Pyruvate Carboxylase from a Thermophilic *Bacillus*

STUDIES ON THE SPECIFICITY OF ACTIVATION BY ACYL DERIVATIVES OF COENZYME A AND ON THE PROPERTIES OF CATALYSIS IN THE ABSENCE OF ACTIVATOR

By SUSAN M. LIBOR,* TRICHUR K. SUNDARAM* and MICHAEL C. SCRUTTON†

* Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, U.K., and

† Department of Biochemistry, University of London King's College, Strand, London WC2R 2LS, U.K.

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1. Oxaloacetate synthesis catalysed by pyruvate carboxylase from a thermophilic *Bacillus* in the absence of acetyl-CoA required addition of high concentrations of pyruvate, MgATP^{2-} and HCO_3^- , and at 45°C occurred at a maximum rate approx. 20% of that in the presence of a saturating concentration of acetyl-CoA. The apparent K_m for HCO_3^- at pH 7.8 was 400 mM without acetyl-CoA, and 16 mM with a saturating activator concentration. The relationship between reciprocal initial rate and reciprocal MgATP^{2-} concentration was non-linear (convex-down) in the absence of acetyl-CoA, but the extent of deviation decreased as the activator concentration was increased. The relationship between reciprocal initial rate and reciprocal pyruvate concentration was non-linear (convex-down) in the presence or absence of acetyl-CoA. 2. The optimum pH for catalysis of oxaloacetate synthesis was similar in the presence or absence of acetyl-CoA. The variation with pH of apparent K_m for HCO_3^- implicated residue(s) with pK_a 8.6 in catalysis of the activator-independent oxaloacetate synthesis. 3. Linear Arrhenius and van't Hoff plots were observed for the temperature-dependence of oxaloacetate synthesis in the absence of acetyl-CoA over the range 25–55°C. E_a (activation energy) was 56.3 kJ/mol and $\Delta H^\ddagger$ (HCO_3^-) (enthalpy of activation) was –38.6 kJ/mol. In the presence of acetyl-CoA, biphasic Arrhenius and van't Hoff plots are observed with a change of slope at 30°C in each case. E_a was 43.7 and 106.3 kJ/mol above and below 30°C respectively. 4. Incubation of *Bacillus* pyruvate carboxylase with trinitrobenzenesulphonate caused specific inactivation of acetyl-CoA-dependent catalytic activity associated with the incorporation of 1.3 ± 0.2 trinitrophenyl residues per subunit. Activator-independent catalysis and regulatory inhibition by L-aspartate were unaffected. The rate of inactivation of acetyl-CoA-dependent catalysis by trinitrobenzenesulphonate was specifically decreased by addition of acetyl-CoA and other acyl-CoA species, but complete protection was not obtained. 5. All alkylacyl derivatives of CoA tested activated *Bacillus* pyruvate carboxylase; acetyl-CoA was the most effective. The apparent K_a exhibited a biphasic relationship with acyl-chain length for the straight-chain homologues. Certain long-chain acyl-CoA species showed additional activation at a high concentration. Weak activation occurred on addition of CoA or adenosine 3',5'-bisphosphate, but carboxyacyl-CoA species and derivatives containing a modified phosphoadenosyl group were inhibitory. Thioesters of CoA with non-carboxylic acids, e.g. methanesulphonyl-CoA, serve as activators of the thermophilic *Bacillus* and *Saccharomyces cerevisiae* pyruvate carboxylases, but as inhibitors of pyruvate carboxylases obtained from chicken and rat liver. 6. α -Oxoglutarate mimics the effect of L-aspartate as a regulatory inhibitor of the pyruvate carboxylases from both the thermophilic *Bacillus* and *Saccharomyces cerevisiae*. L-Glutamate was ineffective in both cases.

Pyruvate carboxylases which catalyse reaction (1) have been isolated in a homogeneous or near-homogeneous state from eight different sources, and

partially purified preparations of this enzyme have been obtained from many other organisms (see Scrutton & Young, 1972; Utter *et al.*, 1975).

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Those enzymes that have been studied in detail show a striking degree of resemblance with respect to the properties of the catalytic site such as metal-ion specificity, nucleotide specificity, presence of biotinyl residues, and in most instances apparent K_m values for all three substrates (Scrutton & Young, 1972). Further, except for inducible pyruvate carboxylases that have been isolated from *Pseudomonas citronellolis* (Seubert & Remberger, 1961; O'Brien *et al.*, 1977) and *Azotobacter vinelandii* (Scrutton & Taylor, 1974), all these enzymes have been characterized as large proteins (mol.wt. approx. 500000), which are composed of four apparently identical polypeptide chains (Barden *et al.*, 1975; Utter *et al.*, 1975). Thus in contrast with certain other biotin carboxylases, the catalytic components of most pyruvate carboxylases (i.e. the carboxylation site, the biotinyl residue and the transcarboxylation site; Barden *et al.*, 1972) together with the effector site(s) all appear to be carried on a single polypeptide chain.

The pyruvate carboxylases obtained from different sources which are subject to 'fine control' differ in at least three important respects. Although all of these enzymes except that isolated from *Aspergillus niger* are activated by acetyl-CoA (cf. Scrutton & Young, 1972), the proportion of the catalytic potential that can be expressed in the absence of the activator ranges from essentially zero for the enzyme from chicken liver (Scrutton & Utter, 1967) to approx. 100% for the enzyme from *Saccharomyces cerevisiae* (Tolbert, 1970). Further, although modification of the nucleotide portion of the acetyl-CoA molecule causes loss of the allosteric activation potential in all cases examined thus far, each of the three enzymes that have been studied in detail exhibits a unique pattern of specificity for activation by homologues of acetyl-CoA (Fung, 1971; Tolbert, 1970; Scrutton, 1974a). The enzymes vary also in the nature of the dicarboxylic acid which acts as an inhibitory effector (Cazzulo & Stoppani, 1968; Cazzulo *et al.*, 1970; Scrutton & White, 1974) and in the mode of organization of the four polypeptide chains within the tetramer (Valentine *et al.*, 1966; Utter *et al.*, 1975). Comparable studies of the properties of catalysis in the absence of activator and of the specificity of acyl-CoA activation have not been reported for the pyruvate carboxylase from a thermophilic *Bacillus*, the only well-characterized bacterial pyruvate carboxylase, although some preliminary observations suggested that this enzyme might be dissimilar in these properties to the other pyruvate carboxylases (Cazzulo *et al.*, 1970). Data from more detailed studies, which are reported here, fulfil this expectation and apparently indicate a relationship between the properties of the activator and regulatory inhibitor sites.

Materials and Methods

Purification of pyruvate carboxylase

The thermophilic *Bacillus* used in this investigation has been described as *Bacillus stearothermophilus* (Cazzulo *et al.*, 1970, 1971). However, more recent data, especially from DNA-hybridization studies, suggest that it may not belong to the species *stearothermophilus* (L. L. Campbell, personal communication). Details of the methods used for growth of the bacterium and for preparation of the cell-free extracts have been presented (Cazzulo *et al.*, 1970). For most of the studies described pyruvate carboxylase was purified by a modification of the method of Cazzulo *et al.* (1970), which was primarily designed to eliminate the step involving fractional re-extraction of the precipitated protein with $(\text{NH}_4)_2\text{SO}_4$. The cell-free extract was prepared, nucleic acids were removed with protamine sulphate and protein was precipitated with $(\text{NH}_4)_2\text{SO}_4$ as described by Cazzulo *et al.* (1970). The precipitated protein was dissolved in a minimum volume of 20 mM-potassium phosphate buffer, pH 6, and, after overnight dialysis against this buffer, and removal of precipitated protein, was applied to a column (50 cm $\times$ 1.5 cm) of phosphocellulose equilibrated with the same buffer. The column was washed with the phosphate buffer until no protein was detectable in the effluent. The pyruvate carboxylase was then eluted from the phosphocellulose column with 6 column volumes of phosphate buffer increasing linearly in concentration from 0.02 M to 0.5 M. The enzyme emerged at a phosphate concentration of approx. 0.25 M at the trailing edge of a broad peak of protein. The protein in the active fractions was precipitated by addition of solid $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of 75% saturation at 25°C. The precipitate was dissolved in 50 mM-Tris/HCl buffer (pH 7.5) and then dialysed overnight against this buffer. After removal of the precipitated protein by centrifugation at 38000g for 10 min, the preparation was fractionated on a column (40 cm $\times$ 2 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. The column was washed with the Tris/HCl buffer after absorption of the enzyme and then eluted with 5 column volumes of the same buffer containing a linear gradient of 0–0.6 M-KCl. The enzyme was eluted in a broad peak at approx. 0.3 M-KCl. The protein in the active fractions was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ as described above and the precipitate obtained was dissolved in a small volume (2–5 ml) of 50 mM-Tris/HCl buffer, pH 7.6, containing 0.15 M-KCl. This fraction was then subjected to gel filtration through a column (40 cm $\times$ 2 cm) of Sephadex G-200 or Sepharose 6B equilibrated with the Tris/HCl/KCl buffer. The fractions containing pyruvate carboxylase at the highest specific activity were pooled and precipitated as described above. For the best enzyme prepara-

