Biochimica et Biophysica Acta, 293 (1973) 125–133

BBA 66794

STEREOSELECTIVE REDUCTION OF PYRUVATE BY SODIUM BOROHYDRIDE CATALYZED BY PYRUVATE KINASE

TIMOTY M. PHILLIPS, GEORGE W. KOSICKI AND DONALD E. SCHMIDT, JR
Department of Chemistry, University of Windsor, Windsor, Ontario (Canada)

(Received August 4th, 1972)

SUMMARY

Sodium borohydride reduction of pyruvate, carried out in the presence of rabbit muscle pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), Mg^{2+} or Mn^{2+}, and Na^{+}, produces lactic acid in which the D-isomer is predominant. No stereoselective reduction of pyruvate is seen when Ca^{2+} is used as divalent metal ion. Pyruvate kinase-catalyzed reduction of pyruvate by $NaBH_4$ does not occur in the absence of added divalent metal ion; a racemic mixture of lactic acid is produced by reduction. The activity of pyruvate kinase from rabbit muscle, alone or in the presence of phosphoenolpyruvate, ADP, or pyruvate, is not affected by $NaBH_4$. Saturating amounts of phosphoenolpyruvate abolish stereoselective reduction of pyruvate. However, saturating amounts of ADP do not abolish stereoselective reduction. Pyruvate kinase, previously inactivated with trinitrobenzene sulfonate is incapable of catalyzing the formation of excess D-lactic acid during the reduction.

INTRODUCTION

The reduction of pyruvate by $NaBH_4$ in the active site of oxaloacetate decarboxylase (EC 4.1.1.3) has been used to indicate the stereochemistry of binding of pyruvate to this enzyme. The technique also yields information about the mechanism of this enzymic reaction[1].

Using this technique, we have studied the stereochemistry of binding of pyruvate to pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) and the effect of metal ions and of certain small organic molecules on the ability of pyruvate kinase to catalyze the stereoselective reduction of pyruvate by $NaBH_4$. A preliminary account of this study has been published[2].

MATERIALS AND METHODS

Materials

Pyruvate kinase (rabbit muscle) and lactate dehydrogenase (pig heart) (EC 1.1.1.27) were commercial preparations (C. F. Boehringer). Sodium pyruvate, phos-

phoenolpyruvate (sodium salt), and NADH (disodium salt) were purchased from C. F. Boehringer. D-Lactic acid (Grade D-1, crystalline) and ADP (Grade 1, sodium salt, 95–100%) were purchased from Sigma Chemical Co. Acetylpyridine NAD was purchased from P-L Biochemicals. Na_2 EDTA, $CaCl_2$, $MgCl_2$, $MnCl_2$, KCl, NaCl, $NaBH_4$, and triethanolamine were reagent grade (Fisher). Tris was Fisher primary standard. Trinitrobenzene sulfonate was purchased from Pierce Chemical Co. Bovine serum albumin (crystalline A Grade) from Calbiochem was used. Silica gel plates for thin-layer chromatography contained fluorescent indicator and were manufactured by E. Merck, Darmstadt.

Assay of pyruvate kinase activity

The activity of pyruvate kinase was measured by following the production of pyruvate from phosphoenolpyruvate using a coupled lactate dehydrogenase system and measuring the decrease in absorbance at 340 nm. Each cuvette contained 50 μmoles triethanolamine·HCl (pH 7.6); 8.9 μmoles Na_2EDTA; 67 μmoles KCl; 7.3 μmoles $MgCl_2$; 0.16 μmole phosphoenolpyruvate; 0.16 μmole ADP; 0.06 μmole NADH; and approximately 0.36 unit of lactate dehydrogenase made up to a final volume of 1.00 ml with deionized water. After incubation of the above system for 2 or 3 min at 30 °C, pyruvate kinase was added.

