hence could not be measured because of experimental limitations.

The data for the pH effect on Vₘₐₓ,ₜₐₜ for T298A in the presence of Fru-6-P₂ and with Mn²⁺ or Mg²⁺ (Fig. 2, A and B) were fit to Equation 5. This equation describes the model where only two ionizable groups are required for catalytic activity, Kₐₐ and Kₐₐ. The resulting pKₐ values are similar to the values for pKₐ and pKₐ, obtained with the wild type YPK (Table II). The results indicate that the pKₐ is 6.4–6.9 was lost upon removal of the hydroxyl group at position 298.

Ligand Binding to Wild Type and Thr-298 Mutants of YPK

The effect of the mutations at Thr-298 of YPK on the binding of Mn²⁺, PEP, and Fru-6-P₂ to various YPK complexes was quantitated by measuring the quenching of the intrinsic tryptophan fluorescence as a function of ligand concentration. The steady-state fluorescence data were fit to Equations 6 or 7. The dissociation constants, Kₐₐ, maximal quenching, Qₘₐₓ, and Hill coefficients, nᵢₜ, for ligand interactions with the enzyme complexes were obtained from appropriate fits to the data and are reported in Table III.

The fluorescence response of apo wild type YPK and apo Thr-298 mutants to Mn²⁺ follows simple saturation behavior that were fit to Equation 6. The Kₐₐ,Mₐ₄ and Qₘₐₓ values do not...
change significantly upon mutation of Thr-298 to Ser or Ala. The interaction of Mn\(^{2+}\) to the YPK-PEP complex of wild type and Thr-298 mutants shows cooperative binding, and the data were fit to Equation 7. The presence of saturating PEP decreases the \(K_{D,\text{Mn}^{2+}}\) by 700-fold with wild type YPK, 180-fold with T298S, and 25-fold with T298A. A significant increase in the cooperativity of Mn\(^{2+}\) binding with T298S was observed (\(n_H = 5\)). The interaction of Mn\(^{2+}\) with wild type YPK and Thr-298 mutants in the presence of saturating Fru-6-P\(_2\) could not be measured by steady-state fluorescence because no additional change in the intensity of the fluorescence emission spectra of the YPK-Fru-6-P\(_2\) or YPK-PEP-Fru-6-P\(_2\) complexes was observed upon the addition of Mn\(^{2+}\).

Binding of Mg\(^{2+}\) to YPK complexes of wild type YPK and the Thr-298 mutants could not be monitored by steady-state fluorescence. Titration of Mg\(^{2+}\) to apo YPK and YPK-PEP complexes did not result in measurable quenching of the fluorescence emission spectra of these complexes. These data also indicate that the binding of the two activating cations, Mg\(^{2+}\) and Mn\(^{2+}\), induce different conformational responses of YPK.

Titration of the apo forms of the enzymes with PEP resulted in significant quenching of the tryptophan fluorescence (\(Q_{\text{max}} < 20\%\)). PEP binds to apo wild type YPK and apo Thr-298 mutants in a hyperbolic manner (data not shown) and with similar \(K_D\) values. Fig. 3, A and B, shows data for the fluorescence response of T298S and T298A to PEP in the presence of 15 mM Mn\(^{2+}\). The response of both Thr-298 mutants is sigmoidal, and the data were fit to Equation 7. With Mn\(^{2+}\) as the activating cation and in the absence of Fru-6-P\(_2\), neither Thr-298 mutant showed kinetic cooperativity with PEP as the variable substrate (Table I). The \(K_{D,\text{PEP}}\) was increased 2-fold with T298S-Mn\(^{2+}\) and 70-fold with T298A-Mn\(^{2+}\), relative to wild type YPK-Mn\(^{2+}\). The interaction of PEP with the YPK-Mg\(^{2+}\) complexes of wild type YPK and the two Thr-298 mutants is described by a simple hyperbola (Equation 6). \(K_{D,\text{PEP}}\) to the enzyme-Mg\(^{2+}\) complex was not significantly affected upon mutation of Thr-298 to Ser or Ala. PEP binding to the YPK-Mg\(^{2+}\) complex is weaker than binding to the YPK-Mn\(^{2+}\) complex by 280-fold with wild type YPK, 150-fold with T298S, and 3-fold with T298A. The binding of PEP to the YPK-Fru-6-P\(_2\) complexes could not be monitored by steady-state fluorescence for the same reason that the binding of Mn\(^{2+}\) to these complexes could not be measured; quenching of YPK by Fru-6-P\(_2\) precludes further quenching.

Binding of Fru-6-P\(_2\) to the apo forms of wild type YPK and Thr-298 mutants resulted in a large quenching of the intrinsic

---

**TABLE II**

| Divalent activator | YPK | p\(K_A\) | p\(K_B\) | p\(K_C\) | \(a^a\) |
|---------------------|-----|---------|---------|---------|------|
| Mn\(^{2+}\)         | Wild type | 5.5 ± 0.1 | 6.4 ± 0.3 | 8.8 ± 0.2 | 0.35 ± 0.05 |
| T298S | 5.6 ± 0.3 | 6.1 ± 0.6 | 9.1 ± 0.2 | 0.48 ± 0.18 |
| T298A | 5.2 ± 0.1 | 8.5 ± 0.1 |             |         |
| K240M\(^b\) | 5.3 ± 0.1 | 7.0 ± 0.1 |             | 0.23 ± 0.02 |
| Mg\(^{2+}\)         | Wild type | 5.6 ± 0.2 | 6.9 ± 0.3 | 8.5 ± 0.5 | 0.27 ± 0.12 |
| T298S | 5.3 ± 0.2 | 6.5 ± 0.3 |             | 0.44 ± 0.07 |
| T298A | 5.5 ± 0.1 | 8.4 ± 0.1 |             |         |

\(^a\) Proportionality factor defined by Equation 3.

\(^b\) From Bollenbach et al. (13). The p\(K_A\) values for the Mn\(^{2+}\)- and Fru-6-P\(_2\)-activated wild type YPK were determined using Equation 3. For the Mn\(^{2+}\)- and Fru-6-P\(_2\)-activated K240M the p\(K_A\) values were calculated using Equation 4.
The parameters were determined by steady-state fluorescence titrations as described under “Experimental Procedures.” WT, T298S, and T298A are wild type, T298S, and T298A YPK (0.05–0.07 mg/ml), respectively, in the presence of 200 mM KCl. Final concentrations of other ligands were 5 mM MnCl₂ or 15 mM MgCl₂ and 5 mM PEP, unless otherwise specified. With T298A YPK, final [Mn/Mg/Cl] was 15 mM. All ligand concentrations are saturating.