tions (specific activity approx. 40 units/mg of protein) the modified procedure gave a 270-fold purification from the cell-free extract with a 27% recovery of enzyme activity.

For a few of the studies described the enzyme was purified by the avidin-Sepharose affinity-chromatography procedure described by Libor *et al.* (1975b).

Before use in the studies described, the enzyme was equilibrated by dialysis or gel filtration with 50 mM-Tris/HCl or Hepes (K^+ salt), pH 7.2–7.6, containing 0.15 M-NaCl (or -KCl), 0.1 mM-EDTA and phenylmethanesulphonyl fluoride (10 μ g/ml).

Pyruvate carboxylases from rat, chicken liver and *Saccharomyces cerevisiae* were prepared as described previously (Scrutton *et al.*, 1969, 1970; Scrutton & White, 1974).

Pyruvate carboxylase activity was assayed by measuring the initial rate of decrease in A_{340} when oxaloacetate synthesis was measured in the presence of malate dehydrogenase and NADH as described previously (Scrutton *et al.*, 1969). All assays were performed at 45°C unless otherwise indicated. Protein was estimated from the A_{280} by using ϵ 428 $\text{mm}^{-1} \cdot \text{cm}^{-1}$.

Inactivation by trinitrobenzenesulphonate

The enzyme preparations used, except where otherwise specified, had a specific activity of 20–30 units/mg of protein. The specific activities of the best preparations of this enzyme are in the region of 40 units/mg of protein at 45°C. The purity of such preparations is estimated to be in excess of 90% on the basis of analysis by polyacrylamide-gel electrophoresis in the presence and absence of sodium dodecyl sulphate. For estimation of the rate of inactivation the incubation system contained 50 mM-Tris/HCl, pH 7.4–7.5, 0.1–0.15 M-NaCl, 0.1 mM-EDTA, and trinitrobenzenesulphonate and enzyme as indicated. Acetyl-CoA, other acyl-CoA species and L-aspartate were also included as indicated. The reaction was initiated by the addition of trinitrobenzenesulphonate and the mixture was incubated at room temperature (20°C). At suitable time intervals 10 μ l portions were withdrawn and assayed for pyruvate carboxylase activity in the absence of acetyl-CoA or in the presence of a saturating concentration of the activator. The assay system for oxaloacetate synthesis in the absence of acetyl-CoA contained (in 1 ml): Tris/HCl, pH 7.8, 200 μ mol; sodium pyruvate, 8 μ mol; ATP, 4 μ mol; MgCl_2 , 7 μ mol; KHCO_3 , 400 μ mol; NADH, 0.2 μ mol; malate dehydrogenase, 20 units. The course of inactivation, which followed first-order kinetics for at least two half-lives or 1 h (when inactivation was slow), was monitored for an appropriate period and expressed in terms of k' , the pseudo-first-order rate constant.

Acetyl-CoA and propionyl-CoA were prepared by reaction of CoA (free acid) dissolved in 0.1 M- NaHCO_3 with a 50% molar excess of acetic anhydride and propionic anhydride respectively. The preparation was then adjusted to pH 2.5, extracted with 5 vol. of diethyl ether and in some instances was further purified by chromatography on DEAE-Sephadex A-25 (Cl^- form) followed by gel filtration on Sephadex G-10 as described by Fung (1971). All other acyl derivatives of CoA and related analogues were obtained from either P-L Biochemicals (Milwaukee, WI 53205, U.S.A.) or Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BK, U.K., and were purified when necessary by chromatography followed by gel filtration as described above. Purity was checked by t.l.c. on polyethyleneimine paper.

The concentrations of stock solutions of acyl-CoA species were determined by measurement of the A_{412} in the presence of hydroxylamine and 5,5'-dithiobis-(2-nitrobenzoate) (Cazzulo *et al.*, 1970) and in some cases also from the A_{259} (ϵ 15.4 $\text{mm}^{-1} \cdot \text{cm}^{-1}$). The ratio of the concentrations determined by these two procedures was also used as an index of the purity of the acyl-CoA preparation.

Source of chemicals

Trinitrobenzenesulphonic acid, ATP and sodium pyruvate were obtained from Sigma, and NADH and malate dehydrogenase (pig heart) from Boehringer (London), Lewes, East Sussex, U.K. For assays performed at the higher temperatures, malate dehydrogenase purified by Mr. I. Wright, University of Manchester Institute of Science and Technology, from the extreme thermophilic bacterium, *Thermus aquaticus*, was used. Buffer reagents, KHCO_3 and MgCl_2 (AnalaR reagents) were obtained from BDH Chemicals, Poole, Dorset, U.K.

Results

Properties of catalysis in the absence of activator

Cazzulo *et al.* (1970) have reported that, under standard assay conditions, pyruvate carboxylase from the thermophilic *Bacillus* catalyses oxaloacetate synthesis in the absence of acetyl-CoA at approx. 2% of the rate observed in the presence of a saturating concentration of this activator. In view of the pronounced effect of increased substrate concentration on the extent of activator-independent catalysis observed for the pyruvate carboxylases isolated from *S. cerevisiae* and rat liver (Tolbert, 1970; Scrutton & White, 1973) the properties of such catalysis for the *Bacillus* enzyme have been characterized. The relevant data, some of which are presented in Figs. 1–3, demonstrate that the properties of acti-

Fig. 1. Reciprocal initial rate of oxaloacetate synthesis measured as a function of reciprocal HCO_3^- concentration in the presence of a series of fixed concentrations of acetyl-CoA

(a) A primary plot of the values of reciprocal initial velocity (v) as a function of reciprocal $[\text{HCO}_3^-]$. The assay system contained, in 1.0 ml: Tris/HCl, pH 7.8, 200 μmol ; sodium pyruvate, 8 μmol ; ATP, 2 μmol ; MgCl_2 , 5 μmol ; NADH, 0.2 μmol ; 20 units of malate dehydrogenase; KHCO_3 and acetyl-CoA as indicated, and pyruvate carboxylase. After equilibration of the system at 45°C, the reaction was initiated by the addition of an appropriate amount (2.5–12.5 μg) of pyruvate carboxylase (specific activity 27.5 units/mg of protein). The initial reaction rate (v) is expressed as μmol of oxaloacetate synthesized/min per 25 μg of enzyme. Acetyl-CoA concentrations were: ●, 0 μM ; ▲, 0.2 μM ; ■, 0.4 μM ; ○, 0.8 μM ; △, 2 μM ; □, 4 μM . (b) A secondary plot of the values of apparent $V_{\max}$ (expressed as μmol of oxaloacetate synthesized/min per mg of protein) (▲) and apparent K_m for HCO_3^- (●) from (a) plotted as a function of acetyl-CoA concentration. The plot includes values of apparent K_m and $V_{\max}$ at 1 μM -acetyl-CoA for which, in the interests of clarity, the primary data are not shown in (a).

vator-independent catalysis differ very markedly from those observed in the presence of activator. For example, the apparent K_m for HCO_3^- observed in the absence of acetyl-CoA (400 mm) is 25 times that observed in the presence of a saturating concentration of this activator (16 mm) (Fig. 1). Both the apparent K_m for HCO_3^- and the apparent $V_{\max}$ are sensitive functions of acetyl-CoA concentration, but over different ranges. The apparent K_m changes most markedly when the acetyl-CoA concentration is increased from 0 to 2 μM , with little change in apparent $V_{\max}$. The increase in $V_{\max}$ then occurs with a further increase in [acetyl-CoA] from 2 to 4 μM , but without much concomitant decrease in the apparent K_m (Fig. 1). However, no deviation from linearity is observed for the double-reciprocal plot of initial velocity/versus $[\text{HCO}_3^-]$ under the conditions used (Fig. 1).