Reduction procedure

To 4.0 ml of 0.25 M triethanolamine·HCl (pH 7.6) containing the noted amounts of Na_2 EDTA, metal salts, substrates, and pyruvate kinase of a specific activity within the range of 130–140 units/mg of enzyme were added $NaBH_4$ (8.0 ml of 0.03 M solution in $1 \cdot 10^{-3}$ M NaOH) and sodium pyruvate (8.0 ml of 0.1 M solution adjusted to pH 6.0 with solid potassium bicarbonate). The reduction was carried out at room temperature; a peristaltic pump was used to maintain the slow, constant rate of addition of $NaBH_4$ and sodium pyruvate over a period of 40 to 80 min. The reaction solution was acidified with concentrated HCl to destroy excess $NaBH_4$ and to inactivate the enzyme. Lactic acid was analyzed as described below.

The buffer used for enzyme assay and borohydride reduction contained 8.9 mM Na_2 EDTA. This chelating agent is required to prevent precipitation of Mn^{2+} from solution as $Mn(OH)_2$. The enzyme is catalytically active even though the concentration of EDTA exceeds the concentration of divalent metal ion added to the buffer. This was reported by Wood[3] and confirmed under identical conditions in our experiments. When no divalent metal ion is added and when a large excess of EDTA (28.9 mM) is used, the enzyme is not catalytically active and stereoselective reduction is not observed. Control experiments were conducted in the absence of either pyruvate kinase or divalent metal ion; these experiments indicate that changes in activity of pyruvate kinase were independent of the presence of EDTA under the conditions of our assay. Rather, the activity of pyruvate kinase was observed to depend on the type of divalent metal ion present.

Purification of lactic acid

Lactic acid was purified from the reduction mixtures as follows. The acidified reduction mixtures were evaporated to dryness. The residue was extracted with four 20-ml portions of diethyl ether. The ethereal solutions were then evaporated to

a sticky residue which was dissolved in 2 ml of 2 M NaOH. Each of these 2-ml samples was then applied to three silica gel plates and developed for 2 h in 150 ml of a solution of ethanol–ammonium hydroxide–water (80:4:16, by vol.). The lactate bands were visually located by ultraviolet light and were scraped from the plates. Lactic acid was extracted from the silica gel by suspending the silica gel in 5 ml of water and then centrifuging. This was followed by two washes of the silica gel with water. These aqueous solutions were acidified and concentrated before enzyme assay for L-lactic acid and optical rotatory dispersion measurement.

Assay of L-*lactic acid*

Quantitative measurement of L-lactic acid was accomplished using an enzyme assay method. L-Lactic acid is converted to pyruvate in the presence of lactate dehydrogenase and 3-acetylpyridine NAD. This analog of NAD greatly favors the formation of pyruvate in the oxidation of L-lactic acid by lactate dehydrogenase. The reduction of the coenzyme is recorded as an increase in absorbance at 365 nm. A standard solution of L-lactic acid is assayed to determine the efficiency of the assay method. Each cuvette contained 100 μmoles of Tris–HCl (pH 9.0), 0.57 μmole of 3-acetylpyridine NAD (made up in 0.1 M Tris–acetate (pH 8.3)), and 45 units of pig heart lactate dehydrogenase (made up in 0.02 M potassium phosphate (pH 7.5), and containing 1 mg/ml of bovine serum albumin) made up to a final volume of 1.00 ml with deionized water. After incubation of the above system for 2 or 3 min at 30 °C, an aliquot containing L-lactic acid was added.

Optical rotatory dispersion measurement

Optical rotatory dispersion measurements were carried out on the aqueous acidified lactic acid solutions using a Cary 60 spectropolarimeter. The amount of excess lactic acid isomer is calculated from the dispersion spectrum of a standard solution of D-lactic acid. Based on the sensitivity of the instrument and the specific rotation of D-lactic acid, the ORD data allows the detection of one μmole of excess D-lactic acid per ml or greater.

RESULTS

With a variety of metal ions, pyruvate kinase is stable in the presence of sodium borohydride. Retention of enzymic activity also occurs to a large extent if $NaBH_4$ treatment is carried out using pyruvate kinase which has been preincubated with pyruvate, phosphoenolpyruvate, or ADP (Table I).