### Table III

| Ligand | YPK | Qₘₐₓ | Kᵦ | nₜ | Ligand | YPK | Qₘₐₓ | Kᵦ | nₜ |
|--------|-----|------|----|----|--------|-----|------|----|----|
| Mn²⁺ | WT⁺ | 9.3 ± 0.5 | 7160 ± 930 | 1 | FBP | WT⁺ | 52.0 ± 0.8 | 321 ± 7 | 2.4 ± 0.1 |
|      | T298S | 14.7 ± 0.5 | 5524 ± 704 | 1 | T298S | 53.2 ± 0.3 | 30.5 ± 0.4 | 2.8 ± 0.1 |
|      | T298A | 8.6 ± 0.3 | 4491 ± 501 | 1 | T298A | 50.8 ± 0.6 | 150 ± 3 | 2.4 ± 0.1 |

Mn²⁺

| WT-PEP⁺ | 22.2 ± 0.3 | 9.9 ± 0.2 | 2.2 ± 0.1 |
| T298S-PEP⁺ | 20.5 ± 0.3 | 30.6 ± 0.4 | 5.0 ± 0.3 |
| T298A-PEP⁺ | 21.4 ± 0.3 | 184 ± 5 | 2.9 ± 0.2 |

FPW⁻

| WT⁻ | 25.0 ± 0.2 | 1100 ± 190 | 1 | FBP⁻ | WT⁻ | 47.7 ± 0.1 | 50.4 ± 0.9 | 2.2 ± 0.1 |
| T298S | 25.9 ± 0.8 | 745 ± 66 | 1 | T298S-PEP⁻ | 51.1 ± 0.3 | 98.0 ± 1.1 | 1.9 ± 0.1 |
| T298A | 20.0 ± 0.4 | 930 ± 53 | 1 | T298A-Mn²⁺⁻ | 44.8 ± 0.4 | 95.5 ± 1.3 | 2.7 ± 0.1 |

PEP

| WT-Mn²⁺⁻ | 34.0 ± 2.0 | 9.0 ± 0.3 | 2.0 ± 0.1 |
| T298S-Mn²⁺⁻ | 35.2 ± 0.2 | 14.4 ± 0.2 | 1.5 ± 0.1 |
| T298A-Mn²⁺⁻ | 35.5 ± 0.7 | 637 ± 22 | 2.0 ± 0.1 |

PEP

| WT-Mg²⁺⁻ | 35.0 ± 0.9 | 2810 ± 165 | 1 | FBP⁻ | WT-Mg²⁺⁻ | 51.4 ± 0.5 | 145 ± 3 | 3.1 ± 0.2 |
| T298S-Mg²⁺⁻ | 30.9 ± 0.8 | 2215 ± 161 | 1 | T298S-Mn²⁺⁻⁻ | 45.5 ± 1.0 | 175 ± 0.6 | 2.0 ± 0.1 |
| T298A-Mg²⁺⁻ | 22.4 ± 0.5 | 1737 ± 113 | 1 | T298A-Mn²⁺⁻⁻ | 48.6 ± 0.5 | 88.1 ± 1.2 | 2.7 ± 0.1 |

Fluorescence Spectra of YPK Complexes

The conformational response of wild type YPK and of the two Thr-298 mutants to ligand binding was analyzed by fluorescence spectroscopy. The single tryptophan, Trp-452, was excited at 295 nm. Fig. 4, A and B, shows the results of emission scans of T298S and T298A, respectively. Each enzyme was sequentially titrated with Mg²⁺⁻, PEP, and Fru-6-P⁻. The apo form of the two mutants in the presence of buffer and KCl had an emission maximum at ~334 nm. Upon saturation of T298S with Mg²⁺⁻, the emission maximum was shifted to the red by ~4 nm, and the maximal fluorescence quenching was 7%. The addition of kinetically saturated amounts of Mg²⁺⁻ to T298A did not cause a change in the emission spectrum of Thr-452. Mg²⁺⁻ binding to wild type YPK did not cause significant changes in the fluorescence emission spectrum (17). Saturation of the YPK-Mg²⁺⁻ complex with PEP caused 25% fluorescence quenching in T298S and 16% quenching and a 4 nm red shift in T298A. In wild type YPK, addition of PEP to the YPK-Mg²⁺⁻ complex resulted in 23% quenching and a 2 nm red shift. In both Thr-298 YPK mutants, addition of Fru-6-P⁻ to the YPK-Mg²⁺⁻-PEP complex caused a large red shift (~12 nm in T298S and 10 nm in T298A), a total fluorescence quenching of 37%, and a broadening of the spectra. The overall fluorescence quenching of 50% observed for the wild type YPK-

### Fig. 3. Interactions of PEP with YPK-Mn²⁺⁻.

The binding of PEP to YPK-Mn²⁺⁻ was measured by steady-state fluorescence quenching at 334 nm. A, the interaction of PEP with T298S-Mn²⁺⁻. The data are best fit to Equation 7 (—), which describes a sigmoidal response. For comparison purposes the fits of the data to Equation 6 (–—), which describes a rectangular hyperbola, is also shown. B, the interaction of PEP with T298A-Mn²⁺⁻. The data are best fit to Equation 7. The best fit parameters are listed in Table III.

tryptophan fluorescence (Qₘₐₓ ~50%). The interaction of Fru-6-P⁻ with the apo forms of the enzymes was cooperative, with Hill coefficients of 2.4–2.8. Fru-6-P⁻ binding to apo T298S was 10-fold tighter than binding to wild type YPK, and Fru-6-P⁻ binding to apo T298A was 2-fold tighter. The interaction of Fru-6-P⁻ with the wild type and Thr-298 mutants in the presence of saturating PEP remained unchanged (n_H = 2.3 to 2.7). The K_D,Fru-6-P⁻ decreased 1.5-fold with wild type YPK-PEP and increased 4-fold with T298S-PEP and T298A-PEP compared with Fru-6-P⁻ binding to the apo forms of these enzymes. The interaction of Fru-6-P⁻ with the YPK-Mg²⁺⁻ (Mn²⁺⁻ or Mg²⁺⁻) complex of wild type and Thr-298 mutants was enhanced compared with binding to apo YPK or YPK-PEP complexes of these enzymes, and the positive cooperativity remained unchanged (n_H = 2.0–3.1). Saturation of wild type YPK and Thr-298 mutants with both Mn²⁺⁻ and PEP reduced but did not abolish the positive cooperativity in Fru-6-P⁻ binding (n_H = 1.2 to 1.5). Fru-6-P⁻ binds significantly tighter to the ternary YPK-Mn²⁺⁻-PEP complex than to either of the respective binary complexes of wild type and Thr-298 mutants. Hill coefficients of 1.3–1.4 were observed for the interaction of Fru-6-P⁻ with the YPK-Mg²⁺⁻-PEP complex of wild type and T298S but was significantly greater with T298A (n_H = 2.7).