By contrast, in the absence, and at low concentrations, of acetyl-CoA the double-reciprocal plot of initial velocity versus $[\text{MgATP}^{2-}]$ becomes markedly non-linear, having a convex-down form as compared

with the essentially linear relationship observed in the presence of saturating activator concentration (Fig. 2). The data suggest that the apparent $V_{\max}$ is not markedly increased on addition of acetyl-CoA when, as in this experiment, the studies are performed in the presence of saturating concentrations of HCO_3^- and pyruvate. The data of Fig. 2 cannot, however, be explained by occupation of the activator site by MgATP^{2-} , since no activation is observed when this nucleotide is added at concentrations in the range 10–20 mm to a standard assay mixture lacking acetyl-CoA.

For pyruvate the double-reciprocal plot of initial rate versus substrate concentration is non-linear (convex-down) even in the presence of saturating concentrations of acetyl-CoA, as has been observed for other pyruvate carboxylases (Taylor *et al.*, 1969; McClure *et al.*, 1971). However, this non-linearity is much increased in the absence and at low concentrations of acetyl-CoA (Fig. 3), and it is notable that, in contrast with the results obtained with MgATP^{2-} (Fig. 2), the apparent $V_{\max}$ varies very markedly with

Fig. 2. Reciprocal initial rate of oxaloacetate synthesis as a function of reciprocal MgATP^{2-} concentration at a series of fixed concentrations of acetyl-CoA

The assay system and other details were similar to those described for Fig. 1 with the following modifications to the assay system: KHCO_3 , $400 \mu\text{mol}$; Mg^{2+} , $3 \mu\text{mol}$; and the concentration of MgATP^{2-} as indicated. It was assumed that under these conditions the concentration of MgATP^{2-} is equivalent to the concentration of ATP added to the system. Acetyl-CoA concentrations were: $\bullet$, $0 \mu\text{M}$; $\blacktriangle$, $0.2 \mu\text{M}$; $\blacksquare$, $0.4 \mu\text{M}$; $\circ$, $0.8 \mu\text{M}$; $\triangle$, $40 \mu\text{M}$.

Fig. 3. Reciprocal initial rate of oxaloacetate synthesis as a function of reciprocal pyruvate concentration at a series of fixed concentrations of acetyl-CoA

The assay system and other details were similar to those described for Fig. 1, except for the following modifications to the assay system: KHCO_3 , $400 \mu\text{mol}$; ATP, $4 \mu\text{mol}$; MgCl_2 , $7 \mu\text{mol}$; sodium pyruvate, as indicated. Acetyl-CoA concentrations were: $\bullet$, $0 \mu\text{M}$; $\blacktriangle$, $0.4 \mu\text{M}$; $\circ$, $2.0 \mu\text{M}$; $\triangle$, $40 \mu\text{M}$.

acetyl-CoA concentration when pyruvate is the variable substrate.

From assays at saturating concentrations of HCO_3^- , MgATP^{2-} and pyruvate, which were obtained from Figs. 1–3, the maximum rate of oxaloacetate synthesis in the absence of acetyl-CoA is estimated to be 20% of that in the presence of a saturating concentration of this activator. Oxaloacetate synthesis in the absence of activator is, however, as sensitive to inhibition by L-aspartate as the reaction in the presence of acetyl-CoA, indicating that interaction between the catalytic and regulatory inhibitor sites is not dependent on occupancy of the activator site.

We have also characterized the temperature- and pH-dependence of oxaloacetate synthesis in the absence of acetyl-CoA, since in many instances an allosteric effector causes marked changes in one or both of these parameters. To this end we have measured the changes in apparent K_m and V_{max} with pH and temperature using HCO_3^- as the variable substrate, since this is the only reaction component that exhibits classical kinetic behaviour in the absence of acetyl-CoA (Fig. 1).

The data obtained with pH as the experimental variable are shown in Fig. 4. The optimum pH for catalysis of oxaloacetate synthesis in the absence of acetyl-CoA is obtained as 8.4, a value that does not differ significantly from that observed in the presence of activator (8.3) (Cazzulo *et al.*, 1970). The variation of apparent K_m (expressed here as $\text{p}K_m$) with pH implicates a residue (or residues) with $\text{p}K_a$ 8.6 in catalysis of the activator-independent reaction. These data are similar to those obtained in studies on the effect of pH on activator-independent synthesis of oxaloacetate by rat liver pyruvate carboxylase, which implicated a residue (or residues) with $\text{p}K_a$ 8.3 in catalysis of this reaction (Scrutton, 1974a).

When the temperature is varied marked differences are observed in the behaviour of oxaloacetate synthesis in the absence of acetyl-CoA and in the presence of a saturating concentration of this activator. The Arrhenius plot for the maximum rate of oxaloacetate synthesis in the absence of acetyl-CoA is linear over the temperature range 25 – 55°C (Fig. 5a). The data obtained below 25°C are less reliable, since the apparent K_m for HCO_3^- becomes very unfavourable and consequently it is very difficult to saturate the reaction system with this component. The activation energy estimated from the slope of this plot is 56.3 kJ/mol . In the presence of a saturating concentration of acetyl-CoA a biphasic plot is observed with a break at 30°C . From the two slopes values of 46.3 and 106.3 kJ/mol may be calculated for the activation energy at temperatures above and below 30°C respectively. The van't Hoff plot of the variation of apparent K_m for HCO_3^- with reciprocal absolute temperature is linear and of positive slope over the

Fig. 4. Effect of pH on apparent $V_{\max}$ and apparent K_m (HCO_3^-) for oxaloacetate synthesis in the absence of acetyl-CoA

The values for apparent $V_{\max}$ (▲) (expressed as μmol of oxaloacetate/min per mg of protein) and apparent K_m (expressed here as pK_m) (HCO_3^-) (●) were derived from double-reciprocal plots of data obtained from measurements of initial reaction rate at 45°C in the following system (1 ml): Hepes (K^+ salt), $200\mu\text{mol}$; sodium pyruvate, $8\mu\text{mol}$; ATP, $4\mu\text{mol}$; MgCl_2 , $7\mu\text{mol}$; KHCO_3 , $3\text{--}800\mu\text{mol}$; NADH, $0.2\mu\text{mol}$; malate dehydrogenase, 20 units; pyruvate carboxylase (specific activity 20 units/mg of protein), $12.5\text{--}25\mu\text{g}$. The pH of the reaction mixture, lacking the enzyme, was adjusted and measured at room temperature (20°C). After equilibration at 45°C the reaction was initiated by addition of enzyme. Several random checks indicated that the pH at the end of the assay was essentially the same as that observed initially.

