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THE PROTON TRANSFER REACTIONS CATALYZED BY YEAST PYRUVATE KINASE

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Summary

1. The proton-transfer reactions of yeast pyruvate kinase (EC 2.7.1.40) were studied. Proton transfer from C-3 of phosphoenolpyruvate to water occurs only in the presence of the phosphoryl-acceptor ADP. Proton transfer from C-3 of pyruvate to water occurs only in the presence of ATP. However, the proton transfer in the latter case occurs 10—100 times faster than phosphoryl transfer; this supports a mechanism in which proton transfer precedes phosphoryl transfer in the reverse reaction of pyruvate kinase.

2. The characteristics of proton-transfer reactions of yeast pyruvate kinase were compared with those previously reported for rabbit muscle pyruvate kinase (Robinson, J.L. and Rose, I.A. (1972) J. Biol. Chem. 247, 1096—1105). The pH-profiles and the divalent cation dependencies were similar for Fru-1,6-P_2-activated yeast pyruvate kinase and the muscle enzyme. Pyruvate enolization by yeast pyruvate kinase has an absolute requirement for ATP in contrast to enolization by the muscle enzyme which proceeds when ATP is replaced by P_i or other dianions.

3. Fructose-1,6-bisphosphate was shown to affect the catelytic steps of yeast pyruvate kinase in addition to the binding of substrates. Its role depends on the divalent cation used to activate the enzyme.

Introduction

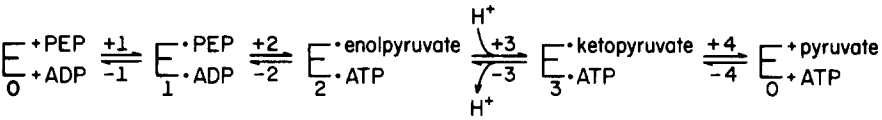
Pyruvate kinases (EC 2.7.1.40) display interspecies and intertissue differences in their regulatory properties [1,2]. Rabbit muscle pyruvate kinase obeys

Buffers used through this study were: MES between pH 5.8 and 6.9, TES from pH 6.9 to 7.9, TAPS from pH 7.9 to 9.4 and glycine from pH 9.4 to 10.0. All buffers were adjusted to the indicated pH with tetramethylammonium hydroxide unless otherwise indicated.

Abbreviations: ADP-CH_2-P, β,γ-methylene-adenosine triphosphate; AMP-CH_2-P, α,β-methylene-adenosine diphosphate; MES, 2(*N*-morpholino)ethane sulfonate; TES, *N*-tris(hydroxymethyl)-methyl-2-amino ethane sulfonate; TAPS, tris(hydroxymethyl)methylaminopropane sulfonate.

Michaelis-Menten kinetics while yeast pyruvate kinase exhibits sigmoidal kinetics with respect to phosphoenolpyruvate and is allosterically activated by fructose-1,6-bisphosphate [3,4]. It is of interest to compare the activities of these two pyruvate kinases to see what elements of reactivity have been maintained throughout their evolution.

The primary, physiologically important reaction catalyzed by pyruvate kinase is the conversion of phosphoenolpyruvate and ADP to pyruvate and ATP. The overall reaction involves two elements: the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, and the transfer of a proton from water to form the methyl group of pyruvate. The equilibrium of the reaction is strongly pH-dependent and lies far in the direction of pyruvate formation even at high pH [5]. Previous work has shown that the two partial reactions of rabbit muscle pyruvate kinase can be separated; secondary kinase reactions (6—8) involve phosphoryl transfer only, while the enolization of pyruvate [9,10] involves only proton transfer. Furthermore, Robinson and Rose [10] have shown that the transfer of a phosphoryl group from phosphoenolpyruvate to ADP precedes the transfer of a proton from water to enolpyruvate, as shown in the scheme:



The preceding paper [11] establishes the existence of secondary kinase reactions of yeast pyruvate kinase which involve only phosphoryl transfer. The present paper examines the proton transfer reactions of yeast pyruvate kinase.

Materials and Methods

Enzymes. Yeast pyruvate kinase was isolated and stored at 4°C as an ammonium sulfate slurry as previously described [11]. The activity of the enzyme measured in the presence of 1 mM Fru-1,6-P_2 remained constant at 380 units/mg protein. The activity in the absence of Fru-1,6-P_2, originally equal to the activity in its presence when measured at 10 mM phosphoenolpyruvate, decreased approximately 40% upon storage for longer than 6 months, this probably indicated a partial oxidation of the enzyme [12]. Attempts to reactivate the enzyme by treatment with 2-mercaptoethanol or dithiothreitol were unsuccessful.

