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SECONDARY KINASE REACTIONS CATALYZED BY YEAST PYRUVATE KINASE *

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Summary

1. Yeast pyruvate kinase (EC 2.7.1.40) catalyzes, in addition to the primary, physiologically important reaction, three secondary kinase reactions, the ATP-dependent phosphorylations of fluoride (fluorokinase), hydroxylamine (hydroxylamine kinase) and glycolate (glycolate kinase).

2. These reactions are accelerated by fructose-1,6-bisphosphate, the allosteric activator of the primary reaction. With Mg^{2+} as the required divalent cation, none of these reactions are observed in the absence of fructose-bisphosphate. With Mn^{2+}, fructose-bisphosphate is required for the glycolate kinase reaction, but merely stimulates the other reactions.

3. The effect of other divalent cations and pH on the three secondary kinase reactions was also examined.

4. Results are compared with those obtained for muscle pyruvate kinase and the implications of the results for the mechanism of the yeast enzyme are discussed.

Introduction

The primary, physiologically important reaction catalyzed by rabbit muscle pyruvate kinase (EC 2.7.1.40) involves phosphoryl transfer from phosphoenolpyruvate to ADP, forming ATP and enolpyruvate, and proton transfer from water to enolpyruvate forming ketopyruvate. The phosphoryl and proton

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Abbreviations used are: MES, 2(*N*-morpholino)ethane sulfonate; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulfonate; TAPS, tris(hydroxymethyl)methylaminopropane sulfonate.

transfers are likely to be sequential during the course of the primary reaction [1]. Proton transfer can proceed in the absence of phophoryl transfer, as in the enolization of pyruvate activated by P_i-like dianions [2]. Furthermore, phosphoryl transfer can be separate and independent of proton transfer, as in three secondary kinase reactions: the fluorokinase reaction, in which ATP phosphorylates fluoride ion [3]; the hydroxylamine kinase reaction, whereby ATP phosphorylates the oxygen of hydroxylamine [4]; and the glycolate kinase reaction, in which the hydroxyl of glycolate is phosphorylated by ATP [5]. Each of these reactions requires, as does the primary reaction, a monovalent and divalent cation activator (e.g. K^+ or NH_4^+ and Mg^{2+}, Mn^{2+}, or Co^{2+} respectively). In addition, the fluorokinase and hydroxylamine kinase reactions require bicarbonate as an effector.

Yeast pyruvate kinase, unlike the rabbit muscle enzyme, it susceptible to allosteric activation by Fru-1,6-P_2 [6,7]. As such, it represents a class of pyruvate kinases subject to feedforward regulation, a class which includes the predominant liver enzyme in mammals [8,9], the enzyme from many tissues in lower animals [10—13] and from some bacteria [14,15]. The present communication reports the existance of the three secondary kinase reactions catalyzed by yeast pyruvate kinase, their requirements for Fru-1,6-P_2 and other characteristics. An accompanying paper [16] deals with the proton transfer reactions catalyzed by yeast pyruvate kinase.

Experimental procedure

The preparation of yeast pyruvate kinase was based on the procedure of Röschlau and Hess [17]. The procedure was modified in the following ways: Red Star Baker's Yeast (obtained fresh locally) was used as starting material, the extract was fractionated between 45 and 50% saturated ammonium sulfate, rather than 40—50%, valine (1 mM) was included in all chromatography buffers (suggested by the effects of valine in renaturing the yeast enzyme [18]), chromatography on DEAE-cellulose was at pH 7.0 instead of 6.5, and the G-100 Sephadex chromatography was replaced by G-200. The enzyme was not crystallized but was homogeneous on polyacrylamide gel electrophoresis in presence and absence of sodium dodecyl sulfate and on cellulose acetate sheets. The following activities were not present at more than 0.01% that of pyruvate kinase, aldolase, fructose diphosphatase, hexokinase, adenosine triphosphatase, adenylate kinase, phosphoenolpyruvate phosphatase, pyruvate decarboxylase, adenosine diphosphatase, and lactate dehydrogenase. A specific activity of 382 μmol ATP synthesized/min per mg protein was obtained, which approaches that recently reported [19,20]. The kinetic parameters of the forward reaction agree quantitatively with those reported by Haeckel et al. [21] and Hunsley and Suelter [7]. The extinction coefficient at 280 nm used for the purified enzyme was that of the latter investigators.

