ACTIVATION OF PYRUVATE CARBOXYLASE FROM *ASPERGILLUS NIDULANS* BY ACETYL COENZYME A

Stephen A. OSMANI and Michael C. SCRUTTON
Department of Biochemistry, King's College, Strand, London, WC2R 2LS, England

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1. Introduction

Activation by an acyl derivative of coenzyme A is observed for pyruvate carboxylases isolated from a wide range of organisms [1]. An exception to this generalisation has been described for pyruvate carboxylases from *Pseudomonas citronellolis* and *Azotobacter vinelandii* which are insensitive to activation or inhibition by any of the metabolites which typically regulate the activity of this enzyme [2–4]. Additionally pyruvate carboxylase from *Aspergillus niger* has been described to be fully active in the absence of acetyl-CoA but sensitive to inhibition by L-aspartate [5,6]. We have confirmed these observations for pyruvate carboxylase obtained from the related fungus *Aspergillus nidulans* but have shown that activation by acetyl-CoA can be demonstrated in the presence of the regulatory inhibitors L-aspartate or α-oxoglutarate [7].

We have now established conditions in which activation of *A. nidulans* pyruvate carboxylase by acetyl-CoA can be observed in the absence of a regulatory inhibitor. These data, which are presented here, demonstrate that pyruvate carboxylase from *A. nidulans* carries an acyl-CoA activator site whether or not other effectors are present and indicate that the pattern of activator–substrate interaction is unlike that described for other pyruvate carboxylases.

2. Methods

Aspergillus nidulans R21 pyC^+ was grown on glucose as sole carbon source and the mycelium was harvested and stored as in [7]. Partially purified pyruvate carboxylase was obtained as in [7] and this preparation was further purified to homogeneity as in [8]. The purified enzyme which gave a single protein band when subjected to polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulphate was used for these studies.

Pyruvate carboxylase was assayed by measurement of oxaloacetate production in the presence of malate dehydrogenase and NADH as in [9] except that 100 mM K^+ Hepes (pH 7.4) was used as buffer for the studies on activation by acetyl-CoA. All reagents were prepared in water that had been boiled and then cooled in the presence of a CO_2 adsorbent (Ascarite). The pH of the assay system was adjusted to pH 7.4 using 0.1 M KOH if necessary before addition of the enzyme and was checked after completion of the assay.

Acetyl-CoA was prepared [9] and assayed [10] as described.

3. Results

Initial studies demonstrated that although activation by acetyl-CoA could not be observed at pH 7.8 such activation could be demonstrated at pH 7.4 provided that non-saturating concentrations of pyruvate and $MgATP^{2-}$ were present in the assay system. No significant activation was observed if all substrates were present at saturating concentration or if a non-saturating concentration of HCO_3^- was used in the presence of saturating concentrations of pyruvate and $MgATP^{2-}$ (table 1). Furthermore, similar results were obtained in the presence of 120 mM K^+ and in the absence of added K^+ indicating that this cation has no significant influence on the extent of activation.

Measurement of the variation of initial velocity

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Table 1
Extent of activation by acetyl-CoA in the presence of saturating and non-saturating concentrations of the substrates

Substrate concentrations (mM)			Activation ratio (V + 0.2 mM Acetyl-CoA) / (V − Acetyl-CoA)
Pyruvate	$MgATP^{2-}$	HCO_3^-	
5.0	2.0	20.2	1.0
5.0	2.0	0.2	1.1
5.0	0.2	0.2	1.6
0.1	2.0	20.2	3.2
0.1	2.0	0.2	3.0
0.1	0.2	20.2	4.1
0.1	0.2	0.2	4.2

Pyruvate carboxylase activity was assayed at pH 7.4 as in section 2. No significant difference was observed in the activation ratios when the studies were performed using K^+ Hepes and $KHCO_3$ or using Na^+ Hepes and $NaHCO_3$

with acetyl-CoA concentration in the presence of non-saturating concentrations of all 3 substrates demonstrated that the concentration of acetyl-CoA used (0.2 mM) for the studies described in table 1 was saturating. The relationship between initial velocity and acetyl-CoA concentration is hyperbolic (h = 1.0) under all conditions tested and the activation constant ($K_{act.}$), which shows no significant variation with the concentration of any of the 3 substrates, is obtained as 0.8 ± 0.1 μM. This is illustrated in fig.1 for data points obtained in the presence of 0.1, 0.2 and 0.35 mM pyruvate. The apparent $K_{act.}$ determined here is 60-fold more favourable than that reported for activation by acetyl-CoA at pH 7.4 in the presence of 5 mM L-aspartate (50 μM) [7]. This relationship is that predicted on the basis of the competitive nature of the interaction between the effects of acetyl-CoA and L-aspartate on catalysis by *A. nidulans* pyruvate carboxylase [7].

