Role of Lysine 240 in the Mechanism of Yeast Pyruvate Kinase Catalysis†

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ABSTRACT: Site-directed mutagenesis was used to change Lys 240 of yeast pyruvate kinase (Lys 269 in muscle PK) to Met. K240M has an absolute requirement for FBP for catalysis. K240M is 100- and 1000-fold less active than wild-type YPK in the presence of Mn^{2+} and Mg^{2+}, respectively. Steady-state fluorescence titration data suggest that the substrate PEP binds to K240M with the same affinity as it does to wild-type YPK. The rate of phosphoryl transfer in K240M has been decreased >1000-fold compared to wild-type YPK. The detritiation of 3-[^{3}H]pyruvate catalyzed by YPK occurs at a rate significantly greater than the spontaneous rate. Detritiation of pyruvate by wild-type YPK occurs as a divalent metal- and FBP-dependent process requiring ATP. There is no detectable detritiation of pyruvate catalyzed by K240M. The solvent deuterium isotope effect on k_{cat} is 2.7 ± 0.2 and 1.6 ± 0.1 for the wild type and for K240M YPK, respectively. This suggests that the isotope sensitive step in the PK reaction does not involve Lys 240 and that the enolpyruvate intermediate is still protonated by K240M. Isotope trapping was used to characterize enolpyruvate protonation by K240M. While there was enrichment of the methyl protons of pyruvate from labeled solvent formed by catalysis with muscle PK and wild-type YPK, only background levels of tritium were trapped with K240M. In K240M, the proton donor exchanges protons with the solvent at a higher rate relative to turnover than does the proton donor in wild-type YPK. The pH−rate profile of K240M exhibits the loss of a pK_a value of 8.8 observed with wild-type YPK. The above data and recent crystal structure data suggest that Lys 240 interacts with the phosphoryl group of phosphoenolpyruvate and helps to stabilize the pentavalent phosphate transition state during phosphoryl transfer. Phosphoryl transfer is highly coupled to proton transfer, or Lys 240 also affects enolate protonation.

Yeast pyruvate kinase (YPK)[1] is a key regulatory enzyme in glycolysis. PK catalyzes the net conversion of phosphoenolpyruvate (PEP) and adenosine 5′-diphosphate (ADP) to pyruvate and ATP. YPK is activated by several divalent metal cations and by fructose 1,6-bisphosphate (FBP). The net reaction catalyzed by pyruvate kinase occurs in two steps. Phosphoryl transfer occurs by an apparent S_N2 mechanism from PEP to ADP with an inversion of configuration at the phosphoryl group, to yield ATP and enolpyruvate (*1*). The second step involves the protonation of the enolate by an active site proton donor to give pyruvate. This is demonstrated by the fact that PK will catalyze the enolization of bound pyruvate without phosphoryl transfer (*2*−*4*). This activity has an absolute requirement for ATP as a cofactor with YPK and MPK; ATP may be substituted for inorganic phosphate, fluorophosphate, and methyl phosphonate with the muscle enzyme (*2*, *3*). PK will also catalyze the ketonization of enolpyruvate, generated in situ from the alkaline phosphatase hydrolysis of PEP (*5*). Enolpyruvate, which is the common species in both partial reactions, has been shown by chemical methods to be a tightly bound intermediate in the overall PK reaction (*6*). The X-ray crystal structure of the cat muscle enzyme (*7*, *8*) indicated that Lys 269 (Lys 240 YPK numbering) was in position to act as the proton donor to the enolpyruvate. Subsequent crystallographic data obtained from the rabbit muscle enzyme (*9*, *10*) and from the yeast enzyme (*11*) demonstrated that the methyl group of pyruvate could be oriented such that Thr 327 would lie above the 2-*si* face of the double bond of the enolate intermediate. These structures show the position of Lys 269 on the 2-*re* face of the double bond. Because the enolpyruvate is protonated stereospecifically from the 2-*si* face (*5*, *12*), Lys 269 is not in a position to directly donate a proton to the enolpyruvate C-3, according to the most recent crystal structure data**.** Thus, the role of Lys 269 in pyruvate kinase remains uncertain. Larsen and co-workers (*9*, *10*) have suggested that Lys 269, in its protonated state, could stabilize the pentavalent transition state of the phosphoryl transfer reaction. This is proposed to occur via stabilization of the γ-phosphoryl group of ATP, which in turn bridges the two divalent cations in the active site (*13*). Because the role that this conserved active site residue plays in catalysis is ambiguous, Lys 240 (Lys 269 in muscle enzyme) has been changed to a methionine by site-directed mutagenesis in an effort to determine its role in catalysis in the pyruvate kinase reaction.

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[1] Abbreviations: BrPEP, (*Z*)-bromophosphoenolpyruvate; dGDP, deoxyguanosine 5′-diphosphate; FBP, fructose 1,6-bisphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; LDH, L-lactate dehydrogenase; M^{2+}, divalent metal cation; MES, 2-(*N*-morpholino)-ethanesulfonic acid; MPK, muscle pyruvate kinase; NADH, reduced nicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TAPS, 3-[*N*-tris-(hydroxymethyl)methylamino]propanesulfonic acid; YPK, yeast pyruvate kinase.

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EXPERIMENTAL PROCEDURES

Materials. Muscle pyruvate kinase and L-(+)-lactate dehydrogenase were purchased from Boehringer-Mannheim. Wild-type and K240M yeast pyruvate kinase were purified according to the method of Mesecar and Nowak (*14*). PEP, ADP, Dowex 1 chloride (400 Mesh), and buffers were purchased from Sigma. Tritiated water (5 Ci/mL) was from New England Nuclear. Deuterium oxide (99.9%) was from Cambridge Isotope Laboratories. The Altered Sites mutagenesis kit was purchased from Promega, and the mutagenic oligonucleotide was purchased from Genosys.

Site-Directed Mutagenesis and Cell Growth. A 1402 bp fragment of the YPK gene was cloned into the pSelect vector. Mutagenesis reactions were performed in the pSelect vector according to the Promega Altered Sites manual. The 751 bp fragment containing the desired mutation in the YPK gene was excised from the pSelect vector with *Bgl*II and *BstE*II. The 751 bp cassette was cloned into the yeast shuttle vector, pPYK101, that contains the entire PK gene. The mutated gene was sequenced by dideoxy sequencing to verify the presence of the desired mutation. The pPYK101 was transformed into the pyruvate kinase-deficient yeast strain, pyk1-5 (*15*), using a lithium acetate protocol.