The enzyme was stable for over six months as a precipitate in 80% saturated ammonium sulfate at 5°C and enzyme so stored was used within six months of preparation. The enzyme was unstable upon dilution in buffer, with valine (1mM), bovine serum albumin (1 mg/ml), and glycerol (50%) providing increasing protection against inactivation. The diluent used routinely contained 50%

glycerol, 0.1 mM EDTA, and 10 mM MES (pH 6.2). The enzyme was stable for at least a week at 5°C when stored under these conditions. All assays were performed in the presence of 1 mg/ml bovine serum albumin to ensure stability of the enzyme during the course of the assays. Assays of the primary reaction were performed at 26°C using spectrophotometric coupling to lactate dehydrogenase.

The [γ-^{32}P]ATP was prepared according to the method of Glynn and Chappell [22] and stored in aliquots at −22°C until needed. The preparation was spectrally pure and less than 2% of the label was in the β-position, assessed by reaction with hexokinase and glucose, followed by polyethyleneimine-cellulose chromatography. All secondary kinase reactions were measured at 30°C as ^{32}P transferred from [γ-^{32}P]ATP to [^{32}P]-non-nucleotide (not charcoal adsorbable), as described by Switzer [23]. Experimental values were corrected for controls omitting either fluoride, hydroxylamine, glycolate or enzyme, as no differences were observed between any of these controls. For each secondary reaction, the assay was shown to be linear with time and enzyme concentration in the region used. Between 50 000 and 100 000 cpm of [γ-^{32}P]ATP were used in each assay. The time intervals used routinely were 15 to 30 min.

All other components were the best grade available commercially and were used without further purification.

Results

Yeast pyruvate kinase was tested for its ability to catalyze the fluoro-, hydroxylamine, and glycolate kinase reactions in the presence of either Mg^{2+} or Mn^{2+}. The results of such a study (Table I) indicate that yeast pyruvate kinase catalyzes all reactions in the presence of Fru-1,6-P_2, but no reaction is observed in the absence of Fru-1,6-P_2 for any of the Mg^{2+}-dependent reactions or for Mn^{2+}-dependent glycolate kinase. In all other Mn^{2+}-dependent reactions, Fru-1,

TABLE I

VELOCITY OF YEAST PYRUVATE KINASE SECONDARY KINASE REACTIONS

Effects of fructose-bisphosphate and Mg^{2+} or Mn^{2+}.

	μmol ATP consumed/min per mg		
	Fru-1,6-P_2	Mg^{2+}	Mn^{2+}
Fluorokinase [a]	+	0.277	0.085
	—	<0.001	0.043
Hydroxylamine kinase [a]	+	0.344	0.836
	—	<0.001	0.691
Glycolate kinase [b]	+	0.063	0.279
	—	<0.001	<0.001

[a] Conditions: 100 mM TAPS (pH 8.0) 200 mM $KHCO_3$, 5 mM ATP, 15 mM $MgCl_2$, or 5 mM $MnCl_2$, 100 mM NH_2OH or 32 mM KF, 0.5 mg bovine serum albumin, 137 μg yeast pyruvate kinase, 2 mM Fru-1,6-P_2 where indicated (final vol. 0.5 ml).

