Biochimica et Biophysica Acta, 384 (1975) 120—126

BBA 67448

CHEMICAL SPECIFICITY OF PYRUVATE KINASE FROM YEAST

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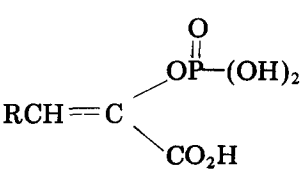
(Received August 12th, 1974)
(Revised manuscript received October 28th, 1974)

Summary

Three analogs of phosphoenolpyruvic acid: (Z)-phosphoenol-3-fluoropyruvate, (Z)-phosphoenol-3-bromopyruvate and (Z)-phosphoenol-α-ketobutyrate were found to be substrates for yeast pyruvate kinase (ATP : pyruvate (Z)-*O*-phosphotransferase, EC 2.7.1.40) with maximal velocities much greater than those found for rabbit muscle pyruvate kinase. The analogs exhibited sigmoidal kinetics, which become hyperbolic upon addition of the allosteric effector, fructose 1,6-diphosphate. Moreover, the reaction of (Z)-phosphoenol-3-bromopyruvate with ADP to produce bromopyruvic acid and ATP irreversibly inhibited the enzyme with a half-life of 32 min.

Introduction

Muscle pyruvate kinase has been shown to be a chemically specific enzyme reacting extremely slowly with substrate analogs of phosphoenolpyruvic acid and exhibiting hyperbolic kinetics [1—3]. On the other hand, yeast and liver pyruvate kinases exhibit sigmoidal kinetics with respect to phosphoenolpyruvate and are allosterically affected by fructose 1,6-diphosphate [4,5]. In light of the differences in kinetics due to the differences in biochemical function of muscle and liver, we decided to study the substrate specificity of yeast pyruvate kinase with the hope of elucidating the topography at the active site.



R = CH_3, Br, F

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Each of the analogs above was tested as a potential substrate for the yeast pyruvate kinase reaction. Recent work by Hess et al. [5], reporting that bromopyruvate irreversibly inhibits pyruvate kinase by alkylation of three sulfhydryl groups per subunit of enzyme, enhanced our interest in (Z)-phosphoenol-3-bromopyruvate. Since (Z)-phosphoenol-3-bromopyruvate is a substrate for yeast pyruvate kinase, the bromopyruvate generated is at the active site and hence it was hoped that only one of the three sulfhydryl groups would be alkylated. This could provide information about the amino acids at the active site which has thus far been elusive.

Materials and Methods

Pyruvate kinase (ATP : pyruvate (Z)-*O*-phosphotransferase, EC 2.7.1.40) from yeast was isolated by the procedure described by Hess et al. [6] to a specific activity ranging from 30 to 50 μmol/min per mg at 25° C as determined by the coupled-assay procedure of Tietz and Ochoa [7]. Attempts to further purify the enzyme using the Cibracon Blau affinity chromatography method [8] were unsuccessful. The yeast was purchased from Sigma, YSC-1 No. 71C-8511. Protein was determined by the Lowry procedure using lysozyme as a standard. Chemicals used as starting materials for the syntheses were all purchased from commercial sources in the highest purity available and the synthesis of analogs themselves carried out as described previously [1,2].

Assay procedures

The activity of pyruvate kinase, with substrate analogs of phosphoenolpyruvic acid, was measured using the coupled assay procedure with excess lactate dehydrogenase [7]. All assays were run at 25° C. The standard assay mixture contained 10 mM ADP, 30 mM $MgSO_4$, 0.05 M potassium phosphate (pH 6.0), 0.25 mM NADH, 77 I.U. of desalted lactate dehydrogenase with 1—10 λ of stock solution of pyruvate kinase (3 mg/ml), diluted to a final volume of 1 ml.

The assay solutions used to determine the allosteric effects of fructose 1,6-diphosphate contained 0.5 mM ADP, 2.5 mM $MgSO_4$, 0.05 M potassium phosphate (pH 6.0), 1.0 mM fructose 1,6-diphosphate, 0.25 mM NADH, 77 I.U. of desalted lactate dehydrogenase, 1—10 λ of a stock solution of pyruvate kinase (approx. 3 mg/ml), diluted to a final volume of 1 ml.