In summary, the mutation of threonine 298 to serine or to alanine altered binding of Mn²⁺⁻ to the YPK-PEP complex, binding of PEP to the YPK-Mn²⁺⁻ complex, and binding of Fru-6-P⁻ to all of the respective enzyme complexes (Table III).

### Tryptophan Fluorescence

The conformational response of wild type YPK and of the two Thr-298 mutants to ligand binding was analyzed by fluorescence spectroscopy. The single tryptophan, Trp-452, was excited at 295 nm. Fig. 4, A and B, shows the results of emission scans of T298S and T298A, respectively. Each enzyme was sequentially titrated with Mg²⁺⁻, PEP, and Fru-6-P⁻. The apo form of the two mutants in the presence of buffer and KCl had an emission maximum at ~334 nm. Upon saturation of T298S with Mg²⁺⁻, the emission maximum was shifted to the red by ~4 nm, and the maximal fluorescence quenching was 7%. The addition of kinetically saturating amounts of Mg²⁺⁻ to T298A did not cause a change in the emission spectrum of Thr-452. Mg²⁺⁻ binding to wild type YPK did not cause significant changes in the fluorescence emission spectrum (17). Saturation of the YPK-Mg²⁺⁻ complex with PEP caused 25% fluorescence quenching in T298S and 16% quenching and a 4 nm red shift in T298A. In wild type YPK, addition of PEP to the YPK-Mg²⁺⁻ complex resulted in 23% quenching and a 2 nm red shift. In both Thr-298 YPK mutants, addition of Fru-6-P⁻ to the YPK-Mg²⁺⁻-PEP complex caused a large red shift (~12 nm in T298S and 10 nm in T298A), a total fluorescence quenching of 37%, and a broadening of the spectra. The overall fluorescence quenching of 50% observed for the wild type YPK-
Role of Thr-298 in Pyruvate Kinase Catalysis

Regardless of the activating divalent cation, the total fluorescence quenching in the fully ligated YPK-Mn$^{2+}$-PEP-Fru-6-P$_2$ complex was ~38% with the two Thr-298 mutants and 52% with wild type YPK. These results indicate that the environments surrounding Trp-452 in the fully ligated complex are similar in the two Thr-298 mutants regardless of the divalent cation but are different from the environment around Trp-452 in wild type YPK. It is evident from Fig. 4 that the two Thr-298 mutants show differences in the responses to M$^{2+}$ binding and subsequent PEP titration. Small conformational changes introduced at the catalytic site upon mutation of Thr-298 to Ser or to Ala are reflected in differences in quenching of the maximal fluorescence emission of Trp-452.

Partial Reactions

The effects of the Thr-298 mutations on the two partial reactions catalyzed by YPK were quantitatively assessed to determine the relative influence of Thr-298 on each catalytic step.

**Phosphoryl Transfer**—The effects of mutation of Thr-298 to Ser or Ala on the phosphoryl transfer half-reaction were determined by measuring the glycolate kinase activity with wild type and the two Thr-298 mutants. Glycolate kinase activity is a secondary kinase reaction where PK catalyzes the ATP-dependent phosphorylation of glycolate (15, 26). Because glycolate lacks the C-3 vinyl group, glycolate kinase reflects phosphoryl transfer in the absence of proton transfer. The rates of the glycolate kinase reaction were measured in the presence of Fru-6-P$_2$ and with Mn$^{2+}$ or with Mg$^{2+}$ as the cation activator, at pH 7.5. The initial velocity response to ATP concentration with wild type YPK and the two Thr-298 mutants followed Michaelis-Menten kinetics. The rate constant for phosphoryl transfer, $v_\text{p}$, by wild type YPK was 60.5 ± 5.5 (100%) and 49.5 ± 5.5 (82%) with wild type YPK. These results indicate that the environments surrounding Trp-452 in the fully ligated complex are similar in the two Thr-298 mutants regardless of the divalent cation but are different from the environment around Trp-452 in wild type YPK. It is evident from Fig. 4 that the two Thr-298 mutants show differences in the responses to M$^{2+}$ binding and subsequent PEP titration. Small conformational changes introduced at the catalytic site upon mutation of Thr-298 to Ser or to Ala are reflected in differences in quenching of the maximal fluorescence emission of Trp-452.

**Detritiation of Pyruvate**—The second half-reaction of pyruvate kinase, the proton transfer to enolpyruvate, was moni-

---

**TABLE IV**

Rate constants for phosphoryl transfer catalyzed by wild type, T298S, and T298A YPK

| Complex                      | $v_p$ [min$^{-1}$] |
|------------------------------|--------------------|
| Wild type-Mn$^{2+}$-Fru-6-P$_2$ | 60.5 ± 5.5 (100%)  |
| T298S-Mn$^{2+}$-Fru-6-P$_2$    | 49.5 ± 5.5 (82%)   |
| T298A-Mn$^{2+}$-Fru-6-P$_2$    | 13.2 ± 1.1 (22%)   |
| Wild type-Mg$^{2+}$-Fru-6-P$_2$| 8.7 ± 1.7 (100%)   |
| T298S-Mg$^{2+}$-Fru-6-P$_2$    | 8.3 ± 1.1 (95%)    |
| T298A-Mg$^{2+}$-Fru-6-P$_2$    | 1.4 ± 1.1 (16%)    |

$v_p$ is the turnover number of the glycolate kinase reaction and is defined as micromoles of phosphoglycolate formed per min/µM of enzyme.
The rates of YPK-catalyzed pyruvate enolization were measured as described previously by Bollenbach et al. (13). Reaction mixtures consisted of 100 mM TAPS (pH 8.0), 4% glycerol, 200 mM KCl, 100 mM 3-[1-3H]pyruvate (75,170 dpm/µmol pyruvate in the experiments with wild type YPK and T298S or 72,900 dpm/µmol pyruvate in the experiments with T298A), 2 mM ATP, and 100–300 µg of YPK in 100 mM HEPES (pH 7.5). The divalent metal was either 10 mM MnCl₂ or 15 mM MgCl₂, as indicated. The concentration of Fru-6-P₂, when present, was 1 mM.