[b] Conditions: 100 mM TAPS (pH 7.8), 100 mM KCl, 5 mM ATP, 15 mM $MgCl_2$ or 5 mM $MnCl_2$, 25 mM glycolate, 0.5 mg bovine serum albumin, 137 μg yeast pyruvate kinase, 2 mM Fru-1,6-P_2 where indicated (final vol. 0.5 ml).

TABLE II

SPECIFICITY OF FLUOROKINASE REACTION FOR DIVALENT CATION

Conditions: 100 mM TES (pH 7.5), 200 mM $KHCO_3$, 10 mM ATP, 32 mM KF, 2 mM Fru-1,6-P_2, 0.5 mg bovine serum albumin, 133 μg yeast pyruvate kinase, and either 15 mM $MgCl_2$, 15 mM $CoCl_2$, 15 mM $NiCl_2$, 5 mM $MnCl_2$, or 1 mM $Zn(acetate)_2$ (final vol. 0.5 ml).

Divalent cation	μmol ATP consumed/min per mg
Mg^{2+}	0.156
Mn^{2+}	0.100
Co^{2+}	0.001
Ni^{2+}	0.013
Zn^{2+}	0.021

6-P_2 proved stimulatory. Each of the secondary kinase reactions proceeds at less than 1% the rate of the primary reaction. Despite these low velocities, these activities are not attributable to a contaminating enzyme in view of their dependence on Fru-1,6-P_2. For each reaction, the divalent cation requirement, pH dependence, and Fru-1,6-P_2 activation were further characterized.

Fluorokinase

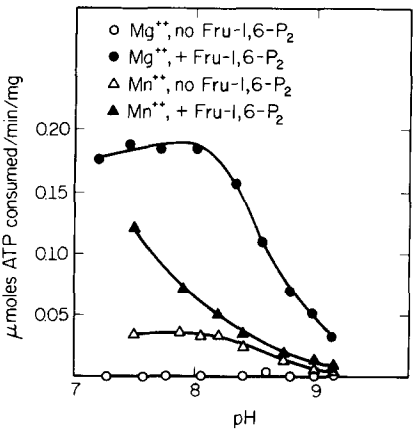
Various divalent cations were tested for their ability to support the fluorokinase reaction (Table II). The reaction showed a preference for Mg^{2+} with M^{2+} exhibiting a lower rate, followed by Zn^{2+} and Ni^{2+}. Co^{2+} showed little or no activity. The pH-profiles of the Mg^{2+}- and Mn^{2+}-dependent fluorokinase reactions are shown in Fig. 1. The pH-profiles were not extended below pH 7 because of CO_2 evolution. The profiles are distinct and indicate that in the presence of Fru-1,6-P_2, the rate with Mg^{2+} exceeds that with Mn^{2+} at each pH value. Fru-1,6-P_2 is absolutely required in the Mg^{2+}-dependent reaction across the pH range tested, while it merely stimulates the Mn^{2+}-dependent reaction. The bicarbonate dependence of the reaction was also examined at pH 7.9 with Mg^{2+} and Fru-1,6-P_2; 24% of the rate is observed in the absence of added bicarbonate. An absolute requirement cannot be ruled out because no effort was extended to assure that all solutions were stringently free from bicarbonate. The effect of divalent ion concentration on fluorokinase was also examined and Fig. 2 shows that while Mn^{2+} is more effective at lower concentration, the maximal rate

TABLE III

SPECIFICITY OF HYDROXYLAMINE KINASE REACTION FOR DIVALENT CATION

Conditions: 100 mM TES (pH 7.5), 200 mM $KHCO_3$, 100 mM NH_2OH, 5 mM ATP, 2 mM Fru-1,6-P_2, 0.5 mg bovine serum albumin, 274 μg yeast pyruvate kinase (55 μg for Mn^{2+} assay), and either 15 mM $MgCl_2$, 15 mM $CoCl_2$, 15 mM $NiCl_2$, 5 mM $MnCl_2$ or 5 mM $Zn(acetate)_2$ (final vol. 0.5 ml).