Results

The activity and kinetic nature of the pyruvate kinase interaction with three substrate analogs, (Z)-phosphoenol-3-bromopyruvate, (Z)-phosphoenol-3-fluoropyruvate, and (Z)-phosphoenol-α-ketobutyrate was determined. In particular, concentrations of the analogs ranging from 10 mM to 0.01 mM were incubated with the standard assay mixture and decrease in absorbance of NADH was followed spectrophotometrically using a Cary 14. The amount of pyruvate kinase used varied from 1 to 10 λ depending upon the stock solution (approx. 3 mg/ml) prepared that day. ADP (0.01 M) was required for maximal velocity in all cases. This result is similar to that described by Haeckel et al.

TABLE I

ACTIVITY OF PYRUVATE KINASE FROM YEAST WITH SUBSTRATE ANALOGS

K_m is the concentration of substrate required to reach half-maximal velocity.

All assay mixtures contained 10 mM ADP, 30 mM $MgSO_4$, 0.05 M potassium phosphate (pH 6.0), 0.25 mM NADH, 77 I.U. desalted lactate dehydrogenase, 1—10 λ of a stock solution of pyruvate kinase (2.0 mg/ml, spec. act. 50 μmol/min per mg) diluted to a final volume of 1 ml. All assays were run at 25° C.

Analog	V (μmol/min per mg)	% relative rate (phosphoenolpyruvate = 100%)		K_m
		Yeast pyruvate kinase	Rabbit muscle pyruvate kinase [1]	
Phosphoenolpyruvate	50	100	100	$2.1 \cdot 10^{-3}$
(Z)-Phosphoenol-3-bromopyruvate	16.8	30	0.19	$2.0 \cdot 10^{-3}$
(Z)-Phosphoenol-3-fluoropyruvate	2.5	5.0	0.23	$4.0 \cdot 10^{-4}$
(Z)-Phosphoenol-α-ketobutyrate	0.14	0.3	0.065	$5.0 \cdot 10^{-4}$

[5]. The results of these assays are presented in Table I. K_m value is the concentration of substrate required to reach half-maximal velocity under standard conditions. At high substrate concentrations, greater than 5 mM, substrate inhibition was observed in all cases.

Two control experiments, as described previously [1], were performed to show that the pyruvate kinase preparation was not contaminated with phosphatase and that the analogs were not hydrolyzed non-enzymatically. Product studies using polyethylenimine-cellulose thin-layer chromatography [1,2], substantiated the substrate nature of phosphoenolpyruvate analogs.

In addition, other studies were carried out to determine the effects of fructose 1,6-diphosphate on the kinetics of the reaction. Non-saturating conditions were used where 0.5 mM ADP and 2.5 mM $MgSO_4$ replaced 10 mM ADP and 30 mM $MgSO_4$ in the standard assay mixture. In the case of all three analogs, the presence of 1.0 mM fructose 1,6-diphosphate dramatically increased the rate of the reaction and changed the sigmoidal curvature exhibited by the interaction of substrate analogs and pyruvate kinase, to a hyperbolic curvature (Figs 1a and 1b).

During the kinetics studies, it was noticed that pyruvate kinase in the presence of (Z)-phosphoenol-3-bromopyruvate seemed to lose activity much more rapidly than loss of enzyme activity due to dilution. As a result of this observation and the paper reported by Hess et al. implicating a sulfhydryl group at the active site of pyruvate kinase, we decided to continue our studies on the interaction of pyruvate kinase and (Z)-phosphoenol-3-bromopyruvate. A sulfhydryl group has a pK_a of approx. 9.0 and is a better nucleophile at pH 7.5 than at pH 6.0. Inhibition studies were therefore carried out at pH 7.5 so that covalent bond formation between sulfhydryl group and bromopyruvate, generated by substrate turnover, would be facilitated.

(Z)-Phosphoenol-3-bromopyruvate (concentrations ranging from $1 \cdot 10^{-2}$ to $1 \cdot 10^{-4}$ M) was incubated at 25° C in a solution containing 0.01 M potassium phosphate (pH 7.5), 10 mM ADP, 30 mM $MgSO_4$ and 20 λ of pyruvate kinase

