INHIBITION OF RAT LIVER PYRUVATE CARBOXYLASE BY ACETOACETYL-CoA

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1. Introduction

Fung and Utter [1] have reported that inhibition of chicken liver pyruvate carboxylase by acetoacetyl-CoA possesses properties suggestive of the existence of a distinct site (or sites) on the enzyme which bind this metabolite. Thus, inhibition by acetoacetyl-CoA is non-competitive with respect to acetyl-CoA as well as to the substrates and, in addition, the presence of acetoacetyl-CoA causes modification of the initial velocity/[acetyl-CoA] profile with n_H for acetyl-CoA decreasing to a value of 1.0 in the presence of saturating concentrations of the inhibitor [1,2]. Since β-hydroxybutyryl-CoA is a weak activator of chicken liver pyruvate carboxylase [1], the contrasting effects of this metabolite pair appeared to provide a mechanism whereby the mitochondrial NAD/NADH ratio might modulate the activity of this pyruvate carboxylase [1]. In view of the interest in this possible regulatory mechanism, and as an alternative approach to assessment of the significance of the inhibition, we have examined the effect of acetoacetyl-CoA on the catalytic activity of rat liver pyruvate carboxylase. The data obtained suggest that, although acetoacetyl-CoA also appears to interact at a unique site or sites, this acyl-CoA is a much less potent inhibitor of the rat liver enzyme and, in addition, in this instance has no effect on the shape of the initial velocity/[acetyl-CoA] profile.

2. Methods

Rat liver pyruvate carboxylase was purified and assayed as described previously [3]. The preparations employed in this study had specific activities in the range 10–15 units/mg.

Acetoacetyl-CoA thiolase was assayed, in the direction of acetoacetyl-CoA cleavage, as described by Stern [4], and β-hydroxybutyryl-CoA dehydrogenase, as described by Lynen and Wieland [5]. In addition direct tests were performed in the assay system to establish that both the pyruvate carboxylase and malate dehydrogenase preparations were not significantly contaminated with acetoacetyl-CoA thiolase or β-hydroxybutyryl-CoA dehydrogenase. For β-hydroxybutyryl-CoA dehydrogenase it was shown that no detectable rate of NADH oxidation occurred on addition of either pyruvate carboxylase (20 μg) or malate dehydrogenase (25 μg) to a standard pyruvate carboxylase assay system lacking acetyl-CoA but containing up to 1 mM acetoacetyl-CoA. For acetoacetyl-CoA thiolase the absence of significant activity was established by showing that no increase in the rate of NADH oxidation occurred when up to 1 mM acetoacetyl-CoA was added to a standard pyruvate carboxylase assay system lacking acetyl-CoA but containing 0.05 mM CoASH and 20 μg pyruvate carboxylase.

Inactivation of rat liver pyruvate carboxylase by

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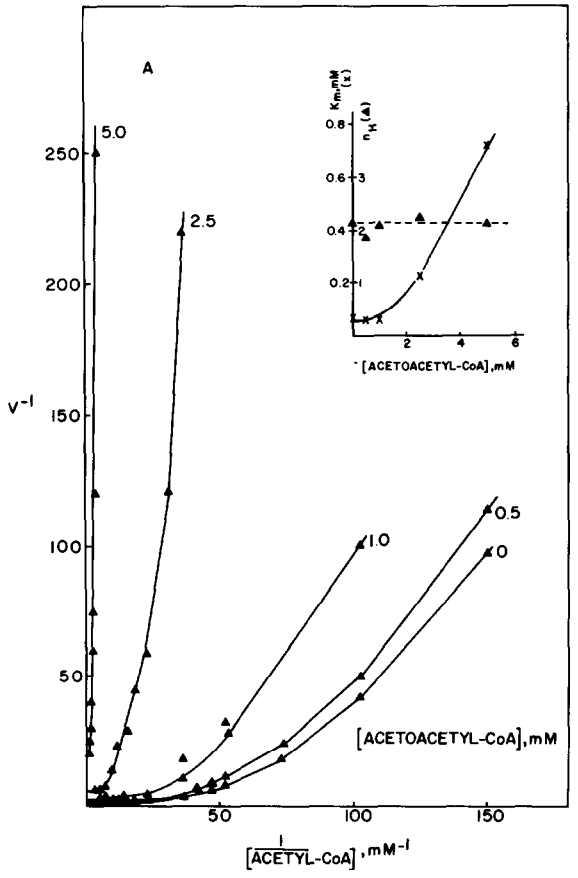
trinitrobenzenesulfonate was performed essentially, as described by Scrutton and White [6].

