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THE ENZYMATIC DECARBOXYLATION OF HYDROXYPYRUVATE ASSOCIATED WITH PURIFIED PYRUVATE DECARBOXYLASE FROM WHEAT GERM

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Key Word Index—*Triticum vulgare*; Gramineae; wheat; pyruvic decarboxylase; hydroxypyruvate; glycollate; glycolaldehyde.

Abstract—Highly purified pyruvic decarboxylase (EC 4.1.1.1) from wheat germ catalyses the decarboxylation of hydroxypyruvate. A kinetic analysis of the activity of the enzyme with pyruvate and hydroxypyruvate as substrates suggests that a single enzyme is involved. The kinetics of decarboxylation are autocatalytic. The time lag before maximum activity is reached is affected by the concentration of hydroxypyruvate and the pH. The question whether or not hydroxypyruvate is a natural substrate for the enzyme remains unresolved, but it may be significant that at physiological pH (*ca* 7.5) the enzyme shows optimum activity with hydroxypyruvate, but negligible activity with pyruvate.

INTRODUCTION

Hydroxypyruvate acts as a precursor of glycollate and oxalate in rats [1, 2] and a similar reaction sequence has been suggested to occur in plants [3]. Hydroxypyruvate is readily autoxidized and decarboxylated under the mild conditions normally used in enzyme studies, and in the presence of hydrogen peroxide, hydroxypyruvate yields glycollate and CO_2 [4]. This route could be particularly important in plants at higher temperatures due to the unequal effects of increased temperature upon the activities of glycollate oxidase which generates hydrogen peroxide, and catalase which removes it. On the other hand, a specific hydroxypyruvate decarboxylase (EC 4.1.1.40) has been isolated from mammalian brain [5]. The enzyme requires the presence of a divalent metal ion and is stimulated by primary amines. The decarboxylation of hydroxypyruvate to form 'active glycolaldehyde' [2-(1,2-dihydroxyethyl)thiamine pyrophosphate] is catalysed by pyruvic dehydrogenase (EC 1.2.4.1) [6]. Transketolase also decarboxylates hydroxypyruvate to yield 'active glycolaldehyde', which is transferred to an acceptor aldehyde [7, 8].

Pyruvate decarboxylase is a cytosolic enzyme which decarboxylates a number of higher homologues at rates which decrease with increasing chain length [9]. Glyoxylic acid is also a substrate but the activity is extremely low due to the slow release of formaldehyde from hydroxymethyl thiamine pyrophosphate which is formed as an intermediate [10]. In this paper we examine the possibility that pyruvate decarboxylase (EC 4.1.1.1) catalyses the decarboxylation of hydroxypyruvate.

RESULTS

Purification of pyruvic decarboxylase from wheat germ

The purification procedure which produced a 180-fold purification (Table 1) was based on the method of Singer [11] but was modified by introducing affinity chromatography on a thiamine-pyrophosphate-Sepharose gel [12]. An alternative to the affinity chromatography step was prolonged dialysis (in excess of 48 hr) of clear solutions of the enzyme against magnesium acetate (pH 6, 20 mM). The precipitate, which increased with time of dialysis, was dissolved in potassium phosphate buffer (pH 7, 50 mM) and centrifuged at 20000 *g* for 30 min. The clear supernatant contained the decarboxylase.

Time course of product formation

The decarboxylation of hydroxypyruvate by wheat germ pyruvic decarboxylase involves a distinct lag period before the enzyme attains its maximum rate. The decarboxylation of pyruvate catalysed by pyruvic decar-

Table 1. Purification of pyruvic decarboxylase from wheat germ (75 g)

Step	Volume (ml)	Protein (mg/ml)	Enzyme activity (units/ml)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Extraction	400	70	0.5	0.007		100
Affinity chromatography	36	1.3	1.7	1.31	187	30.6

boxylase shows a similar lag [13]. We define this lag period as the time (t_1) taken to make a given amount of product (P) after the reaction has reached the steady state, minus the time taken to make the same amount of product if the enzyme had been in the fully active form from time t_0.

Effect of substrate concentration and pH *on the duration of the lag*

The duration of the lag was found to decrease with increasing concentrations of hydroxypyruvate (Fig. 1) and to increase with increasing pH over the range 6.5–7.5 (Fig. 2). When hydroxypyruvate and pyruvate were incubated together with the enzyme, the duration of the lag was decreased by increasing the concentration of either or both substrates (Table 2).