To prepare the enzyme for use, it was centrifuged from the ammonium sulfate suspension, dissolved to a concentration of 10 mg protein/ml in 50% (v/v) glycerol containing 10 mM MES (pH 6.2) and 0.1 mM EDTA, and dialyzed 20 h against the same glycerol buffer. The enzyme retained full activity when stored in the glycerol buffer at 4°C for at least 12 days. Enzyme concentration was determined spectrophotometrically, using $E_{280}^{1\,mg/ml} = 0.58$ [12].

Lactate dehydrogenase was purchased from Boehringer Mannheim Corp. Mannheim, Germany). It was dialyzed against 20 mM TES (pH 7.6), 0.1 mM EDTA to remove the ammonium sulfate before use.

Radioactive compounds. [3-^{3}H]Pyruvate was labeled from ^3H_2O using the proton-exchange reaction catalyzed by 2-keto-3-deoxy-6-phosphogluconate as described by Meloche [13]. [3-^{3}H]Phosphoenolpyruvate was prepared from

[3-^{3}H] pyruvate by phosphoenolpyruvate synthease as described by Robinson and Rose [10] except that the reaction was treated with acid-washed charcoal (to remove adenine nucleotides) and with hydrogen peroxide (to destroy any remaining pyruvate) just prior to column chromatography of the product. [γ-^{32}P] ATP was prepared according to the exchange method of Glynn and Chappell [14]. The radioactivity of each sample was determined in 10 ml of 25% Triton X-114 in xylene containing 5 g/l diphenyloxazole as described by Anderson and McClure [15]. ^2H_2O was purchased from Bio-Rad and contained less than 600 cpm ^{3}H/ml. Other chemicals were the best grade commercially available.

Pyruvate kinase activity. The velocity of the forward reaction of yeast pyruvate kinase was measured in the presence of the following: 100 mM MES (pH 6.2), 150 mM KCl, 15 mM $MgCl_2$, 10 mM ADP, 10 mM phosphoenolpyruvate, 1 mM Fru-1,6-P_2, 0.2—0.3 mM NADH, 0.003 mg lactate dehydrogenase and approx 0.1 units of pyruvate kinase (total vol. 1.0 ml). The decrease in absorption at 340 nm was followed continuously on a Gilford 240 recording spectrophotometer thermostatted at 30° C.

Partition of tritium from [3-^{3}H] phosphoenolpyruvate between pyruvate and water. The ratio of tritium from [3-^{3}H] phosphoenolpyruvate appearing in water to that appearing in pyruvate, called R_T, was measured in the following reactionmixture: 100 mM buffer, 150 mM KCl, 2 mM ADP, 0.1 mM [3-^{3}H] phosphoenolpyruvate ($1.5 \cdot 10^6$ cpm/mM), divalent cation and Fru-1,6-P_2 as indicated in text, 0.2—0.3 mM NADH, 0.003 mg lactate dehydrogenase and 0.01—0.05 mg yeast pyruvate kinase (total vol. 1.0 ml). Reactions were followed to completion at 30° C by monitoring the A_{340}. In addition to providing a means of following the pyruvate kinase reaction, the lactate dehydrogenase system provided a trap which immediately removed pyruvate from the reaction mixture. Tritium released into the water was determined as previously described [10] except 25 mM sodium citrate (pH 6) was used to stop the reaction. Tritium released to water was corrected for a control without enzyme. Total counts were made on an aliquot of each sample, and pH was verified following each reaction. R_T, the ratio of counts in water to counts in pyruvate (lactate), was calculated by dividing the volatile counts by the total counts less the volatile counts.

Velocity of pyruvate enolization. The velocity of pyruvate enolization was followed as the rate of exchange of tritium from [3-^{3}H] pyruvate into water (v_{xT}) in the following reaction mixture: 100 mM buffer, 150 mM KCl, 100 mM [3-^{3}H] pyruvate (1500 cpm/mM), 2 mM ATP, divalent cation and Fru-1,6-P_2 as indicated, and 0.1—0.5 mg yeast pyruvate kinase (total volume 1.0 ml). A nonenzymic blank was run for each sample. Reactions were incubated at 30° C for a fixed time (15—120 min). Reactions were stopped by adding 0.1 ml samples to 0.4 ml of 25 mM sodium citrate (pH 6.0). Tritium released to water was determined as previously described [10]. Calculation of exchange rates from the three equivalent positions of the pyruvate methyl group was made from the equation [10]:

$$v_{xT} = -3\,[\text{pyruvate}]\,(\text{time} \cdot \text{mg enzyme})^{-1}\,\ln(1\text{-fraction exchanged})$$