† νₚ is the rate of PK catalyzed detriment of 3-[^3H]pyruvate and is defined as µmol of tritium exchanged into water/min/mg protein. ‡ Taken from Bollenbach et al. (13).

Removal of the functional group at this position (T298A) should decrease the reaction rate of proton transfer. The isotope effect on kₚ on all catalytic steps up to and including the first irreversible step of the reaction. The first irreversible step is presumed to be phosphoryl transfer from PEP to ADP. The values for D(kₑₚ/Kₚ) are listed in Table VI. The isotope effect on kₑₚ/Kₚ was similar for the Mn²⁺- and Mg²⁺-activated wild type YPK, respectively. The values for D(kₑₚ/Kₚ) for T298S were similar to those for wild type YPK. The isotope effect on kₑₚ/Kₚ was eliminated for T298A regardless of the metal activator.

**Proton Inventory**

Proton inventory studies were performed for the Mn²⁺- and for the Mg²⁺-activated wild type YPK and for the two Thr-298 mutants in the presence of Fru-6-P₂ (13). The study was undertaken to disect the individual contributions to the observed net SIE in wild type YPK and for the Thr-298 mutants. The proton inventories with Mn²⁺- and Fru-6-P₂-activated wild type YPK and Thr-298 mutants were all linear, and the data were fit to Equation 8 (Fig. 5A). A linear proton inventory is indicative of a solvent-sensitive step involving a single proton in the transition state that contributes to the SIE. The calculated fractionation factors, φ₊, were 0.36, 0.47, and 0.64 for wild type YPK, T298S, and T298A, respectively (Table VI). With Mg²⁺ as the activator, wild type YPK gave a linear proton inventory effect. The T298S and T298A mutants show a downward curvature in the proton inventory plots (Fig. 5B). The data in Fig. 5B were fit to Equation 8 for wild type YPK and to Equation 9 for the two Thr-298 mutants. Equation 9 describes contributions of a proton in the transition state and a proton in the reactant state to the observed isotope effect. This is the simplest model that gives the best fit to the data obtained with the Mg²⁺-activated Thr-298 mutants. The values obtained for the fractionation factors are listed in Table VI. The fractionation factors are significantly less than 1 with wild type YPK and the two Thr-298 mutants. Such low fractionation factors are distinct and suggest that the proton responsible for the observed overall solvent isotope effect in each case may be derived from a metal-bound water. The nonlinear proton inventory data for T298S and T298A can alternatively be fit by assuming two protons are involved in the transition state. If this model is used, equally good fits are obtained. For T298S the fit to this model gives φ₊₁ = 0.21 and φ₊₂ = 1.64. With T298A, the fit for such a model gives φ₊₁ = 0.43 and φ₊₂ = 1.36. In this model, one fractionation factor is still quite low, whereas the value of φ₊ for the second proton is >1 indicating perhaps a hindered proton that binds tighter than proton binding in bulk water.

Table VI summarizes the theoretical values for the solvent isotope effect on kₑ obtained from the ratio of the experimentally measured value for kₑ in H₂O (n = 0), (kₑ)₀, and the fitted value for kₑ in D₂O (n = 1), (kₑ), to Equation 8 or 9.

**DISCUSSION**

Threonine 298 is located at the active site of pyruvate kinase based on the recent x-ray crystal structures of both the yeast (11) and the muscle (12) enzymes. The orientation of Thr-298 relative to the 2-st face of PEP, determined by modeling PEP relative to phosphoglycolate or pyruvate, suggests a putative role in the protonation of the enolate of pyruvate at the C-3 position. Thr-298 was mutated to serine and to alanine in YPK in an attempt to clarify its role in catalysis. If Thr-298 is the proton donor in yeast pyruvate kinase, substitution with serine
is expected to result in minor alterations in net catalytic activity and a reaction where the enzyme still retains the proton donating ability. Mutation of Thr-298 to alanine would be expected to abolish completely the proton donating ability and hence to eliminate the net catalytic activity of the enzyme. It is possible that phosphoryl transfer might remain "normal," but the enolate of pyruvate is released from the enzyme as the second product. The enolate would then undergo protonation in solution.

The T298S mutant of YPK is catalytically active with minor alterations in the kinetic constants as expected. The effect of T298S on the $K_{\text{cat}}$, $K_{\text{m,PEP}}$, and $k_{\text{cat}}/K_{\text{m,PEP}}$ are minor. This conservative mutation results in small alterations in steady-state kinetic responses except with Mn$^{2+}$ as the activator. In the presence of Mn$^{2+}$ but in the absence of Fru-6-P$_2$, this mutant does not demonstrate cooperative kinetics with PEP.

The T298A mutant results in a catalytically active enzyme that was superficially not anticipated. The value for $k_{\text{cat}}$ is decreased by about an order of magnitude relative to wild type. The $k_{\text{cat}}$ value for the Mg$^{2+}$-activated enzyme is doubled in the presence of Fru-6-P$_2$ compared with the value in its absence (Table I). An increase in the total Mg$^{2+}$ concentration to 22 mM in the kinetic assay with T298A in the absence of Fru-6-P$_2$ did not change the value for $k_{\text{cat}}$. The presence of Fru-6-P$_2$ induces a significant decrease in the $K_{\text{m,PEP}}$ and an increase in $k_{\text{cat}}$ for the Mg$^{2+}$-activated T298A resulting in a 20-fold increase in catalytic efficiency. The apparent $K_{\text{m}}$ for Mg$^{2+}$ with T298A has been measured to be $\approx 10.4$ mM in the absence of Fru-6-P$_2$ (data not shown). The modification of Thr-298 to alanine at the active site of YPK must cause an alteration at the catalytic site such that in the absence of Fru-6-P$_2$, a less active conformation of the enzyme is induced. This altered active conformation at the catalytic site in T298A is less competent to accommodate Mg$^{2+}$ and PEP binding than that with wild type or T298S YPK. This conclusion is reinforced by the fluorescence emission spectra of T298S and T298A sequentially titrated with Mg$^{2+}$, PEP, and Fru-6-P$_2$ (Fig. 4, A and B). The T298A mutation decreases the $k_{\text{cat}}/K_{\text{m,PEP}}$ significantly relative to the value for wild type YPK.