temperature range studied ($25\text{--}55^\circ\text{C}$) for oxaloacetate synthesis in the absence of acetyl-CoA but is non-linear in the presence of this activator (Fig. 5b). The $\Delta H^\ddagger$ values obtained from the slopes of the van't Hoff plots are -38.6kJ/mol for oxaloacetate synthesis in the absence of acetyl-CoA, and -52.1 and -101.6kJ/mol above and below 30°C respectively in the presence of a saturating concentration of this activator. It is noteworthy that, when acetyl-CoA is present, a break occurs in both the van't Hoff and Arrhenius plots at about 30°C and in the context of the thermophilic growth habit of this *Bacillus*, both the activation energy and the K_m for HCO_3^- become more favourable at the higher temperatures. Further, the results show notable similarities and differences compared with those of the studies of this type performed with rat liver pyruvate carboxylase (Scrutton, 1974a). The Arrhenius plots are of a similar form for both enzymes, but for acetyl-CoA-

dependent catalysis the break occurs at 25°C for rat liver pyruvate carboxylase. If, as suggested by Raison (1973), the biphasic nature of the Arrhenius plot indicates a phase transition, this occurs at a significantly higher temperature for the *Bacillus* enzyme, as might be expected for an enzyme obtained from a thermophilic organism. The van't Hoff plot obtained for rat liver pyruvate carboxylase is linear but of negative slope for oxaloacetate synthesis in the absence of acetyl-CoA and is biphasic in the presence of a saturating concentration of this activator, with the break occurring at the same temperature as in the Arrhenius plot (Scrutton, 1974a). Thus $\Delta H^\ddagger$ has a positive value for activator-independent catalysis by rat liver pyruvate carboxylase, in contrast with the negative values for this parameter observed for the *Bacillus* enzyme (Fig. 5b).

Specific inactivation of acetyl-CoA-dependent oxaloacetate synthesis on incubation with trinitrobenzenesulphonate and the effects of acyl derivatives of CoA on the rate of this inactivation

As observed for several other pyruvate carboxylases (Scrutton & White, 1973; Ashman *et al.*, 1973) incubation of the *Bacillus* enzyme with trinitrobenzenesulphonate causes specific inactivation of oxaloacetate synthesis dependent on acetyl-CoA without a concomitant impairment of catalytic capacity which is not dependent on this activator (Fig. 6a). The extent of inhibition by L-aspartate of oxaloacetate synthesis in the absence of acetyl-CoA also remains unaffected under conditions that cause almost total desensitization to activation by acetyl-CoA (Fig. 6a), further indicating the specificity of this modification.

The rate and properties of the inactivation process were unaffected by the inclusion of a thiol compound in the system, indicating that it was not due to a reaction between trinitrobenzenesulphonate and cysteine residues in the enzyme. However, the spectral changes that occurred during this process were characteristic of a reaction between trinitrobenzenesulphonate and lysine residues in the enzyme paralleling the findings made for other pyruvate carboxylases (Keech & Farrant, 1968; Scrutton & White, 1973; Scrutton *et al.*, 1977). An incorporation of 1.3 ± 0.2 trinitrophenyl residues per subunit (136000 daltons) was estimated from the change in absorption spectrum at a time when there was a 97% decrease in the rate of oxaloacetate synthesis dependent on acetyl-CoA. The enzyme used in this experiment had a specific activity of 40 units/mg of protein and hence was at least 90% pure. The estimate of incorporation of trinitrophenyl residues was based on absorption coefficients of $1.05 \times 10^4 \text{M}^{-1} \cdot \text{cm}^{-1}$ for trinitrophenyl-lysine at 367 nm (Coffee *et al.*, 1970) and of $4.28 \times 10^5 \text{M}^{-1} \cdot \text{cm}^{-1}$ for *Bacillus* pyruvate

Fig. 5. Effect of variation of temperature on apparent V_{max} . (a) and the apparent K_m (HCO_3^-) (b) for oxaloacetate synthesis in the presence and absence of acetyl-CoA

The values for apparent V_{max} , (expressed as μmol of oxaloacetate/min per mg of protein) and apparent K_m were obtained from double-reciprocal plots of data from initial-reaction-rate measurements in the following systems (1 ml). Oxaloacetate synthesis in the absence of acetyl-CoA: Hepes (K^+ salt), pH 7.6, 200 μmol ; sodium pyruvate, 8 μmol ; ATP, 4 μmol ; $MgCl_2$, 7 μmol ; $KHCO_3$, 20–1000 μmol ; NADH, 0.2 μmol ; malate dehydrogenase, 20 units. Oxaloacetate synthesis in the presence of acetyl-CoA: Hepes (K^+ salt), pH 7.6, 200 μmol ; sodium pyruvate, 4 μmol ; ATP, 2 μmol ; $MgCl_2$, 5 μmol ; acetyl-CoA, 0.04 μmol ; $KHCO_3$, 2–100 μmol ; NADH, 0.2 μmol ; malate dehydrogenase, 20 units. After equilibration at the required temperature, the reaction was initiated by the addition of malate dehydrogenase followed by pyruvate carboxylase (2.5–12.5 μg). When the assay temperature was greater than 45°C, malate dehydrogenase used was from *T. aquaticus*; up to 45°C pig heart malate dehydrogenase was used. Control experiments demonstrated that the pH of the assay system did not vary significantly over the range of temperature used in this study. Symbols: ●, apparent V_{max} . (a) or apparent K_m (b), no acetyl-CoA; ○, apparent V_{max} . (a) or apparent K_m (b), +acetyl-CoA.

carboxylase at 280 nm (Libor *et al.*, 1975a). On continued incubation with trinitrobenzenesulphonate the extent of incorporation of trinitrophenyl groups gradually increased, indicating reaction at other lysine residues.

Additional insight into the nature of the role of the lysine residues that react with trinitrobenzenesulphonate is provided by the effects observed on the rate of inactivation by this group-specific reagent when various components of the pyruvate carboxylase reaction are added to the incubation system. Addition of the substrates (pyruvate, $MgATP^{2-}$, HCO_3^-) has no effect on the rate of inactivation, but this rate is dramatically decreased in the presence of acetyl-CoA and certain of its homologues (Fig. 6 and Table 1). A sigmoidal relationship is observed between inactivation rate and acetyl-CoA concentration, but the protection afforded against inactivation is not complete (Fig. 6). The residual rate of inactivation of the enzyme–acetyl-CoA complex, which is expressed

as k_2/k_1 in Table 1, is observed for all acyl derivatives of CoA that have been tested in this system. Analysis of the data according to the Hill equation (Scrutton *et al.*, 1977) has yielded values for K_d , the acyl-CoA concentration required to produce 50% decrease in the inactivation rate, and for h , the Hill coefficient. Except for the K_d for acetyl-CoA, the values for both these parameters obtained in the inactivation-rate studies (Table 1) are in reasonable agreement with those observed in initial-rate studies (Table 2). For acetyl-CoA, a direct comparison cannot be made since, at the protein concentrations used in these studies (Fig. 6b), the concentration of binding sites calculated on the basis of a subunit mol.wt. of 136000 (1–2 μM) is appreciable as compared with the acetyl-CoA concentration. When a correction is applied for the bound ligand, the K_d determined from the inactivation-rate analysis is in better agreement with the apparent K_d .

These studies therefore demonstrate that, like

Fig. 6. Inactivation by trinitrobenzenesulphonate of oxaloacetate synthesis dependent on the presence of acetyl-CoA catalysed by pyruvate carboxylase

(a) The incubation mixture contained: Tris/HCl, pH 7.4, 50 mM; EDTA, 0.1 mM; NaCl, 0.1 M; pyruvate carboxylase (specific activity 24 units/mg of protein), 250 μg/ml; trinitrobenzenesulphonate, 0.25 mM. Acetyl-CoA (0.8 mM) and L-aspartate (10 mM) were included as indicated. The rate of inactivation was measured as described in the Materials and Methods section. ○, Oxaloacetate synthesis independent of acetyl-CoA; □, oxaloacetate synthesis independent of acetyl-CoA in the presence of 10 mM-L-aspartate; ●, oxaloacetate synthesis dependent on activation by acetyl-CoA; ▲, oxaloacetate synthesis dependent on activation by acetyl-CoA, but with enzyme exposed to trinitrobenzenesulphonate in the presence of acetyl-CoA. (b) The incubation mixture was similar to that for (a) with the following modifications: the pH of the Tris/HCl buffer was 7.5, the concentrations of NaCl and trinitrobenzenesulphonate were 0.15 M and 0.5 mM respectively and the acetyl-CoA concentration was varied as indicated.