Velocity of phosphoryl exchange at equilibrium. The velocity of exchange

between [γ-^{32}P] ATP and phosphenolpyruvate (v_{xP}) was measured in reaction mixtures that had been allowed to come to equilibrium. Reaction mixtures included: 100 mM buffer, 150 mM KCl, 100 mM pyruvate, 2 mM ATP, divalent metal ion and Fru-1,6-P_2 as indicated, and 0.001—0.002 mg/ml yeast pyruvate kinase. After the reaction mixtures had been incubated for 1 h at 30°C, [γ-^{32}P] ATP was added to give a specific activity of about $3 \cdot 10^5$ cpm/millimole. Samples (0.5 ml) were removed, and the reaction stopped by rapid addition of the samples into plastic tubes containing 0.5 ml of 5% perchloric acid. Samples were treated with acid-washed Norit Charcoal and soluble counts determined as described by Switzer [16] with the exception that 50 mM potassium phosphate (pH 7) replaced pyrophosphate as the buffer for the "carrier". To determine the equilibrium of phosphoryl exchange, the same procedure described above was used except the [γ-^{32}P] ATP was added at the beginning of the incubation and 0.1 mg/ml of yeast pyruvate kinase was added. Equilibrium was reached in 1—5 minutes, depending on reaction conditions. Calculation of velocity of phosphoryl exchange was made from the equation

$$v_{xP} = -\frac{[\text{ATP}]\,[\text{phosphoenolpyruvate}]}{[\text{ATP} + \text{phosphoenolpyruvate}]}\,(\text{time} \cdot \text{mg enzyme})^{-1} \cdot \ln\,(1 - \text{fraction exchanged})$$

Primary hydrogen isotope effect in pyruvate enolization. The primary hydrogen isotope effect in the enolization of pyruvate was determined by measuring the rates of exchange of the methyl protons and tritium from [3-^{3}H] pyruvate with deuterium in ^2H_2O in a single reaction mixture as has been described previously [10]. Reaction mixtures consisted of: 50 mM Tris · Cl, 150 mM KCl, 100 mM [3-^{3}H] pyruvate (1500 cpm/mM), 2 mM ATP, divalent cation and Fru-1,6-P_2 as indicated and 0.2—0.6 mg/ml yeast pyruvate kinase in ^2H_2O. The enzyme for this experiment was centrifuged out of the ammonium sulfate suspension, dissolved in a concentration of 10 mg protein/ml in 10% (v/v) glycerol containing 10 mM K-MES (measured "pH", 6.3) and 0.1 mM EDTA in ^2H_2O. The enzyme was used immediately (without dialysis). Proton exchange was followed on a Varian A60 nuclear magnetic resonance spectrophotometer thermostatted at 30° C as the decrease in the relative area of the pyruvate methyl group signal to that of the methylene group of Tris. Tritium exchange was followed as production of tritiated water as described above. No hydrogen exchange was observed in the absence of enzyme as measured either by disappearance of pyruvate methyl group signal in the NMR or by tritium exchange into water. Values for p^{2}H were the measured "pH" + 0.4 [17]. All tabular and graphical data represent averages of replicate determinations.

Results

Reaction sequence. One objective of this work was to determine the order of the phosphoryl transfer and proton transfer reactions of yeast pyruvate kinase and to compare the mechanism with that already established for muscle pyruvate kinase (see Scheme 1). Isotope exchange experiments were used to investigate the partial reactions involved.

In order for tritium from [3-^{3}H] phosphoenolpyruvate to appear in water, a proton must be added to the methyl carbon of [3-^{3}H] phosphoenolpyruvate (or enolpyruvate), rotation around the C_2-C_3 bond must occur and a tritium must be abstracted in the reverse of the first step. It is possible that the protonation of phosphoenolpyruvate might precede phosphoryl transfer or that the phosphoryl group might be transferred to a site on the enzyme. In either case one would expect tritium exchange between [3-^{3}H] phosphoenolpyruvate and water in the absence of ADP. Alternatively, phosphoryl transfer to ADP could precede or occur concomitantly with proton transfer to enolpyruvate, in which case one would expect tritium exchange between [3-^{3}H] phosphoenolpyruvate and water to be dependent upon ADP, as has been noted with rabbit muscle pyruvate kinase [10].