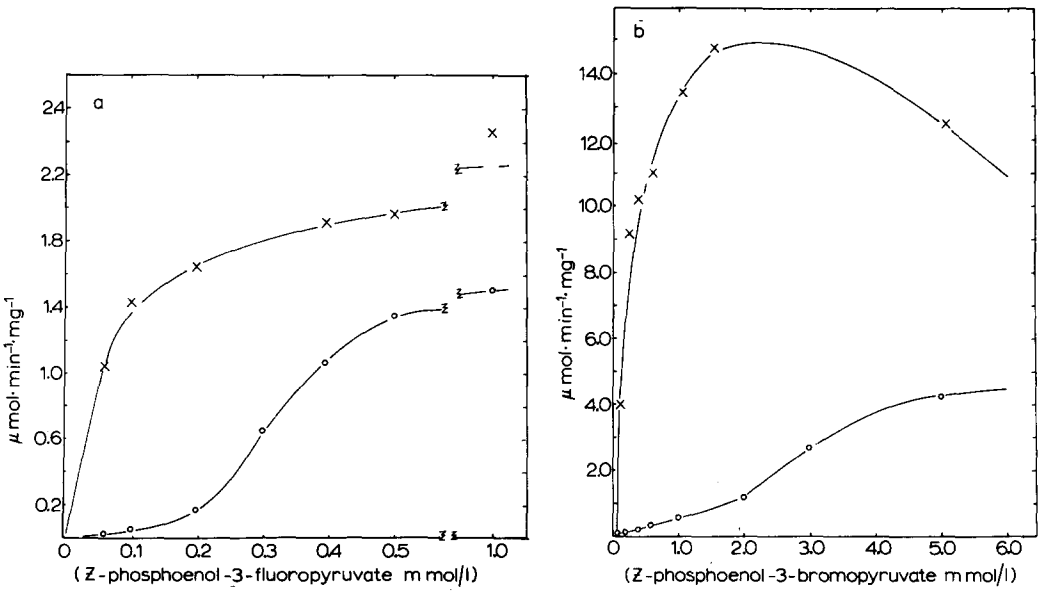

Fig. 1. (Z)-Phosphoenol-3-fluoropyruvate and (Z)-phosphoenol-3-bromopyruvate interaction with pyruvate kinase. Assay conditions: 0.5 mM ADP, 2.5 mM $MgSO_4$, 0.05 M potassium phosphate (pH 6.0), 0.25 mM NADH, 77 I.U. of desalted lactate dehydrogenase, (a) (Z)-phosphoenol-3-fluoropyruvate, 10 λ of pyruvate kinase (3.7 mg/ml, spec. act. 50 μmol/min per mg) diluted to a final volume of 1 ml. (b) (Z)-phosphoenol-3-bromopyruvate, 5 λ of pyruvate kinase (2.9 mg/ml, spec. act. 50 μmol/min per mg) diluted to a final volume of 1 ml. o———o, in absence of fructose 1,6-diphosphate; X———X, in presence of fructose 1,6-diphosphate.

from a stock solution (3 mg/ml), and diluted to a final volume of 1 ml. Aliquots were taken from this incubation mixture at fixed intervals and added to the standard assay mixture. When high substrate concentrations were employed (>5 mM) there was an initial burst due to the built-up bromopyruvate followed by turnover due to reaction with pyruvate kinase. Inactivation of pyruvate kinase increased with time until after 60 min only 5% of the initial activity remained. During this time period a blank was run under identical conditions in which phosphoenolpyruvate replaced (Z)-phosphoenol-3-bromopyruvate. After 60 min 98% of its initial activity remained.

The problem of initial burst due to built-up bromopyruvate was eliminated by removal of bromopyruvate using a Sephadex G-25 gel filtration column. The inhibition studies with 3 and 5 mM (Z)-phosphoenol-3-bromopyruvate (incubated with 0.01 M potassium phosphate (pH 7.5), 10 mM ADP, 30 mM $MgSO_4$ and 20 λ of pyruvate kinase (3 mg/ml)) were repeated. Aliquots were taken every 10 min and transferred to a Sephadex G-25 column pre-equilibrated with 0.01 M potassium phosphate buffer (pH 6.0). The fractions collected contained pyruvate kinase freed from bromopyruvate. The fractions were then assayed for activity. As a control experiment a sample of pyruvate kinase incubated with phosphoenolpyruvic acid in place of the analog was treated in the same manner.

Both inhibition studies gave similar results. These results are displayed in Fig. 2 and show and initial lag period followed by pseudo first-order kinetics.