Divalent cation	μmol ATP consumed/min per mg
Mg^{2+}	0.054
Mn^{2+}	0.723
Co^{2+}	0.028
Ni^{2+}	0.001
Zn^{2+}	0.039



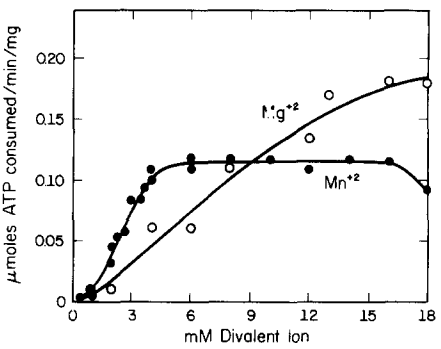


Fig. 1. Fluorokinase activity as a function of pH. Conditions: 100 mM buffer (TES from pH 7.2 to 8 and TAPS from pH 8 to 9.2), 10 mM ATP, 200 mM $KHCO_3$, 32 mM KF, 15 mM $MgCl_2$, 0.5 mg bovine serum albumin, 135 μg yeast pyruvate kinase, and 2 mM Fru-1,6-P_2 where indivcated (final vol. 0.5 ml). For Mn^{2+}-dependent activity, 5 mM ATP and 5 mM $MnCl_2$ were substituted.

Fig. 2. Fluorokinase activity as a function of divalent ion concentration. Conditions: 100 mM TES (pH 7.4), 200 mM $KHCO_3$, 10 mM ATP, 32 mM KF, 2 mM Fru-1,6-P_2, 0.5 mg bovine serum albumin, 133 μg yeast pyruvate kinase and either $MnCl_2$ or $MgCl_2$, varied as indicated (final vol. 0.5 ml).

attained with Mg^{2+} is greater. The concentrations routinely used in these studies (15 mM Mg^{2+} and 5 mM Mn^{2+}) are shown to be optimal.

Hydroxylamine kinase

Various divalent cations were tested for their ability to promote the hydroxylamide kinase reaction (Table III). A difference in pH accounts for the differences between Tables I and III. These results at pH 7.5 indicate that Mn^{2+} is by far superior, followed by Mg^{2+}, Co^{2+}, and Zn^{2+}. Ni^{2+} showed little or no activity. Examination revealed that 5 mM Mn^{2+}, 5 mM ATP, 100 mM NH_2OH, 200 mM $KHCO_3$, and 2 mM Fru-1,6-P_2 were the practical optima for the reaction, although the hydroxylamine was not saturating. The requirement for bicarbonate was nearly absolute as less than 2% of the activity was measured in the absence of added bicarbonate (pH 7.6, Mn^{2+} and Fru-1,6-P_2). The pH-

TABLE IV

DEPENDENCE OF GLYCOLATE KINASE REACTION ON DIVALENT METAL ION

Conditions: 100 mM TAPS (pH 8.2), 100 mM KCl, 10 mM ATP, 25 mM glycolate, 2 mM Fru-1,6-P_2 where indicated, 0.5 mg bovine serum albumin, 134 μg yeast pyruvate kinase, and 15 mM $MgCl_2$, 15 mM $MnCl_2$, 15 mM $CoCl_2$, 15 mM $NiCl_2$, or 5 mM $Zn(acetate)_2$ (final vol. 0.5 ml).