The requirements for the transfer of tritium from [3-^{3}H] phosphoenolpyruvate to water catalyzed by yeast pyruvate kinase are shown in Table I. The reactions were studied under conditions that gave significant exchange during the complete reaction. In the absence of ADP, no tritium appeared in the water. Neither P_i nor AMP could substitute for ADP to promote tritium exchange with water. These results are consistent with a mechanism in which phosphoryl transfer must precede or accompany proton transfer.

To investigate the mechanism of yeast pyruvate kinase further, the detritiation of [3-^{3}H] pyruvate was studied. It has been shown for muscle pyruvate kinase that enolization of pyruvate can occur in the absence of phosphoryl transfer [9,10]. The requirements for the detritiation (enolization) of [3-^{3}H] pyruvate catalyzed by yeast pyruvate kinase are presented in Table II. Yeast pyruvate kinase shows an absolute requirement for ATP in the enolization of pyruvate. The reaction is inhibited by the addition of P_i to the complete system. No enolization of pyruvate is seen when P_i or NO_3^- are substituted for ATP, nor can other adenine nucleotides replace ATP. The small reaction seen with ADP was due to a contamination of approximately 5% ATP in the ADP. Even ADP-CH_2-P, a close structural analog of ATP, did not promote pyruvate enolization. Thus the requirements for the enolization of pyruvate by yeast pyruvate kinase are

TABLE I

REQUIREMENTS FOR CONVERSION OF TRITIUM FROM [3-^{3}H] PHOSPHOENOLPYRUVATE TO ^3H_2O BY YEAST PYRUVATE KINASE

The complete reaction mixture was as described in Materials and Methods, with 1 mM Fru-1,6-P_2. Additions were: 25 mM KP_i, pH 8.8; 5 mM AMP. Reaction mixtures were incubated 30 min at 30° C. In the complete system the reaction was complete within 15 min at pH 9.3 and in 2 min at pH 8.3. No net forward reaction was observed in the absence of ADP.

Reaction conditions	Tritium released to water (%)	
	pH 9.3 5 mM $MgCl_2$	pH 8.3 5 mM $MnCl_2$
Complete	18.7	32.2
— enzyme	1.5	1.8
— ADP	1.6	1.2
— ADP + P_i	1.4	1.3
— ADP + AMP	1.6	1.1
— ADP, + AMP + P_i	1.4	1.2

TABLE II

REQUIREMENTS FOR DETRITIATION OF [3-^{3}H]PYRUVATE BY YEAST PYRUVATE KINASE

The complete reaction mixture and experimental conditions were as described in Materials and Methods, with 5 mM $MgCl_2$ and 1 mM Fru-1, 6-P_2. Reactions were carried out at pH 8.4. Non-enzymic controls have been subtracted from the values presented.

Conditions	v_{xT} (μmol/min per mg protein)
Complete	9.7
Complete + 25 mM P_i	1.4
— ATP	0.07
— ATP + 100 mM P_i	0.05
— ATP + 100 mM NO_3^-	0.05
— ATP + 5 mM AMP	0.20
— ATP + 5 mM AMP, 30 mM P_i	0.26
— ATP + 5 mM ADP	1.26 *
— ATP + 5 mM ADP, 30 mM P_i	0.36 *
— ATP + 2 mM AMP-CH_2-P	0.14
— ATP + 2 mM ADP-CH_2-P	0.12

* 5% contamination of ADP with ATP accounts for the observed v_{xT}.

more stringent than for muscle pyruvate kinase. This experiment does not support a mechanism requiring the enolization of pyruvate in the absence of phosphoryl transfer.