Transformed pyk1-5 containing the pPYK101 with the wild-type YPK gene was grown on rich medium containing the following (per liter): 10 g of yeast extract, 20 g of bactopeptone, and 2% glucose. Transformed pyk1-5 containing pPYK101 with the K240M mutation was grown on glycerol/ethanol minimal medium containing the following (per liter): 6.68 g of yeast nitrogen base, 10 g of succinic acid, 4 g of NaOH, 0.5% casamino acids, 0.001% adenine, 0.001% methionine, 2% glycerol, and 2% ethanol. Cells were harvested and the wild-type YPK and K240M purified as described previously (*14*).

Pyruvate Kinase Assay. Pyruvate kinase was assayed according to the method of Bücher and Pfleiderer (*16*). The change in absorbance at 340 nm was measured as a function of time using a Gilford 240 spectrophotometer. Typical assays contained in 1 mL 100 mM MES (pH 6.2), 4% glycerol, 200 mM KCl, either $MgCl_2$, $CoCl_2$, or $MnCl_2$, 5 mM PEP, 5 mM ADP, 1 mM FBP, 175 μM NADH, 20 μg of LDH, and YPK (1.5 μg of the wild type or 15 μg of K240M). The specific activity of pyruvate kinase is expressed as micromoles of NADH oxidized per milliliter per minute per milligram of protein. The concentration of PK was determined by its absorbance at 280 nm. The extinction coefficients that were used were as follows: $\epsilon_{280} = 0.51$ mL/mg and $\epsilon_{280} = 0.54$ mL/mg for yeast pyruvate kinase and rabbit muscle pyruvate kinase, respectively. The specific activity was determined prior to all experiments.

Steady-state reaction rates were determined by measuring the slope of a line drawn tangent to the reaction progress curve. The initial velocity data were then fit to both the Michaelis−Menten equation:

$$v/V_{max} = 1/(1 + K_m/[S]) \quad (1)$$

and the Hill equation:

$$v/V_{max} = 1/[1 + (K_m/[S])^{n_H}] \quad (2)$$

for the determination of the apparent Michaelis constants (K_m), Hill coefficients (n_H), and maximal velocity (V_{max}). The Hill equation was used to check for cooperativity.

pH Measurements and Titrations. The acetic acid, MES, HEPES, PIPES, Tris, triethanolamine, Bis-Tris Propane, and TAPS buffers were all titrated to the desired pH by using either potassium hydroxide or hydrochloric acid. The pH of the kinetic assays, containing all of the assay components except the lactate dehydrogenase and pyruvate kinase, was determined before the reactions were initiated. The rate equation describing the effect of pH on the apparent V_{max} of wild-type YPK was derived and is described by eq 3:

$$V_{max,app} = \frac{V_{max}\left(1 + \frac{\alpha[H^+]}{K_B}\right)}{1 + \frac{[H^+]^2}{K_A K_B} + \frac{[H^+]}{K_B} + \frac{K_C}{[H^+]}} \quad (3)$$

Equation 3 represents the situation where more than one form of the enzyme−substrate complex can yield products. This situation arises when the protonation or deprotonation of a group in the ES complex simply changes the rate of the reaction instead of preventing the reaction. Since the data indicate that a change in the rate of the reaction is taking place only in the pH range of 6−8, the maximal velocity in this plateau region, V_{max}', can be related to the maximal velocity, V_{max}, by a proportionality factor, α, where $V_{max}' = \alpha V_{max}$. The value of α can be either greater than or less than 1 depending on whether protons are inhibiting or activating the reaction. The ionization constant for the group that determines the value of α is K_B. Equation 3 also describes two groups in the ES complex that upon ionization prevent the reaction from occurring: protonation of a group in the acidic range having an ionization constant K_A and deprotonation of a group in the basic range having an ionization constant K_C.

Synthesis of 3-[^{3}H]Pyruvate. 3-[^{3}H]Pyruvate was synthesized according to the method of Rose (*2*). The sodium salt of pyruvic acid was dissolved in tritiated water (110 mCi) to a concentration of approximately 1 M and sealed in a tube under reduced pressure. The vessel was heated to 135 °C for 90 min. The solution was allowed to cool, placed in a distillation apparatus, and lyophilized under high vacuum. The residue was washed with 4 mL of water and relyophilized. The final product contained approximately 4% residual counts from tritiated water.

Rate of Pyruvate Enolization. The enolization of pyruvate was monitored as the time-dependent exchange of tritium from 3-[^{3}H]pyruvate into water according to Rose (*2*). The following modifications were made. Reaction mixtures consisted of 100 mM TAPS (pH 8.0), 4% glycerol, 200 mM KCl, 100 mM 3-[^{3}H]pyruvate (9360 dpm/μmol of pyruvate), 2 mM ATP, and 100−200 μg of pyruvate kinase in 100 mM HEPES (pH 7.5). The divalent metal was either 15 mM $MgCl_2$ or 10 mM $MnCl_2$. FBP, when present, was at a concentration of 1 mM. The final volume was 1 mL, and the pH was 7.7. Reactions were initiated by the addition of 3-[^{3}H]pyruvate. Aliquots (150 μL) were removed from the reaction mixture at selected time points, added to 350 μL of H_2O, and loaded onto a short bed of Dowex 1 acetate resin. The eluent was collected in a disposable 20 mL scintillation vial. The column was washed with three additions of 500

μL of H_2O. The combined effluent and washes were counted to determine total volatile counts. Controls containing no enzyme were run parallel to each experiment to determine background exchange rates. Background rates were subtracted from observed rates to obtain v_H, the PK-catalyzed rate of exchange of methyl protons of pyruvate into water. v_H is defined as micromoles of protons in water per minute per milligram of protein.

Isotope Trapping of Substrate Proton. Isotope trapping experiments were performed according to Rose and Kuo (*17*) with the following modifications. Ammonium sulfate suspensions of muscle and of yeast PK were desalted prior to use on a Bio Gel P6DG column. Muscle PK was exchanged into 100 mM MES (pH 6.2), 4% glycerol, and 200 mM KCl. Yeast PK was exchanged into 200 mM MES (pH 6.2), 25% glycerol, and 400 mM KCl. The presence of KCl in the exchange buffer caused K240M to precipitate and was therefore omitted when the mutant was desalted. The desalted enzyme was concentrated in an Amicon stirred cell concentrator to >30 mg/mL.