Effect of pH *and substrate concentration on the rate of decarboxylation of hydroxypyruvate*

The effect of pH on the V_{max} for hydroxypyruvate decarboxylation together with the effect of pH on the rate at two concentrations of hydroxypyruvate is shown in Figs. 2 and 3. The data for V_{max} vs pH indicates a pH optimum at 7.5. However, at lower concentrations of substrate, an increased rate of decarboxylation at acid pH was apparent. This effect can be attributed to the increased affinity for the substrate at lower pH values. The effect of varying the substrate concentration on the rate of decarboxylation is shown in Fig. 1. At low pH (6.5), Michaelian kinetics were observed, but at higher values of pH (7.5), sigmoid kinetics were found.

Substrate specificity of wheat germ pyruvic decarboxylase

If a single enzyme exhibiting Michaelian kinetics is active with pyruvate and hydroxypyruvate, then the

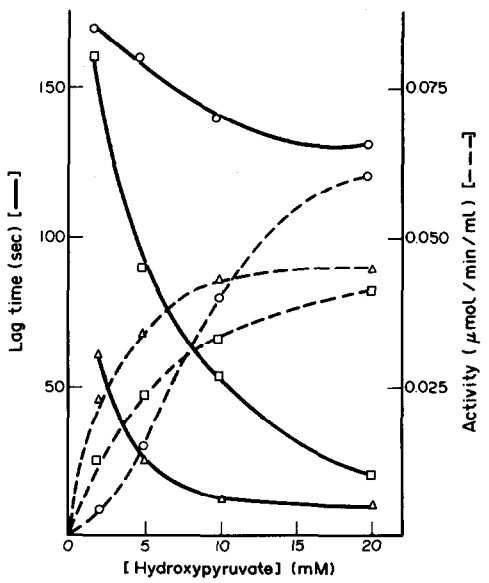


Fig. 1. The effect of varying pH and substrate concentration (hydroxypyruvate) on the rate of the reaction and the lag time. (– – –) Rate of reaction; (——) lag time; (○) pH 7.5; (□) pH 7.0; (Δ) pH 6.5.

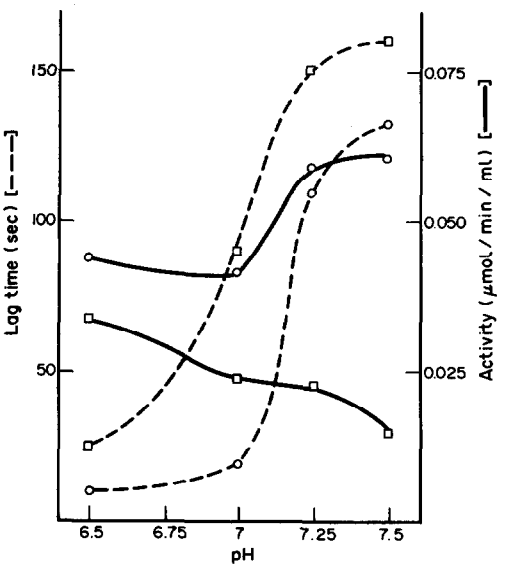


Fig. 2. The effect of pH on the rate of the reaction and on the lag time of the reaction at two concentrations of hydroxypyruvate (5 and 20 mM). (– – –) Lag time; (——) rate of reaction; (○) 20 mM hydroxypyruvate; (□) 5 mM hydroxypyruvate.

Table 2. The effect of varying concentrations of mixed substrates (pyruvate and hydroxypyruvate) on the lag time of the reaction*

Substrate concentration (mM)		Lag time t_1
Pyruvate	Hydroxypyruvate	(sec)
10	10	72
10	5	96
5	10	126
5	5	144

*Lag time is defined as the time (t_1) taken to make a given amount of product after the reaction has reached the steady state, minus the time taken to make the same amount of product if the enzyme had been in the fully active state at time t_0. Activity was measured at pH 7 under standard conditions as described in the Experimental.

activity V_t with both substrates (S_1 and S_2) is given by the equation:

$$V_t = \frac{V_{max_1}[S_1]}{[S_1] + K_{m_1}\left(1 + \frac{[S_2]}{K_{m_2}}\right)} + \frac{V_{max_2}[S_2]}{[S_2] + K_{m_2}\left(1 + \frac{[S_1]}{K_{m_1}}\right)}$$

If the observed activity with mixed substrates involves two specific enzymes, the activity with both substrates is given by:

$$V_t = V_1 + V_2$$

where V_1 and V_2 are the rates of reaction with substrates S_1 and S_2.