The requirement of ADP for detritiation of [3-^{3}H] phosphoenolpyruvate and the requirement of ATP for detritiation of [3-^{3}H] pyruvate suggested the possibility that phosphoryl transfer and proton transfer occur simultaneously on the yeast enzyme. Another possible explanation is that the events occur sequentially, but strict steric requirements at the enzyme active site necessitate the presence of all substrates for the partial reactions to occur. To investigate these possibilities, the rate of tritium exchange between [3-^{3}H] pyruvate and water (v_{xT}) was compared with the rate of phosphoryl exchange between [γ-^{32}P] ATP and phosphoenolpyruvate (v_{xP}). Under the conditions necessary to measure the rate of pyruvate enolization, macroscopic equilibrium was reached within 2 min, so the v_{xT} measured was the equilibrium rate of tritium transfer from pyruvate to water. In order to achieve a conveniently measurable rate of phosphoryl transfer from ATP to phosphoenolpyruvate, the amount of enzyme added to the reaction mixture was reduced 50—100 fold. So that both reactions would be measured under equilibrium conditions, the complete reaction mixture for v_{xP} determinations was pre-incubated for 1 h at 30°C. Then a small amount of [γ-^{32}P] ATP of very high specific radioactivity was added, and the rate of exchange of radioactive ATP with phosphoenolpyruvate was determined. A comparison of v_{xT} and v_{xP} is shown in Table III. In the presence of Fru-1,6-P_2, with Mg^{2+} as the divalent cation, the rate of tritium exchange was 2—3 times the rate of phosphoryl exchange. With Mn^{2+} as the divalent cation, in the presence of Fru-1,6-P_2, the rate of proton transfer was 30 times the rate of phosphoryl transfer. With Mn^{2+} as the divalent cation in the absence of Fru-1,6-P_2, there was very little phosphoryl transfer and equilibrium was not reached within 1 h. Even so, the rate of ^{3}H exchange was approx. 2 orders of magnitude × rate of phosphoryl exchange (data not shown).

The rate of tritium exchange between [3-^{3}H] pyruvate and water may greatly

TABLE III

RATE COMPARISON BETWEEN PROTON TRANSFER AND PHOSPHORYL TRANSFER CATALYZED BY YEAST PYRUVATE KINASE

Rates determined at equilibrium, v_{xT} from tritium exchange from [3-^{3}H]pyruvate into water, v_{xP} from ^{32}P exchange from [γ-^{32}P]ATP into phosphoenolpyruvate. Reaction conditions and experimental procedure were as described in Materials and Methods with 5 mM divalent cation and 1 mM Fru-1, 6-P_2.

Divalent cation	pH	v_{xT}	v_{xP}	v_{xT}/v_{xP}
		(μmol/min per mg protein)		
Mg^{2+}	9.3	13.4	4.3	3.1
Mg^{2+}	8.5	5.1	3.1	1.6
Mn^{2+}	8.5	30.6	0.95	32

underestimate the rate of proton exchange. To compare the rate of proton exchange to the rate of phosphoryl exchange, the primary tritium isotope effect on pyruvate enolization must be taken into account. The tritium isotope effect on hydrogen exchange was measured in mixtures of [3-^{1}H]pyruvate and [3-^{3}H]-pyruvate which were incubated in a medium of ^2H_2O under conditions that catalyze pyruvate enolization. The rates of exchange of the methyl protons and tritium from pyruvate with ^2H_2O were measured (Table IV). With Mg^{2+} (5 mM) as the divalent cation a tritium isotope effect of 6.1 was measured at p^{2}H 9.1. In order to measure proton exchange by NMR in the presence of the paramagnetic divalent cation, Mn^{2+}, both low p^{2}H and low Mn^{2+} concentration had to be used. At 0.5 mM $MnCl_2$ and p^{2}H 7.3, a tritium-isotope effect of 4.3 was observed.

The measured tritium-isotope effect confirms that the values determined for v_{xT} underestimate by 4 to 6-fold the actual rate of pyruvate enolization catalyzed by yeast pyruvate kinase. Thus, referring to Table III, the rate of pyruvate enolization catalyzed by yeast pyruvate kinase is actually 12—18 times the rate of phosphoryl transfer with Mg^{2+} as the divalent cation. If the isotope effect measured at 0.5 mM Mn^{2+} can be applied at high divalent cation concentrations, the rate of pyruvate enolization catalyzed by yeast pyruvate kinase with Mn^{2+} as the divalent cation is two orders of magnitude greater than the rate of phosphoryl transfer. These results establish that in the back reaction pyruvate enolization takes place much more rapidly than phosphoryl transfer, and that the order of events is consistent with that shown in Scheme 1.

Roles of divalent cation and Fru-1,6-P_2. Another objective of this research was to study the role of the divalent cation and of Fru-1,6-P_2 in the yeast pyruvate kinase reaction. Pyruvate kinase is absolutely dependent on divalent

TABLE IV

ISOTOPE EFFECT FOR PYRUVATE ENOLIZATION CATALYZED BY YEAST PYRUVATE KINASE

Reaction conditions and experimental procedure were as described in Materials and Methods.