Conditions of the pulse varied but typically contained, in 6 μL, 1−1.5 nmol of pyruvate kinase sites (determined by A_{280}), 50 mM MES (pH 6.2), 4% glycerol, 100 mM KCl, 5 mM ADP, and ^3H_2O (typically $\sim 10^9$ cpm). The divalent metal was 15 mM $MgCl_2$, 10 mM $MnCl_2$, or 15 mM $CoCl_2$. The FBP concentration was either 0 or 1 mM. When yeast PK was used, the concentration of glycerol in the pulse was increased to 25%, to prevent precipitation of the enzyme. The chase solution typically contained 100 mM MES (pH 6.2), 4% glycerol, 200 mM KCl, 10 mM PEP, and 5 mM ADP in H_2O in a volume of 2 mL. The divalent metal was 15 mM $MgCl_2$, 10 mM $MnCl_2$, or 15 mM $CoCl_2$. The FBP concentration was either 0 or 1 mM. The reaction was quenched after approximately 2 s with 160 μL of 1 M TCA; the mixture was incubated on ice for 5 min and neutralized with 75 μL of 2 M triethanolamine base.

The reaction mixture was diluted to 5 mL and placed on a 2 mL Dowex 1 chloride column. The column was washed with H_2O until effluent counts came to background. Pyruvate was eluted with 20 mM HCl, and 2 mL fractions were collected. Pyruvate was quantitated enzymatically with muscle PK and LDH (*14*). Pyruvate typically eluted in fractions 4−10. In experiments where trapped counts were converted from pyruvate to lactate, lactate was isolated from PEP by chromatography on Dowex 1 formate. Lactate was eluted with 100 mM potassium formate, and 2 mL fractions were collected. Lactate was quantitated enzymatically with LDH in glycine/hydrazine buffer at pH 9.0 according to the method of Gutemann and Wahlefeld (*16*). Fractions containing lactate were diluted with 100 mM citrate (pH 6.0) and lyophilized overnight. Parallel controls were run for each experiment. PEP was eluted with 400 mM potassium formate and quantitated as described above.

Solvent Deuterium Isotope Effect on YPK and K240M. All buffers, divalent metals, and substrates were exchanged in 99.9% D_2O and lyophilized. This process was repeated three times. The samples were redissolved in D_2O to give the desired concentrations. The pD of MES was adjusted to 6.2 using KOD. NADH was dissolved in MES buffer, as prepared above, immediately prior to use. Assays (1 mL) were prepared and covered with Parafilm prior to use. Assays were performed in triplicate as described previously.

Glycolate Kinase Rate Measurements. The glycolate kinase reaction in wild-type YPK and in K240M was assessed in MES (pH 6.3 and 6.99), HEPES (pH 7.5 and 8.0), and TAPS (pH 8.5) buffers. The pH of the complete kinetic assay was determined after completion of the reaction. The glycolate kinase reaction was performed in 100 mM buffer, 4% glycerol, 200 mM KCl, 50 mM glycolate, 10 mM ATP, and 5 mM FBP. The divalent metal was either 50 mM $MgCl_2$, 10 or 20 mM $MnCl_2$, or 25 mM $CoCl_2$. The enzyme was wild-type YPK or K240M (approximately 350 μg). The final assay volume was 1 mL. Reactions were initiated by the addition of enzyme [diluted in HEPES (pH 7.5)]. The reaction was quenched by the addition of a 100 μL aliquot from the reaction mix to 30 μL of cold 2.5 M $HClO_4$ and incubated on ice for 5 min. Aliquots were taken at selected time points ranging from 1 to 60 min. The solution was neutralized by the addition of 30 μL of 1.25 M K_2CO_3. The resulting precipitate was removed by centrifugation. The supernatant was diluted 10-fold and filtered for HPLC analysis.

ADP and ATP were separated chromatographically on a Beckman 421 Liquid Chromatograph equipped with a Beckman 334 Gradient System and a 25 cm Rainin Microsorb MV C_{18} column with a pore size of 300 Å. Integration and plotting of chromatograms were carried out on a Spectra-Physics SP4290 integrator. Samples were eluted isocratically using 100 mM phosphate buffer (pH 5.5) containing 8 mM tetrabutylammonium hydrogen sulfate and 20% methanol.

RESULTS

Cell Growth and Purification of K240M. K240M was constructed, expressed, and purified using the same procedure as for wild-type YPK. The pyruvate kinase-deficient *Saccharomyces cerevisiae* strain, pyk1-5, containing the pPYK101 plasmid with the K240M mutation was unable to grow on medium containing glucose as the primary carbon source. These cells were instead grown on glycerol/ethanol medium. Forty liters of glycerol/ethanol medium yielded approximately 100 g of cells. The final yield of pure K240M was approximately 200 mg. The enzyme was judged to be >95% pure as determined by SDS−PAGE. Initial kinetic studies for quantitating the activity of K240M YPK show an absolute requirement for FBP for activity. This indicates that the final preparation of pure K240M was free from contamination by trace amounts of wild-type YPK.

Steady-State Kinetics. Steady-state kinetic studies were performed for the wild type and for K240M YPK under identical conditions. The kinetic responses of the wild type and K240M YPK to concentrations of PEP, ADP, and divalent metal were fit to eqs 1 and 2. A summary of the resulting steady-state kinetic parameters is listed in Table 1. The divalent metal specificity has changed from $Mg^{2+} > Co^{2+} > Mn^{2+}$ in the wild type to $Co^{2+} > Mn^{2+} > Mg^{2+}$ in K240M, based on k_{cat} values. Compared to that of wild-type YPK, the turnover rates have decreased by 1000-, 100-, and 200-fold with K240M activated by Mg^{2+}, Mn^{2+}, and Co^{2+}, respectively. The $K_{m,PEP}$ changes 10−100-fold depending on the divalent activator. Changes in the $K_{m,ADP}$ are 2−3-fold depending on the divalent metal. These data indicate that Lys 240 affects the interaction of PEP with the enzyme but

Table 1: Steady-State Kinetic Parameters for Wild-Type and K240M YPK[a]

divalent activator	YPK	k_{cat} (min^{-1})	$K_{m,PEP}$ (mM)	$k_{cat}/K_{m,PEP}$ (M^{-1} min^{-1})	$K_{m,ADP}$ (mM)	$K_{m,app,M^{2+}}$ (mM)
Mg^{2+}	wild-type	13380 ± 650	0.31 ± 0.05	(4.32 ± 0.21) × 10^7	1.10 ± 0.11	1.44 ± 0.07
	K240M	13.5 ± 1.1	4.26 ± 0.49	(3.17 ± 0.26) × 10^3	3.67 ± 0.76	17.9 ± 2.2
Mn^{2+}	wild-type	3900 ± 110	0.021 ± 0.003	(1.86 ± 0.05) × 10^8	0.24 ± 0.01	0.31 ± 0.02
	K240M	42.2 ± 1.1	0.50 ± 0.04	(8.44 ± 0.22) × 10^4	0.49 ± 0.03	3.00 ± 0.27
Co^{2+}	wild-type	7910 ± 110	0.050 ± 0.003	(1.58 ± 0.02) × 10^8	0.41 ± 0.03	0.40 ± 0.03
	K240M	120 ± 4	4.40 ± 0.38	(2.73 ± 0.09) × 10^4	1.01 ± 0.19	5.49 ± 0.51

[a] Steady-state kinetics were measured as described in Experimental Procedures with either PEP, ADP, or divalent metal as the variable substrate and with either wild-type YPK or the K240M mutant.