The nature of substrate–activator interaction in pyruvate carboxylase from *A. nidulans* was characterised further by examining the relationship between initial velocity and substrate concentration in the presence and absence of a saturating concentration of acetyl-CoA. Data obtained in studies of this type are summarised in fig.2. At pH 7.4 and in the presence of saturating concentrations of $MgATP^{2-}$ and HCO_3^- the relationship between reciprocal initial velocity and reciprocal pyruvate concentration is non-linear (h = 1.3) in the absence of acetyl-CoA but becomes linear (h = 1.0) in the presence of this activator. In addition $[S]_{0.5}$ for pyruvate decreases from 0.78 mM in the absence of acetyl-CoA to 0.3 mM in the presence of 0.2 mM acetyl-CoA but the addition of this activator has no effect on the apparent V_{max} (fig.2A). In contrast, when $MgATP^{2-}$ is used as the variable

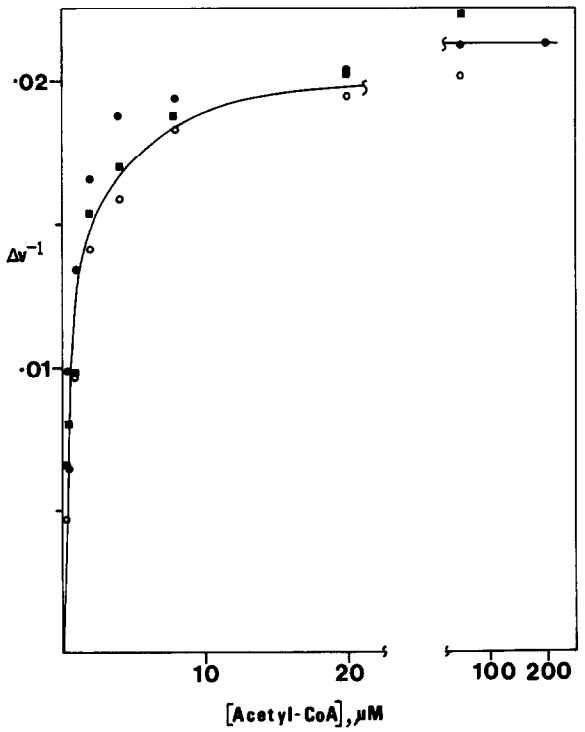
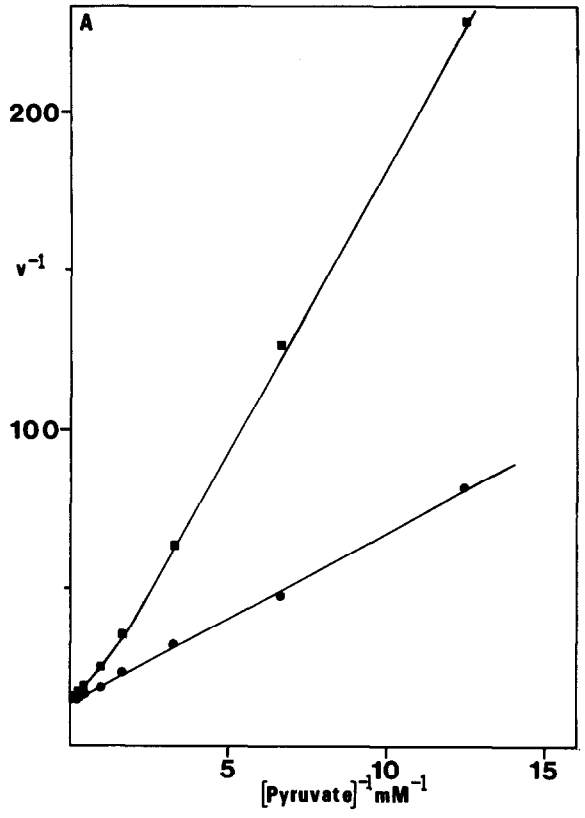
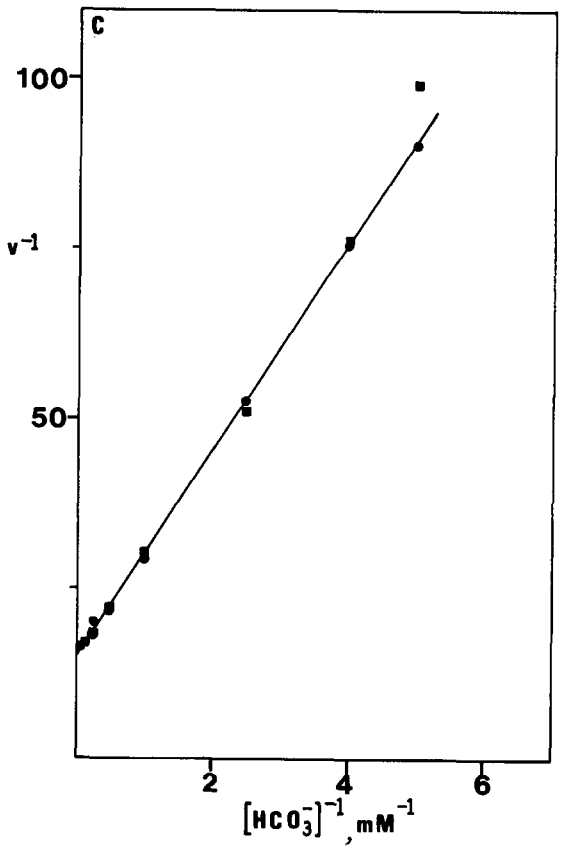