Metal ion	μmol ATP consumed/min per mg	
	+ Fru-1,6-P_2	— Fru-1,6-P_2
Mg^{2+}	0.105	0.001
Mn^{2+}	0.439	0.001
Co^{2+}	0.332	0.001
Ni^{2+}	0.001	0.001
Zn^{2+}	0.001	0.001

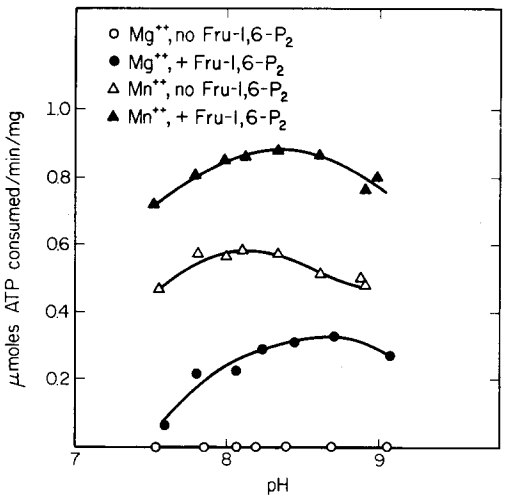


Fig. 3. Hydroxylamine kinase activity as a function of pH. Conditions: 100 mM buffer (TES between pH 7.5 and 8; TAPS between pH 8 and 9), 15 mM $MgCl_2$, 5 mM ATP, 100 mM NH_2OH, 200 mM $KHCO_3$, 0.5 mg bovine serum albumin, 137 μg yeast pyruvate kinase, and 2 mM Fru-1,6-P_2 where indicated (final vol. 0.5 ml). For Mn^{2+}-dependent activity, 5 mM $MnCl_2$ and 55 μg enzyme were substituted.

profiles for the hydroxylamine kinase reaction in the presence of Mg^{2+} and Mn^{2+} are shown in Fig. 3. In the case of the Mg^{2+}-dependent reaction, the pH-optimum is much sharper and the requirement for Fru-1,6-P_2 extends across the pH range. At each pH value, the rates with Mn^{2+} exceed those with Mg^{2+}.

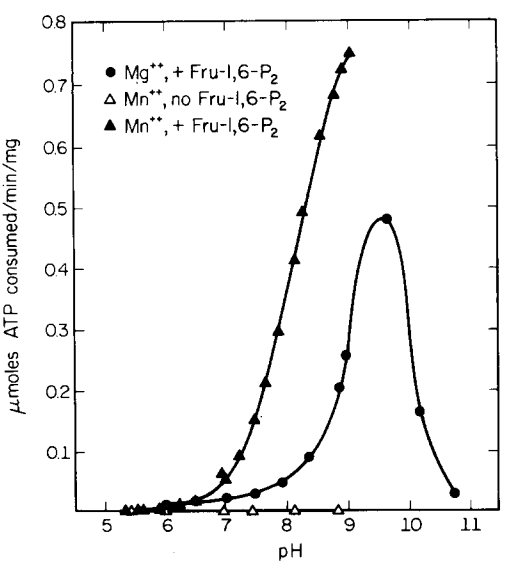


Fig. 4. Glycolate kinase activity as a function of pH. Conditions: 100 mM buffer (MES was used between pH 5.3 and 7; TES between pH 7 and 8; TAPS between 8 and 9; and cyclohexylpropane sulfonate between 9 and 11), 100 mM KCl, 15 mM $MgCl_2$, 10 mM ATP, 25 mM glycolate, 0.5 mg bovine serum albumin, 133 μg yeast pyruvate kinase, and 2 mM Fru-1,6-P_2 where indicated (final col. 0.5 ml).

Glycolate kinase

The rate of the glycolate kinase reaction in the presence of various divalent cations is compared in Table IV. Mn^{2+} is the most effective, followed by Co^{2+} and Mg^{2+}. Ni^{2+} and Zn^{2+} show little or no activity. No activity is observed in the absence of Fru-1,6-P_2 for any of these metals. The pH-profiles for the Mg^{2+}- and Mn^{2+}-dependent reactions are shown in Fig. 4. With Mg^{2+}, a sharp optimum is observed around pH 9.5. With Mn^{2+} the rate increases with increasing pH to a maximum above pH 9 (measurements above which were prevented by precipitation of the cation). The pH-profile with Co^{2+} (not shown) displays a broad peak centered around pH 9 and peak activity of 0.41 μmol ATP consumed/min per mg. Of the three cations, Mn^{2+} produces the highest rates, whereas Mg^{2+} and Co^{2+} produce peak rates of 60% and 50% respectively of that with Mn^{2+} at pH 9.

