Biochem. J. (1990) 266, 305–308 (Printed in Great Britain)

Inactivation of pyruvate decarboxylase by 3-hydroxypyruvate

Greg THOMAS, Russell DIEFENBACH and Ronald G. DUGGLEBY*
Department of Biochemistry, University of Queensland, St. Lucia, Queensland 4067, Australia

Pyruvate decarboxylase from *Zymomonas mobilis* is inhibited by 3-hydroxypyruvate, which can also act as a poor substate. While catalysing the decarboxylation of this alternative substrate, the enzyme undergoes a progressive but partial inactivation over several hours. The extent of inactivation depends upon the pH and upon the concentration of 3-hydroxypyruvate. After partial inactivation and removal of unchanged 3-hydroxypyruvate, enzymic activity recovers slowly. We suggest that inactivation results from accumulation of enzyme-bound glycollaldehyde, which is relatively stable, possibly because it is dehydrated to form an acetyl group.

INTRODUCTION

Pyruvate decarboxylase (EC 4.1.1.1) catalyses the conversion of pyruvate into acetaldehyde and is one of a handful of enzymes that require thiamin diphosphate as a cofactor. This enzyme occurs free in some organisms that can ferment sugar to ethanol. The decarboxylation is similar to that of the first stage of the reaction catalysed by the pyruvate dehydrogenase multienzyme complex, but it liberates the acetaldehyde formed rather than using it for reductive acetylation of lipoyl groups. The enzymes from yeast [1–3], wheat germ [3–5] and the bacterium *Zymomonas mobilis* [6,7] have received particular attention.

We have been interested in the last of these and, while investigating its susceptibility to substrate analogues, discovered an unusual effect of 3-hydroxypyruvate. This compound is a very poor substrate and a good inhibitor, but prolonged incubation of the enzyme with this compound causes a progressive partial inactivation. We describe our observations in the present paper.

EXPERIMENTAL

Cultures of *Z. mobilis* strain ZM6 (A.T.C.C. 29191) were obtained from Dr. Lindsay Sly (Culture Collection, Microbiology Department, University of Queensland). Cells were grown, harvested and lysed, and pyruvate decarboxylase was purified, as described by Neale *et al.* [7], except that a final purification step by gel filtration on Bio-Gel P-300 was added. Other enzymes, as well as all reagents, were obtained from commercial sources. One unit of enzyme activity is defined as the quantity that catalyses the formation of 1 μmol of product/min.

Pyruvate decarboxylase activity was assayed by measuring the rate of change in absorbance at 340 nm at 30 °C in 50 mM-Mes/KOH buffer, pH 6.5. Assay mixtures contained 5 mM-$MgCl_2$, 0.5 mM-thiamin diphosphate, 0.15 mM-NADH, 67 units of yeast alcohol dehydrogenase/ml and, except when there was a specific reason to vary its concentration, pyruvate was used at 5 mM. Reactions were started by the addition of pyruvate decarboxylase. Inhibition of pyruvate decarboxylation by substrate analogues, and the activity with alternative substrates, was determined by using a similar procedure.

Inactivation of pyruvate decarboxylase was performed at 30 °C in 50 mM-Mes/KOH buffer, pH 6.5 containing 5 mM-$MgCl_2$ and 0.5 mM-thiamin diphosphate. After preincubation of the enzyme in this mixture for 45–75 min, 3-hydroxypyruvate was added at concentrations ranging from 0 to 15 mM. Samples were removed at intervals and pyruvate decarboxylase activity was determined as described above. A similar procuedure was followed when inactivation was determined at other pH values, except that the buffer was 100 mM-Tris/HCl at pH 7.5 and at pH 8.5.

The concentration of 3-hydroxypyruvate was determined at 30 °C in 50 mM-Tes/NaOH buffer, pH 7.5, containing 0.25 mM-NADH and up to 0.2 mM-3-hydroxypyruvate; the concentration of 3-hydroxypyruvate was calculated from the change in absorbance at 340 nm 9 min after the addition of 10 units of pig heart (band 1) lactate dehydrogenase/ml. The concentration of acetate was detemined enzymically with acetyl-CoA thiokinase, citrate synthase and malate dehydrogenase in a preceding indicator procedure [8].

Non-linear-regression analysis was performed by using the DNRP53 computer program [9].