Acetoacetyl-CoA was synthesised from diketene and CoASH, as described by Wieland and Rueff [7], and was then purified by chromatography on DEAE-Sephadex A-25 (Cl^-) and gel filtration on Sephadex G-10 essentially as described by Fung [8]. Acetoacetyl-CoA was assayed enzymically, using β-hydroxybutyryl-CoA dehydrogenase.

3. Results

Preliminary experiments established that acetoacetyl-CoA is ineffective when tested as an activator of rat liver pyruvate carboxylase, but did act as an inhibitor of this enzyme. The data obtained in studies designed to define the properties of inhibition by acetoacetyl-CoA are summarised in figs.1 and 2.



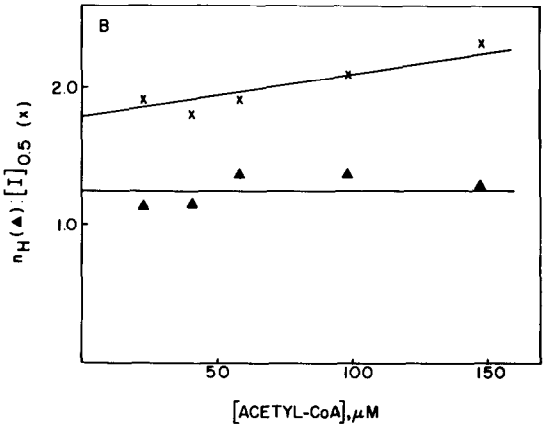


Fig.1(A) Initial velocity of oxalacetate synthesis measured as a function of acetyl-CoA concentration at a series of changing fixed concentrations of acetoacetyl-CoA. The assay system contained 100 mM K^+ HEPES, pH 7.2, 5 mM pyruvate, 2 mM ATP, 5 mM $MgCl_2$, 50 mM $KHCO_3$, 3.3 μg malate dehydrogenase, 0.15 mM NADH and the concentrations of acetyl-CoA and acetoacetyl-CoA as indicated in a total volume of 0.5 ml. After equilibration the 25°C reactions were initiated by addition of 5 μg rat liver pyruvate carboxylase (specific activity = 10.8 units/mg) and initial rate (expressed as ΔA_{340} nm/min 5 μg enzyme) was determined from the decrease in absorbance at 340 nm. In the inset K_m ($[A]_{0.5}$) and n_H obtained from analysis of these data according to the Hill equation:

$$\text{Log}_{10}\ \frac{V}{V_m - V} = n_H \log_{10}\ (\text{acetyl-CoA}) - \log_{10} K$$