The elimination of the alcohol function at the active site affects the steady-state interaction between PEP and free enzyme. Mn$^{2+}$-activated T298S and T298A do not exhibit homotropic kinetic cooperativity with PEP as the variable substrate in the absence of Fru-6-P$_2$. PEP binding to the YPK-Mn$^{2+}$ complex is cooperative, however ($n_H$ of 1.5 and 2.0 for T298S-Mn$^{2+}$ and T298A-Mn$^{2+}$, respectively). The metal activators elicit different kinetic behavior for both Thr-298 mutants. Mutation of the active site Thr-298 to serine or alanine results primarily in $k_{\text{cat}}$ effects indicating alterations in the catalytic process. The steady-state kinetic data with T298S and T298A support an important but not a critical function of Thr-298 in YPK catalysis. The turnover rate with T298A, which is still significant, suggests that Thr-298 is not the direct proton donor in the YPK-catalyzed reaction.

Small conformational alterations introduced at the active site of yeast PK by mutation of Thr-298 to serine or alanine trigger changes at the allosteric site that is located more than 40 Å away (11). These changes are also translated in an alteration of the allosteric response of the mutant enzymes. The altered allosteric response with the T298S and T298A YPK mutants is not surprising in the light of the data reported by Rigden et al. (27) on the allosterically regulated PK from the

### Table VI

| Divalent activator | YPK | $D_{k_{\text{cat}}/K_{\text{m,PEP}}}$ | $D_{k_{\text{cat}}}$ | $D_{k_{\text{cat}}}$ | $\langle f \rangle$ | $\langle \rho \rangle$ |
|-------------------|-----|-------------------------------|-------------------|-------------------|----------------|----------------|
| Mn$^{2+}$         | Wild type | 2.2 ± 0.4                      | 2.7 ± 0.1          | 3.7 ± 0.3         | 0.27 ± 0.02 | 0.65 ± 0.01  |
|                   | T298S     | 2.2 ± 0.3                      | 2.2 ± 0.1          | 2.1 ± 0.1         | 0.47 ± 0.02 | 0.65 ± 0.01  |
|                   | T298A     | 0.9 ± 0.2                      | 1.4 ± 0.1          | 1.6 ± 0.01        | 0.65 ± 0.01 | 0.65 ± 0.01  |
| Mg$^{2+}$         | Wild type | 2.4 ± 0.2                      | 5.2 ± 0.1          | 7.1 ± 1.5         | 0.14 ± 0.03 | 0.27 ± 0.03  |
|                   | T298S     | 1.9 ± 0.2                      | 2.1 ± 0.1          | 3.0 ± 0.7         | 0.15 ± 0.03 | 0.34 ± 0.02  |
|                   | T298A     | 1.1 ± 0.1                      | 1.3 ± 0.1          | 1.8 ± 0.1         | 0.15 ± 0.03 | 0.60 ± 0.03  |

- Value calculated from the ratio of $k_{\text{cat}}$ in H$_2$O and in D$_2$O.
- Value determined from the fit of $V_{\text{max,app}}$ to Equation 8.
- Value determined from the fit of $V_{\text{max,app}}$ to Equation 9.

![Fig. 5. Proton inventory plots for wild type, T298S, and T298A YPK. Initial velocities were measured at pH 6.2 (L = H, D) and at saturating concentrations of the substrate PEP (5 mM) and of all ligands (4 mM MnCl$_2$ or 15 mM MgCl$_2$, 5 mM ADP, 1 mM Fru-6-P$_2$, and 200 mM KC1).](http://www.jbc.org/)

<http://www.jbc.org/Downloaded from>
Role of Thr-298 in Pyruvate Kinase Catalysis

important group with a pKa for an ionization with pKb.

the absence of net phosphoryl transfer (6). The rates of the PK-catalyzed deprotonation (enolization) of pyruvate in the presence of ATP. This reaction occurs in the catalytic site where phosphoryl transfer does occur, although these changes do not extend into the nucleotide-binding site. The kinetic studies of glycolate kinase activity indicate that the interaction of ATP with the enzyme is unaffected by mutation of Thr-298.

The rates of detritiation of pyruvate measured with wild type YPK and the Thr-298 mutants are also metal-dependent. Mn2+ is the preferred activator compared with Mg2+. Mutation of Thr-298 to serine or alanine results in a decrease in the rate of pyruvate enolization relative to wild type enzyme (Table V). The T298A mutant catalyzes pyruvate enolization at 13–42% relative to wild type YPK. Loss of the functional group at position 298 in yeast pyruvate kinase decreases but does not eliminate the enolization reaction. Mutation of Thr-298 to serine or alanine alters the rates of both the phosphoryl transfer and proton transfer steps in the PK-catalyzed reaction. In neither reaction is the effect proportional to the effect on the overall reaction, nor are the effects parallel for the two partial reactions. Both mutations of Thr-298 affect pyruvate enolization to a greater extent than they affect phosphoryl transfer. These results strongly suggest that Thr-298 is not the direct proton donor in the PK-catalyzed reaction but may play a role in the proton transfer step. The results summarized in Table V can be explained by a role for the divalent activator both in stabilizing the enolate intermediate and in fostering the process of proton transfer. Based on the above results, the two steps in the net catalytic reaction, phosphoryl transfer and pyruvate enolization, appear to be coupled. Such a conclusion was also reached from the studies of the K240M mutant of YPK (13).

