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KINETIC ASPECTS OF REGULATION OF PYRUVIC DECARBOXYLASE

P. KENWORTHY and D. D. DAVIES
School of Biological Sciences, University of East Anglia, Norwich, England

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Key Word Index—Wheat germ; decarboxylation; enzyme interconversion.

Abstract—The decarboxylation of pyruvate catalysed by pyruvic decarboxylase (EC 4.1.1.1) from wheat germ is shown to be autocatalytic. Evidence is presented which suggests that the enzyme exists in an active and an inactive form—the latter being converted into the active form in the presence of pyruvate and by low pH. It is suggested that the relatively slow interconversion of the two forms of the enzyme may represent a time buffering system to prevent the decarboxylation of pyruvate in response to transient changes in pH.

INTRODUCTION

Pyruvic decarboxylase of higher plants has an acid pH optimum and would be expected to be virtually inactive at the neutral or slightly alkaline pH of cytoplasm. Davies *et al.* [1] have proposed that anaerobiosis leads to the production of excess acid (lactate or malate) and the local associated fall in pH activates pyruvic decarboxylase leading to the eventual production of ethanol. However, should the pH of the cytoplasm suddenly fall due to a reaction not associated with anaerobiosis, then the expected decarboxylation of pyruvate may not be physiologically advantageous to the plant and appropriate control mechanisms may be involved. Sigmoid kinetics associated with pyruvic decarboxylase were first reported by Davies and Corbett [2] for the wheat germ enzyme and similar kinetics were later reported for the yeast enzyme [3–5]. We have examined the response of wheat germ pyruvic decarboxylase to a wide range of possible allosteric effectors without finding responses which might be considered physiologically significant. However, in this paper we report the presence of a time lag in the response of wheat germ pyruvic decarboxylase to a sudden change in pH or pyruvate concentration and briefly discuss the possible mechanism and physiological significance of the lag.

RESULTS

Time course of product formation

Since all enzymes must bind their substrates before forming products, a lag phase (transient state) must precede the steady state. In most cases the duration of the lag phase is less than the time taken to mix enzyme and substrate and initiate the recording of product formation. However, in the case of wheat germ pyruvic decarboxylase a distinct lag period was observed before the enzyme attained its maximum rate (Fig. 1). The lag period may be characterised in two ways:

(a) The time taken for the enzyme to reach maximum velocity (t_{vf}), but this value cannot be determined accurately.

(b) The time (t') 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 at time t_0.

This value (t') will be used as the measure of the lag throughout this paper and can be obtained by extrapolating the linear part of the rate curve to $P = 0$.

Coupled assays can produce apparent lags when the activity of the second enzyme in the sequence is rate

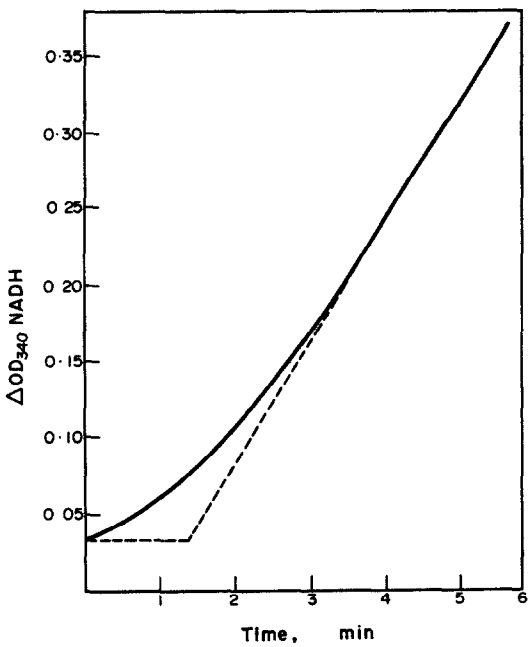

Fig. 1. Product vs time curves for pyruvic decarboxylase. Coupled assay with alcohol dehydrogenase cuvettes contained enzyme (20 μg) Na pyruvate (5 μmol), thiamine pyrophosphate (1 μmol), $MgCl_2$ (1 μmol), NADH (0·4 mg) Na Pi buffer (pH 6·0, 200 μmol) alcohol dehydrogenase (40 units) in a final vol of 3 ml.

limiting. A simple theoretical treatment of coupled assays has been presented by McClure [6] in which the second enzyme is assumed to show first order kinetics. A treatment which does not make the simplifying assumption of the first order kinetics has been presented by Stoner and Cornish-Bowden [7]. In the coupled assay for pyruvic decarboxylase, theoretical considerations indicate that the activity was not limited by alcohol dehydrogenase and increasing the amount of alcohol dehydrogenase had no effect on the overall activity. Furthermore the existence of the lag was confirmed by the titrimetric and dimedone assays.