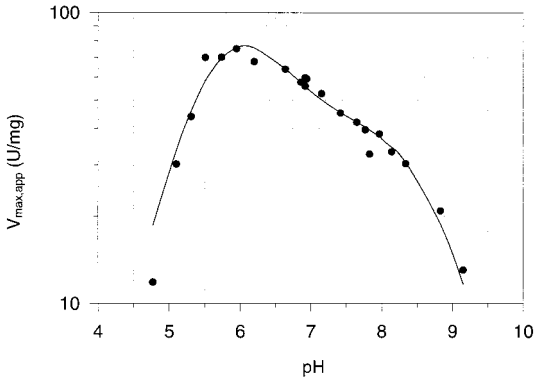

FIGURE 1: pH effects on $V_{max,app}$ for wild-type YPK. The activity of YPK was measured in the presence of 10 mM $MnCl_2$, 5 mM ADP, and 5 mM FBP. $V_{max,app}$ was obtained from fits of velocity vs PEP concentration to eq 1. The data were fit to a kinetic model describing the ionization of three groups in the ES complex (eq 3).

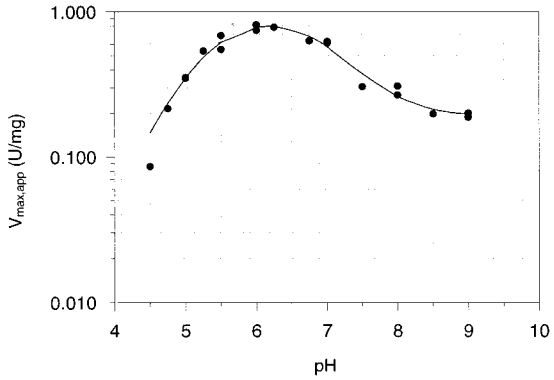

FIGURE 2: pH effects on $V_{max,app}$ for K240M YPK. The activity of K240M was measured in the presence of 10 mM $MnCl_2$, 5 mM ADP, and 5 mM FBP. $V_{max,app}$ was obtained from fits of velocity vs PEP concentration to eq 2. The data were fit to a kinetic model describing the ionization of two groups in the ES complex (eq 4).

plays only a minor role in the interaction of ADP with the enzyme. Ligand binding was assessed by steady-state fluorescence titrations with wild-type and K240M YPK. Titration data indicate that the dissociation constant describing the binding between PEP and wild-type apo-YPK (638 ± 53 μM) is similar to the dissociation constant for the interaction between PEP and apo-K240M YPK (587 ± 46 μM). The steady-state interaction of PEP with YPK, as measured by relative K_m and k_{cat}/K_m values, has been affected by the mutation, but the thermodynamic interactions between PEP and the enzyme are unaffected. The $K_{m,M^{2+}}$ that is reported is an apparent value based on the total concentration and not corrected for the binding of M^{2+} by ADP in solution. The $K_{m,M^{2+}}$ changes significantly on mutation of Lys 240, indicating an effect on the cation interaction with YPK by Lys 240. The large effect of the K240M mutation on k_{cat} indicates that Lys 240 plays a role in transition-state stabilization.

pH Effects on Catalysis by Wild-Type and K240M YPK. The influence of pH on the $V_{max,app}$ for FBP- and Mn^{2+}-activated YPK was measured over the pH range of 4.8−9.1, and the results are plotted in Figure 1. The shape of the curve in Figure 1 indicates that more than two ionizations are needed to describe the $V_{max,app}$ data. The deprotonation of a group with a pK_a of 5.5 and the protonation of a group with a pK_a of 8.5 are needed for catalytic activity. In addition, the ionization of a group or groups in the pH range of 6−8 alters the catalytic activity. The curvature in this portion of the profile indicates that a plateau in the $V_{max,app}$ data may occur, indicating that deprotonation of a group or groups changes the rate-limiting step. The data in Figure 1 were fit to eq 3, and the resulting pK_a values that describe the curve are as follows: pK_A = 5.50 ± 0.13, pK_B = 6.35 ± 0.24, and pK_C = 8.78 ± 0.16 with an α of 0.35. It should be noted that pK_a values obtained from kinetic studies should be interpreted with care. They may not be thermodynamic values reflecting the pK_a of a specific ionizable group (*19*).

The influence of pH on $V_{max,app}$ for the Mn^{2+}- and FBP-activated K240M was measured over the pH range of 4.5−9.0, and the results are plotted in Figure 2. The $V_{max,app}$ on the basic limb of the pH profile decreases to a plateau of approximately 0.2 unit/mg. A comparison of the basic region of the pH profiles of wild-type YPK and K240M indicates that a basic pK_a has been lost on mutation of Lys 240. The data in Figure 2 were fit to equation 4:

$$V_{max,app} = \frac{V_{max}\left(1 + \frac{\alpha K_B}{[H^+]}\right)}{1 + \frac{[H^+]}{K_A} + \frac{K_B}{[H^+]}} \quad (4)$$

This equation describes only two ionizations, K_A and K_B, in the ES complex and gives a better statistical fit to the data in Figure 2 than does eq 3. The resulting pK_a values are as follows: pK_A = 5.25 ± 0.07 and pK_B = 6.99 ± 0.11 with an α of 0.81. The pK_C of 8.78 is lost and may represent the ionization of Lys 240 in the catalytic process.

