PYRUVATE KINASE CATALYZED PHOSPHORYLATION OF GLYCOLATE

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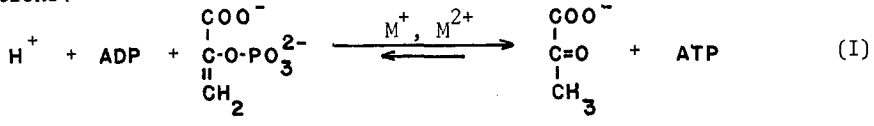
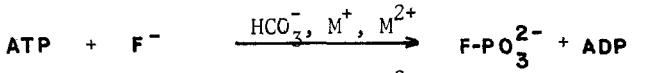
SUMMARY

Rabbit muscle pyruvate kinase has been found to catalyze the phosphorylation of the hydroxyl group of glycolate by ATP. The products were characterized as P-glycolate and ADP by NMR spectroscopy and chromatography respectively. The maximal velocity is of the same order of magnitude as that for the phosphorylation of pyruvate, the "normal" reverse reaction of this enzyme. The apparent K_M for glycolate is 2.3 mM and the reaction is apparently analogous to the other known side reactions of this enzyme. The product might be produced in any system with a moderate level of pyruvate kinase and low phosphatase activity.

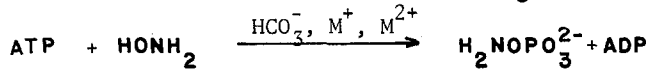
Rabbit muscle pyruvate kinase (E.C. 2.7.1.40) catalyzes the following reactions:



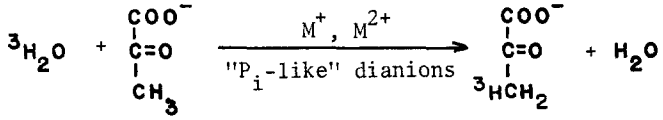
(I)



(II) (1)



(III) (2)



(IV) (3)

Reactions (II) and (III) are analogous to the reverse of the normal physiological reaction (I), whereas reaction (IV) apparently involves formation of enolpyruvate, a potential intermediate in the overall reaction (3). In fact, although (II-IV) and the reverse of (I) display different divalent cation requirements, the maximal velocities for these reactions are all in the range

of 1-10 μmoles/min/mg protein at 30^0 and pH's between 7 and 8. Hence, a fairly narrow range of phosphorylation (or proton exchange) rate constants must be operative in the overall phosphoryl transfer mechanism.

Other substitutes for the three-carbon substrate in reaction (I) have been investigated. A number of workers (4-8) have shown that substitution at C-3 of pyruvate or P-enolpyruvate results in pseudosubstrates with little or no ability to participate in phosphoryl transfer. Changes at the carboxylate group apparently eliminate the ability of the molecule to bind and act as either substrate or inhibitor of the enzyme. A two-carbon candidate, oxalate, was shown to be a strong inhibitor of the normal reaction and apparently was not phosphorylated (9). These observations suggested the likelihood that a C-2 hydroxyl analog might be phosphorylated by the enzyme. This work reports the catalysis by pyruvate kinase of the phosphorylation of glycolate by ATP.

MATERIALS AND METHODS

Pyruvate kinase was isolated from frozen rabbit muscle (Pel-Freeze Biologicals, Rogers, Ark.) by the method of Teitz and Ochoa (1) as described previously (10). The preparation had a specific activity of ca. 225 μmoles of product formed per minute per mg protein at pH 7.5 and 30° as measured by the coupled lactic dehydrogenase assay (10). Tris, ATP and ADP were purchased from Sigma, glycolic acid from Calbiochem and the tricyclohexylammonium salt of phosphoglycolic acid from General Biochemicals. Reactions were monitored with a Beckman DB spectrophotometer for the lactic dehydrogenase assay, or with a Radiometer pH-stat (TTT 1c/SBR2C). Proton NMR spectra were recorded on a Varian HR-220 spectrometer after two lyophilizations of the materials and dilution in ^2H_2O.

RESULTS

Addition of microgram quantities of pyruvate kinase (dialyzed against 0.1 M $(CH_3)_4NCl$, 0.05 M $(CH_3)_4$-N^+ N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate pH 7.5) to a solution of: $(CH_3)_4N^+$-glycolate, ATP, Mg^{2+} and K^+ results

in (1) the glycolate-dependent production of ADP as measured by NADH oxidation through the lactic dehydrogenase system when this is added either with P-enolpyruvate after a fixed time, or simultaneously with the glycolate; and (2) the release of a proton, measured by the amount of NaOH required for the pH-stat to maintain a constant pH. Figure 1 shows NMR spectra which identify

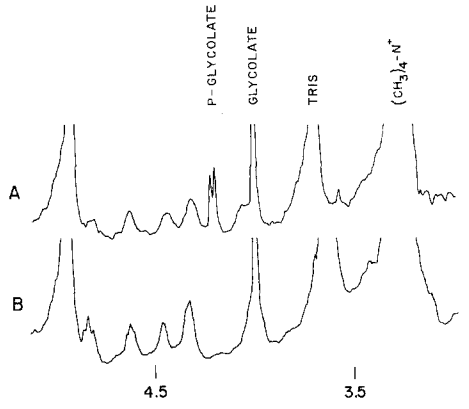


Figure 1. NMR spectra of P-glycolate formation reaction. 220 MHz NMR spectra traced from 10-transient Fourier transformed output, with chemical shifts referenced to $(CH_3)_4N^+$ (3.267 ppm downfield from DSS) A. 100 mM KCl, 50 mM Tris HCl, 50 mM $(CH_3)_4$-N-glycolate, 20 mM $MgCl_2$, 20 mM ATP, pH ca. 8.5 plus 0.6 mg pyruvate kinase. B. As in A minus pyruvate kinase.

the product of the reaction as P-glycolate. The characteristic ^{31}P-splitting of the methylene protons and the chemical shift of this group match those obtained for a known sample of tricyclohexyl ammonium P-glycolate. Thin layer chromatography on PEI-cellulose acetate sheets developed in 1 M LiCl, showed ADP to be the chief nucleotide product.