RESULTS AND DISCUSSION

Pyruvate decarboxylase was tested for its susceptibility to inhibition by a range of substrate analogues (Table 1). Glyoxylate was found to be a strong inhibitor, consistent with earlier observations on the yeast enzyme [1]. The second most effective inhibition was seen with 3-hydroxypyruvate, which also proved to be a weak substrate. Although the maximum velocity observed with this alternative substrate was less than 1% of that seen with pyruvate, the affinity for 3-hydroxypyruvate (K_m 0.584 ± 0.047 mM) is somewhat higher than that for pyruvate (K_m 0.947 ± 0.064 mM). The inhibition by this compound was shown to be competitive with pyruvate (Fig. 1), with an inhibition constant of 0.558 ± 0.045 mM, equal to the K_m, as expected for inhibition by an alternative substrate.

We attempted to exploit this binding to protect the active site for some chemical modification studies. To our surprise, controls in which the enzyme was incubated

* To whom correspondence should be addressed.

Table 1. Inhibition of pyruvate decarboxylase by substrate analogues

Each compound was tested at 5 mM for its ability to inhibit the decarboxylation of 5 mM-pyruvate. Results are given as the percentage inhibition of the rate observed in an assay with pyruvate alone. Values in parentheses represent the effectiveness of the compound as a substrate (at 5 mM) as a percentage of the rate observed with pyruvate.

Compound	Inhibition (%)
Glyoxylate	98
3-Hydroxypyruvate (0.6)	60
2-Oxo-4-methylvalerate (0.1)	33
2-Oxobutyrate (28.0)	26
3-Bromopyruvate (0.3)	26
3-Fluoropyruvate	13*
Acetone	12
2-Oxo-3-methylvalerate	11
2-Oxo-3-methylbutyrate	0
2-Oxoglutarate	0
Oxamate	0
Propionate	0

* At 1 mM-pyruvate.

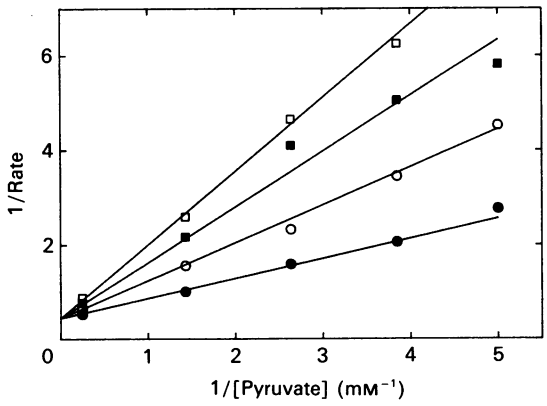

Fig. 1. Inhibition of pyruvate decarboxylase by 3-hydroxypyruvate

The enzyme was assayed at 3-hydroxypyruvate concentrations of 0 mM (●), 0.5 mM (○), 1.0 mM (■) and 1.5 mM (□). Rates are expressed as the change in absorbance per 10^3 s and are plotted in double-reciprocal form with each point representing the average of duplicate measurements. The lines represent the best fit of the equation for competitive inhibition to the data.

with 3-hydroxypyruvate showed a marked inactivation over a period of a few hours. The extent of inactivation depends on the concentration of 3-hydroxypyruvate to some extent (Fig. 2), but a substantial fraction of the enzyme remained active after incubation with 15 mM-3-hydroxypyruvate (results not shown), which is 25 times the inhibition constant and which should be saturating. The extent of inactivation also depends upon the pH (Fig. 3), with little or no effect at alkaline pH values; inactivation was most pronounced at pH 6.5, but even under these conditions it did not exceed 50%.

The results in Table 1 indicate that 3-hydroxypyruvate

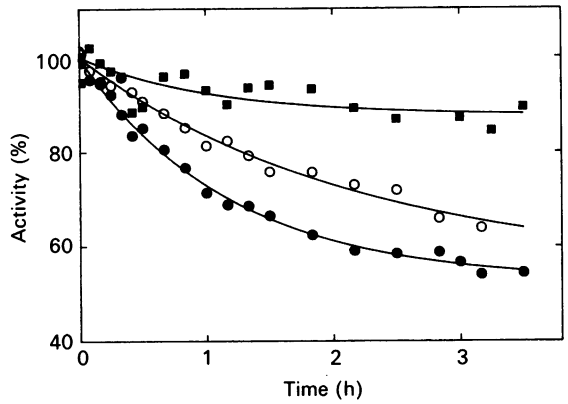

Fig. 2. Effect of 3-hydroxypyruvate concentration on the inactivation of pyruvate decarboxylase

The enzyme was incubated with 3-hydroxypyruvate at 5.0 mM (●), 1.0 mM (○) or 0.5 mM (■), and samples were withdrawn at intervals for assay of residual activity. Results are expressed as a percentage of the activity before addition of 3-hydroxypyruvate.