Phosphoryl Transfer. Glycolate kinase activity was measured with wild-type and K240M PK to determine the effects of mutation on the phosphoryl transfer half-reaction (Table 2). The glycolate kinase reaction, the ATP-dependent phosphorylation of glycolate, is a secondary kinase reaction catalyzed by pyruvate kinase (*20*, *21*). This reaction represents the phosphoryl transfer half-reaction in the absence of proton transfer since glycolate lacks a C-3. In the absence of FBP, the v_P was 11.4 min^{-1}. The velocity response to

Table 2: Glycolate Kinase Activity of Wild-Type and K240M YPK

complex	v_P[a] (min^{-1})	complex	v_P[a] (min^{-1})
YPK−Mn^{2+}	11.4 ± 0.54	K240M−Mg^{2+}−FBP	<0.05
YPK−Mn^{2+}−FBP	74.8 ± 1.1	K240M−Co^{2+}−FBP	<0.05
K240M−Mn^{2+}−FBP	<0.05		

[a] v_P is the turnover number of the glycolate kinase reaction and is defined as phosphoglycolate formed per minute per micromole of enzyme.

Table 3: Rates of Detritiation of 3-[^{3}H]Pyruvate by Pyruvate Kinase[a]

complex	v_T (μmol of $^3H^+$ min^{-1} mg^{-1})
MPK−Mn^{2+}	3.67 ± 0.035
YPK−Mg^{2+}	<0.003
YPK−Mg^{2+}−FBP	0.135 ± 0.003
YPK−Mn^{2+}	0.626 ± 0.050
YPK−Mn^{2+}−FBP	0.810 ± 0.090
YPK−Mn^{2+}−FBP and ADP*	<0.003
K240M−Mn^{2+}−FBP	<0.003

[a] The rates of PK-catalyzed pyruvate enolization were measured as described (Experimental Procedures) with 2 mM ATP as the cofactor and with either 15 mM $MgCl_2$ or 10 mM $MnCl_2$ as the activating divalent metal. In the reaction denoted with an asterisk, ATP is substituted with 10 mM ADP. v_T is the rate of catalyzed tritium release from pyruvate normalized per milligram of enzyme.

ATP concentration was sigmoidal. The rate of the glycolate kinase reaction in the presence of Mn^{2+} and FBP was 74.8 min^{-1} at pH 7.5, and the velocity response to ATP concentration obeyed Michaelis−Menten kinetics. The $K_{m,ATP,app}$ decreased from 2.4 mM in the presence of FBP to 1.1 mM in the absence of FBP. A nonenzymatic blank 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. K240M has no measurable glycolate kinase activity in the presence of FBP and either Mn^{2+}, Mg^{2+}, or Co^{2+} as activators at pH 7.5. There is a significant pH-dependent increase in the rate of the glycolate kinase reaction with wild-type YPK above pH 7.0 (*20*). In an attempt to increase the sensitivity of the measurement of the glycolate kinase reaction with K240M, experiments were performed in the pH range of 6.0−8.5 and with increasing concentrations of K240M. No glycolate kinase activity was detected in this pH range. The mutation of Lys 240 has eliminated glycolate kinase activity in YPK. This response is not expected if Lys 240 serves as the proton donor for the enolpyruvate intermediate.

Detritiation of Pyruvate. The proton transfer half-reaction in PK can be monitored via the enzyme-catalyzed exchange of methyl protons of pyruvate into solvent (pyruvate enolization) (*2*). The rate of pyruvate enolization was measured for wild-type and K240M YPK. All the rates that are shown are net rates obtained following subtraction of the rate of spontaneous detritiation of pyruvate in solution. The rate of catalysis of pyruvate enolization was higher with muscle pyruvate kinase than with the yeast enzyme under similar conditions (Table 3). The yeast enzyme requires a divalent metal ion, FBP, and ATP for enolization. The detritiation rates for FBP-activated YPK in the presence of Mn^{2+} and Mg^{2+} were 0.810 ± 0.090 and 0.135 ± 0.003 μmol of $^3H^+$ min^{-1} mg^{-1}, respectively, making Mn^{2+} a 6-fold better activator of pyruvate enolization than Mg^{2+} in the presence of FBP. The rate of exchange with Mn-activated wild-type YPK decreases to 0.626 ± 0.050 μmol of $^3H^+$ min^{-1} mg^{-1} in the absence of FBP, a 20% decrease in the rate measured in the presence of FBP. There is no detectable detritiation by Mg^{2+}-activated YPK in the absence of FBP. The effects of the K240M mutation on the proton transfer reactions in YPK were measured (Table 3). K240M YPK, activated by Mn^{2+} and FBP, demonstrates no detectable detritiation of pyruvate. Lys 240 influences both the phosphoryl transfer and proton transfer steps in the PK-catalyzed reaction.

Table 4: Trapping of Protons of Pyruvate Kinase by Isotope Trapping

complex	equiv of protons trapped/ mol of pyruvate[d]
MPK−Mg^{2+}	1.3
MPK−Mn^{2+}	2.9
MPK−Co^{2+}[a]	3.0
MPK−Mn^{2+}−LDH[b]	3.0
YPK−Mg^{2+}	0.37
YPK−Mg^{2+}−FBP	0.55
YPK−Mn^{2+}	1.5
YPK−Mn^{2+}−FBP	1.0
YPK−Mn^{2+}−FBP[c]	0
YPK−Co^{2+}	0.48
YPK−Co^{2+}−FBP	1.4
K240M−Mn^{2+}−FBP	0
K240M−Co^{2+}−FBP	0
YPK−Mn^{2+}−BrPEP−FBP	0

[a] From ref *17*. The pulse contained enzyme, cation as indicated, ADP, and FBP when indicated. The chase contained PEP. [b] LDH was used to trap free pyruvate after release from MPK. [c] The pulse contained YPK−Mn^{2+}, PEP, and FBP. The chase contained ADP. [d] Errors in measured values are ≤10%.

Isotope Trapping. Isotope trapping was used to characterize the proton donation step in wild-type and K240M YPK. The rate of pyruvate enolization is diagnostic of the proton transfer half-reaction of PK, but isotope trapping is diagnostic of proton transfer in the net PK-catalyzed reaction. In the isotope trapping experiment, an equilibrium complex is formed between the enzyme, a combination of its cofactors, and either PEP or ADP, but not both. This prevents catalytic turnover. An aliquot of ^3H_2O with high specific radioactivity is added to this complex, labeling the proton donor on the enzyme. This constitutes the pulse phase. The pulse is then added to a chase phase that consists of all the substrates and cofactors necessary for turnover and dilutes the ^3H label. The product traps the radiolabeled proton in pyruvate.