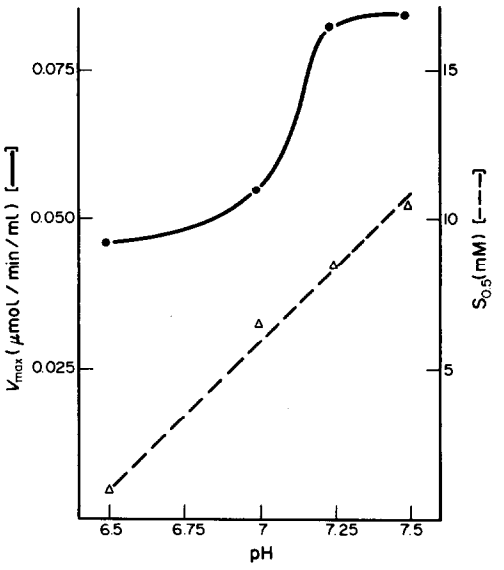


Fig. 3. The effect of pH on the V_{max} for hydroxypyruvate decarboxylation and on the concentration of substrate (hydroxypyruvate) producing half-maximum velocity. (Note, at low pH this concentration corresponds to K_m, but at high pH to $S_{(0.5)}$). (△- - -△) $S_{(0.5)}$ (mM); (●- - -●) V_{max}.

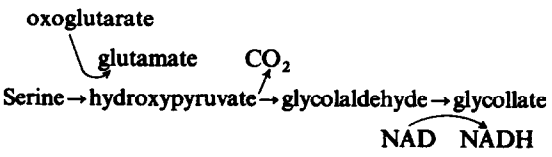
At pH 6.5, the kinetics of decarboxylation were approximately Michaelian and the observed rate of decarboxylation of mixed substrates was compared with the rate calculated, assuming one or two enzymes are involved (Table 3). At pH 7, the kinetics of decarboxylation were sigmoid and the simple equation for mixed substrates [14] cannot be applied. However, if two enzymes are involved, the rate with both substrates present should still be additive. The results presented in Table 3 are not consistent with two enzymes being involved.

DISCUSSION

The present study establishes that an enzyme is present in wheat germ which decarboxylates hydroxypyruvate, and suggests that this activity is an inherent property of pyruvate decarboxylase. All preparations of pyruvic decarboxylase examined by us have been observed to decarboxylate pyruvate and hydroxypyruvate at a constant ratio. The kinetic analysis of reactions with two substrates suggests that a single enzyme is involved, although it must be admitted that at pH 7, when the kinetics of decarboxylation are sigmoid, the argument is less well founded.

The kinetics of decarboxylation catalysed by the purified wheat germ pyruvic decarboxylase with hydroxypyruvate as substrate closely resemble those reported with pyruvate [13]. Thus the duration of the lag before maximum catalytic activity was attained, was found to decrease by increasing either or both substrates. This result is consistent with a single inactive enzyme which is activated by either substrate.

The next question involves the physiological significance of the decarboxylation of hydroxypyruvate. At physiological pH (*ca* 7–7.5), pyruvic decarboxylase has little or no activity with pyruvate [15]. However, with hydroxypyruvate as substrate, the enzyme shows a slightly alkaline pH optimum (Figs. 2 and 3). It should be noted that as the pH is raised from 6.5 to 7.5, the kinetics of decarboxylation change from Michaelian at pH 6.5 to sigmoid at pH 7.5 and the $S_{(0.5)}$ value (i.e. the concentration of substrate necessary to produce 50% of V_{max}) increases as the pH increases from 6.5 to 7.5 (Fig. 3). Thus at pH 7.5 pyruvic decarboxylase shows the kinetic characteristics consistent with the controlled decarboxylation of hydroxypyruvate and with the proposition that pyruvic decarboxylation is involved in the production of glycollate as shown below:



EXPERIMENTAL

Materials. Na pyruvate, thiamine pyrophosphate, hydroxypyruvate, nucleotides, alcohol dehydrogenase and aldehyde dehydrogenase were obtained from Sigma. DEAE-Sepharose and AH-Sepharose were obtained from Pharmacia. *p*-Nitrobenzoylazide was obtained from Eastman. All other chemicals

Table 3. Comparison between the observed rate of the reaction with various concentrations of mixed substrates (pyruvate hydroxypyruvate) and the calculated rates assuming one or two enzymes*

Substrate concentration (mM)		pH 6.5			pH 7.0	
S_1 pyruvate	S_2 hydroxypyruvate	V_t observed (μmol/min/ml)	V_t calculated for one enzyme	V_t calculated for two enzymes	V_t observed (μmol/min/ml)	V_t calculated for two enzymes
10	10	0.025	0.026	0.052	0.02	0.031
10	5	0.035	0.033	0.052	0.023	0.029
5	10	0.015	0.017	0.050	0.013	0.022
5	5	—	—	—	0.011	0.021

*The activity was measured at pH 6.5 or 7.0 under standard conditions as described in the Experimental.

were of the best grade available commercially. Fresh, untreated wheat germ was a gift from J. and J. Colman Ltd., Norwich.