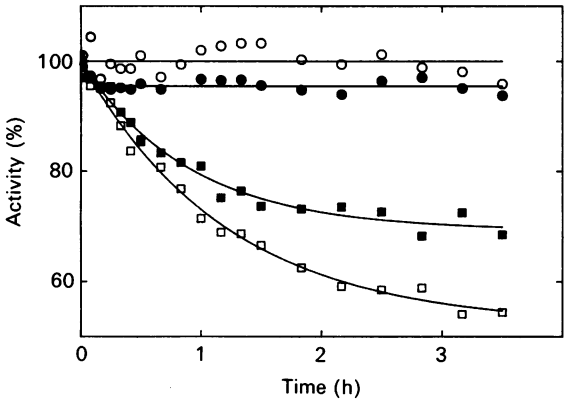

Fig. 3. Effect of variation of pH on the inactivation of pyruvate decarboxylase by 3-hydroxypyruvate

The enzyme was incubated with 5.0 mM-3-hydroxypyruvate at pH values of 8.5 (○), 7.5 (●), 6.5 (□) or 5.5 (■), and samples were withdrawn at intervals for assay of residual activity. Results are expressed as a percentage of the activity before addition of 3-hydroxypyruvate.

is a very poor substrate of pyruvate decarboxylase; however, these experiments were performed at the low enzyme concentrations and over the short time periods needed to measure the decarboxylation of pyruvate, the normal substrate. In contrast, the experiments of Fig. 2 used much higher enzyme concentrations, and under these conditions catalysis was detectable. Over the period of a few hours during which partial inactivation occurred, a substantial fraction of the 3-hydroxypyruvate was utilized (Table 2). It should be noted that the inactivation is not being caused by the glycollaldehyde formed during the reaction, as the enzyme was found to be stable when incubated with this compound (results not shown). However, it appears that inactivation accompanies catalysis; the apoenzyme formed by removal of thiamin diphosphate and Mg^{2+} was stable to treatment with 3-hydroxypyruvate.

Table 2. Utilization of 3-hydroxypyruvate by pyruvate decarboxylase

The enzyme was incubated with 5 mM-3-hydroxypyruvate at 30 °C, and samples were removed at intervals and assayed for 3-hydroxypyruvate and for residual enzymic activity.

Time (min)	Activity (%)	Concn. of 3-hydroxypyruvate (mM)
0	100.0	5.02
2	99.0	4.99
35	95.1	4.56
66	87.2	4.21
109	79.7	3.79
168	69.4	3.54
227	59.7	3.21

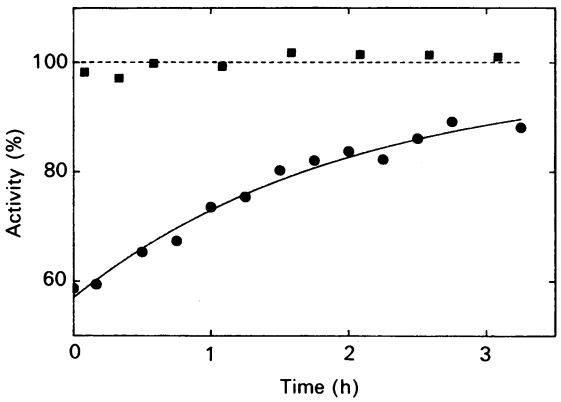

Fig. 4. Re-activation of pyruvate decarboxylase by removal of 3-hydroxypyruvate

The enzyme was incubated with 5.0 mM-3-hydroxypyruvate for 190 min, by which time it had lost 47% of its initial activity. After removal of small molecules by gel filtration on Sephadex G-25, the enzyme was incubated further under the same conditions, except for the omission of 3-hydroxypyruvate; samples were withdrawn at intervals for assay for residual activity (●). The ■ symbols are for a control sample that was never exposed to 3-hydroxypyruvate, and results are expressed as a percentage of the average activity of this control.

A puzzling feature of the data shown in Fig. 2 is the fact that activity declines to a plateau, which is unaffected by further incubation or by higher concentrations of 3-hydroxypyruvate. The cessation of inactivation is not due to exhaustion of 3-hydroxypyruvate (Table 2). Hence the plateau may represent an equilibrium, at which further inactivation is balanced by spontaneous re-activation. In order to examine this possiblity, partially inctivated enzyme was separated from 3-hydroxypyruvate and glycollaldehyde; the activity was slowly recovered and reached nearly full activity after several hours (Fig. 4). Re-activation could also be induced by raising the pH to 8.5 for a short period (results not shown).