Table 1. Effects of acyl derivatives of CoA on the rate of inactivation of *Bacillus* pyruvate carboxylase by trinitrobenzenesulphonate

The parameters for acetyl-CoA were derived from a Hill plot of the data presented in Fig. 6(b). The parameters for the other acyl-CoA species were derived from similar experiments in which the concentration of pyruvate carboxylase (specific activity 24 units/mg of protein) was 100 μg/ml. k_1 and k_2 are respectively the rate constants of inactivation observed in the absence of added acyl-CoA and in the presence of a concentration of acyl-CoA that afforded maximum protection against inactivation.

Ligand	K_d (μM)	h	k_2/k_1
Acetyl-CoA	10	2.9	0.01
<i>n</i> -Butyryl-CoA	47	2.0	0.02
<i>n</i> -Hexanoyl-CoA	250	1.5	0.01
<i>n</i> -Octanoyl-CoA	80	1.7	0.01

other pyruvate carboxylases, the *Bacillus* enzyme can be desensitized to acyl-CoA activation by trinitrophenylation of the ε-amino group of a specific reactive lysine residue in each protomer.

Specificity for activation of oxaloacetate synthesis by acyl derivatives of CoA and related compounds

Previous studies on the activation specificity of pyruvate carboxylase from the thermophilic *Bacillus* have shown that acetyl-CoA is a very effective activator, but that activation is also caused by CoA at high concentration (Cazzulo *et al.*, 1970). In view of the apparent species specificity in the properties of acyl-CoA activation it was decided to define this parameter for the *Bacillus* enzyme. Some relevant data are summarized in Table 2. A wide range of acyl-CoA species are able to activate this bacterial pyruvate carboxylase, but the relationship between efficiency of activation and the structure of the acyl group is complex. For the straight-chain homologues an increase in chain length from two to 14 carbon atoms causes a progressive decrease in apparent V_{max} . However, the apparent K_A increases by two orders of magnitude with an increase in acyl-chain length from two to five carbon atoms and then decreases by one order of magnitude with an increase in chain length to 12 carbon atoms (Table 2). Rather similar behaviour is observed for h which decreases from 2.9 to 1.3 as activator acyl-chain length increases

Table 2. *Specificity for activation of pyruvate carboxylase from the thermophilic Bacillus by acyl derivatives of CoA*

The assay system was as described in the Materials and Methods section but with variable concentrations of the acyl-CoA under study over the range 0.2–10.0 apparent K_A . The apparent V_{max} was then estimated from a plot of $1/v$ versus $1/[acyl-CoA]^2$ or $1/[acyl-CoA]^3$. The apparent K_A and the Hill coefficient (h) were then obtained by plotting the data according to the equation:

$$\log \frac{v}{V_{max}-v} = \log K - h \log [acyl-CoA]$$

where K is a complex constant. Derivatives in which the adenine nucleotide portion of the CoA molecule is deleted or modified, e.g. acetyl-pantetheine, acetyl-3'-dephospho-CoA, acetyl-deamino-CoA, or in which the acyl moiety contains a carboxyl group, e.g. methylmalonyl-CoA, succinyl-CoA (3-carboxypropionyl-CoA), glutaryl-CoA, act as inhibitors of *Bacillus* pyruvate carboxylase. The inhibition typically exhibits competitive characteristics with respect to acetyl-CoA as the varied component and $[I]_{0.5}$ values are in the range 0.1–1.0 mM, except for acetyl-pantetheine.

Acyl-CoA	Apparent K_A (pH 7.8) (μM)	h	Relative V_{max} *
Acetyl-CoA	2	2.9	100
Propionyl-CoA	10	2.9	100
Isobutyryl-CoA	74	2.7	83
n-Butyryl-CoA	24	2.4	98
Crotonyl-CoA	40	2.1	75
Isovaleryl-CoA	59	2.6	93
α -Methylcrotonyl-CoA	710	2.0	18
β -Methylcrotonyl-CoA	110	2.5	52
n-Valeryl-CoA	180	1.7	65
n-Hexanoyl-CoA	150	1.3	54
n-Heptanoyl-CoA	150	1.3	28
n-Octanoyl-CoA	130	1.4	24
n-Nonanoyl-CoA	84	1.4	23
n-Decanoyl-CoA	31	1.5	24
n-Undecanoyl-CoA	28	1.5	25
n-Dodecanoyl-CoA	18	1.6	23
Benzoyl-CoA	380	1.3	17
CoA	950	1.4	12
Adenosine 3',5'-bisphosphate	5020	1.0	6

* Under the assay conditions used (5 mM-pyruvate, 2 mM-ATP, 5 mM-MgCl₂, 50 mM-KHCO₃, pH 7.8), the rate of oxaloacetate synthesis observed in the absence of an activator is $1.8 \pm 0.2\%$ of V_{max} for acetyl-CoA.

from two to six carbon atoms and then increases more slowly to a value of 1.7 for an acyl-chain length of 13 carbon atoms (Table 2).

The presence of a double bond (crotonyl-CoA) or of a side-chain methyl group on the β -carbon atom

(isovaleryl-CoA) causes only a minimal increase in apparent K_A or decrease in relative V_{max} , as compared with the unsubstituted homologue (n-butyryl-CoA). However, a more striking change, especially in the apparent K_A , is observed when the side-chain methyl group is present on the α -carbon atom (isobutyryl-CoA versus propionyl-CoA), suggesting that bulky groups are less well tolerated in the immediate vicinity of the thioester bond. This conclusion is borne out by the properties observed for activation by α -methylcrotonyl (tiglyl)- and β -methylcrotonyl-CoA. The α -methyl derivative is a much weaker activator on the basis of all three parameters listed in Table 2 and in fact is less effective than would be predicted on the basis of the effects of the individual modifications. Thus the α,β -double bond and the α -methyl group individually cause 1.7- and 7.4-fold increases in the apparent K_A , leading to a predicted increase of 13-fold for the α -methylcrotonyl derivative; however, the observed increase is 30-fold (Table 2).

The characteristics of activation by benzoyl-CoA (Table 2) are also consistent with this suggested spatial restriction in interaction between the activator site and the region of the thioester bond.

CoA derivatives containing acyl chains longer than 13 carbon atoms also activate the *Bacillus* pyruvate carboxylase. The data for palmitoyl-CoA are shown in Fig. 7(a), which illustrates the non-linear relationship between reciprocal initial rate and reciprocal acyl-CoA concentration generally observed with all activators tested (h above 1) (Table 2). However, at high palmitoyl-CoA concentration (over 30 μM) a further increase in initial rate is seen (Fig. 7a). This is apparently a specific effect, since it is observed with oleoyl-CoA, palmitoleoyl-CoA and to a lesser extent with tetradecanoyl-CoA but not with stearoyl-CoA, which in fact inhibits in this higher concentration range (Fig. 7b). This secondary activation by palmitoyl-CoA and other analogues may be explained in terms of micelle formation by these acyl-CoA species. Similar behaviour has been reported for acyltransferases involved in triacylglycerol and phospholipid biosynthesis (Barden & Cleland, 1969). This explanation is supported by the finding that the catalytic potential of *Bacillus* pyruvate carboxylase can be increased by incubation with concentrations of those long-chain acyl-CoA species that cause this additional activation in initial-rate studies (cf. Fig. 7a). This effect is illustrated for palmitoyl-CoA in Fig. 8. In this experiment a system containing buffer, the acyl-CoA at the concentration indicated and pyruvate carboxylase (2–5 $\mu g/ml$) was incubated at the assay temperature (45°C). At the time indicated, the ingredients for the assay of enzyme activity, including acetyl-CoA (100 μM), were added. When palmitoyl-CoA was present initially at a concentration exceeding 30 μM , the catalytic potential increased rapidly during the first 30–60 s of the

Fig. 7. Reciprocal initial rate of oxaloacetate synthesis as a function of reciprocal palmitoyl-CoA (a) or stearoyl-CoA (b) concentration

The assay system (1.0 ml) was as described in the Materials and Methods section, except that acetyl-CoA was omitted and the concentration of palmitoyl-CoA (a) or stearoyl-CoA (b) was as indicated. The acyl-CoA was added to the system equilibrated to 45°C immediately before pyruvate carboxylase (2–5 μg ; specific activity 16.7 units/mg). In the Figure Δv (the increase in initial velocity due to addition of the acyl-CoA) is expressed as $\Delta A_{340}/\text{min}$ per 5 μg of enzyme.