Divalent cation	p^{2}H	v_{xH}	v_{xT}	v_{xH}/v_{xT}
		(μmol/min per mg protein)		
5 mM Mg^{2+}	9.1	68.2	11.2	6.1
0.5 mM Mn^{2+}	7.3	7.4	1.7	4.3

cations for activity. Fru-1,6-P_2 is potent allosteric activator of yeast pyruvate kinase.

The effects of divalent cation and Fru-1,6-P_2 on the partition of tritium from [3-^{3}H] phosphoenolpyruvate between water and pyruvate, R_T, were determined as a function of pH (Fig. 1). In all cases R_T was low at low pH. Near pH 6.2, the pH-optimum of yeast pyruvate kinase [18], the value of R_T depended upon the divalent cation, but not on the presence or absence of Fru-1,6-P_2. With increasing pH, R_T increased dramatically in the presence of Fru-1,6-P_2 with either Mg^{2+} or Mn^{2+}. Only slight increases in R_T were seen in the absence of the allosteric activator; it must be noted that the concentration of phosphoenolpyruvate used in determining R_T was 0.10 mM, where effects of Fru-1,6-P_2 would be enhanced. R_T increases at lower pH and reached much higher values with Mn^{2+} than with Mg^{2+} as the divalent cation. Data could not be obtained above pH 9 for Mn^{2+}, due to precipitation of the cation.

The effects of divalent cations and Fru-1,6-P_2 on the ATP-activated anolization of pyruvate as a function of pH are shown in Fig. 2. With Mg^{2+} as the divalent cation, enolization of pyruvate was completely dependent upon Fru-1,6-P_2. In its presence there was no enolization below pH 7, but v_{xT} increased with increasing pH between 7 and 9.5. At higher pH, v_{xT} dropped off sharply, presumably due to inactivation of the enzyme. With Mn^{2+} as the divalent cation, the rate of enolization was greater at all pH values than when

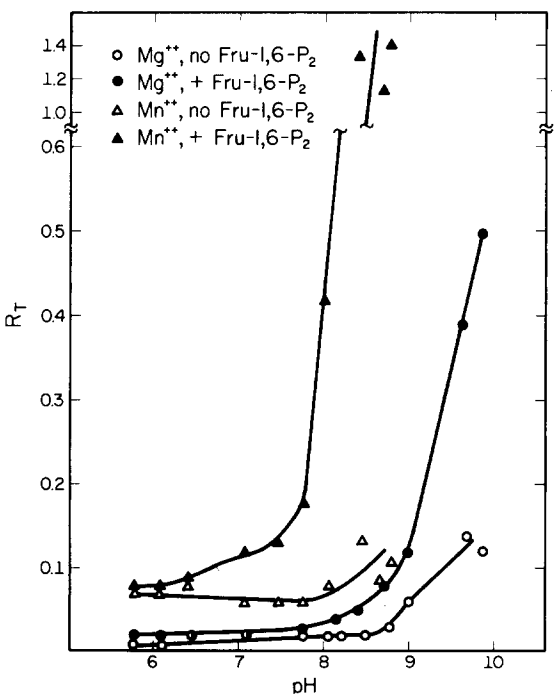


Fig. 1. Partitioning of tritium from [3-^{3}H] phosphoenolpyruvate between water and pyruvate during the pyruvate kinase reaction. Effects of pH, divalent cation and Fru-1,6-P_2. The reaction conditions and experimental procedure were as described in Materials and Methods. Non-enzymic controls have been subtracted. R_T is the ratio of tritium in water to tritium in pyruvate.

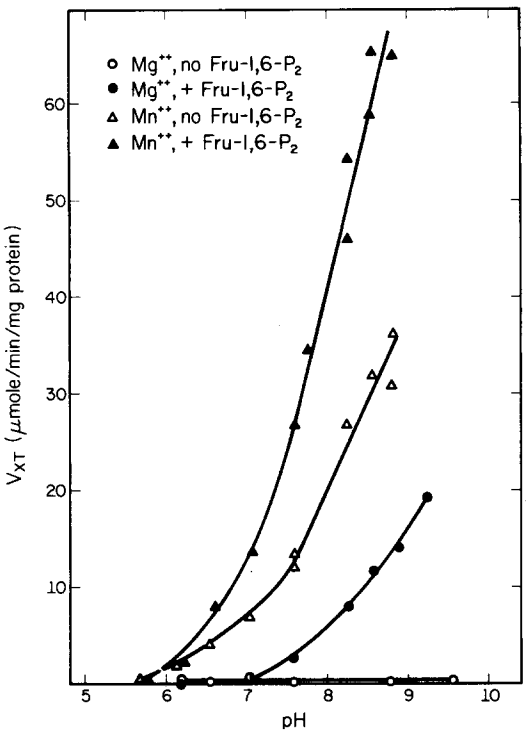


Fig. 2. Velocity of pyruvate detritiation by yeast pyruvate kinase. Effects of pH, divalent cation and Fru-1,6-P_2. The reaction conditions and experimental procedure were as described in Materials and Methods. Non-enzymic controls have been subtracted. v_{XT} is the velocity of tritium released from [3-^{3}H]pyruvate to water in μmol of hydrogen/min per mg protein.