Isotope trapping with MPK was performed as a control using the conditions described by Rose and Kuo (*17*). With Mg^{2+} and Mn^{2+} as divalent metal activators, 1.3 and 2.9 equiv of protons were trapped per mole of pyruvate, respectively. The value of 1.3 agrees with the values of 1.15 and 1.25 obtained previously in the presence of Mg^{2+} (*17*, *22*). The trapping of three protons is the physical limit for these experiments, since pyruvate contains three protons in which the label could appear. Significantly fewer protons were trapped with YPK than with muscle PK (Table 4). With Mg^{2+} as the activator, 0.37 and 0.55 PK equiv of protons per mole of pyruvate were trapped in the absence and presence of FBP, respectively. With Mn^{2+}, the number of protons trapped in the absence and presence of FBP was 1.5 and 1.0, respectively. No protons were trapped with K240M in the presence of either Mn^{2+} or Co^{2+} as the activator. This could occur if the rate of exchange of the

Table 5: Solvent Deuterium Isotope Effects for Wild-Type and K240M YPK

complex	solvent	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\times 10^6$ M^{-1} min^{-1})	$^Dk_{cat}$	$^D(k_{cat}/K_m)$
YPK−PEP−FBP[a]	H_2O	12.4 ± 1.7	3467 ± 110	280 ± 46	2.7 ± 0.2	2.2 ± 0.7
YPK−PEP−FBP[a]	D_2O	10.3 ± 1.2	1296 ± 33	126 ± 18		
YPK−BrPEP−FBP	H_2O	4.25 ± 0.50	68.8 ± 2.8	16 ± 2	1.1 ± 0.1	0.84 ± 0.2
YPK−BrPEP−FBP	D_2O	3.37 ± 0.40	64.4 ± 2.8	19 ± 4		
K240M−PEP−FBP	H_2O	318 ± 15	57.9 ± 1.1	18 ± 1	1.6 ± 0.1	1.0 ± 0.2
K240M−PEP−FBP	D_2O	219 ± 17	38.6 ± 1.2	18 ± 2		

[a] D. Resiga, unpublished data.

protons on the donor with the solvent is faster than the rate of proton trapping by pyruvate during turnover of the enzyme ($k_{off,H^+} \gg k_{cat}$) (*23*). This hypothesis was supported by experiments with wild-type YPK in the pulse and the presence of the slow substrate BrPEP (*24*) in the chase. The pulse was the same in both experiments. No protons were trapped with BrPEP in the chase. A step (or steps) prior to proton transfer by PK to bromoenolpyruvate must be much slower than k_{off,H^+} for the labeled proton. The labeled proton exchanges with solvent before it incorporates into the product bromopyruvate. A similar mechanism ($k_{off,H^+} \gg k_{cat}$) probably accounts for the lack of trapping of label when K240M is the enzyme and PEP is the substrate.

Solvent Deuterium Isotope Effects. If the phosphoryl transfer step is exclusively affected by mutation of Lys 240 to methionine, then the rate of phosphoryl transfer should decrease relative to other rates in the catalytic process, including proton transfer. A useful measure of relative rates of solvent-exchangeable proton transfer in enzymes is the solvent isotope effect. Solvent isotope effects were measured for wild-type and K240M YPK with PEP as the variable substrate. The results of these studies are summarized in Table 5. The isotope effect on k_{cat} ($^Dk_{cat}$) for wild-type YPK where PEP or the slow substrate BrPEP was the varied substrate was 2.7 ± 0.2 or 1.1 ± 0.1, respectively. These values are significantly different, and indicate that the rate of the isotope-sensitive step with BrPEP as the substrate is significantly less rate-limiting than that with PEP as the substrate. The $^Dk_{cat}$ is 1.6 ± 0.1 with K240M YPK. The isotope effect on k_{cat}/K_m [$^D(k_{cat}/K_m)$], the term that includes all the catalytic steps up to and including the first irreversible step of the reaction, was also measured. The $^D(k_{cat}/K_m)$ decreased from 2.2 ± 0.7 to 0.84 ± 0.18 for the variable substrates PEP and BrPEP, respectively, with wild-type YPK. In K240M, the isotope effect on k_{cat}/K_m was eliminated. Mutation of Lys 240 to methionine affects the interaction of PEP with the enzyme, consistent with the results of steady-state kinetics experiments, but has only partially destabilized the transition state of the isotope-sensitive step.

DISCUSSION

Lys 240 was mutated to methionine in yeast pyruvate kinase to determine its role in catalysis. The k_{cat} for K240M decreases approximately 100-, 200-, and 1000-fold compared to wild-type YPK activated by Mn^{2+}, Co^{2+}, and Mg^{2+}, respectively. There is no measurable PK activity with K240M in the absence of FBP. The modification of Lys 240 at the active site of YPK must cause an alteration at the active site such that the binding of the heterotropic activator, FBP, is required to induce the active conformation of the enzyme. The K_m for ADP has changed only 2−3-fold, indicating that Lys 240 has a minor influence on the interaction of ADP with the enzyme. There is a 1 order of magnitude increase in the K_m of PEP and the divalent metal compared to that of wild-type YPK. The elimination of the active site Lys 240 diminishes the extent of interaction of the cation with the YPK−PEP complex. Pulsed spin-echo EPR studies of bound Mn^{2+} in the PK−Mn−PEP complex gave a Mn^{2+} signal with superhyperfine coupling to ^{14}N (*25*). This coupling was attributed to a lysine residue, an amino acid residue not normally found as a ligand to enzyme-bound cations.

Binding of Mn^{2+} and PEP was assessed in the muscle PK apoenzyme in which the active site lysine was chemically modified by trinitrophenyl benzene. This modified form of the enzyme was able to bind Mn^{2+} and PEP with the same affinity as the unmodified enzyme, but modification caused a 95% decrease in k_{cat} (*26*). Comparison of k_{cat} values for the wild type and K240M indicates that lysine 240 contributes approximately 3−4 kcal/mol of transition-state stabilization to the net reaction.

The phosphoryl transfer step of the PK-catalyzed reaction can be measured independent of the proton transfer step by the measurement of PK-catalyzed secondary kinase reactions. The fluorokinase, the glycolate kinase, and the hydroxycarbamate kinase reactions are PK-catalyzed, ATP-dependent phosphorylation reactions of fluoride ion, glycolate, and hydroxycarbamate, respectively (*20*, *21*, *27*). Glycolate kinase activity and proton exchange rates were measured to independently determine the chemical step(s) affected by the Lys 240 mutation. No glycolate kinase activity was observed at pH 7.5 with K240M. In light of the strong pH dependence of this reaction catalyzed by wild-type YPK (*20*) and muscle PK (*21*), assays with K240M were performed between pH 6.3 and 8.0. At each pH, either Mg^{2+}, Mn^{2+}, or Co^{2+} was the divalent metal activator. Glycolate kinase activity under all conditions studied was below the limit of detection of the assay (<0.05 min^{-1}). This represents a 1000-fold decrease in glycolate kinase activity compared to that catalyzed by wild-type YPK. The Lys to Met mutation has affected the phosphoryl transfer activity in this enzyme. This explains the significant decrease in the specific activity of the enzyme. In the net PK-catalyzed reaction, phosphoryl transfer from PEP does occur at a significant rate because K240M has net catalytic activity. The loss in transition-state stabilization for the net reaction decreases by 3−4 kcal/mol, depending on the specific divalent cation, and it is the phosphoryl transfer step that appears to be primarily affected. Transition-state stabilization of the pentavalent phosphate in the K240M-catalyzed reaction still occurs but is also influenced by the enzyme-bound divalent cation. The influence of this cation is partially dependent on Lys 240.