There are several reasons to believe that inactivation and catalysis are related processes. First, 3-hydroxypyruvate is both an alternative substrate and a competitive inhibitor, with its Michaelis constant equal to its inhibition constant. Thus it is unlikely that 3-hydroxypyruvate binds anywhere but at the active site, so that inactivation must result from events occurring at the catalytic centre. Secondly, inactivation is most pronounced at pH 5.5 and 6.5, where the enzyme is most active [7]. And, thirdly, reconstitution of the enzyme for catalytically incompetent apoenzyme shows that this is not inactivated by 3-hydroxypyruvate.

From these observations, we propose the following kinetic model to explain the partial inactivation of pyruvate decarboxylase (E) during conversion of 3-hydroxypyruvate (3-HP) into glycolladehyde (GA):

$$E + 3\text{-HP} \overset{\text{Fast}}{\rightleftharpoons} E\cdot 3\text{-HP} \overset{\text{Slow}}{\longrightarrow} \begin{matrix}\text{E-GA} \\ +CO_2\end{matrix} \overset{\text{Slow}}{\longrightarrow} E + GA$$

First, it is known that 3-hydroxypyruvate behaves as a conventional competitive inhibitor, causing immediate inhibition of pyruvate decarboxylation without the necessity for prolonged preincubation. Thus the initial binding phase must be fast. Secondly, as an alternative substrate, 3-hydroxypyruvate has a low maximum velocity, so that there must be at least one slow step after binding. If there are two successive slow steps, a fraction of the enzyme will gradually accumulate (presumably as enzyme-bound dihydroxyethylthiamin diphosphate) until a steady state is reached in which the rates of formation and breakdown of this intermediate are equal.

The reason for the stability of the enzyme-bound dihydroxyethylthiamin diphosphate are unclear, since the corresponding hydroxyethylthiamin diphosphate, which formed during pyruvate decarboxylation, rapidly dissociates to release acetaldehyde. It may be that the presence of the hydroxy group on C-3 allows the initial decarboxylation product, instead of gaining H^+ to form $-CHOH-CH_2OH$, to lose OH^- to become $-C(OH)=CH_2$, which will at once tautomerize to form an acetyl group. A reaction of this type would not be without precedent, as wheat-germ pyruvate decarboxylase has been shown to catalyse decarboxylation and dehalogenation of 3-fluoropyruvate, to form stoichiometric amounts of acetate [5]. A similar dehalogenation of both 3-fluoropyruvate and 3-bromopyruvate by the *Escherichia coli* pyruvate dehydrogenase complex has also been described [10,11], and for this enzyme 3-bomopyruvate also acts as an inactivator. Further, the isolated pyruvate decarboxylase component of this complex is inactivated by 3-bromopyruvate, which has been proposed [11] to result from formation of a thiamin diphosphate-bound acetyl group, which is then irreversibly transferred to a cysteine residue in the enzyme. However, such a transfer would appear to be unlikely in the case of *Z. mobilis* pyruvate decarboxylase, as the inactivation is readily reversed by incubation in the absence of 3-hydroxypyruvate or by raising the pH.

REFERENCES

1. Uhlemann, H. & Schellenberger, A. (1976) FEBS Lett. **63**, 37–39
2. Kuo, D. J., Dikdan, G. & Jordan, F. (1986) J. Biol. Chem. **261**, 3316–3319
3. Ullrich, J. (1982) Ann. N.Y. Acad. Sci. **378**, 287–305

4. Zehender, H., Trescher, D. & Ullrich, J. (1987) Eur. J. Biochem. **167**, 149–154
5. Gish, G., Smyth, T. & Kluger, R. (1988) J. Am. Chem. Soc. **110**, 6230–6234
6. Bringer-Meyr, S., Schimz, K.-L. & Sahm, H. (1986) Arch. Microbiol. **146**, 105–110
7. Neale, A. D., Scopes, R. K., Wettenhall, R. E. H. & Hoogenraad, N. J. (1987) J. Bacteriol. **169**, 1024–1028
8. Beutler, H.-O. (1984) in Methods of Enzymatic Analysis (Bergmeyer, H. U., ed.), 3rd edn., vol. 6, pp. 639–645, Verlag Chemie, Weinheim, Deerfield Beach and Basel
9. Duggleby, R. G. (1984) Comput. Biol. Med. **14**, 447–455
10. Apfel, M. A., Ikeda, B. H., Speckhard, D. C. & Frey, P. A. (1984) J. Biol. Chem. **259**, 2905–2909
11. Lowe, P. N. & Perham, R. N. (1984) Biochemistry **23**, 91–97

Received 24 October 1989/29 November 1989; accepted 13 December 1989