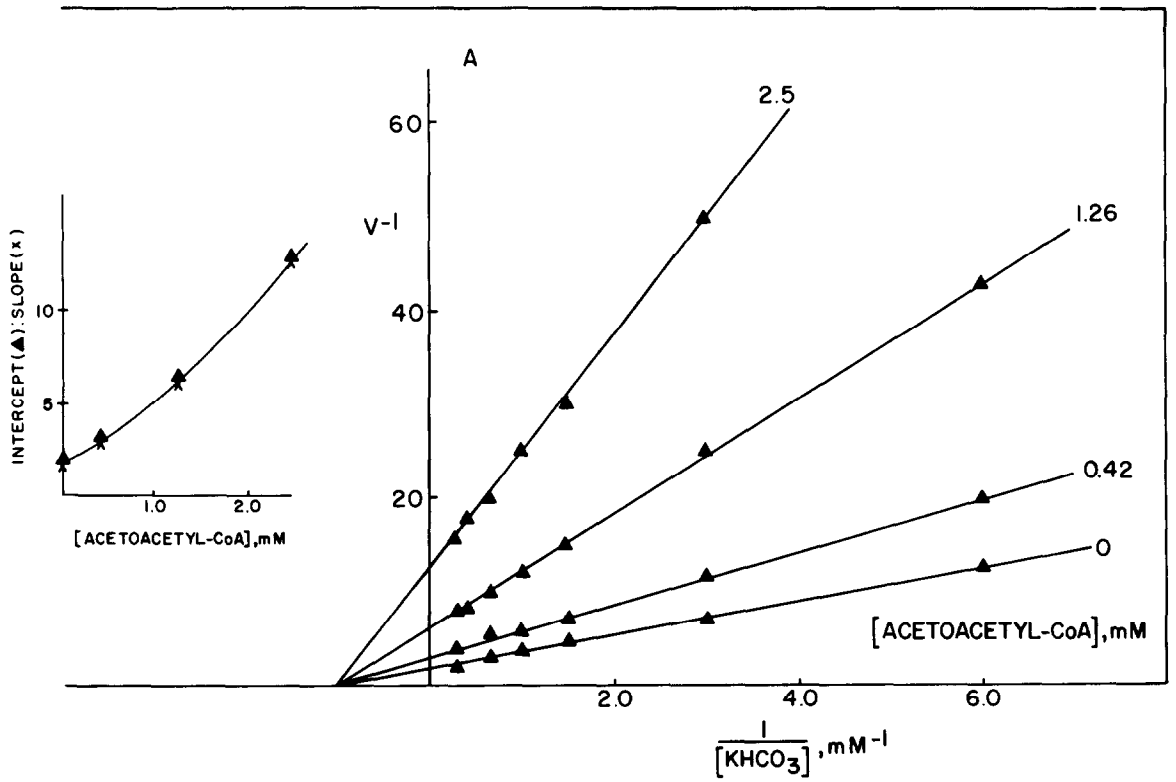
are plotted as a function of acetoacetyl-CoA concentration. (B) Initial velocity of oxalacetate synthesis measured as a function of acetoacetyl-CoA concentration at a series of changing fixed concentrations of acetyl-CoA. Initial rates were obtained as described in A, and the data were analysed according to the Hill equation:

$$\text{Log}_{10}\ \frac{V_o - V_i}{V_i - V_{sat.}} = n_H \log_{10}\ (\text{acetoacetyl-CoA}) - \log_{10} K$$

to yield $[I]_{0.5}$ and n_H.

When initial velocity is measured as a function of [acetyl-CoA], inhibition by acetoacetyl-CoA is clearly non-competitive (fig.1A) as is the case with chicken liver pyruvate carboxylase [2,8]. However, the presence of acetoacetyl-CoA causes no significant alteration in the sigmoid nature of the initial velocity/[acetyl-CoA] profile, as indicated by the invariance of n_H (the Hill coefficient) over a range of 0–2.5 mM acetoacetyl-CoA (fig.1A, inset). The apparent K_A for acetyl-CoA shows some dependence on acetoacetyl-CoA concentration but this is observed only in the presence of higher concentrations of the inhibitor. Conversely, when initial rate is measured as a function of [acetoacetyl-CoA] at a series of changing fixed concentrations of acetyl-CoA and the data analysed according to the Hill equation, it is apparent that both the values for $[I]_{0.5}$ and n_H which characterise this inhibitor are essentially independent of the acetyl-CoA concentration (fig.1b).