Fig. 8. Effect of incubation of *Bacillus pyruvate carboxylase* at 45°C in the presence or absence of acetyl-CoA or palmitoyl-CoA

The initial incubation system contained, in 0.5 ml, 200 μmol of Hepes (K^+ salt), pH 7.8, the concentration of acetyl-CoA or palmitoyl-CoA as indicated and pyruvate carboxylase (2.4 μg ; specific activity 16.7 units/mg). The system was equilibrated to 45°C before addition of pyruvate carboxylase and was incubated at that temperature. At the times indicated the residual catalytic activity was assessed by addition of 0.5 ml of a complete assay system containing a saturating concentration of acetyl-CoA (50 μM). In (a) the change in residual catalytic activity is plotted as a function of the time of initial incubation at 45°C in the presence of: buffer [200 mM-Hepes (K^+ salt), pH 7.8] ($\circ$); buffer + 50 μM -acetyl-CoA ($\bullet$); buffer + 46.5 μM -palmitoyl-CoA (Δ); buffer + 123 μM -palmitoyl-CoA ($\square$). In (b) the maximum increase in catalytic activity achieved (after 30–60 s of initial incubation) as determined from plots similar to those shown in (a) is plotted as a function of palmitoyl-CoA concentration.

initial incubation and then declined slowly. No significant increase in catalytic potential was, however, observed if acetyl-CoA was present during the initial incubation. It is notable that both acetyl-CoA and palmitoyl-CoA protect the *Bacillus* pyruvate carboxylase against the rapid inactivation that occurs during the initial incubation if these ligands are not present (Fig. 8).

This observation agrees with the earlier finding (Cazzulo *et al.*, 1970) that, although the thermophilic pyruvate carboxylase possesses greater innate thermostability than its mesophilic counterparts, it is quite labile at temperatures that favour the growth of the *Bacillus*, but can be considerably stabilized by acetyl-CoA and other reaction components.

Data qualitatively similar to those presented for palmitoyl-CoA in Fig. 8 have been obtained with tetradecanoyl-CoA and palmitoleoyl-CoA at appropriate concentrations. With oleoyl-CoA the initial rapid increase in catalytic potential is followed by a rapid decrease in enzyme activity, owing presumably to failure of this acyl-CoA to afford effective protection against thermal inactivation. Stearoyl-CoA neither causes the initial increase in catalytic potential nor protects the enzyme against inactivation caused by incubation at 45°C.

Properties of interaction of pyruvate carboxylases from the thermophilic Bacillus and from other organisms with non-carboxylic acyl thioesters of CoA

As a further probe for differences in activator-site specificity we have examined the effects of methanesulphonyl-CoA, cacodyl-CoA [(CH₃)₂As-CoA] and cacodylyl-CoA [(CH₃)₂AsO-CoA] on oxaloacetate synthesis by pyruvate carboxylases obtained from the thermophilic *Bacillus* and from three other organisms. The data obtained in these studies are summarized in Table 3 and representative examples of the primary data are shown as Hill plots in Fig. 9. The enzymes from *S. cerevisiae* and *Bacillus* which are activated by long-chain acyl-CoA species, e.g. palmitoyl-CoA, and are subject to regulatory inhibition by L-aspartate are activated by all three non-carboxylic acyl-CoA species tested (Fig. 9a; Table 3). The properties of activation, which is reversible in all cases, are generally similar to those observed for other acyl-CoA species, although the non-carboxylic analogues are less effective than the corresponding acyl-CoA.

In contrast, these analogues are either ineffective, or act as inhibitors of, the two vertebrate liver pyruvate carboxylases tested, which do not show activation by palmitoyl-CoA and which are inhibited by

Table 3. *Regulatory site specificity of four pyruvate carboxylases*

The assay systems for the thermophilic *Bacillus* and *S. cerevisiae* pyruvate carboxylases were as described for Fig. 9(b), and similar procedures were used to determine and analyse the relationship between initial rate and activator concentration. The assay systems for rat and chicken liver pyruvate carboxylases were as described previously (Scrutton *et al.*, 1969) except that a non-saturating acetyl-CoA concentration was used (2 μM for chicken liver; 20 μM for rat liver). The data were obtained and analysed as described for Fig. 9(a).

Pyruvate carboxylase from:						
Expt. (a) Inhibition	Chicken liver*			Rat liver†		
	[I] _{0.5} (mM)		<i>h</i>	[I] _{0.5} (mM)		<i>h</i>
Methanesulphonyl-CoA	0.12	1.4		0.24	1.6	
Cacodyl-CoA [As(III)]	50% inhibition at 0.36mM†			25% inhibition at 0.95mM		
Cacodylyl-CoA [As(V)]	0.17	1.5		0% inhibition at 0.5mM		
Pyruvate carboxylase from:						
Expt. (b) Activation	<i>S. cerevisiae</i> §			Thermophilic <i>Bacillus</i>		
	<i>K_A</i> (mM)	<i>h</i>	Rel. <i>V_{max}</i>	<i>K_A</i> (mM)	<i>h</i>	Rel. <i>V_{max}</i>
Methanesulphonyl-CoA	1.14	1.0	74	0.24	1.8	25
Cacodyl-CoA [As(III)]	0.28	1.0	78	0.28	2.2	81
Cacodylyl-CoA [As(V)]	0.20	1.0	44	0.15	2.3	43

* The concentration of acetyl-CoA was 2 μM.

† Inhibition was not competitive with respect to acetyl-CoA.

‡ The concentration of acetyl-CoA was 20 μM.

§ Under the assay conditions used the apparent *K_A* for acetyl-CoA was 30 μM.

|| Expressed relative to *V_{max}*, for acetyl-CoA=100.

Fig. 9. Interaction of various pyruvate carboxylases with methanesulphonyl-CoA and cacodylyl-CoA (a) The inhibition of chicken liver pyruvate carboxylase by methanesulphonyl-CoA (O) and cacodylyl-CoA (Δ). Acetyl-CoA was present at a fixed concentration equal to the apparent K_A ($=2\mu\text{M}$) and the concentration of the inhibitory analogue was varied over the indicated range. Other conditions were as described in the Materials and Methods section. The assays were performed at 25°C . The kinetic data were analysed by the empirical equation shown below:

$$-\log \frac{v_0 - v_1}{v_1 - v_{\text{sat.}}} = \log K - n \log [\text{acyl-CoA}]$$

v_0 is the initial rate observed in the absence of added inhibitor, v_1 is the rate observed in the presence of a given concentration of inhibitor, and $v_{\text{sat.}}$ is the rate observed in the presence of a saturating concentration of inhibitor. $v_{\text{sat.}}$ was approx. zero in these studies. $[I]_{0.5}$ is defined as the concentration of inhibitor required to give $v_0 - v_1/v_1 - v_{\text{sat.}} = 1.0$. K is a complex constant. (b) Activation of pyruvate carboxylases from *S. cerevisiae* (□) and the thermophilic *Bacillus* (Δ) by methanesulphonyl-CoA. For *S. cerevisiae* pyruvate carboxylase the assay system contained, in 0.5 ml, 100 mM-Tris/HCl, pH 7.8, 10 mM-sodium pyruvate, 3.3 mM-sodium ATP, 6.7 mM-MgCl₂, 10 mM-NaHCO₃, 1.5 mM-(NH₄)₂SO₄, 1 unit of malate dehydrogenase, 0.15 mM-NADH and the indicated concentration of methanesulphonyl-CoA. For pyruvate carboxylase from the thermophilic *Bacillus* the assay system contained in 0.5 ml, 100 mM-Hepes (K⁺ salt), pH 7.8, 5 mM-sodium pyruvate, 4 mM-sodium ATP, 6 mM-MgCl₂, 50 mM-NaHCO₃, 1 unit of malate dehydrogenase, 0.15 mM-NADH and the indicated concentration of methanesulphonyl-CoA. The reaction was initiated by addition of 2–5 munits of the appropriate enzyme and initial velocity measured as described in the Materials and Methods section at 45°C (*Bacillus*) or 25°C (*S. cerevisiae*). $V_{\text{max.}}$ was determined from a plot of $1/v$ versus $1/[\text{methanesulphonyl-CoA}]$ (*S. cerevisiae*) or $1/v$ versus $1/[\text{methanesulphonyl-CoA}]^2$ (thermophilic *Bacillus*). The data were then analysed according to the Hill equation.

