SUBSTRATE ACTIVATION OF PYRUVATE CARBOXYLASE BY PYRUVATE*

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SUMMARY

A kinetic analysis of pyruvate carboxylase isolated from sheep liver and using pyruvate as the variable substrate revealed non-Michaelis Menten kinetic. Double reciprocal plots were biphasic and R_S values of 222 were obtained. Hill plots prepared from the initial velocity data showed that at low pyruvate concentrations, the slope of the line varied from 1.0 to 0.7 and then decreased to 0.4 at 0.8 x 10^{-3}M-pyruvate. With increasing pyruvate concentrations, the slope increased to a value of 1.0. This type of kinetic behaviour is typical of a system in which there is negative cooperativity with respect to ligand binding with concurrent progressive substrate activation.

The fact that acetyl-CoA is essential for the activity of vertebrate pyruvate carboxylase (pyruvate:CO_2 ligase (ADP), EC. 6.4.1.1.) and that the activation is a homotropic allosteric effect (Barritt *et al.*, 1966; Scrutton and Utter, 1967) has led to the hypothesis that the activity of the enzyme *in vivo* is controlled by the intracellular concentration of acetyl-CoA. The studies reported here using pyruvate carboxylase isolated from sheep liver suggest that, in addition to the control exerted by acetyl-CoA, there is a negative-type cooperativity in the binding of pyruvate to this enzyme.

EXPERIMENTAL

Pyruvate carboxylase was purified from freeze-dried mito-

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chondria isolated from sheep liver. The unpublished procedure involved extraction of the freeze-dried material, precipitation with $(NH_4)_2SO_4$ followed by precipitation with polyethylene glycol. The soluble protein was then chromatographed twice on DEAE-cellulose columns, the first at pH 6.5 and the second at pH 7.5. Elution of the enzyme was effected by a KCl concentration gradient.

Enzymic activity was measured by a modification of the method described by Gailiusis et al. (1964). The standard assay mixture (total volume 0.50 ml) contained in micromoles/ml; 60, tris-HCl buffer, pH 8.4; tris-pyruvate, varied as required; 1.25, ATP; 4, $MgCl_2$; 5, sodium bicarbonate (6×10^5 c.p.m. per μmole); 0.13, acetyl-CoA and enzyme. Reaction mixtures were incubated at 30° for 4 min. and stopped by the addition of saturated 2,4-dinitrophenyl-hydrazine HCl. Using these conditions the reaction velocity was linear for all of the pyruvate concentrations used. After centrifuging to remove precipitated protein, aliquots of the supernatant were applied (in triplicate) to Whatman 3MM filter-paper squares (1" x 1"), dried and counted in a Packard scintillation spectrometer using the channels ratio method to correct for quenching.

RESULTS AND DISCUSSION

When initial velocities were measured and plotted as a function of pyruvate concentration, a pronounced deviation from a rectangular hyperbola was consistently observed (Fig. 1). Double reciprocal plots obtained from these experiments exhibited a biphasic effect and the R_s value (i.e., the ratio of the pyruvate concentration at 90% and 10% of the saturation velocity) of 222, far exceeded the value of 81 which is expected for a classical Michaelis-Menten hyperbola. These data provide strong evidence for negative cooperative binding of pyruvate to the enzyme

(Levitzki and Koshland, 1969). A Hill plot prepared from the initial velocity data presented in Fig. 1 is presented in Fig. 2. It can be seen that the slope of the line varies with pyruvate concentration. At very low levels of pyruvate, the slope varies

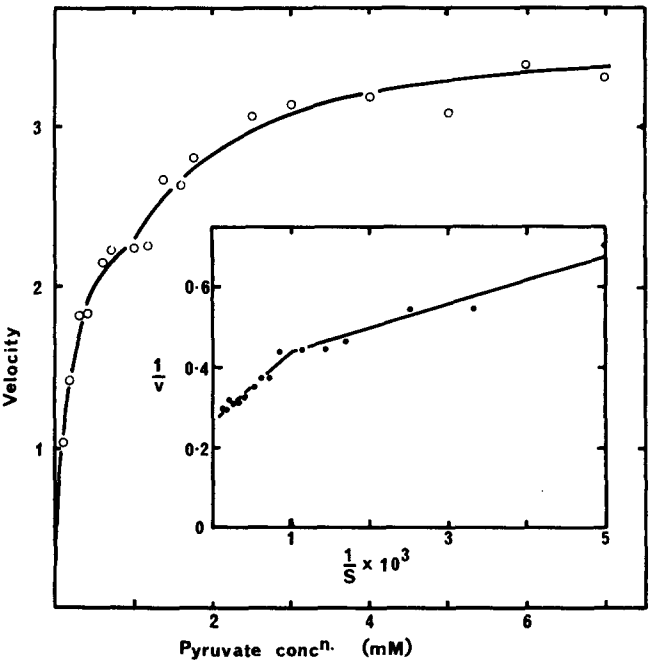

Fig. 1. Velocity plotted as a function of pyruvate concentration. The points marked are experimental values. Inset: reciprocal of velocity plotted as a function of the reciprocal of the pyruvate concentration. The curves were drawn from the kinetic constants obtained by fitting the data for separate parts of the biphasic plots to a computer program for a rectangular hyperbola using the method of least squares (Wilkinson, 1961). These values were; apparent K_m 1.5 x 10^{-4}M and 6.2 x 10^{-4}M.