The single tryptophan residue (Trp-452) of yeast pyruvate kinase provides a unique probe for monitoring local conformational changes in the vicinity of the Fru-6-P2 site. The intrinsic fluorescence of Trp-452 is quenched upon ligand binding, allowing for the measurement of thermodynamic dissociation constants of ligands from various YPK complexes (16, 28). Binding of Mn2+ and PEP to apo YPK and of PEP to the YPK-Mg2+ complex has not been affected by mutation of Thr-298 at the active site to serine or alanine. The binding of Mn2+ to the YPK-PEP complex is 3- and 20-fold weaker in T298S and T298A, respectively, relative to wild type YPK. The Kd of PEP from the YPK-Mn2+ complex is increased by 2- and 70-fold in T298S and T298A, respectively, compared with wild type YPK. It is possible that the thermodynamically "preferred" ordered pathway occurring in wild type YPK, where either PEP or Mn2+ binds first to form the YPK-Mn2+-PEP complex before ADP binds (16), is changed to a more ordered pathway in T298A, with PEP binding first followed by Mn2+. These results suggest that the mutations of Thr-298 affect the conformational response of YPK to substrate (and activator) binding. PEP binding to the YPK-Mn2+ complex of both Thr-298 mutants is cooperative. Although kinetic cooperativity with PEP is no longer observed with both mutants in the presence of Mn2+ and in the absence of Fru-6-P2, cooperative binding of PEP to the YPK-Mn2+ complex occurs. Similar behavior was observed with the slow substrate Br-PEP for Mn2+-activated wild type YPK in the absence of Fru-6-P2 (30). In the latter case, it was proposed that the cooperative binding of Br-PEP to wild type YPK.

The pH dependence of kcat for wild type YPK is described by the same three pKa values regardless of the activating cation (Table II). Bollenbach et al. (13) showed that the catalytically important group with a pKa of 8.8 has been lost on mutation of Lys-240 to methionine. The mutation of Thr-298 to serine had little effect on the pH dependence of kcat when Mn2+ was the divalent activator. In the pH rate profile for the Mg2+-activated T298S, pKa cannot be determined. This results from a shift of the basic pKb to >9.1 cannot be measured because of experimental limitations. The pH dependence of kcat for Mn2+- and Mg2+-activated T298A indicates that the second ionization with a pKa of 6.4 (Mn2+-YPK) and of 6.9 (Mg2+-YPK), respectively, has been lost on mutation. It is this ionization that is responsible for modulation of kcat in the YPK-catalyzed reaction. Although it may be tempting to attribute pKb = 6.4–6.9 to Thr-298, it is likely that Thr-298 is important for an ionization with pKa = 6.5. Cieland (25) has cautioned against the temptation to interpret such pKa values as intrinsic thermodynamic values.

Glycolate kinase activity and proton exchange rates were measured to determine independently how each of the two specific chemical steps in the reaction may be affected by the alteration of Thr-298. The phosphoryl transfer step in pyruvate kinase can be measured independently by the “glycolate kinase” activity of PK, one of several PK-catalyzed secondary kinase reactions (15, 26, 29). The second half-reaction catalyzed by YPK seems to be complex and involves both catalytic and regulatory functions.

The pH dependence of kcat for wild type YPK is described by the same three pKa values regardless of the activating cation (Table II). Bollenbach et al. (13) showed that the catalytically important group with a pKa of 8.8 has been lost on mutation of Lys-240 to methionine. The mutation of Thr-298 to serine had little effect on the pH dependence of kcat when Mn2+ was the divalent activator. In the pH rate profile for the Mg2+-activated T298S, pKa cannot be determined. This results from a shift of the basic pKb to >9.1 cannot be measured because of experimental limitations. The pH dependence of kcat for Mn2+- and Mg2+-activated T298A indicates that the second ionization with a pKa of 6.4 (Mn2+-YPK) and of 6.9 (Mg2+-YPK), respectively, has been lost on mutation. It is this ionization that is responsible for modulation of kcat in the YPK-catalyzed reaction. Although it may be tempting to attribute pKb = 6.4–6.9 to Thr-298, it is likely that Thr-298 is important for an ionization with pKa = 6.5. Cieland (25) has cautioned against the temptation to interpret such pKa values as intrinsic thermodynamic values.

Glycolate kinase activity and proton exchange rates were measured to determine independently how each of the two specific chemical steps in the reaction may be affected by the alteration of Thr-298. The phosphoryl transfer step in pyruvate kinase can be measured independently by the “glycolate kinase” activity of PK, one of several PK-catalyzed secondary kinase reactions (15, 26, 29). The second half-reaction catalyzed by pyruvate kinase, the proton transfer to enolpyruvate, can be measured by the PK-catalyzed deprotonation (enolization) of pyruvate in the presence of ATP. This reaction occurs in the absence of net phosphoryl transfer (6). The rates of the glycolate kinase reaction measured with wild type YPK and the Thr-298 mutants in the presence of Fru-6-P2 are metal-dependent. Mn2+ is a better activator of glycolate kinase than is Mg2+. Mutation of Thr-298 to serine does not affect the glycolate kinase activity. The mutation of Thr-298 to alanine results in a 5-fold decrease of the glycolate kinase activity compared with wild type YPK. Phosphoryl transfer precedes proton transfer in the PK-catalyzed reaction, and the two reactions were suggested to be decoupled from each other in the wild type enzyme.

The T298A mutant catalyzes pyruvate enolization at 13–42% relative to wild type YPK. Loss of the functional group at position 298 in yeast pyruvate kinase decreases but does not eliminate the enolization reaction. Mutation of Thr-298 to serine or alanine alters the rates of both the phosphoryl transfer and proton transfer steps in the PK-catalyzed reaction. In neither reaction is the effect proportional to the effect on the overall reaction, nor are the effects parallel for the two partial reactions. Both mutations of Thr-298 affect pyruvate enolization to a greater extent than they affect phosphoryl transfer. These results strongly suggest that Thr-298 is not the direct proton donor in the PK-catalyzed reaction but may play a role in the proton transfer step. The results summarized in Table V can be explained by a role for the divalent activator both in stabilizing the enolate intermediate and in fostering the process of proton transfer. Based on the above results, the two steps in the net catalytic reaction, phosphoryl transfer and pyruvate enolization, appear to be coupled. Such a conclusion was also reached from the studies of the K240M mutant of YPK (13).
YPK is masked by a late slow kinetic step and therefore cooperativity in kinetics is not observed. The possibility of kinetic factors in the cooperative response of T298S was investigated by measuring the velocity response to variable [PEP] in a temperature range from 8 to 40 °C. The apparent Hill coefficients for PEP do not change significantly over the temperature range studied. This suggests that if there is a kinetic step (or steps) that attenuates the cooperative response of PEP by the Mn2+-activated enzyme in the absence of Fru-6-P, then this step is not temperature-sensitive. It is possible that in the presence of Mn2+ but in the absence of Fru-6-P, the two Thr-298 mutants already acquire the active conformation that is induced in wild type YPK by Fru-6-P binding. The divalent cations Mg2+ and Mn2+ elicit different conformational effects on the enzyme (Table III). Binding of the allosteric activator Fru-6-P to all the enzyme complexes studied has been altered upon mutation of the active site Thr-298. The effects are metal-dependent, reinforcing the fact that the coupling between the Fru-6-P and PEP sites in yeast PK is modulated through the enzyme-bound metal (16, 28). Again, it is evident that small conformational changes introduced at the active site of YPK by mutation of Thr-298 to serine or alanine can induce long range effects at the Fru-6-P-binding site situated more than 40 Å away.