Inhibition studies, analogous to those described above, were also carried

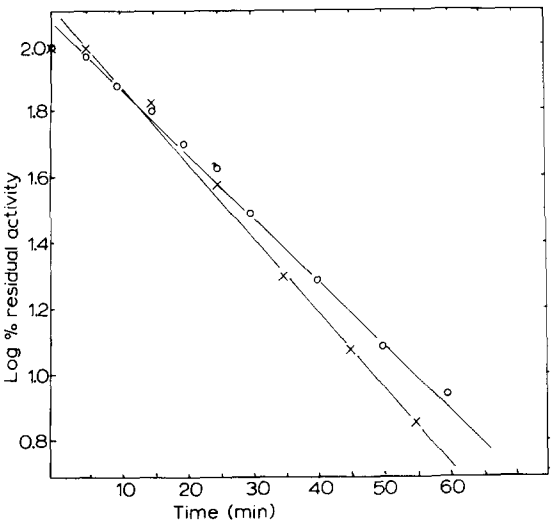

Fig. 2. Inhibition studies with (Z)-phosphoenol-3-bromopyruvate. Incubation mixture, at 25°C, contained 10 mM ADP, 30 mM $MgSO_4$, 0.05 M potassium phosphate (pH 7.5), and 20 λ of pyruvate kinase from a stock solution (2.9 mg/ml, spec. act. 40 μmol/min per mg) to a final volume of 1 ml. Aliquots (10 λ) from this mixture were taken at intervals and assayed for pyruvate kinase activity as described in the results. ○———○, 3 mM (Z)-phosphoenol-3-bromopyruvate; X———X, 5 mM (Z)-phosphoenol-3-bromopyruvate.

out at pH 6.0. The rate of formation of bromopyruvate from 5 mM (Z)-phosphoenol-3-bromopyruvate at pH 6.0 is 2.5 times the rate at pH 7.5. The half-life of inactivation, however, is 60 min in the former case and 32 min in the latter case.

Discussion

The kinetics experiments described in Figs 1a and 1b show that the (Z)-phosphoenol-3-bromopyruvate and (Z)-phosphoenol-3-fluoropyruvate behave much like phosphoenolpyruvic acid. All the analogs, including (Z)-phosphoenol-α-ketobutyrate, exhibit sigmoidal kinetics under non-saturating conditions, which become hyperbolic in the presence of fructose 1,6 diphosphate. The K_m values of these substrate analogs are dramatically influenced by this allosteric effector.

From the kinetics studies described in Table I, the chemical specificity of yeast pyruvate kinase is quite different from rabbit muscle pyruvate kinase [1,2]. While (Z)-phosphoenol-3-bromopyruvate reacts 1/500 the rate of the normal substrate with muscle enzyme [1], it reacts one-third the rate of the normal substrate with yeast enzyme. Both (Z)-phosphoenol-3-fluoropyruvate and (Z)-phosphoenol-αketobutyrate react at greater rates with the yeast enzyme, compared with the enzyme from rabbit muscle.

It should be pointed out that the K_m values for the normal substrate and (Z)-phosphoenol-3-bromopyruvate are very similar. The K_m values for (Z)-phosphoenol-3-fluoropyruvate and (Z)-phosphoenol-α-ketobutyrate are an order of magnitude lower and the rates of reaction are significantly lower. It was expected that (Z)-phosphoenol-3-fluoropyruvate would behave much like

phosphoenolpyruvate because of their steric similarities. H and F are approximately the same size. The apparent difference in K_m values and rates of reaction may be due to the difference in electronegativities of H and F.

In addition to (Z)-phosphoenol-3-bromopyruvate being the most reactive substrate, it is the only analog which generates a reactive alkylating agent at the active site of pyruvate kinase. However, several pieces of evidence from the inhibition studies seem to indicate the bromopyruvate, generated at the active site, behaves as a non-specific alkylating agent. As illustrated in Fig. 2, there is an initial lag time of inhibition of pyruvate kinase with (Z)-phosphoenol-3-bromopyruvate followed by a pseudo first-order reaction. These results are very similar to those reported by Hess et al. [5] and Coon et al. [9] who used non-specific sulfhydryl alkylating agents and bromopyruvate. In addition, at pH 6.0 where bromopyruvate is generated more rapidly, the inhibition studies showed a decreased rate of inactivation. It appears from these data that bromopyruvate generated at the active site dissociates very quickly before being attacked by a nucleophile at the active site. Sulfhydryl-group titrations with 5,5′-thiobis(2-nitrobenzoic acid) must be carried out on the extensively purified protein before definite conclusions can be drawn.

Furthermore, under conditions in which the yeast enzyme was totally inactivated, similar experiments performed with the rabbit muscle pyruvate kinase (Stubbe, J.A. and Kenyon, G.L., unpublished) showed no noticeable inactivation. This result may be due to the low turnover rate of (Z)-phosphoenol-3-bromopyruvate with muscle enzyme.