Fig.1. Activation of *A. nidulans* pyruvate carboxylase by acetyl-CoA in the presence of various concentrations of pyruvate. The assay system contained, in 0.5 ml, 100 mM K^+ Hepes (pH 7.4), 0.1 (■), 0.2 (●) or 0.35 (○) mM Na-pyruvate, 0.3 mM $MgATP^{2-}$, 2 mM Mg^{2+}, 2.2 mM $KHCO_3$, acetyl-CoA at the concentrations indicated, 5 μg malate dehydrogenase and 0.15 mM NADH. After equilibration to 25°C the reaction was initiated by addition of 0.4 μg pyruvate carboxylase (spec. act. 22 units/mg) and V determined from the initial rate of the decrease in absorbance at 340 nm. In the figure ΔV is expressed as $\Delta A_{340\ nm} \cdot min^{-1} \cdot \mu g\ enzyme^{-1}$ and is obtained by subtracting the rate observed in the absence of acetyl-CoA from the rate observed in the presence of this activator. The plots of $1/\Delta V$ versus 1/[acetyl-CoA] were linear for all 3 concentrations of pyruvate and gave estimates of the apparent $K_{act.}$ for acetyl-CoA as 1.06 μM (0.1 mM pyruvate), 0.60 μM (0.2 mM pyruvate) and 0.83 μM (0.35 mM pyruvate). The activation ratios (expressed as V_{max}/V in absence of acetyl-CoA) were 2.6 (0.1 mM pyruvate), 2.0 (0.2 mM pyruvate) and 1.8 (0.35 mM pyruvate). At higher pyruvate concentrations the extent of activation was insufficient to allow an accurate determination of these parameters.