The effect of Mg^{2+} *and thiamine pyrophosphate on the time course of pyruvate decarboxylation*

The existence of a lag period during which Mg^{2+}, thiamine pyrophosphate and yeast pyruvic decarboxylase combine to form an active complex has been reported [8]. We have confirmed these results for the wheat germ enzyme. The lag time becomes independent of thiamine pyrophosphate and Mg when the concentrations of both are in excess of 0·16 mM. Concentrations of thiamine pyrophosphate above 0·7 mM are inhibitory. To ensure that the enzyme was in the active form it was preincubated with $MgCl_2$ (0·33 mM) and thiamine pyrophosphate (0·33 mM) for 45 minutes before the addition of pyruvate to start the reaction.

The effect of pyruvate concentration on the rate of decarboxylation

The previously reported [2] sigmoid relationship between velocity and pyruvate concentration was confirmed using the coupled assay and the titrimetric assay. The Hill value (nH) calculated from the data of the coupled assay was 1·66 at pH 6·0 and from the titrimetric assay was 2·4 at pH 5·85. It should be noted that the velocities used in the plots are *not* initial velocities but steady state velocities i.e. after the lag period is completed. To obtain values for initial velocities it is necessary to analyse the data from the product versus time curves.

(a) *Kinetic analysis.* If we assume that the initial and steady state velocities correspond to two forms of the enzyme, then these velocities may be related by the equation [9]

$$v_t = v_f + (v_o - v_f)e^{-k't} \qquad (1)$$

where v_t is the velocity at time t; v_o is the velocity at time t_o; v_f is the maximum velocity; k' is a complex rate constant.

The overriding assumption is that the substrate level remains constant during the time of the conversion from one form of the enzyme to another.

The integrated form of the equation is

$$P_t = v_f^t - 1/k'(v_f - v_o)(1 - e^{k't}) \qquad (2)$$

so that extrapolation of the linear portion of a product vs time plot to $P = 0$ gives $t = v_f - v_o/k'v_f$ as illustrated in Fig. 1.

By drawing a number of tangents to the curve of product vs time, a corresponding number of values of v_t are obtained. Values of v_t obtained from the type of curve shown in Fig. 1 can be presented as a plot of log $(v_f - v_t)$ against time. The intercept at $t = 0$ gives log $v_f - v_o$, v_f is obtained from the linear portion of Fig. 1, v_o can be calculated. These initial rate (v) values are plotted against pyruvate concentration in Fig. 2 and show considerable scatter.

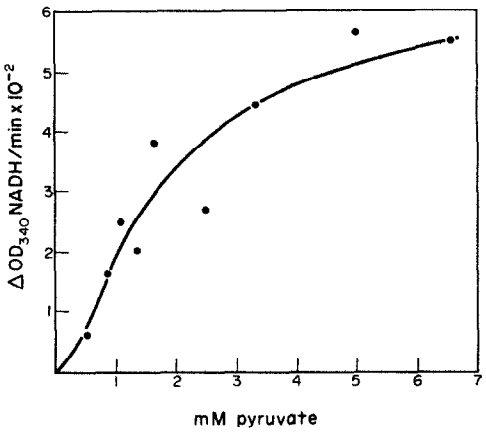

Fig. 2. Initial rate vs substrate plot for pyruvate decarboxylase. (The values were obtained by the method of tangents). Assay conditions as in Fig. 1.