Inhibition studies attempted with (Z)-phosphoenol-3-fluoropyruvate, which generates fluoropyruvate at the active site, showed no inactivation. The fact that the bromo group is a much better leaving group than the fluoro group, and that fluoropyruvate is similar in size to pyruvate and may dissociate very rapidly from the active site (K_m pyruvate = $1 \cdot 10^{-2}$ M) [3], accounts for these observations.

Conclusions

One should note, in conclusion, that when designing a K_{cat} inhibitor [10] such as (Z)-phosphoenol-3-bromopyruvate, it is necessary to consider both K_i, rate of covalent bond formation, and K_d, rate of dissociation from the enzyme inhibitor complex. In the case of yeast pyruvate kinase, K_d is quite large and no nucleophile is available in the immediate vicinity of the phosphoenolpyruvate binding site.

However, there are other phosphoenolpyruvate-requiring systems which might benefit from study with (Z)-phosphoenol-3-bromopyruvate, where the K_d is small or K_i is large. Deoxy-3-arabinoheptulosonate-7-phosphate synthetase is irreversibly inhibited by bromopyruvate acting as a phosphoenolpyruvate analog [11—13]. Enolpyruvylshikimate-3-phosphate synthetase [14] and UDP-*N*-acetylglucosamine enolpyruvyltransferase [15] are thought to involve pyruvyl intermediates generated at the active site prior to product formation. Moreover, nucleophiles are thought to be adjacent to these phosphoenolpyruvate binding sites. In all three cases, (Z)-phosphoenol-3-bromopyruvate is of potential use in identifying amino acid residues at active sites of these enzymes.

Finally, the results of these experiments clearly indicate that there is a difference in chemical specificities between the yeast enzyme and muscle enzyme. These differences are evident when studying the relative reaction rates of the analogs with the enzymes and the inhibition studies with (Z)-phosphoenol-3-bromopyruvate. It is of interest to note that pyruvate kinase from normal and undifferentiated cancerous liver differ with respect to both kinetics and reactivity with non-specific sulfhydryl alkylating agents. Differences in chemical specificities of these isoenzymes may exist as in the case of yeast and rabbit muscle enzyme.

Acknowledgements

We thank John A. Lowe and Gordon J. Sata for preparative assistance. We also wish to thank Ciba-Geigy, Basel, Switzerland, for Cibracon Blau. This work was supported in part by a grant from the Alfred P. Sloan Foundation to Williams College.

References

1 Stubbe, J.A. and Kenyon, G.L. (1971) Biochemistry 10, 2669—2677
2 Stubbe, J.A. and Kenyon, G.L. (1972) Biochemistry 11, 338—345
3 Boyer, P.D. (1973) The Enzymes, Vol. 8, p. 353, 3rd edn, Academic Press, New York
4 Tanaka, T., Imamura, K., Nishina, T., Nakashima, K. and Miwa, S. (1973) J. Biochem. Tokyo 74, 1165—1172
5 Haeckel, R., Hess, B., Lauterborn, W. and Wöster, K.H. (1968) Hoppe-Seyler's Z. Physiol. Chem. 349, 699—714
6 Wieker, H.J. and Hess, B. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 1877—1893
7 Tietz, A. and Ochoa, S. (1958) Arch. Biochem. Biophys. 78, 477—493
8 Röschlau, P. and Hess, B. (1972) Hoppe-Seyler's Z. Physiol. Chem. 353, 441—443
9 Flashner, M., Hollenberg, P.F. and Coon, M.J. (1972) J. Biol. Chem. 247, 8114—8121
10 Rando, R.R. (1974) Science 185, 320—324
11 Staub, M. and Dénes, G. (1967) Biochim. Biophys. Acta 139, 519—521
12 DeLeo, A.B. and Sprinson, D.B. (1968) Biochem. Biophys. Res. Commun. 32, 873—877
13 DeLeo, A.B., Dayan, J. and Sprinson, D.B. (1973) J. Biol. Chem. Tokyo 248, 2344—2353
14 Bondinell, W.E., Vnek, J., Knowles, P.F., Sprecher, M. and Sprinson, D.B. (1971) J. Biol. Chem. 246, 6191—6196
15 Cassidy, P.J. and Kahan, F.M. (1973) Biochemistry 12, 1364—1373