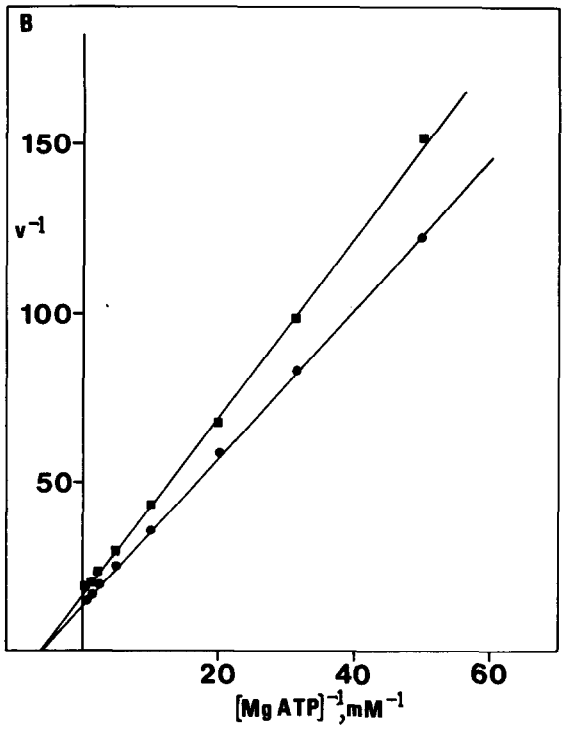


Fig.2. (A) Relationship between reciprocal initial velocity and reciprocal pyruvate concentration for *A. nidulans* pyruvate carboxylase in the presence (•) and absence (▪) of acetyl-CoA. The assay system contained, in 0.5 ml, 100 mM K^+ Hepes (pH 7.4), Na-pyruvate at the concentrations indicated, 10 mM $MgATP^{2-}$, 2 mM Mg^{2+}, 20.2 mM $KHCO_3$, 0 (▪) or 200 (•) μM acetyl-CoA, 5 μg malate dehydrogenase and 0.15 mM NADH. After equilibration to 25°C the reaction was initiated by addition of 0.4 μg pyruvate carboxylase (spec. act. 8.3 units/mg) and V determined from the initial rate of decrease in absorbance at 340 nm. In the figure, V is expressed as $\Delta A_{340\ nm} \cdot min^{-1} \cdot 0.4\ \mu g\ enzyme^{-1}$.

Fig.2. (B) Relationship between reciprocal initial velocity and reciprocal $MgATP^{2-}$ concentration for *A. nidulans* pyruvate carboxylase in the presence (•) and absence (▪) of 200 μM acetyl-CoA. The details are as in (A) except that the assay system contained 10 mM pyruvate and the concentration of $MgATP^{2-}$ indicated.

Fig.2. (C) Relationship between reciprocal initial velocity and reciprocal HCO_3^- concentration for *A. nidulans* pyruvate carboxylase in the presence (•) and absence (▪) of 200 μM acetyl-CoA. The details are as in (A) except that the assay system contained 10 mM pyruvate and HCO_3^- at the concentrations indicated.

substrate a similar analysis demonstrates that addition of acetyl-CoA causes an increase in the apparent V_{max} for this substrate but has no effect on the apparent K_m (fig.2B). With HCO_3^- as the variable substrate and in the presence of saturating concentrations of pyruvate and $MgATP^{2-}$ no significant effect of acetyl-CoA on the apparent K_m or V_{max} for this substrate could be detected (fig.2C) in accord with the data of table 1.

4. Discussion

These studies demonstrate that activation by acetyl-CoA of pyruvate carboxylase from *A. nidulans* can be observed under appropriate conditions in the absence of the regulatory inhibitors, thus establishing the relationship of this enzyme to other pyruvate carboxylases which are activated by acetyl-CoA and inhibited by a dicarboxylic acid, e.g., L-aspartate, L-glutamate [1]. A wide spectrum of sensitivity to activation by acetyl-CoA exists however among these enzymes. Thus in the absence of acetyl-CoA pyruvate carboxylases from avian and mammalian liver, respectively, exhibit no significant catalytic activity [11] and 20% maximal catalytic activity in the presence of very high concentrations of all 3 substrates [12,13], whereas pyruvate carboxylase from *Saccharomyces cerevisiae* shows no activity in the absence of acetyl-CoA only when assayed in a system containing non-saturating concentrations of the substrates and K^+ [14,15]. The enzyme from *A. nidulans* extends this spectrum further since as shown here partial activation by acetyl-CoA can be demonstrated only at a pH on the acid side of the pH optimum and in the presence of non-saturating concentrations of the substrates (table 1, fig.2). We have, however, been unable to establish conditions in which pyruvate carboxylase from *A. nidulans* is inactive in the absence of acetyl-CoA. Although pyruvate carboxylases from *S. cerevisiae* and *A. nidulans* resemble each other in that long-chain acetyl derivatives of CoA are more effective activators than acetyl-CoA [7,16], the nature of the activator–substrate interactions differ in these 2 enzymes. The data of fig.2 indicate that activation of *A. nidulans* pyruvate carboxylase results primarily from an effect on the interaction of this enzyme with pyruvate in which homotropic interactions involving this substrate are abolished and the affinity (as indicated by $[S]_{0.5}$) is increased (fig.2A). In contrast, activation of *S. cerevisiae* pyruvate carboxylase by acetyl-CoA results primarily from an increase in the affinity of this enzyme for K^+ and HCO_3^- [14,15]. Since all the pyruvate carboxylases which are activated by acetyl-CoA share a similar sub-unit structure and a similar involvement of lysyl residues at or near the activation site [7,17,18] it will be of interest to probe the molecular basis for these wide variations in the properties of activation.

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