(b) *Computer analysis.* Values of v_o obtained as described above, depend greatly on the accuracy with which tangents can be drawn in the product vs time plot. To reduce the error associated with these estimates, the approximate values obtained by the use of tangents were supplied to a computer programme based on the function

$$P_t v_f t - [(vf - vo)/k'] \qquad (3)$$

By minimising the sum of squares of deviation of the computed values of P_t from the observed results, the programme forms best estimates of v_f, v_o, k' and t by iteration. The output also contains standard deviations of those estimates. Hence the system once established, is faster and gives a better analysis than the graphical approach. However, the programme does require reasonably good initial estimates of v_f, v_o, k' and t.

An alternative procedure for making initial estimates of v_f, v_o, k' and t and avoiding the use of tangents to evaluate k' and v_o was developed from the observation that the ratio v_f/v_o was approximately constant. V_f and t can be measured accurately from the product vs time curve and approximate values for v_o and k' can be calculated. When these values are supplied to the computer, iteration based on equation 3 produces improved value. These values are plotted against substrate concentration (Fig. 3) giving sigmoid plots and a constant ratio v_f/v_o of 5. The concentration of substrate giving half maximum velocity ($s_{0.5}$) was 4·2 mM for the plot of v_f and v_o against pyruvate concentration.

Effect of other factors on the rate of pyruvate decarboxylation and the lag

Boiteux and Hess [5] have reported that potassium phosphate inhibits yeast pyruvic decarboxylase showing negative heterotropic cooperative effects. Potassium phosphate also inhibits wheat germ pyruvic decarboxylase but this inhibition appears to be due to the potassium rather than the phosphate ion (Table 1).

Morpholino ethane sulphonic acid (MES) buffer was used to cover investigate the effect of the pH over the range pH 5·4–6·8. The results (Fig. 4) show that the

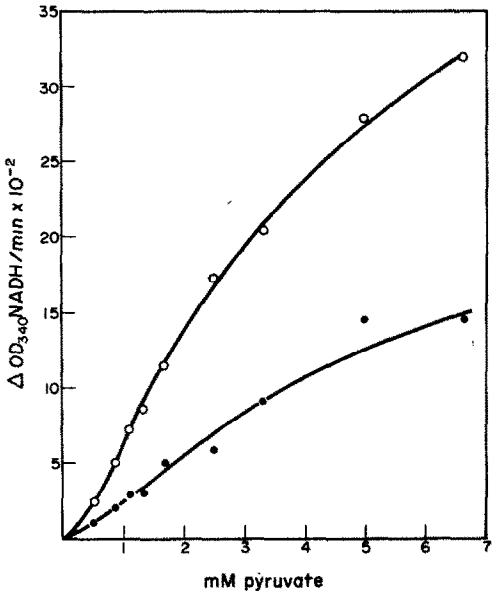

Fig. 3. Plots of initial and steady state rates against substrate concentration for pyruvate decarboxylase. Initial (v_o) rates and steady state (v_f) rates are obtained from a computer analysis. Assay conditions as in Fig. 1. O——O steady state (v_f) rates; ●——● initial (v_o) rates.

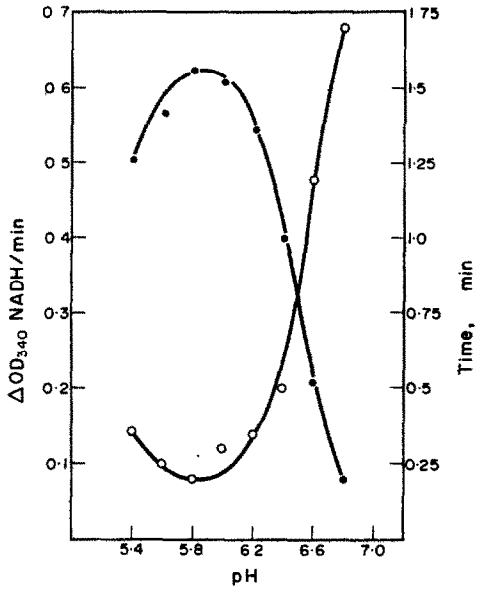

Fig. 4. Effect of pH on enzyme activity lag. Assay conditions as in Fig. 1 except pyruvate concentration was (3·3 mM) and pH was varied with MES buffer as indicated. ●——● steady state velocities; O——O lag time (t').

duration of the lag is shortest at the pH optimum of the enzyme.