The crystal structure data from the rabbit muscle (*9*, *10*) and yeast PK (*11*), in which Lys 240 interacts directly with the phosphoryl group being transferred, are consistent with this hypothesis. Lys 240 is in a position to participate in leaving group activation and stabilization of the pentavalent transition state during phosphoryl transfer. A similar role has also been assigned to the protonated form of Lys 13 of adenylate kinase (*28*, *29*) and Lys 256 of yeast phosphoenolpyruvate carboxykinase (*30*).

The pH dependence of k_{cat} for Mn^{2+}-activated wild-type YPK was fit to a model with three ionizations in the ES complex: $pK_A = 5.35$, $pK_B = 6.35$, and $pK_C = 8.78$. The pH dependence of k_{cat} for K240M indicates that the group with a pK_a of 8.8 has been lost on mutation and that this group plays a role in the catalytic mechanism of YPK.

Solvent deuterium isotope effects were measured to study the effects of proton transfer and the rate-limiting step with wild-type YPK and K240M. The solvent deuterium isotope effect on k_{cat} for K240M is significantly smaller than for wild-type YPK. If the rate of phosphoryl transfer is totally rate-determining for the net reaction catalyzed PK, a solvent deuterium isotope effect approaching a limit of 1.0 is expected. The solvent deuterium isotope effect with MPK decreased to 1.0 with the use of dGDP as a poor phosphoryl acceptor (*31*). The isotope effect for protonation of free enolpyruvate in solution at pD 6.4 is 6.0 (*5*). Because $^{D}k_{cat}$ is 1.6 for K240M, this rules out the reaction mechanism where the enzyme catalyzes phosphoryl transfer and releases enolpyruvate to be protonated by bulk solvent. In this case, an increase in $^{D}k_{cat}$ is expected. The $^{D}(k_{cat}/K_m)$ changes from a large value of 2.2 for wild-type YPK to the absence of an effect in K240M [$^{D}(k_{cat}/K_m) = 1$]. The k_{cat}/K_m isotope effect with K240M with PEP as the substrate or with the wild type and using BrPEP as the substrate indicates that the isotope-sensitive step occurs after the first irreversible step. The first irreversible step is probably phosphoryl transfer. The relative rate of the rate-limiting step(s) changes when BrPEP is the substrate or with the mutation of Lys 240 to methionine. The isotope-sensitive step is probably the same in each of the three cases. These results suggest that the rate of the isotope-sensitive step in the net reaction catalyzed by YPK is not significantly modulated by Lys 240.

ATP serves as a cofactor for PK in catalyzing the exchange of the methyl protons of pyruvate into solvent (pyruvate enolization) (*2*−*4*). Detritiation of pyruvate was used to measure the effects of mutating Lys 240 on the rate of pyruvate enolization by YPK. In the absence of FBP, the rate of detritiation of pyruvate with Mg^{2+} as the divalent activator was not above background. The presence of FBP increased the rate of detritiation significantly. FBP had little effect on the rate of detritiation with Mn^{2+} as the activator. Mg^{2+} is a 3-fold poorer activator of pyruvate enolization than is Mn^{2+}. With muscle PK, the rate of detritiation is 4-fold higher than with YPK. The relative partitioning between pyruvate enolization and pyruvate exchange from PK differs between MPK and YPK and is cation-dependent.

There was no measurable detritiation of pyruvate by K240M. One explanation for this is that the proton donor has been mutated and the enzyme does not catalyze transfer of a proton to the enolpyruvate intermediate in the net reaction. In this case, the solvent deuterium isotope effect would be expected to increase, and this does not occur. A more likely explanation is that Lys 240 participates in both the phosphoryl transfer step and proton transfer to enolpyruvate. Flashner and co-workers (*26*) showed, by trinitrophenyl modification of MPK, that the ϵ-amino group of an active site lysine residue was involved in the detritiation of pyruvate. The ATP-activated detritiation of pyruvate with the modified enzyme was almost completely inhibited. However, the modified enzyme retained the ability to catalyze the P_i-mediated detritiation of pyruvate, indicating that the modified lysine residue was involved in the enolization of pyruvate, but that it was not the active site base that abstracted the proton. Recently, the crystal structure of rabbit muscle PK was solved in the presence of oxalate, ATP, Mg^{2+}, and K^+ (*10*). Oxalate is a structural analogue of the enolpyruvate intermediate. This structure appears to represent a snapshot of the enzyme in an intermediate state during catalysis. In this structure, the ϵ-amino group of Lys 269 is close to the carboxyl oxygen of oxalate that corresponds to the C-2 enolate oxygen of the enolpyruvate intermediate. These data suggest that Lys 269 could promote the enolization of bound pyruvate and participate in leaving group activation (*10*). Removing the electrostatic stabilization in this half-reaction could significantly affect the rate of detritiation, although the extent to which Lys 240 stabilizes this transition state must be smaller than for phosphoryl transfer.