L-glutamate and/or α -oxoglutarate rather than by L-aspartate (Scrutton & White, 1974; Scrutton, 1974a). Since non-classical kinetic behaviour is observed for activation of both these enzymes by acetyl-CoA, the data cannot be analysed by classical procedures. Initially therefore the nature of the inhibition was defined in experiments in which initial rate was measured as a function of acetyl-CoA concentration in the presence and absence of a fixed concentration of the analogue. When competitive behaviour was observed, the inhibition was further characterized by measurement of initial rate as a function of inhibitor concentration in the presence of a fixed concentration of acetyl-CoA approximating to the apparent K_A . These data were then analysed by modified Hill equation (see legend, Fig. 9) to obtain h and $[I]_{0.5}$ (concentration required to achieve 50% inhibition). Typical data for inhibition of chicken

liver pyruvate carboxylase plotted from this equation are shown in Fig. 9(b). Methanesulphonyl-CoA is an effective inhibitor of both enzymes. Cacodyl- and cacodylyl-CoA are generally less effective as inhibitors at the activator sites, and rat liver pyruvate carboxylase is not significantly inhibited by either of these analogues over the range tested (Table 3). In fact, cacodyl-CoA at concentrations less than 1 mM causes significant inhibition only of pyruvate carboxylase from chicken liver, and, as noted in footnote (†) to Table 3, the inhibition by this analogue is not competitive with acetyl-CoA when examined as described above. Hence the effect is attributable at least in part to interaction at other sites.

The relationship between the properties of the allosteric activator and regulatory inhibitor sites observed for pyruvate carboxylases does not, however, appear to extend to the related enzyme,

phosphoenolpyruvate carboxylase. Although this enzyme obtained from *Escherichia coli* is activated by long-chain acyl-CoA species and is subject to regulatory inhibition by L-aspartate (Canovas & Kornberg, 1966; Scrutton, 1974b), methanesulphonyl-CoA and cacodylyl-CoA act as inhibitors of this enzyme, in contrast with the activation observed for the *Bacillus* and *S. cerevisiae* pyruvate carboxylases (Table 3).

Specificity of inhibition of pyruvate carboxylase from the thermophilic Bacillus and S. cerevisiae by dicarboxylic and amino acids

Previous studies have demonstrated that L-aspartate acts as a regulatory inhibitor of these two pyruvate carboxylases (Cazzulo *et al.*, 1970; Cazzulo & Stoppani, 1968). However, the specificity of the effect has not been defined, and is of interest since more recent investigations have shown that pyruvate carboxylase purified from chicken liver is inhibited by α -oxoglutarate and L-glutamate, and that from rat liver by L-glutamate (Scrutton & White, 1974). Neither of these latter pyruvate carboxylases is inhibited by L-aspartate. Table 4 summarizes the data obtained and indicates that neither L-glutamate nor L-alanine is significantly inhibitory to either the *Bacillus* or *S. cerevisiae* enzymes. However, significant inhibition is observed on addition of α -oxoglutarate, with properties suggesting that the effect results from occupancy of the regulatory inhibitor site. For *Bacillus* pyruvate carboxylase $[I]_{0.5}$ for α -oxoglutarate is only twofold greater than that observed for L-aspartate under the same conditions, and the initial velocity/[α -oxoglutarate] profile is significantly sigmoid (h 1.6), although less so than for L-aspartate (h 4.3) (Table 4). Control experiments demonstrated that product inhibition by oxaloacetate obeyed a classical Michaelis-Menten relationship. For pyruvate carboxylase from *S. cerevisiae* α -oxoglutarate is a less potent inhibitor, with $[I]_{0.5}$ an order of magnitude greater than that observed for L-aspartate under comparable conditions (Table 4). When the inhibition is examined in a system lacking acetyl-CoA and containing high concentrations of K^+ and HCO_3^- little co-operativity is observed. However, under conditions in which oxaloacetate synthesis is dependent on addition of acetyl-CoA, and in the presence of a saturating concentration of this activator, the relationship between initial rate and α -oxoglutarate concentration becomes significantly sigmoid (h 2.1) and an associated increase in $[I]_{0.5}$ is observed. These data are similar to those reported previously for the effect of acetyl-CoA on inhibition by L-aspartate (Cazzulo & Stoppani, 1968). In contrast, product inhibition by oxaloacetate shows no similar dependence on acetyl-CoA concentration.

These data therefore suggest that inhibition of the *Bacillus* and *S. cerevisiae* pyruvate carboxylases by

Table 4. *Specificity and properties of inhibition of pyruvate carboxylases from S. cerevisiae and the thermophilic Bacillus by dicarboxylic and amino acids*

The assay system for the thermophilic *Bacillus* pyruvate carboxylase was as described in the Materials and Methods section, except that the acetyl-CoA concentration was 10 μ M. A similar system was used for *S. cerevisiae* pyruvate carboxylase, except that acetyl-CoA was omitted, the $KHCO_3$ concentration was increased to 100 mM and the assays were performed at 25°C. $[I]_{0.5}$ and h were obtained by analysis of the data according to the Hill equation. $[I]_{0.5}$ is the concentration required to give 50% inhibition of the initial rate of oxaloacetate synthesis.

Dicarboxylic or amino acid	Pyruvate carboxylase from			
	Thermophilic <i>Bacillus</i>		<i>S. cerevisiae</i>	
	$[I]_{0.5}$ (mM)	h	$[I]_{0.5}$ (mM)	h
L-Aspartate	4.3	4.3	2.0	1.2
α -Oxoglutarate	8.7	1.6	18.5*	1.3*
			34.0	2.1
L-Glutamate	10% inhibition at 20 mM		11% inhibition at 20 mM	
L-Alanine	17% inhibition at 20 mM		10% inhibition at 20 mM	

* These data were obtained in an assay system in which Hepes (K^+ salt), pH 7.8, was replaced by Tris/HCl, pH 7.8, HCO_3^- (10 mM) was added as $NaHCO_3$, and acetyl-CoA (100 μ M) was added. Under these conditions oxaloacetate synthesis is almost totally dependent on the addition of acetyl-CoA.

α -oxoglutarate results from interaction of this metabolite at effector sites that may be identical with those occupied by L-aspartate. It is notable that L-glutamate appears essentially ineffective as a regulatory inhibitor of both these enzymes, in contrast with its effect on pyruvate carboxylases purified from chicken and rat liver (Scrutton & White, 1974).