Mg^{2+} was the cation. In the presence of Mn^{2+}, there was no strong dependence on Fru-1,6-P_2, but Fru-1,6-P_2 accelerated the rate of pyruvate enolization approximately two-fold over the rate seen in its absence.

Discussion

The results of this study suggest that the reaction sequence of yeast pyruvate kinase is as indicated in Scheme 1, with phosphoryl transfer preceding proton transfer, as has been shown for muscle pyruvate kinase [10]. Two lines of evidence support this conclusion. First, the fact that no proton transfer from phosphoenolpyruvate to water occurs in the absence of a functional phosphoryl acceptor (ADP) suggests that phosphoryl transfer is required for proton transfer from phosphoenolpyruvate. This result does not prove phosphoryl transfer precedes proton transfer, but is a necessary consequence of such a mechanism. Second, the rate of enolization of pyruvate is 1—2 order of magnitude faster than the rate of phosphoryl transfer from ATP to form phosphoenolpyruvate in the reverse reaction. This faster rate would be observed if, in the reverse reaction (from ATP and pyruvate) proton transfer occurred before phosphoryl transfer. Therefore, in the forward reaction proton transfer must follow phosphoryl transfer, as indicated in Scheme 1. The two steps cannot

occur simultaneously because of the differences in their rates, unless the rate-limiting step in the back reaction is release of phosphoenolpyruvate.

The measured tritium-isotope effect is consistent with a mechanism in which proton transfer can precede the rate-limiting step of the reverse reaction. If proton transfer occurred after the rate-limiting step, an isotope effect of 1.0 would be expected. If proton transfer alone were rate limiting, a large tritium isotope effect (>20) would be expected. The observed isotope effect of 4—6 suggests that C-H bond breaking is not solely rate-limiting, but is not completely independent of the rate-limiting step [19].

One of the aims of this study was to compare the proton-transfer reactions of yeast pyruvate kinase with those that have previously been reported for muscle pyruvate kinase [10]. Yeast pyruvate kinase differs from the muscle enzyme in that the yeast enzyme shows cooperative phosphoenolpyruvate binding and allosteric activation by Fru-1,6-P_2 [3,4]. This study shows that the parameters of the proton transfer reactions of yeast pyruvate kinase in the presence of 1 mM Fru-1,6-P_2 (which results in Michaelis-Menten kinetics) are very similar to those of muscle pyruvate kinase. The dependence of R_T on pH and divalent cation are very similar, although the absolute values with Mg^{2+} are lower for the yeast enzyme than for the muscle enzyme. In both cases R_T is low at low pH and increases dramatically with increasing pH. The pH dependence of R_T for Co^{2+}-activated yeast pyruvate kinase and muscle pyruvate kinase is similar to that observed with Mn^{2+} and Mg^{2+} as the divalent cation (data not shown). Since R_T is a measure of the ratio of exchange of tritium from pyruvate to water compared to the release of pyruvate from the enzyme ($R_T = k_{-3}/k_4$ in Scheme 1), these results mean the relative amount of pyruvate enolization that occurs, compared to the release of products, increases with increasing pH and is somewhat dependent on the divalent cation. Very low R_T values were observed in the absence of Fru-1,6-P_2 for all divalent cation investigated, suggesting that it plays a crucial role in allowing significant reversal of the proton transfer step at elevated pH. The role of Fru-1,6-P_2 will be considered in more detail later.

The dependence on pH and divalent cation of the rate of yeast pyruvate kinase-catalyzed pyruvate enolization (v_{xT}) is also very similar (in the presence of Fru-1,6-P_2) to that observed for the muscle enzyme. v_{xT} in the presence of Mn^{2+} rises at slightly higher pH with the yeast enzyme and does not show the plateau observed with the muscle enzyme around pH 8 [10]. The pH-profile for the Mg^{2+}-enzyme is virtually identical for the two enzymes. The pH-profile for Co^{2+}-yeast pyruvate kinase shows very low v_{xT} values with a maximum of 1.2 μmol/min per mg at pH 7.4 (data not shown). The maximum v_{xT} for Co^{2+}-activated muscle pyruvate kinase is also about pH 7.4, but the rate is approximately 10-fold the rate for the yeast enzyme.