Purification of pyruvate decarboxylase from wheat germ. All operations were carried out at 2°. Wheat germ (75 g) was homogenized in a Waring blender with 450 ml K-Pi buffer (pH 5.7, 0.1 M) containing β-mercaptoethanol (5 mM). The homogenate was filtered through 3 layers of muslin, adjusted to pH 5.6 with dilute HOAc, then centrifuged at 20000 *g* for 30 min. The pH of the supernatant was raised to pH 7 with KPi buffer (pH 8, 1 M), before fractionating with $(NH_4)_2SO_4$. The fraction precipitating between 0 and 40% saturation was collected by centrifugation at 23000 *g* for 20 min, dissolved in 40 ml KPi buffer (pH 7, 0.1 M) and dialysed against 5 l. of the KPi buffer (pH 6.5, 50 mM) overnight. After centrifugation, the clear sample was loaded onto a DEAE-Sepharose column (25 mm × 300 mm), previously equilibrated with KPi buffer (pH 6.5, 25 mM). When the sample had passed through the column, the column was washed with 50 ml of the buffer used for equilibration. The enzyme was then eluted with a linear gradient of KCl (0–0.7 M) in 400 ml of the same buffer. Fractions (5 ml) were collected and assayed for pyruvate decarboxylase. The combined active fractions were dialysed for 5 hr against KPi buffer (pH 5.8, 50 mM), then clarified by centrifuging at 30000 *g* for 20 min. The clear supernatant was applied to a column (1.5 × 12 cm) of thiamine pyrophosphate-Sepharose previously equilibrated with KPi buffer (pH 5.8, 50 mM). When 15 ml of the sample had passed into the column, the column was washed with 30 ml of the equilibrating buffer. This procedure was repeated until all the extract had been applied to the column. After the final wash, the enzyme was eluted with KPi buffer (pH 7.5, 0.1 M) and the active fractions were combined.

Preparation of thiamine pyrophosphate-Sepharose. The method of ref. [12] was modified as follows. AH-Sepharose (aminohexyl-Sepharose) was washed on a glass filter with 0.5 M NaCl soln. The gel was then washed with H_2O and reswollen. The washed gel was suspended in a soln of Na borate (0.2 M, pH 9.3) containing 40% dimethylformamide and treated for 1 hr at room temp. with *p*-nitrobenzoylazide (0.07 M). The gel was then washed extensively with 50% dimethylformamide and then reduced by a soln containing Na dithionite (0.1 M) and $NaHCO_3$ (0.5 M, pH 8.5) for 40 min at 40°. The effectiveness of this procedure was demonstrated by the red-orange colour produced upon reaction with Na trinitrobenzene sulphonate. The gel was washed with 50% dimethylformamide, suspended in 0.5 M HCl and diazotized by treating for 7 min at 4° with $NaNO_2$ (0.1 M). The gel was immediately reacted with thiamine pyrophosphate (4 g in 30 ml of cold saturated Na borate at pH 8.6) and the pH was adjusted to 8.6 with NaOH. The resulting reddish-orange substituted-Sepharose was stirred slowly overnight at 4°, washed extensively, and stored in H_2O at 4°.

Spectrophotometric assay of pyruvate decarboxylase. During purification, enzyme activity was measured by the method of ref. [10] except that oxamate (50 mM) was added to inhibit lactate dehydrogenase [15]. After purification, activity with pyruvate and hydroxypyruvate as substrates was measured by coupling the decarboxylase with aldehyde dehydrogenase. The concns of substrates and pH were varied as indicated in the Results, but in all assays, the NAD concn was 1 mg/ml, thiamine pyrophosphate was 0.5 μmol/ml, $MgCl_2$ was 0.5 μmol/ml, the buffer was KPi (0.15 M) and 2 units of aldehyde dehydrogenase were used. Before the reaction was started, the enzyme was incubated with thiamine pyrophosphate (0.33 mM) and $MgCl_2$ (0.33 mM) at 22° for 45 min. The temp. of the assay was 25° and the rate of reaction was not increased by further additions of aldehyde dehydrogenase. Hydroxypyruvate is a relatively unstable compound and freshly prepared solns must be used for kinetic studies. Under these conditions there is a negligible blank reaction.

Protein was determined by the method of ref. [16].

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