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from 1.0 to 0.7 and then decreases to 0.4 in the region of transition from low to high enzymic activity (i.e. 1.0 x 10^{-3}M-pyruvate) and then increases to 1.0 at high pyruvate concentrations. This type of plot is identical to that predicted by Levitzki and Koshland (1969) for a system in which there is negative cooperativity with respect to binding but positive

cooperativity with respect to catalysis.

An alternate explanation for the above data is that in the enzyme preparation there exist two enzymes, one functional at

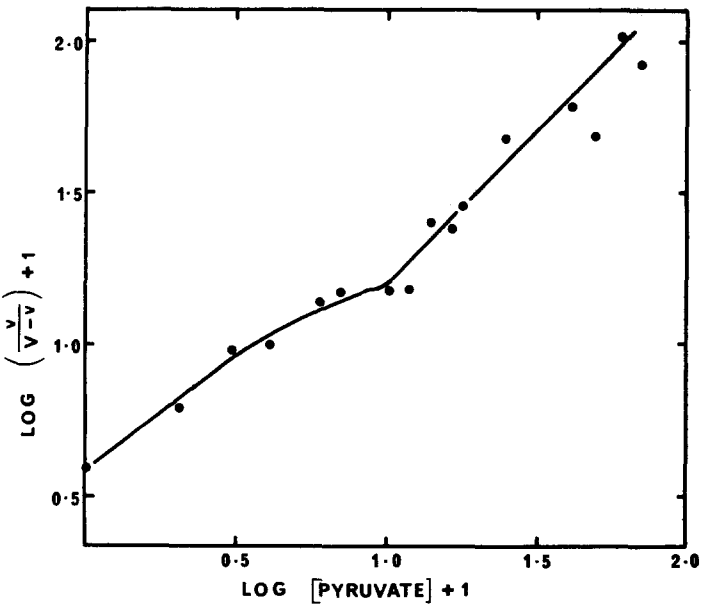

Fig. 2. Hill plot for the data presented in Fig. 1. The points marked are experimental values and the curves were drawn as described in Fig. 1.

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low and the other functional at high pyruvate concentrations. However, against this explanation is the fact that the substrate activation is present at all stages of the purification procedure. Also, the R_s values and the ratios of the two apparent K_m and V_{max} values remain constant throughout the purification procedure (Table 1).

The effect of varying certain parameters of the reaction mixture was examined and shown to have very little effect on the relative kinetic characteristics. For example, when the pH was varied between pH 7.5 and 9.0, it appeared that the negative cooperativity was more pronounced as the pH was shifted away from the optimum (pH 8.4). However, when these curves were normal

ised they were found to be very similar to each other. Similar

TABLE 1

COMPARISON OF THE KINETIC PROPERTIES AT VARIOUS STAGES OF THE PURIFICATION

Stage of Purification	R_s value	Ratio of the two		Inflection point of double reciprocal plot	Spec. Act.
		K_m's	V_{max}'s		
				mM	
Polyethylene glycol step	239	5.65	1.74	0.94	1.4
DEAE-cellulose column pH 6.5	276	5.91	1.69	0.93	3.5
DEAE-cellulose column pH 7.5	294	6.32	1.62	1.11	5.1
G200 Sephadex column	222	5.94	1.88	0.84	6.6

results were obtained when the reaction velocity was examined over a ten-fold range of fixed levels of either acetyl-CoA or $MgATP^{2-}$.

Although it is widely accepted that the activity of pyruvate carboxylase is controlled by the cellular level of acetyl-CoA, Williamson et al. (1968) have pointed out that from the known cellular levels of acetyl-CoA and the apparent K_m values for acetyl-CoA for the vertebrate enzymes, it seems unlikely that acetyl-CoA exerts a fine control over the enzymic activity. The evidence presented here indicates that at least with the sheep liver enzyme, the pyruvate concentration can have a profound influence on the level of enzymic activity. Although negative cooperativity with respect to the binding of pyruvate with subsequent substrate activation appears to be the most likely explana-

tion for the observed kinetics, the existence of two separate independent pyruvate binding sites with different physical and catalytic properties is not excluded. However, the functional significance of these results is not affected by the explanation. The apparent K_m value for pyruvate in the lower activity range is 1.5×10^{-4}M which is approximately three-fold higher than the reported physiological levels of pyruvate in the ruminant liver (Ballard and Hanson, 1968; Baird et al., 1968). Thus it would seem that the activity represented by the upper portion of the velocity curve would be used by the enzyme only when a large influx into the liver of pyruvate precursors occurred.

This communication emphasises the complexity of the control of pyruvate carboxylase which exhibits positive cooperativity with respect to acetyl-CoA (Barritt et al., 1966; Scrutton and Utter, 1967), $MgATP^{2-}$ and Mg^{2+} (Keech and Barritt, 1967), and negative cooperativity with respect to pyruvate.

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