Primary reaction

In view of the variety of responses to divalent ions exhibited by the secondary kinase reactions, their effects on the primary reaction were examined. With Mg^{2+}, the kinetic parameters obtained compare favorably with those for other preparations of yeast pyruvate kinase (Table V). The enzyme exhibits sigmoid kinetics with respect to phosphoenolpyruvate in the absence of Fru-1,6-P_2 and hyperbolic kinetics in its presence. K-type [24] of activation is observed with the $S_{0.5}$ value for phosphoenolpyruvate changing by a factor of 20—30. With Mn^{2+}, however, hyperbolic kinetics are observed in the presence and absence of Fru-1,6-P_2 (Table VI); the $S_{0.5}$ and Hill slopes are unaffected by the presence of Fru-1,6-P_2, while the V is increased. A V-type [24] of activation is observed. Table VI also compares kinatic parameters for the other divalent cations. In all cases except Mn^{2+}, Fru-1,6-P_2 affects the $S_{0.5}$ value and Hill slope. The V for the Co^{2+} reaction is increased by Fru-1,6-P_2, while the rates observed with Ni^{2+} and Zn^{2+} are quite low in the presence and absence of Fru-1,6-P_2; the low level of Zn^{2+} used gave the optimal rates for that metal ion. Mn^{2+} appears to be unique among the divalent cations in making the primary reaction less sensitive to the allosteric modifier, Fru-1,6-P_2.

TABLE V

COMPARISON OF Mg^{2+}-ACTIVATED PRIMARY REACTION FOR VARIOUS YEAST PYRUVATE KINASE PREPARATIONS

In this preparation, the conditions were: 100 mM MES (pH 6.2), 10 mM ADP, variable amounts of phosphoenolpyruvate, 150 mM KCl, 15 mM $MgCl_2$, 0.2 mM NADH, 3 units of lactate dehydrogenase, 1 mg bovine serum albumin, 0.12 μg yeast pyruvate kinase and 2 mM Fru-1,6-P_2 where appropriate (total vol. 1.0 ml). $S_{0.5}$ and n_H are with respect to phosphoenolpyruvate.

Parameter	This preparation	Hunsley and Suelter [7]	Haeckel et al. [21]	Aust et al. [19]
V (μmol/min per mg)	400	219	200	400—500
n_H (— Fru-1,6-P_2)	2.6	2.85	2.6	—
n_H (+ Fru-1,6-P_2)	0.95	0.94	0.91	—
$S_{0.5}$ (— Fru-1,6-P_2) (mM)	3.2	2	3.6	—
$S_{0.5}$ (+ Fru-1,6-P_2) (mM)	0.11	0.125	0.12	0.099

TABLE VI

KINETIC PARAMETERS OF PRIMARY REACTION AS A FUNCTION OF DIVALENT METAL

Conditions: 100 mM MES (pH 6.2), 150 mM KCl, 10 mM ADP, variable levels of phosphoenolpyruvate, 0.2 mM NADH, 1 mg bovine serum albumin, 3 units of lactate dehydrogenase, appropriate quantities of yeast pyruvate kinase, 2 mM Fru-1,6-P_2 where indicated, and either 15 mM $MgCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$ or 0.15 mM $Zn(acetate)_2$ (total vol. 1.0 ml).