When initial rate is measured as a function of the concentration of each of the substrates (pyruvate, $MgATP^{2-}$, HCO_3^-) varied individually at a series of changing fixed concentrations of acetoacetyl-CoA, classical non-competitive inhibition is also observed. This is illustrated in fig.2 for $MgATP^{2-}$ and HCO_3^- as the variable substrates. A similar relationship is observed when pyruvate is the variable substrate provided that the studies are conducted under conditions where then reciprocal initial rate/reciprocal [pyruvate] relationship approaches linearity [9]. In all cases the presence of acetoacetyl-CoA causes no modification of the classical initial velocity/[substrate] relationship observed in the absence of this inhibitor. Secondary plots of the intercepts and slopes as a function of acetoacetyl-CoA concentration are shown in the insets to figs.2A and B. These replots demonstrate that minimal, if any, deviation from linearity is observed in accord with the mean value of n_H (= 1.3) for acetoacetyl-CoA obtained from the initial rate/[inhibitor] profile (fig.1B). The apparent K_i derived from the linear intercept and slope replots (inset, fig.2) (1.4 mM) is in reasonable agreement with the mean $[I]_{0.5}$ value obtained from fig.1B (1.8 mM).



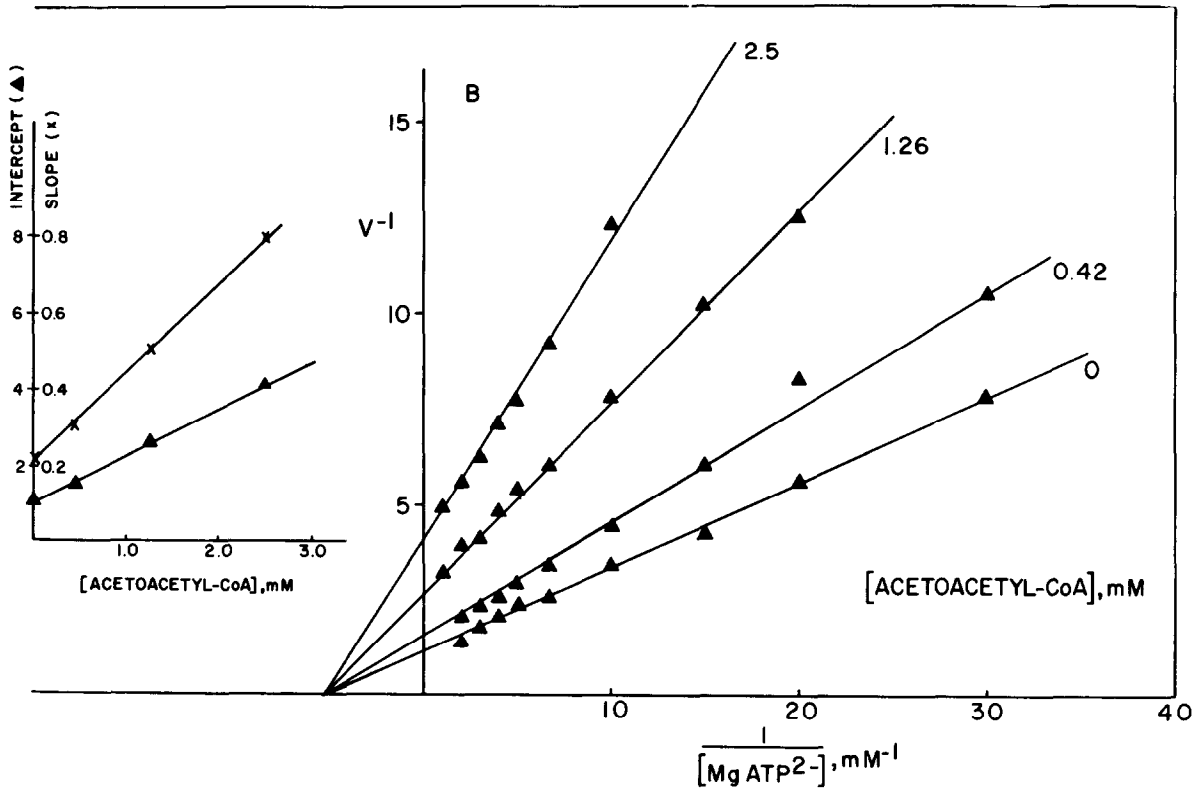


Fig.2. Initial velocity of oxalacetate synthesis measured as a function of HCO_3^- (A) and $MgATP^{2-}$ (B) Concentration at a series of changing fixed concentrations of acetoacetyl-CoA. The assay system contained, in a total volume of 0.3 ml, 100 mM K^+ HEPES pH 7.2, 5 mM pyruvate, 2 mM ATP (A) or as indicated (B), 5 mM $MgCl_2$ (A) or 3 mM + as indicated (B), 50 mM $KHCO_3$ (B) or as indicated (A), 0.15 mM acetyl-CoA, 3.3 μg malate dehydrogenase and 0.15 mM NADH. After equilibrium to 25°C the reaction was indicated by addition of 5 μg pyruvate carboxylase (specific activity = 10.8 units/mg) and the initial rate (expressed as ΔA_{340} nm/min/5 μg) was determined from the decrease in absorbance at 340 nm. In the inset slopes and intercepts (arbitrary units) are plotted as a function of acetoacetyl-CoA concentration.

We have also examined the effect of incubation with trinitrobenzene sulphonate (TNBS) on the extent of inhibition by acetoacetyl-CoA of oxalacetate synthesis in the presence and absence of acetyl-CoA. As shown in fig.3, inhibition by acetoacetyl-CoA is observed in the absence of acetyl-CoA as well as in the presence of the activator, and the extent of inhibition of both activities is unaffected by incubation of the enzyme with trinitrobenzene sulphonate under conditions which result in preferential inactivation of acetyl-CoA-dependent oxalacetate synthesis (fig.3) [6]. Furthermore, when acetoacetyl-CoA is added to the initial incubation at concentrations up to 5-fold in excess of the observed K_i no significant decrease in the rate of inactivation by TNBS is observed in marked contrast to the decrease in inactivation rate which occurs in the presence of acetyl-CoA [6]; and of other acyl-CoAs, e.g. glutaryl-CoA, which act as competitive inhibitors with respect to acetyl-CoA in initial rate studies.

4. Discussion

The evidence presented here provides strong indirect evidence for the presence of a specific site (or sites) on rat liver pyruvate carboxylase which interact with acetoacetyl-CoA. This conclusion, which is in accord