Discussion

The results presented here taken together with earlier studies on this and other pyruvate carboxylases (cf. Scrutton & Young, 1972; Utter *et al.*, 1975) demonstrate that the regulatory properties of the carboxylase from the thermophilic *Bacillus* are unique but can in many respects be considered as a combination of features observed previously for this enzyme isolated from other sources. For example, the maximum extent of catalytic activity which can be expressed in the absence of the activator is similar to

that reported for rat and sheep liver pyruvate carboxylases, and the kinetic characteristics of activator-independent oxaloacetate synthesis by the *Bacillus* pyruvate carboxylase also closely resemble those described for the two mammalian liver enzymes (Scrutton & White, 1972; Ashman *et al.*, 1972; Scrutton, 1974a). On the other hand, this bacterial pyruvate carboxylase is comparable with the enzyme obtained from *S. cerevisiae* in its regulatory inhibition by L-aspartate (Cazzulo & Stoppani, 1968; Cazzulo *et al.*, 1970) and in observation of activation by the methanesulphonyl, cacodyl and cacodylyl derivatives of CoA (Table 3), although differences of detail are observed between the two enzymes in the kinetic characteristics of these effects. As shown in Table 2 and Fig. 7, the *Bacillus* enzyme is susceptible to activation by acyl-CoA species over a wider range of acyl-chain length than for the vertebrate liver enzymes. For example, long-chain acyl-CoA species such as palmitoyl-CoA, palmitoleoyl-CoA and oleoyl-CoA activate the *Bacillus* pyruvate carboxylase, but not the liver enzymes. In this respect the *Bacillus* carboxylase resembles the enzyme from *S. cerevisiae* (Tolbert, 1970). Although the complex kinetic behaviour observed for activation of the thermophile enzyme by long-chain acyl-CoA species (Fig. 7) renders precise comparison difficult, these acyl-CoA species are clearly less effective than acetyl-CoA with respect to both the concentration

required for half-maximal activation and apparent V_{max} . In fact, for both the bacterial and the liver enzymes, acetyl-CoA is the most effective activator.

Fig. 10 summarizes the variation of apparent K_A and of h with acyl-chain length for the pyruvate carboxylases from chicken liver, rat liver, thermophilic *Bacillus* and *S. cerevisiae*, systems in which this property has been examined in detail. The parameters for activation of the *Bacillus* enzyme by long-chain acyl-CoA are derived only from data obtained below the critical micelle concentration (see below). The *Bacillus* pyruvate carboxylase resembles the chicken and rat liver enzymes in showing a marked increase in apparent K_A as the length of the acyl chain is increased from two to four or five carbon atoms, whereas over this same range there is a decrease in apparent K_A for the *S. cerevisiae* enzyme. However, when the acyl-chain length exceeds five carbon atoms a decrease in apparent K_A , which continues for the *S. cerevisiae* pyruvate carboxylase, is observed also for the *Bacillus* enzyme, whereas there is little change in this parameter for the rat liver carboxylase over the range where activation is seen (Fig. 10). No direct comparison is possible with the chicken liver pyruvate carboxylase, since acyl-CoA species in which the acyl-chain length exceeds four carbon atoms inhibit this enzyme competitively with respect to acetyl-CoA (Fung, 1971; Scrutton, 1974a). However, $[I]_{0.5}$, the

Fig. 10. Variation of K_A (a) and h (b) for activation of various pyruvate carboxylases by acyl derivatives of CoA as a function of acyl-chain length

Source of enzyme: Δ , rat liver; $\circ$, chicken liver; $\circ$, *S. cerevisiae*; Δ , *Bacillus*. The additional data for pyruvate carboxylases other than that from the thermophilic *Bacillus* were taken from Scrutton (1974a) (rat liver), Fung (1971) and Scrutton (1974a) (chicken liver), and Tolbert (1970) and M. C. Scrutton (unpublished work) (*S. cerevisiae*).

concentration required to elicit 50% inhibition, increases slightly with acyl-chain length from five to ten carbon atoms (Scrutton, 1974a).

Fig. 10(b) also shows that h is invariant for rat liver pyruvate carboxylase and shows a small, but significant, increase for the *S. cerevisiae* enzyme as the acyl chain lengthens. By contrast, this parameter decreases for the chicken liver and *Bacillus* carboxylases over the acyl-chain length range of two to six carbon atoms and then increases for the longer acyl-chain derivatives. In the chicken liver system h values for acyl-CoA species of chain length greater than four carbon atoms relate to inhibition of enzyme activity. Similarity between the *Bacillus* and chicken liver enzymes is also evident in the identical values of apparent K_A and of h which characterize their activation by acetyl-CoA (Table 2) (Fung, 1971). The biphasic relationship between apparent K_A , or h , and activator acyl-chain length exhibited by the thermophile pyruvate carboxylase is unusual and suggests the existence of multiple factors which determine activator specificity in this system. The analysis presented also indicates that the factors responsible for control of oxaloacetate synthesis may be finely tuned to the particular requirements of a given organism, with striking interrelationships being nevertheless detectable. Interestingly the *Bacillus* enzyme appears to be a focal point in these relationships, displaying activation properties which, as revealed in Fig. 10, are a blend of features characteristic of the other three pyruvate carboxylases.

The activation by long-chain acyl-CoA species is seen with only those pyruvate carboxylases (*Bacillus* and *S. cerevisiae*) that are inhibited by L-aspartate. Further these properties are also correlated with the observation of activation by non-carboxylic acid thioesters of CoA (Table 3). These observations therefore suggest that a relationship exists between the properties of the allosteric activator and regulatory inhibitor sites, although previous studies (Scrutton & White, 1973) and the data of Fig. 6 clearly indicate that separate sites are involved. The nature of this relationship remains to be explored at the level of enzyme structure. All these pyruvate carboxylases are tetrameric molecules in which all the components of the active site (carboxylation sub-site, biotinyl residue and transcarboxylation sub-site), together with presumably the regulatory sites, are carried on a single polypeptide chain (Barden *et al.*, 1972; Libor *et al.*, 1975a; Frey & Utter, 1977). However, the enzymes may differ in the organization of the constituent polypeptides in the tetramer. Further, antibody prepared against pyruvate carboxylase from chicken liver fails to cross-react with the enzyme from *S. cerevisiae* (cf. Utter *et al.*, 1975).

Two further observations reported here warrant comment. First, the relationship between initial rate and $MgATP^{2-}$ concentration shows apparent nega-

tive co-operative behaviour in the absence, and with the lower concentrations, of activator, but becomes essentially hyperbolic in the presence of concentrations of acetyl-CoA in excess of the apparent K_A (Fig. 2). Similar behaviour has been reported for pyruvate carboxylases from rat and sheep liver (Scrutton & White, 1972; Ashman *et al.*, 1972). These findings appear consistent with the postulate advanced by Frey & Utter (1977), on the basis of binding studies performed on chicken liver pyruvate carboxylase, that this enzyme may exhibit half-of-site reactivity with respect to $MgATP^{2-}$. The initial-rate data here suggest that such half-of-site reactivity might be observed for $MgATP^{2-}$ in the presence of the other substrates, but might be abolished on further addition of acetyl-CoA.

The second point for comment concerns the properties, and possible physiological significance, of the activation of *Bacillus* pyruvate carboxylase by long-chain acyl-CoA species. The atypical behaviour at high concentration of palmitoyl-CoA (Fig. 7a) can be explained by an increase in catalytic potential under these conditions (Fig. 8), which may result from interaction between enzyme and the activator in the micellar state. Although the threshold concentration observed for this effect (30 μM) is somewhat greater than the critical micelle concentration for palmitoyl-CoA as determined by Barden & Cleland (1969) (4 μM), it is well established that this latter parameter can vary widely depending on the experimental conditions and the method of determination used. The presumed formation of micelles is, however, not always associated with an increase in catalytic activity. An increase is seen with palmitoyl-CoA, palmitoleoyl-CoA, tetradecanoyl-CoA and oleoyl-CoA, but not with stearoyl-CoA, which in fact becomes inhibitory in the higher concentration range (Fig. 7b). This apparent specificity suggests that the effect may have some physiological relevance, and it is of interest that similar phenomena have been observed for certain membrane-associated enzymes, e.g. the long-chain acyl-CoA transferase involved in triglyceride synthesis. One may speculate therefore that in the intact *Bacillus* pyruvate carboxylase could be localized on or near the cell membrane, possibly in association with the enzymes of the tricarboxylic acid cycle.

The present results and those from earlier studies (Cazzulo *et al.*, 1970; Sundaram *et al.*, 1971) show that, despite speculation to the contrary (Brock, 1967), the thermophile pyruvate carboxylase can undergo kinetic and allosteric modulations as readily as several of its mesophilic homologues.

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