Isotope trapping experiments were performed to characterize the proton donors in wild-type and K240M YPK. In a simple reaction mechanism, PK is expected to catalyze phosphoryl transfer followed by transfer of a single proton to enolpyruvate and rapid release of pyruvate from the enzyme. If this is the chemical process, 1.0 equiv of protons would be incorporated into pyruvate. Results with MPK agree with those reported by Rose and Kuo (*17*), in which approximately 1.25 equiv of protons was trapped into pyruvate in the presence of the activator Mg^{2+}. The experiment was repeated using Mn^{2+} as divalent cofactor, and 2.9 equiv of protons was trapped. Three equivalents of protons was also trapped in pyruvate by MPK with Co^{2+} as the activator (*17*). Thus with MPK, more than a single proton equivalent is trapped in pyruvate. This can be rationalized as pyruvate product release being at least partially rate-limiting in the overall reaction catalyzed by PK. Robinson and Rose (*3*) showed that during steady-state turnover, the label from tritiated water appears in PEP, indicating that the product form of the enzyme may reverse to the substrate form at a significant rate relative to the rate of product release. In this mechanism, the enolization of the pyruvate product occurs at a significant rate relative to that of its release from the enzyme. This explains the incorporation of label from solvent into PEP and allows the incorporation of more than a single proton from the enzyme into pyruvate from sites adjacent to the proton donor in what Rose has described as a proton relay (*22*). Incorporation of additional protons by enolization of bound pyruvate was distinguished from incorporation of additional protons by release of pyruvate and subsequent binding to PK by trapping the released pyruvate as lactate. If the enzyme released pyruvate before three protons were exchanged, then the isolated lactate would contain less than three trapped protons under the same pulse and chase conditions. Comparison of results with and

without LDH shows that all three protons are incorporated into pyruvate before release from the enzyme.

The maximum number of protons trapped per mole of pyruvate approaches 1.5 with YPK. With MPK, the physical limit of three trapped protons per mole of pyruvate is reached. There are two explanations for the decrease in the number of trapped protons by YPK compared to that by MPK. The relative rate of release of pyruvate from the enzyme may be greater with YPK than with MPK. The rate of enolization of pyruvate is greater in MPK than in YPK (Table 3). The higher product release rate and lower pyruvate enolization rate with YPK would result in fewer protons becoming trapped in pyruvate by YPK. Alternatively, the exchange rate of the substrate proton in the enzyme−substrates complex (YPK−PEP−ADP) could be faster in YPK than in MPK. This would allow partial exchange of the proton of either the donor or relay sites with solvent in the chase before net turnover could trap the protons in pyruvate.

In the presence of FBP and Mg^{2+}, 0.55 equiv of protons was trapped in pyruvate by YPK. In the absence of FBP, this value decreases to 0.37. These values are less than unity. This indicates that there is partitioning of the proton label between trapping (turnover) and exchange from the proton donor. There are two potential routes for proton exchange. The proton may exchange from the enzyme−substrates complex (YPK−PEP−ADP) before phosphoryl transfer, or may exchange from the intermediate complex (YPK−enolpyruvate−ATP). Our data cannot distinguish between these two routes of proton exchange from the enzyme. FBP also modulates the number of protons trapped per mole of pyruvate when either Mn^{2+} or Co^{2+} is the activator. In the net reaction catalyzed by YPK, FBP affects the rate of pyruvate enolization, the rate of the phosphoryl transfer step, and the ratio of tritium label from 3-[^{3}H]PEP appearing in water and pyruvate (*4*). It is the sum of the rates of these processes that determines the number of proton equivalents that will be trapped in pyruvate by YPK. The heterotropic effector FBP affects the rate constants for several of these processes, although it does not significantly affect the net k_{cat} for wild-type YPK regardless of the divalent cation.

No protons were trapped in pyruvate by K240M. A likely explanation is that the turnover of K240M, and therefore trapping of the substrate proton in pyruvate, is much slower than the rate of exchange of the proton from the donor into solvent. Because phosphoryl transfer is slow in K240M, the labeled proton probably exchanges with solvent in the enzyme−substrates complex before the phosphoryl transfer step. The slow turnover of K240M was mimicked in wild-type YPK by using the slow substrate BrPEP as the trapping species in the chase. Because the pulse was the same regardless of whether PEP or BrPEP was in the chase, the proton donor on YPK in both experiments must have been labeled prior to initiating turnover of the enzyme with the chase. There were no protons trapped in bromopyruvate by Mn^{2+}- and FBP-activated YPK. This occurs because the exchange rate of the labeled protons is more rapid than the rate of turnover with BrPEP and supports the hypothesis that the rate of turnover of K240M is much slower than the rate of exchange of the labeled proton from the proton donor.

The identity of the proton donor in the PK-catalyzed reaction is still unknown. Clearly, Lys 240 plays a role in proton transfer from PK to the enolpyruvate intermediate, although it is not the proton donor. Several pieces of evidence suggest that an active site water molecule is the proton donor. The PK-catalyzed enolization of pyruvate is dependent on the presence of both divalent metal and a nucleotide cofactor (*2*, *3*). In the most recent crystal structure of MPK, a water molecule that is in the coordination sphere of Mg^{2+} in the Mg(II)−ATP complex lies in a strategic position above the 2-*si* face of O-1 of oxalate; O-1 of oxalate corresponds to C-3 of the enolpyruvate intermediate. Studies with Cr(III)-ATP as a cofactor for the enolization reaction also implicate the nucleotide-bound metal in the activation of the base involved in proton exchange (*32*). Furthermore, these studies showed that the rate of enolization of pyruvate by MPK correlated with the electronegativity of the nucleotide-bound metal and, consequently, with the pK_a of the metal-bound water molecule.

A comparison between the isotope trapping experiment in which the pulse contained ADP and the experiment in which the pulse contained PEP suggests that ADP must be present in the pulse for the label to be incorporated into pyruvate (Table 4). This is consistent with a Mg(II)ATP-bound water molecule acting as, or being linked to, the proton donor to the enolpyruvate intermediate in the PK−enolpyruvate−MgATP complex. Enzyme turnover is initiated in this experiment by mixing the pulse, which contains PK and tritiated water as a label, with the chase, which contains a large excess of unlabeled water. This excess of unlabeled water dilutes the label in the pulse so that any tritium that is not specifically bound to the proton donor or proton relay sites is exchanged into solvent and is only incorporated into pyruvate at a background level. This background level of tritium incorporation into pyruvate is corrected by a control experiment. In the absence of the putative metal−nucleotide proton donor in the pulse, the chased label would be too dilute to be incorporated into pyruvate at levels greater than background.

In isotope trapping experiments with MPK in the presence of Mg^{2+}, there was no change in the number of protons trapped from pH 6 to 9.5 (*17*). This indicates that the pK_a of the proton donor must be very high. The pK_a of Mg^{2+}-bound water is approximately 12.8 (*33*). These data are all consistent with the hypothesis that a metal-bound water in the metal−nucleotide complex at the active site of PK is the proton donor to the enolpyruvate intermediate in the net PK-catalyzed reaction.

In summary, Lys 240 plays a role in both the phosphoryl transfer and proton transfer half-reactions of yeast pyruvate kinase. It plays a key role in transition state stabilization for phosphoryl transfer. It is unlikely, on the basis of the kinetic data that are presented, and the crystal data that are referenced, that Lys 240 is the direct proton donor to the enolpyruvate intermediate.

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