Pyruvate kinase, in the presence of Mg^{2+}, catalyzes a stereoselective reduction of pyruvate by $NaBH_4$. The resulting solution contains a 10% excess of D-lactic acid (Table II). Omission of either pyruvate kinase or Mg^{2+} in the incubation mixture produces a racemic mixture of lactic acid. Stereoselective reduction occurs if Mn^{2+} is substituted for Mg^{2+} in the incubation mixture. Substitution of Ca^{2+} for Mg^{2+}, however, abolishes the ability of pyruvate kinase to catalyze a stereoselective reduction of pyruvate. In a control experiment, a racemic mixture of lactic acid was incubated with pyruvate kinase; using our isolation procedure, a racemic mixture of lactic acid was obtained. Therefore, the excess D-lactic acid found in the products of pyruvate kinase-catalyzed reduction of pyruvate by $NaBH_4$ is not the result of preferen-

TABLE I

EFFECT OF SODIUM BOROHYDRIDE ON PYRUVATE KINASE ACTIVITY

Two 0.4-ml aliquots of $NaBH_4$ (0.03 M in $1 \cdot 10^{-3}$ M NaOH) or two 0.4-ml aliquots of $1 \cdot 10^{-3}$ M NaOH were added at 2-min intervals to 0.4-ml 0.25 M triethanolamine·HCl (pH 7.6) containing the indicated components. After 2 additional minutes, an aliquot of the reaction mixture was assayed for pyruvate kinase activity using the coupled lactate dehydrogenase assay method. All values in mM unless otherwise indicated.

Reaction No.	*Pyruvate kinase units*	*EDTA*	*Mg^{2+}*	*Mn^{2+}*	*Ca^{2+}*	*K^+*	*Na^+*	*ADP*	*PEP***	*Pyruvate*	*$NaBH_4$*	*Relative activity (%)*
1	32.3	8.9	7.3			67						100
2	32.3	8.9	7.3			67					24	88
3	32.3*	8.9	7.3			67					24	93
4	32.3	8.9	7.3			67				80		108
5	32.3	8.9	7.3			67				80	24	85
6	32.3	8.9	7.3			67		39				89
7	32.3	8.9	7.3			67		39			24	85
8	32.3	8.9	7.3			67			39			95
9	32.3	8.9	7.3			67			39		24	95
10	169	28.9					67			80	24	84
11	169	8.9			7.3	67				80	24	83
12	161	8.9		7.3		67				80	24	73
13	161	8.9		7.3			67			80	24	81

* Added last.
** Phosphoenolpyruvate.

TABLE II

STEREOSELECTIVE REDUCTION OF PYRUVATE CATALYZED BY PYRUVATE KINASE

Each reaction vessel contained the components of the pH 7.6 buffer described in Materials and Methods and 8.9 mM EDTA except for Reaction 7 where no EDTA was present .The contents were analyzed as previously outlined.

Reaction No.	*Pyruvate kinase units*	*Mg^{2+} (mM)*	*Mn^{2+} (mM)*	*Ca^{2+} (mM)*	*K^{+} (mM)*	*Pyruvate μmoles × 10^{-2}*	*$NaBH_4$ μmoles × 10^{-2}*	*Lactic acid added M × 10^{2}*	*L-Lactic acid produced μmoles × 10^{-2}*	*ORD observed at 225 nm (°)*	*Excess D-lactic acid μmoles × 10^{-2}*	**Total lactic acid produced μmoles × 10^{-2}*	*% D-Lactic acid*
1	176	7.3				8.0	2.4		0.50	−0.074	0.23	1.2	59.3
2						8.0	2.4		0.44	0.00	0.0	0.88	50
3	221					8.0	2.4		1.4	0.00	0.0	2.8	50
4	176		7.3			8.0	2.4		0.62	−0.048	0.16	1.4	55.8
5	176			7.3		8.0	2.4		0.64	0.00	0.0	1.28	50
6	140	7.3			67			2.0 D(−) 2.0 L(+)		0.00	0.0	2.24	50
7								4.0 D(−)		−0.232			100

* Total lactic acid = 2 × L + excess D.

tial binding of L-lactic acid to the enzyme. In fact, calculations based on our data indicate that 1000 moles of L-lactic acid would have to bind to each mole of enzyme to observe a 10% excess of D-lactic acid.