Solvent isotope effects on $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$,PEP for the wild type and the two mutants of YPK were measured to investigate the influence of the solvent on proton transfer and net catalysis. These experiments were further analyzed by proton inventory studies in an effort to determine the number of protons “in flight” in the isootope-sensitive step(s) with wild type YPK and the two Thr-298 mutants. A linear proton inventory response ($V_{\text{max}}$ versus the mole fraction of D2O in the solvent of D2O + H2O) can be fit by the linear form of the Gross-Butler equation (Equation 8) that models a single proton in the transition state involved in the isotope-sensitive step in the reaction. Nonlinear responses can be more complex. They can be attributed to a proton in the transition state and in the reactant (Equation 9), multiple protons in the transition state, effects of commitment factors on the intrinsic isotope effects, and other complications. Some of these issues and their treatment have been addressed (23, 24, 33). Because we have limited knowledge about details of the solvent-sensitive steps of wild type YPK and even less information regarding the effects of Mn2+ versus Mg2+ and the effects of the Thr-298 mutants, our interpretation of these results are conservative.

It is feasible that upon alteration of Thr-298, enolpyruvate may be released into solution as the product. The SIE for protonation of free enolpyruvate in solution at pH 6.4 is 6.0 (3). Hence a maximum SIE on $k_{\text{cat}}$ of 6.0 would be expected if the acid/base catalyst is lost by YPK and if protonation of enolpyruvate in solution occurs and is rate-limiting in the catalytic process. The measured values for $\frac{Dk_{\text{cat}}}{k_{\text{cat}}}$ of 1.4 (with Mn2+) and 1.3 (with Mg2+) for T298A rule out this possibility.

The observation of $\frac{Dk_{\text{cat}}}{k_{\text{cat}}}$ values of $>1$ for wild type, T298S, and T298A indicates that the isotope-sensitive step is expressed in this kinetic parameter with each of the enzymes. These values are metal-dependent for wild type YPK and metal-independent for the two mutants. Values for $\frac{Dk_{\text{cat}}}{k_{\text{cat}}}$ > 1 are also observed. The $k_{\text{cat}}/K_m$,PEP term reflects the interaction of PEP to the enzyme up to and including the first irreversible step. This is most likely phosphoryl transfer. The linear response to the proton inventory studies that were obtained with the Mn2+-activated wild type YPK and both Thr-298 mutants indicates a single solvent-related proton in the transition state. The fractionation factor ($\phi$) for each enzyme is significantly less than one suggesting that $^1$H and not $^2$H accumulates at the site of exchange. Values $<1$ for the fractionation factors have been identified with cysteine residues ($\phi = 0.40–0.46$) or metal-bound water (e.g. $\phi = 0.69$). Most other functional groups including alcohols have a $\phi = 1.0$. There is no cysteine residue in the active site of YPK, nor is there any evidence that such a residue plays a role in PK catalysis. Hence, our conclusion is that the proton in question is derived from metal-bound water in the active site of PK. The magnitude of the fraction factor increases in the order wild type $<$ T298S $<$ T298A suggesting that in T298A, the proton in transit does not interact with the donor water in the putative channel as selectively as in wild type YPK. In the case of T298A, the binding of hydroxion at the exchangeable site begins to approach the binding as in bulk water ($\phi$ approaches unity).

The effects observed are cation-dependent. The fractionation factors for the Mn2+-activated enzymes are larger than for the Mg2+-activated enzymes.

The proton inventory data for the two mutants activated by Mg2+ can also be fit with a model of multiple protons in the transition state. A fit assuming two protons in the transition state gives one fractionation factor significantly lower than 1, which is consistent with this proton coming from a metal-bound water. The second proton has a fractionation factor greater than 1 and could reflect a tightly bound water where its effects are manifest in these mutants. This proton might either be that shared by Glu-334 and Ser-332 or a water bound to Glu-334 which is at the end of the channel. Our treatment of the solvent isotope data indicates that water plays a key role in catalysis by YPK. The low value for the fractionation factor suggests the catalytically important water is bound to the metal ion at the catalytic site.

If Thr-298 is part of a water network at the active site that is directly or remotely hydrogen-bonded to a metal-bound water, then the p$K_a$ of 6.4–6.9 that is lost in T298A may be related to the ionization of water in the proposed channel. One of the water molecules in the channel is liganded to the enzyme-bound metal. The remote effect of the metal-bound water on enolpyruvate protonation is consistent with the weak correlation of the rate of pyruvate enolization with the p$K_a$ of metal-bound water. From the semilog plot of the rate constant of pyruvate enolization by muscle PK with the p$K_a$ of metal-bound water, the apparent Hamnett constant is 0.24 ± 0.06 (34). Although a p$K_a$ of 6.4–6.9 is quite low for the ionization of metal-bound water (the p$K_a$ of Mg2+-bound water is ~12.8 (35)), it is important to realize that the p$K_a$ values obtained from kinetic studies do not necessarily reflect microscopic p$K_a$ values but rather group p$K_a$ values. A p$K_a$ of 6.8 has been measured for the Zn2+-bound water in carbonic anhydrase II (36), although the microscopic p$K_a$ of Zn2+-bound water is ~8.7 (25).

An alternative explanation for the lost p$K_a$ of 6.4–6.9 comes from the studies of Larsen et al. (37). The crystal structure of the
Role of Thr-298 in Pyruvate Kinase Catalysis

The hydroxyl group of Thr-298 was proposed to function as an acid/base catalyst in YPK through a relay of bound water and the hydroxyl groups of Ser-332 and Thr-298. It is possible that the pKₐ of 6.4–6.9 could correspond to the carboxylate of Glu-334 and the hydroxyl groups of Ser-332 and Thr-298. It is possible that Thr-298 is an acid/base catalyst in YPK through a relay of bound water.