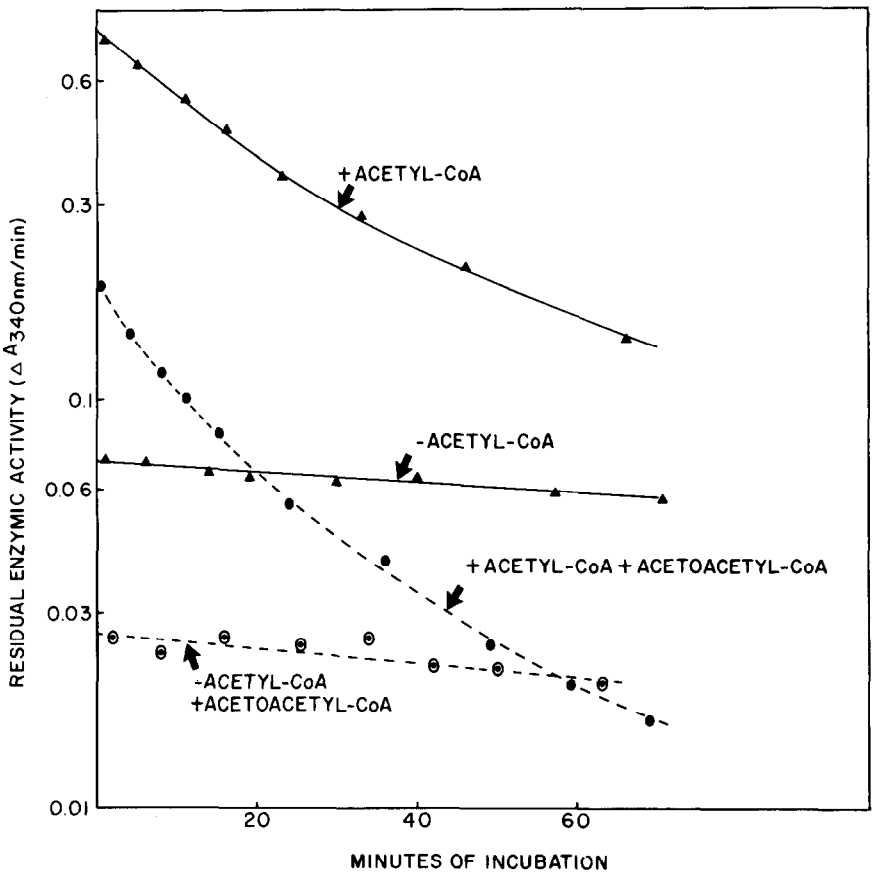


Fig.3. Effect of incubation with trinitrobenzene sulfonate (TNBS) on rates of acetyl-CoA-dependent and -independent oxalacetate synthesis measured in the presence or absence of acetoacetyl-CoA. The incubation system contained, in 0.25 ml, 25 mM K-Na-phosphate, pH 7.2, 50 mM $(NH_4)_2SO_4$, 0.5 mM TNBS and 0.2 mg pyruvate carboxylase (specific activity = 14.1 units/mg). After incubation at 23°C for the times indicated samples were withdrawn for assay of acetyl-CoA-dependent (5 μl) and acetyl-CoA-independent (15 μl) oxalacetate synthesis in the presence (6.0 mM) and absence of acetoacetyl-CoA. The assay systems were as described previously [6,11] except that 100 mM K^+ HEPES pH 7.2 was used as the buffer, the acetyl-CoA concentration was 0.11 mM, and the final volume was 0.5 ml.

with the postulate derived from earlier studies on chicken liver pyruvate carboxylase [1,2] is based primarily on the non-competitive character of the inhibition observed when either acetyl-CoA (fig.1A) or the substrates (fig.2) are the varied reaction components, on the observation of inhibition of acetyl-CoA independent oxalacetate synthesis by acetoacetyl-CoA and the failure to observe a decrease in the extent of this inhibition on incubation with TNBS (fig.3).

However, the effect of occupancy of the postulated acetoacetyl-CoA sites on rat liver pyruvate carboxylase differs in several important respects from that reported previously for the chicken liver enzyme [1,2,8]. Firstly, in the case of rat liver pyruvate carboxylase, the presence of acetoacetyl-CoA has no significant effect on n_H for acetyl-CoA (fig.1A), in contrast to the marked decrease in this parameter which is observed on interaction of acetoacetyl-CoA with chicken liver pyruvate carboxylase [2,8]. The initial rate/[acetoacetyl-CoA] profile is also markedly less sigmoid for rat liver pyruvate carboxylase (n_H = 1.3) (fig.1B) than is the case for the chicken liver enzyme ($n_H \approx 3.0$) [2,8], although for both enzymes

n_H for the inhibitor is insensitive to changes in acetyl-CoA concentration. And finally acetoacetyl-CoA is markedly less potent as an inhibitor of rat liver pyruvate carboxylase (apparent K_i = 1.8 mM) (fig.1B) than is the case for chicken liver pyruvate carboxylase (apparent K_i = 0.17 mM) [2,8]. It should, however, be noted that the ratio of the apparent K_is for acetoacetyl-CoA for these two enzymes is similar to that observed on comparison of the apparent K_as for activation by acetyl-CoA [9–11].

The physiological significance of the inhibition of these pyruvate carboxylases by acetoacetyl-CoA observed in these in vitro studies is, however, still unclear. The data presented both here and previously [1,2] indicate that two pyruvate carboxylases which differ markedly in respect to the properties of activation by acyl derivatives of CoA [8,12] both appear to carry a unique site (or sites) for acetoacetyl-CoA. Although such physiological continuity appears indicative of a significant metabolic role, measurements of acetoacetyl-CoA concentrations in tissue and sub-cellular fractions suggest that the level of this metabolite is probably in the μmolar range and does not exceed 10 μM under any conditions examined thus far [13].

Acknowledgements

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