When pyruvate kinase was treated with 1.0 mM trinitrobenzene sulfonate for 30 min, complete inactivation of the enzyme occurred[4] (Table III). This trinitrobenzene sulfonate-inactivated enzyme was unable to catalyze the stereoselective reduction of pyruvate by $NaBH_4$ (Table IV). Enzyme preincubated with ADP was only partially inactivated by 1.0 mM trinitrobenzene sulfonate after 30 min (Table III); this pyruvate kinase was able to catalyze the formation of some D-lactic acid during the reduction of pyruvate (Table IV). Other workers have suggested that trinitrobenzene sulfonate inactivates pyruvate kinase by reacting with a critical lysine residue in the nucleotide binding site of the enzyme[4]. Adenosine 5'-diphosphate protects pyruvate kinase from inactivation by shielding this critical lysine from attack by trinitrobenzene sulfonate.

TABLE III

EFFECT OF TRINITROBENZENE SULFONATE ON PYRUVATE KINASE ACTIVITY

Pyruvate kinase was incubated at 22 °C for 10 min in 0.08 M triethanolamine·HCl (pH 7.6) containing 0.015 M EDTA and ADP as indicated. Then, an equivalent amount of buffer containing 0.015 M EDTA and 1.0 mM trinitrobenzene sulfonate was added to the enzyme solution and incubation performed in the dark for 30 min at 22 °C. The solution was assayed for enzyme activity. An aliquot of each solution was immediately used as a source of pyruvate kinase for the reduction of pyruvate by $NaBH_4$ (see Table IV).

Reaction No.	*Pyruvate kinase units*	*ADP (mM)*	*Trinitrobenzene sulfonate (mM)*	*Pyruvate kinase activity remaining units*
1	169		1.0	0
2	169	10	1.0	130

When the reduction of pyruvate was performed in the presence of pyruvate kinase, Mg^{2+}, K^+ and saturating amounts of ADP[5], some excess D-lactic acid was produced. Under the conditions of reduction and in the presence of pyruvate kinase, Mg^{2+}, K^+, and saturating amounts of phosphoenolpyruvate, there was no evidence of stereoselective reduction (Table IV).

DISCUSSION

The reduction of pyruvate by $NaBH_4$ takes place in the absence of pyruvate kinase (Table II); however, this reduction is not stereoselective *i.e.* the expected racemic mixture of the D- and L-isomers of lactic acid is produced. The reduction of pyruvate by $NaBH_4$ is, however, stereoselective in the presence of enzyme since an excess of D-lactic acid is produced (Table II). It appears that pyruvate kinase confers an asymmetry on enzyme-bound pyruvate. This enzyme-induced asymmetry allows hydride attack on the carbonyl carbon of pyruvate from only one side of the pyruvate molecule. Most of the pyruvate, however, is not reduced on the enzyme surface but is non-stereoselectively reduced in the bulk of the solution. This non-stereoselective

TABLE IV

EFFECT OF SMALL ORGANIC MOLECULES ON THE BINDING OF PYRUVATE TO PYRUVATE KINASE

Each reaction vessel contained the components of the pH 7.6 buffer described in Materials and Methods and 8.9 mM EDTA, 7.3 mM Mg^{2+}, and 67 mM K^{+}. The contents were analyzed as previously outlined.