Table I shows the relative abilities of various divalent cations to activate the reaction as measured by the pH stat assay. The enzyme concentrations were adjusted so that low absolute rates were measured (ca. 6 mM titrant) because of the considerable product inhibition expected for P-glycolate [i.e. P-glycolate has a low K_i (K_D) for pyruvate kinase (11)]. In the absence of K^+ (+ 100 mM $(CH_3)_4$-N^+) or a divalent cation, the observed velocities were less

Table I. Relative Velocity of Glycolate Phosphorylation*

Additions	pH	$V_{initial}$
100 mM K^+, 5 mM Mn^{2+}	8.0	100
100 mM$(CH_3)_4N^+$, 5 mM Mn^{2+}	"	< 1
25 mM K^+, 5 mM Mn^{2+}	"	41
100 mM K^+, 5 mM Mg^{2+}	"	11
100 mM K^+, 5 mM Co^{2+}	"	24
100 mM K^+, 5 mM Ni^{2+}	"	4
100 mM K^+, 2.5 mM Mn^{2+}	"	80
100 mM K^+, 20 mM Mn^{2+}	"	73
100 mM K^+, 5 mM Mn^{2+}	7.5	37
100 mM K^+, 5 mM Mn^{2+}	8.5	> 180
100 mM K^+, 5 mM Mn^{2+} + 0.2 mM oxalate	8.0	< 1

* pH stat assay, 30°C in the presence of 2 mM ATP and 25 mM $(CH_3)_4N$-glycolate

than 1% that measured in the presence of 100 mM K^+ + 5 mM Mn^{2+}. In the presence of 0.2 mM oxalate, the velocity was less than 1% of that measured in its absence (25 mM glycolate). The kinetic constants for glycolate obtained from the Lineweaver-Burke plot shown in Fig. 2 are a K_M value of 2.3 mM, and a V_{MAX} of approximately 3 μmoles/min/mg.

DISCUSSION

The results demonstrate that the 2-OH group of glycolate can be phosphorylated in a reaction catalyzed by rabbit muscle pyruvate kinase. The similarities of cofactor requirements and of product and oxalate inhibition for the glycolate and normal reactions suggest that the glycolate reaction takes place at the "normal" active site of this enzyme. The velocity is of the same order of magnitude as the other "side" reactions previously mentioned. These observations raise the question of the mechanism whereby the enzyme (and divalent or monovalent cation) adjusts the nucleophilicity of the

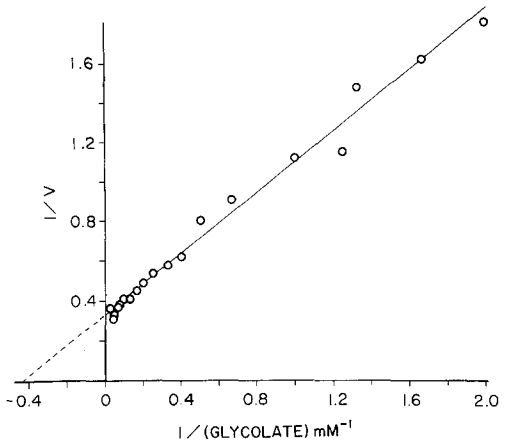


Figure 2. Double reciprocal plot of P-glycolate formation measured by the pH-stat assay. Initial conditions were: KCl, 100 mM; $MnCl_2$, 5 mM; ATP, 2 mM; pH 8.0, 30°. 25-75 µg pyruvate kinase were added to start the reaction. Initial velocities are given as µmoles H^+/min/mg protein required to maintain pH 8.0. Approximate stoichiometry was above 0.9 mole H^+/mole substrate.

attacking group of F^-, $NONH_2$, enolpyruvate or glycolate to approximately the same extent relative to the γ-phosphoryl of ATP. An additional constraint on the reaction scheme is that H_2O cannot substitute as the phosphoryl acceptor. Since only very low ATPase activity has ever been observed (1,2) for pyruvate kinase, the enzyme must effectively block the H_2O attack. However, one H_2O molecule or OH^- may be coordinated to the divalent cation (12). If either of these species are involved in transmitting the polarizing effect of the divalent cation, as has been suggested for the catalysis of ^3H-exchange (3), its coordination must be such that in the absence of a potential substrate, it itself can not act as a phosphoryl acceptor.

At present there is no indication for any physiological significance to the glycolate reaction in mammalian systems. However, the low free energy of hydrolysis of P-glycolate would create a very favorable equilibrium for its formation, while a favorable K_M for glycolate and significant velocity of the enzyme-catalyzed reaction would allow a considerable rate of production under appropriate conditions. It obviously could be found in any sys-

tem with a moderate level of pyruvate kinase and low, or controlled, phosphatase activity. This last factor would be especially important in any consideration of the possibility of P-glycolate formation in the cytoplasm of higher plants.

ACKNOWLEDGEMENTS

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