In conclusion, the identity of the general acid/base in the PK-catalyzed reaction is still unclear. It is unlikely that Thr-298 is the direct proton donor to the enolpyruvate intermediate based on the data presented from this study. Thr-298 plays a role in a late step in the catalytic mechanism involving proton transfer. Our data suggest that the direct proton donor to enolpyruvate in YPK may be a water molecule at the catalytic site that is part of a water channel. One water in this channel is coordinated to the enzyme-bound metal. Thr-298 is suggested as being the amino acid that interacts with the terminal water molecule of the proton circuit. The Thr-298 affects the pKₐ of the water in the channel and therefore its reactivity. The proposal of a water molecule as the ultimate proton donor and as part of a water channel is supported by the x-ray structure of the bis(Mg²⁺)-ATP-oxalate complex of rabbit muscle PK at 2.1 Å resolution, where specific water molecules are indicated (37).

REFERENCES

1. Blattler, W. A., and Knowles, J. R. (1979) Biochemistry 18, 3927–3933
2. Rose, I. A. (1970) J. Biol. Chem. 245, 6052–6056
3. Kuo, D. J., and Rose, I. A. (1978) J. Am. Chem. Soc. 100, 6288–6289
4. Kuo, D. J., O’Connell, E. L., and Rose, I. A. (1979) J. Am. Chem. Soc. 101, 5025–5030
5. Seebohler, S. H., Jaworski, A., and Rose, I. A. (1991) Biochemistry 30, 727–732
6. Rose, I. A. (1980) J. Biol. Chem. 255, 1170–1177
7. Robinson, J. L., and Rose, I. A. (1972) J. Biol. Chem. 247, 1096–1105
8. Ford, S. R., and Robinson, J. L. (1976) Biochim. Biophys. Acta 438, 119–130
9. Muirhead, H., Claydon, D. A., Barford, D., Lorimer, C. G., Fothergill-Gilmore, L. A., Schiltz, E., and Schmitt, W. (1986) EMBO J. 5, 475–481
10. Rose, I. A., and Kuo, D. J. (1989) Biochemistry 28, 9579–9585
11. Jurica, M. S., Messecar, A., Heath, P. J., Shi, W., Nowak, T., and Stoddard, B. L. (1998) Structure 6, 185–210
12. Larsen, T. M., Laughlin, T. L., Holden, H. M., Rayment, I., and Reed, G. H. (1994) Biochemistry 33, 6301–6309
13. Bollenbach, T. J., Messecar, A. D., and Nowak, T. (1999) Biochemistry 38, 9137–9145
14. Rose, I. A., Kuo, D. J., and Warm, J. V. B. (1991) Biochemistry 30, 722–726
15. Dougherty, T. M., and Clesard, W. W. (1985) Biochemistry 24, 5870–5875
16. Messecar, A. D., and Nowak, T. (1997) Biochemistry 36, 6803–6813
17. Bollenbach, T. J. (1999) Catalytic Mechanism and Activation of Yeast Pyruvate Kinase. Ph.D. thesis, University of Notre Dame
18. Messecar, A. D. (1995) Kinetic Responses and Conformational Changes Required for Yeast Kinase Activation and Catalysis. Ph.D. thesis, University of Notre Dame
19. Burke, R. L., Tekamp-Olson, P., and Najarian, R. (1983) J. Biol. Chem. 258, 2193–2201
20. Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) J. Bacteriol. 153, 163–168
21. Teita, A., and Ochoa, S. (1958) Arch. Biochem. Biophys. 78, 477–493
22. Lummry, R., Smith, E. L., and Glantze, R. R. (1951) J. Am. Chem. Soc. 73, 4330–4340
23. Krebs, A. J. (1964) Pure Appl. Chem. 8, 243–258
24. Shorow, K. B., and Shorow, R. L. (1982) Methods Enzymol. 87, 551–606
25. Clesard, W. W. (1977) Adv. Enzymol. Related Areas Mol. Biol. 45, 273–387
26. LeBlond, D. J., and Robinson, J. L. (1976) Biochim. Biophys. Acta 438, 158–158
27. Bigden, D. J., Phillips, S. E. V., Michals, P. A. M., and Fothergill-Gilmore, I. A. (1999) J. Mol. Biol. 291, 615–635
28. Bollenbach, T. J., and Nowak, T. (2001) Biochemistry 40, 13088–13096
29. Weiss, P. M., Hermes, J. D., Dougherty, T. M., and Clesard, W. W. (1984) Biochemistry 23, 4346–4350
30. Lorca, J. P. (1997) Active Site Structure and Conformational Changes in Yeast Pyruvate Kinase. Ph.D. thesis, University of Notre Dame
31. Swindells, J. F., Hardy, R. C., and Goldin, P. E. (1958) Viscosities of sucrose solutions at various temperatures. Supplement to National Bureau of Standards Circular, C440, p. 3, National Bureau of Standards, U. S. Gov.
32. Kirshenbaum, I. (1961) Physical Properties and Analysis of Heavy Water, p. 33, McGraw-Hill Inc., New York
33. Kicik, D. M. (1991) J. Am. Chem. Soc. 113, 8499–8504
34. Gupta, R. K., Oesterling, M., and Mildvan, A. S. (1976) Biochemistry 15, 2881–2887
35. Chaberek, S., Jr., Courtney, R. C., and Mccall, L. A. (1980) J. Am. Chem. Soc. 74, 5057–5060
36. Clesard, W. W., and Cox, J. D. (1999) Annu. Rev. Biochem. 68, 33–57
37. Larsen, T. M., Benning, M. M., Rayment, I., and Reed, G. H. (1998) Biochemistry 37, 6247–6255
38. Gupta, T., Hummer, G., and Garcia, A. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 94, 2135–2138
39. Hishik, T., Shimada, H., Nagano, S., Eerwa, T., Kanaori, Y., Makino, R., Park, S. Y., Adachi, S., Shio, Y., and Ishimura, Y. (2000) J. Biochem. (Tokyo) 128, 965–974
The Proton Transfer Step Catalyzed by Yeast Pyruvate Kinase
Delia Susan-Resiga and Thomas Nowak

J. Biol. Chem. 2003, 278:12660-12671.
doi: 10.1074/jbc.M300257200 originally published online January 31, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300257200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 6 of which can be accessed free at http://www.jbc.org/content/278/15/12660.full.html#ref-list-1