The lag time (t') measured directly from the experimental trace of product vs time agrees very closely with values obtained by calculation via the computer. The effect of pyruvate concentration on the duration of the lag is shown in Fig. 5a. The results shown in Fig. 5b indicate that the duration of the lag is inversely proportional to the concentration of enzyme.

Molecular weight of pyruvic decarboxylase

The possibility that the conversion of pyruvic decarboxylase from an inactive to an active form in the presence of pyruvate involved a change in molecular weight was examined by passing the enzyme through a column of Sepharose 6B previously equilibrated with a solution containing sodium phosphate buffer (0·1 M pH 7·9) TPP (0·1 mM) and $MgCl_2$ (0·1 mM) or with the same solution plus sodium pyruvate (2 mM). The enzyme emerged at

Table 1. Effect of phosphate and potassium on the rate of decarboxylation of pyruvic acid by pyruvic decarboxylase. The enzyme was assayed under standard conditions except that the buffers and ions were varied as indicated (MES 50 μmol was present in all cases). Rates are expressed as a percentage of the rate in the absence of K and PO_4

Tris base (μmol)	Phosphate (μmol)	K (μmol)	Rate
22		—	100
51·6	25	—	100
81·2	50	—	100
110·8	75	—	100
22	25	29·6	91·5
22	50	59·2	80·0
22	75	88·8	75·5
22	100	118·4	67·0

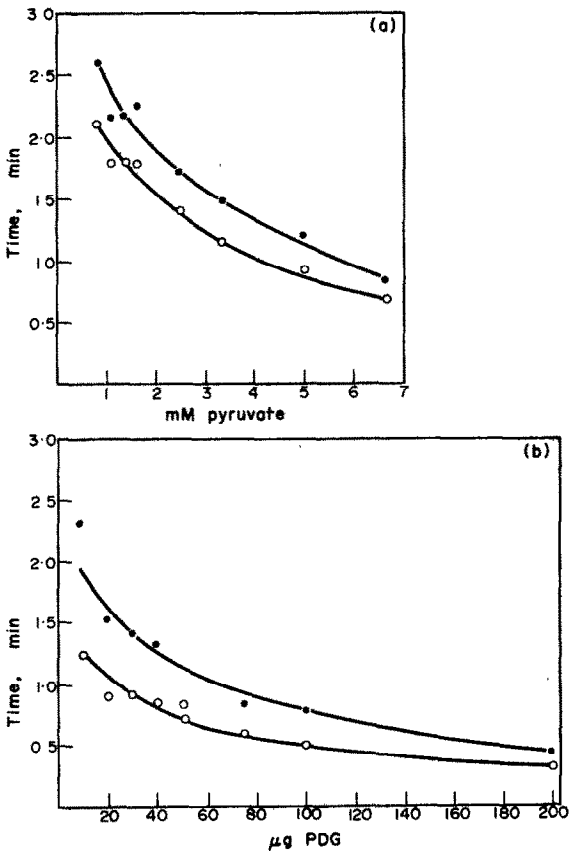

Fig. 5. Effect of substrate and enzyme concentration on the lag. (a) Pyruvate concentration varied. Assay conditions as in Fig. 1. $1/k'$ and t' are improved values obtained by computer analysis. (b) Enzyme concentration varied. Assay conditions as in Fig. 1 except pyruvate concentration (3·3 mM) $1/k'$ and t' obtained by calculation from the method of tangents. O——O t'; ●——● $1/k'$.

the same point indicating that under these conditions incubation with pyruvate did not produce a change in molecular weight. By using markers the MW of pyruvic decarboxylase determined by the method of Andrews [10] was 570000.

DISCUSSION

Pyruvate is a central metabolite and it is highly likely that the partitioning of pyruvate between various pathways is under metabolic control. In the case of pyruvate decarboxylation it appears likely that the major control factor is pH—pyruvic decarboxylase being inactive at pH values above 7. The slow response of pyruvic decarboxylase to sudden changes in pH or pyruvate concentration may represent a time dependent buffering system. Such a system would prevent the decarboxylation of pyruvate in response to transient changes but allow decarboxylation when appropriate physiological conditions are sustained. This teleological explanation is advanced on the assumption that the kinetics reported here for the purified enzyme are related to *in vivo* conditions. We have examined the kinetics of pyruvate decarboxylation throughout the purification of the enzyme and observed the same kinetics at all stages of purification.