Divalent cation	V *		$S_{0.5}$ **		n_H **	
	— Fru-1,6-P_2	+ Fru-1,6-P_2	— Fru-1,6-P_2	+ Fru-1,6-P_2	— Fru-1,6-P_2	+ Fru-1,6-P_2
Mg^{2+}	400	400	3.2	0.11	2.63	0.95
Mn^{2+}	100	126	0.06	0.04	0.85	0.87
Co^{2+}	118	166	0.48	0.03	1.30	0.68
Ni^{2+}	3.5	4.0	4.30	0.07	2.20	1.00
Zn^{2+}	1.5	1.5	9.10	0.22	2.30	1.30

* V is given as μmol/min per mg.

** $S_{0.5}$ and n_H are with respect to phosphoenolpyruvate; $S_{0.5}$ is given in mM.

Discussion

Yeast pyruvate kinase, like the rabbit muscle enzyme, is shown to catalyze three secondary kinase reactions, fluorokinase, hydroxylamine kinase and glycolate kinase. The versatility of the active site seems to have been preserved in these evolutionary divergent species. Fig. 5 illustrates the similarities in structure which account for this versability. Each reaction may be considered a nucleophilic attack on the terminal phosphorus of ATP. For substrates like fluoride ion and hydroxylamine which lack a carboxyl group, bicarbonate appears to be a cofactor, presumably acting by occupying the subsite for the carboxylate of phosphoenolpyruvate or pyruvate (and glycolate). An occupied carboxylate subsite could be essential for conformational changes associated with these catalyses.

While the primary reaction velocities are comparable for the rabbit muscle and yeast enzymes, the secondary kinase reactions of yeast pyruvate kinase are slower than the rabbit enzyme secondary reactions. The velocities of fluorokinase, hydroxylamine kinase, and glycolate kinase are, respectively, 2, 2, and 3 μmol ATP consumed/min per mg rabbit muscle enzyme [3—5]. The corresponding values reported in this paper for the yeast enzyme are 0.2, 0.9, and 0.8. The value for fluorokinase was the most disparate. These lower values suggest that the yeast enzyme is more selective, a characteristic also noted for the pyruvate enolization [16].

The role of the divalent metal ion in catalysis is not indicated in Fig. 5, although proton relaxation-rate measurements suggest the formation of enzyme/metal/substrate bridge complexes for both the muscle [25] and yeast enzymes [26]. The data is consistent with divalent ion coordination to the phosphoryl being transferred. Differences in divalent cation specificity for the kinase reactions catalyzed by the muscle enzyme are observed. Fluorokinase is reported to be activated by Mg^{2+} and Mn^{2+}, but not Zn^{2+} (pH 7.0) [27]; hydroxylamine kinase is active with Zn^{2+}, Co^{2+}, and Mn^{2+} but not Mg^{2+} (pH 6.5)

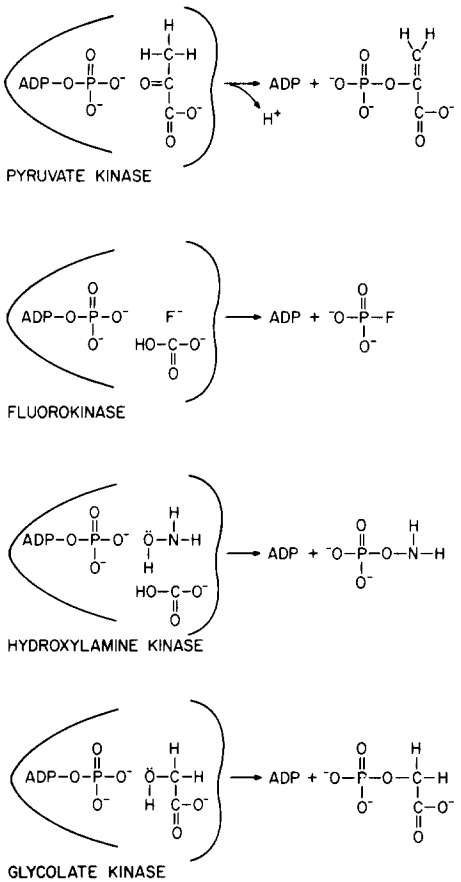


Fig. 5. Comparison of the primary and secondary kinase reactions of pyruvate kinase.