The requirements for pyruvate enolization catalyzed by yeast pyruvate kinase were shown to be more stringent than for the muscle enzyme. Whereas inorganic phosphate or a series of anions were able to replace ATP to promote pyruvate enolization by muscle pyruvate kinase [9], there is a strict requirement for ATP in the yeast pyruvate kinase catalyzed enolization reaction. This strict requirement is observed in spite of the fact that phosphoryl transfer from ATP cannot be required for pyruvate enolization due to the differences in the

relative rates of the two reactions. The fact that neither P_i, nor ADP, nor ADP-CH_2-P could replace ATP in this function suggests a strict steric requirement for ATP in the pyruvate enolization reaction. Thus, although the mechanism by which yeast pyruvate kinase catalyzes its physiologically important reaction is similar to the mechanism of rabbit muscle pyruvate kinase, the yeast enzyme appears more stringent in its substrate requirements.

Another goal of this study was to investigate the role of Fru-1,6-P_2 in the activity of yeast pyruvate kinase. A review of the effect of Fru-1,6-P_2 on different parameters of enzyme activity (Table V) reveals that Fru-1,6-P_2 must be involved in the catalytic steps in addition to substrate-binding steps. When Mg^{2+} is the divalent cation, Fru-1,6-P_2 has a large effect on all parameters measured (except V of the forward reaction). Fru-1,6-P_2 is involved in substrate binding, as evidenced by its effect on $S_{0.5}$ for phosphoenolpyruvate. The effect of Fru-1,6-P_2 on R_T and v_{xT} implies involvement at other steps in the mechanism. Fru-1,6-P_2 could increase R_T by increasing the rate of pyruvate enolization (step −3) and/or by decreasing the rate of product release from the enzyme (step +4). The effect on v_{xT} also suggests that pyruvate binding (step −4) and an enolization (step −3) are accelerated by Fru-1,6-P_2. Since it increases all the partial reactions in the presence of Mg^{2+}, one cannot determine from these data at which steps the Fru-1,6-P_2 is most active.

The effects of Fru-1,6-P_2 on Mn^{2+}-activated yeast pyruvate kinase yield more information. In this case it has no effect on binding of phosphoenolpyruvate as measured by $S_{0.5}$, but affects some of the partial reactions. Fru-1,6-P_2 has a strong effect on R_T and v_{xP} with the Mn^{2+}-activated enzyme, but has only a small effect on v_{xT}. Since any substrate binding effects of Fru-1,6-P_2 should affect v_{xT} and v_{xP} equally, it is clear that at least part of the effect is on a catalytic step of the reaction.

In summary, Fru-1,6-P_2 is involved in the binding of substrates to yeast pyruvate kinase and in addition affects the rates of the catalytic steps of the enzyme. When Mg^{2+} is the divalent cation, Fru-1,6-P_2 greatly enhanced all measured reactions. When Mn^{2+} is used, some steps become much less sensitive to Fru-1,6-P_2 activation; the binding of substrates and the enolization of pyruvate are largely independent of Fru-1,6-P_2 under these conditions. Similarly, the flu-

TABLE V

SUMMARY OF THE EFFECTS OF FRU-1,6-P_2 ON YEAST PYRUVATE KINASE ACTIVITY

Parameter	Effect of Fru-1,6-P_2 with	
	Mg^{2+} as divalent cation	Mn^{2+} as divalent cation
$V_{forward}$ *	no effect	no effect
$S_{0.5}$ *	large decrease	small decrease
R_T	large increase	large increase
V_{xT}	large increase	small increase
V_{xP}	large increase	large increase

* Data from LeBlond and Robinson [11]; $S_{0.5}$ is with respect to phosphoenolpyruvate.

orokinase and hydroxylamine kinase reactions are shown to be little affected by the presence of Fru-1,6-P_2 when Mn^{2+} is the activating divalent cation [11]. Since the sensitivity to Fru-1,6-P_2 appears to depend on the concentration of Mn^{2+}, it is reasonable to suggest that Mn^{2+} activates the partial reactions in a manner that cannot be accomplished with Mg^{2+}

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