Frieden [9, 11] has used the term hysteresis to describe the slow response of enzymes to sudden changes in substrate or effector concentration and has discussed mechanisms which may be involved in the hysteretic response. Unfortunately a number of mechanisms give the same rate equation and it is therefore difficult to distinguish between them. In the case of pyruvic decarboxylase the increase in activity on incubating the enzyme with pyruvate could be due to either (a) the conversion of an inactive form of the enzyme into an active form or (b) the conversion to a more active form of the enzyme (Fig. 6). It should be noted that the two forms of the enzyme considered here do not correspond to the $R \leftrightarrow T$ interconversion postulated for allosteric enzymes [12], since the enzyme exhibits sigmoid kinetics when the lag phase has ended.

If mechanism (a) is correct, then the activity observed immediately pyruvate is added, is due to a certain amount of the enzyme being in the active form. Thus the kinetic constants of the enzyme measured immediately after addition of pyruvate should be the same as the constants measured at the end of the lag phase. If mechanism (b) is correct the kinetic constants for the two forms of the enzyme could well be different so that different kinetic constants would be observed at the beginning and end of the lag phase.

The results illustrated in Fig. 5, the constancy of the ratio v_f/v_o and the equality of the $S_{(0.5)}$ values for pyruvate measured under initial and steady state conditions indicate that the kinetic properties of the enzyme are the same at the beginning and the end of the lag phase. Thus the experimental data is consistent with mechanism (a) and although mechanism (b) is not fully eliminated, it seems perhaps less likely.

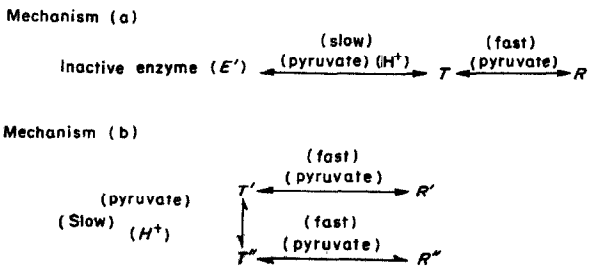

Fig. 6. Possible mechanisms controlling pyruvate decarboxylase activity.

We can say little about the nature of the transition from inactive to active enzyme, except to note that it does not appear to involve association or dissociation of sub-units since we have been unable to detect changes in molecular weight in the presence and absence of pyruvate.

EXPERIMENTAL

Materials. Wheatgerm was a gift from J. & J. Colman Ltd., Norwich. Yeast alcohol dehydrogenase, β-galactosidase, catalase, myoglobin, NADH and yeast pyruvic decarboxylase were obtained from Boehringer Co. Sodium pyruvate and thiamine pyrophosphate were obtained from Sigma Chemical Co. Dimedone was obtained from British Drug Houses and was recrystallised twice from EtOH before use.

Pyruvic decarboxylase. The enzyme was purified from wheat germ as described by Singer [13].

Enzyme assays. Pyruvic decarboxylase was routinely assayed by coupling to alcohol dehydrogenase and measuring the decrease in E_{340} associated with NADH oxidation as described by Davies and Corbett [2]. The formation of acetaldehyde was also measured by reacting in with dimedone [14]. Samples (2 ml) were added to 2 ml of dimedone reagent (dimedone 0·3 g, ammonium acetate 2·5 g, HOAc 0·4 ml and H_2O to a final vol of 100 ml) and heated for exactly 6 min at 100°. The mixture was cooled to 25° and the fluorescence was measured with the exciter at 396 nm and the analyzer at 460 nm. The enzyme was also assayed by a titrimetric method. The assay system contained sodium pyruvate (10 μmol), thiamine pyrophosphate (2 μmol), MgCl, (2 μmol) and H_2O to a final vol of 5·6 ml. pH was carefully adjusted to 5·85, pyruvic decarboxylase (0·2 ml), previously incubated for 45 min with $MgCl_2$ (0·33 mM) and thiamine pyrophosphate (0·33 mM) was added and the pH maintained at pH 5·85 by the addition of HCl (4 mM) from the autoburette of a Radiometer autotitrator.

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