Reaction No.	*Pyruvate kinase units*	*PEP*** *(mM)*	*ADP (mM)*	*Pyruvate μmoles × 10^{-2}*	*$NaBH_4$ μmoles × 10^{-2}*	*L-Lactic acid produced μmoles × 10^{-2}*	*ORD observed 225 nm (°)*	*Excess D-lactic acid μmoles × 10^{-2}*	***Total lactic acid produced μmoles × 10^{-2}*	*% D-Lactic acid*
1	0*			8.0	2.4	0.81	0.00	0.0	1.6	50
2	130*			8.0	2.4	0.88	−0.028	0.22	2.0	55.5
3	580		24	8.0	2.4	0.50	−0.035	0.19	1.2	58.1
4	198	5.0		8.0	2.4	1.56	0.00	0.0	3.1	50

* Pyruvate kinase previously reacted with trinitrobenzene sulfonate was used as a source of pyruvate kinase (see Table III).
** Total lactic acid = 2 × L + excess D.
*** Phosphoenolpyruvate

reduction produces a 50/50 racemic mixture of lactic acid. The excess of D-isomer represents the portion of pyruvate reduced on the enzyme surface.

The configuration of the lactic acid produced by reduction of enzyme-bound pyruvate is an indicator of the stereochemistry of enzyme–pyruvate interaction. D-Lactic acid is the isomer produced if hydride attack on the carbonyl carbon of pyruvate occurs from the 2-si side of the pyruvate molecule[6]. Rose[7] has shown that pyruvate kinase facilitates addition of a proton to C-3 of phosphoenolpyruvate on the 2-si face. Protonation of α-ketobutyrate enol phosphate by pyruvate kinase in deuterium oxide occurs from the 2-si face[8]. Magnetic resonance studies have shown that pyruvate kinase has an 18-fold greater affinity for D-phospholactate than for L-phospholactate[9]. D-Phospholactate is the isomer that would be obtained by reduction of the C–C double bond of phosphoenolpyruvate on the 2-si face of the phosphoenolpyruvate molecule. Phosphoenolpyruvate carboxylase (EC 4.1.1.31), phosphoenolpyruvate carboxytransphosphorylase (EC 4.1.1.38), and phosphoenolpyruvate carboxykinase (EC 4.1.1.32) have the same chirality with respect to phosphoenolpyruvate binding[10]. Pyruvate bound to oxaloacetate decarboxylase is also reduced by $NaBH_4$ on the 2-si face[1].

Enzyme-induced stereoselective reduction takes place only in the presence of divalent cation (Table II). It is interesting to note that Mg^{2+} or Mn^{2+} may function in this regard. Magnesium ion or manganese ion is known to activate the enzyme for the enzymic phosphoryl group transfer reaction[11,12]. Ca^{2+} does not function as a replacement for Mg^{2+} or Mn^{2+} either in phosphoryl group transfer or in stereoselective reduction. There appears to be a close parallel between the ability of the divalent ion to function in enzymic phosphoryl group transfer and enzymic stereoselective reduction.

Pyruvate kinase was completely inactivated by trinitrobenzene sulfonate (Table III). This trinitrobenzene sulfonate-modified enzyme did not facilitate stereoselective reduction (Table IV). Trininitrobenzene sulfonate could accomplish this in several ways. It appears to bind at the nucleotide binding site[4] and by steric bulk effect prevents pyruvate binding, hydride attack on the pyruvate, or BH_4^- binding itself. Prevention of binding of BH_4^- to the active site is an intriguing possibility. In the only other application of this technique, a similar phenomenon was observed. Pyruvate was stereoselectively reduced by $NaBH_4$ in the presence of oxaloacetate decarboxylase[1]. When the reduction of oxaloacetate was attempted in the presence of oxaloacetate decarboxylase, a racemic mixture of malic acid was produced. Apparently, the second carboxyl group of oxaloacetate occupied the positively charged site on the enzyme that BH_4^- occupied during the stereoselective reduction of pyruvate. Likewise for pyruvate kinase, the BH_4^- may have to bind at a positively charged site in the nucleotide site in order to stereoselectively reduce pyruvate. When the nucleotide site is blocked by trinitrobenzene sulfonate, BH_4^- is unable to catalytically reduce the pyruvate on the enzyme. Adenosine 5′-diphosphate does not appear to block BH_4^- binding at the nucleotide binding site as effectively as trinitrobenzene sulfonate since some stereoselective reduction occurs in the presence of saturating levels of ADP.