[28]; glycolate kinase is most active with Mn^{2+}, while Co^{2+} and Mg^{2+} show 24 and 11% the rate with Mn^{2+} (pH 8.2) [5]; for the primary reaction of muscle pyruvate kinase, Mg^{2+}, Mn^{2+}, and Co^{2+} and, to a much smaller extent Ni^{2+}, are effective, while Zn^{2+} is inactive (pH 7.5) [29]. Cottam et al. [4] have emphasized that Zn^{2+} is effective in the hydroxylamine kinase reaction. They reason that it, like Mn^{2+} but unlike Mg^{2+}, readily forms complexes with the nitrogen of hydroxylamine, polarizes the N-O bond, and thus facilitates nucleophilic attack on the terminal phosphoryl of ATP. This generalization clearly cannot be extended to yeast pyruvate kinase, where Zn^{2+} is even less effective than Mg^{2+} in promoting the hydroxylamine kinase reaction (Table III). The other kinase reactions catalyzed by yeast pyruvate kinase show a general similarity in divalent cation specificity to those of the muscle enzyme. The pH-profiles for each secondary kinase reaction reveal differences due to metal ion and indicate that quantitative and qualitative comparisons can be pH-dependent. Studies with the muscle enzyme have largely overlooked the likely possibility that similar differences may exist with that enzyme.

Fructose-1,6-bisphosphate is an allosteric activator of yeast pyruvate kinase. The Fru-1,6-P_2-activated enzyme resembles the muscle enzyme in numerous

ways, including kinetics with respect to phosphoenolpyruvate [6,7]. This paper emphsizes other similarities, namely that the Fru-1,6-P_2-activated yeast enzyme catalyzes the secondary kinase reactions when Mg^{2+} is the divalent cation, while the enzyme unactivated does not. The enzyme is much less dependent on Fru-1,6-P_2 with Mn^{2+} as the metal ion, when only glycolate kinase is shown to require Fru-1,6-P_2 (Table I). As will be shown [16], the phosphorylation of pyruvate similarly requires Fru-1,6-P_2. These differences in activator requirement for the various kinase reactions may relate to the carboxyl-binding subsite of the enzyme and to binding differences between HCO_3^- (which is not attached to the fluoride or hydroxylamine) and the glycolate or pyruvate carboxyl (which is convalently attached to the group being phosphorylated). It has been suggested that Fru-1,6-P_2 modifies the carboxyl-binding subsite [26]. When that subsite is filled with HCO_3^-, as in the fluorkinase and hydroxylamine kinase reactions, modification may be unnecessary for the catalysis when Mn^{2+} is present. When that subsite is filled by the carboxyl of glycolate or pyruvate, phosphorylation of these molecules may require considerable modification at the subsite.

While the study reported in this paper has emphasized the secondary kinase reactions catalyzed by yeast pyruvate kinase, kinetic parameters of the primary reaction (forward velocity) were also examined as a function of divalent metal ion (Table VI). Fru-1,6-P_2 *greatly reduces the* $S_{0.5}$ for phospheoenolpyruvate and corresponding Hill coefficient in the presence of Mg^{2+}, while with Mn^{2+} no such effects are observed; it merely stimulates the V by 26%. Similar behavior has been noted for the pyruvate kinases of *Mucor rouxii* [30] and of several mammalian tissues [31,32]. The failure of Cottam et al. [26] to note this effect with the yeast enzyme must be attributed to their levels of Mn^{2+} (1 mM), which were lower than the ADP used. With Co^{2+}, Fru-1,6-P_2 shifts the V, $S_{0.5}$ and n_H values; with Ni^{2+} and Zn^{2+}, while the rates are low, $S_{0.5}$ and n_H values appear to be primarily altered by Fru-1,6-P_2.

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