Levels of phosphoenolpyruvate sufficient to saturate the enzyme prevent stereoselective reduction of pyruvate. Phosphoenolpyruvate is a competitive inhibitor of pyruvate binding at the active site[5]. Presumably, pyruvate is unable to bind ef-

fectively at the active site and hence cannot be reduced on the enzyme to give an excess of D-lactic acid.

One other possibility exists as to the site of stereoselective reduction of pyruvate. Pyruvate has been shown to bind at an ancillary site on pyruvate kinase; the stereoselective reduction may be occurring here. Two kinds of evidence mitigate against this hypothesis. Firstly, phosphoenolpyruvate has been shown not to bind to the ancillary site; therefore, saturating amounts of phosphoenolpyruvate should not prevent stereoselective reduction at the ancillary site. Saturating amounts of phosphoenolpyruvate do completely block the stereoselective reduction of pyruvate. Secondly, the ancillary site exists even in the absence of divalent metal ion[5]. Since stereoselective reduction clearly depends on the presence of divalent metal ion, it seems unlikely that stereoselective reduction would occur at a site which exists even in the absence of divalent metal ion. On the other hand, binding of pyruvate at the active site depends on the presence of a divalent metal ion[13]. Therefore, abolition of stereoselective reduction upon removal of the divalent metal ion is consistent with reduction occurring at the active site rather than at the ancillary site.

The reduction of pyruvate by $NaBH_4$ in the active site of pyruvate kinase is a true catalysis. Borohydride reduction of pyruvate in solution occurs rapidly; in order that enzyme-catalyzed reduction compete with this process at the low concentration of enzyme used (10^{-6} M), the rate of reduction on the enzyme surface must be much greater than that in solution. The enhanced rate of reduction of pyruvate on the enzyme surface could be a result of enzyme-induced polarization of the carbonyl group of pyruvate; the increased positive charge on the carbonyl carbon atom would make it more susceptible to attack by hydride. Evidence for this effect of pyruvate kinase on enzyme-bound pyruvate was obtained by Rose[13] who studied the enzyme-induced enolization of pyruvate.

ACKNOWLEDGEMENTS

The authors wish to thank Dr C. R. Harmison of the Lafayette Clinic, Detroit, Michigan, for the kind use of the Cary 60 spectropolarimeter and Professor M. J. Coon, Department of Biological Chemistry, The University of Michigan, for his helpful suggestions. Supported by the National Research Council of Canada, Grant No. A 1330.

REFERENCES

1 Kosicki, G. W. and Westheimer, F. H. (1968) *Biochemistry* 7, 4303–4309
2 Phillips, T. M. and Kosicki, G. W. (1970) *Fed. Proc.* 29, 462
3 Wood, T. (1968) *Biochem. Biophys. Res. Commun.* 31, 779–785
4 Hollenberg, P. F., Flashner, M. and Coon, M. J. (1971) *J. Biol. Chem.* 246, 946–953
5 Mildvan, A. S. and Cohn, M. (1966) *J. Biol. Chem.* 241, 1178–1193
6 Hanson, K. R. (1966) *J. Am. Chem. Soc.* 88, 2731–2742
7 Rose, I. A. (1970) *J. Biol. Chem.* 245, 6052–6056
8 Bondinell, W. E. and Sprinson, D. B. (1970) *Biochem. Biophys. Res. Commun.* 40, 1464–1467
9 Nowak, T. and Mildvan, A. S. (1970) *J. Biol. Chem.* 245, 6057–6064
10 Rose, I. A., O'Connell, E. L., Noce, P., Utter, M. F., Wood, H. G., Willard, J. M., Cooper, T. G. and Benziman, M. (1969) *J. Biol. Chem.* 244, 6130–6133
11 Boyer, P. D. (1962) in *The Enzymes* (Boyer, P. D., ed.), Vol. 6, pp. 95–113, Academic Press, London and New York
12 Mildvan, A. S. and Cohn, M. (1965) *J. Biol. Chem.* 240, 238–246
13 Rose, I. A. (1960) *J. Biol. Chem.* 235, 1170–1177