Synthesis of Pyruvate Carboxylase from its Apoenzyme and (+)-Biotin in *Bacillus stearothermophilus*

MECHANISM AND CONTROL OF THE REACTION

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1. Acetyl-CoA acts as a positive allosteric effector in the formation of active pyruvate carboxylase from its apoenzyme, ATP and (+)-biotin which is catalysed by holoenzyme synthetase; this effect is counteracted by L-aspartate. 2. The Hill coefficients (apparent n values) were approximately 2 for acetyl-CoA and 4 for L-aspartate; the n value for each effector remained constant when the concentration of the other effector was varied. 3. Active pyruvate carboxylase was formed also when the apoenzyme was incubated with holoenzyme synthetase and synthetic biotinyl-5'-AMP; acetyl-CoA and L-aspartate affected this process as they did the overall reaction from (+)-biotin and ATP. 4. When hydroxylamine replaced the apoenzyme, holoenzyme synthetase catalysed the formation of biotinylhydroxamate from (+)-biotin and ATP. This reaction was not affected by the allosteric effectors. 5. The apoenzyme was protected against thermal denaturation by acetyl-CoA and, to a lesser degree, by L-aspartate. The holoenzyme synthetase was not markedly protected by these effectors. 6. It is concluded that the allosteric effectors act on the apoenzyme and not the synthetase.

The pathway whereby biotin-dependent carboxylases are formed from their inactive apoenzymes and (+)-biotin has been shown (Siegel, Foote, Christner & Coon, 1963; Lane, Young & Lynen, 1964a; Höpner & Knappe, 1965) to involve two enzymic reactions. In the first (reaction 1), biotin and ATP interact to yield pyrophosphate and biotinyl-5'-AMP; in a second step (reaction 2), this latter compound reacts with the apoprotein to liberate AMP and to produce the enzymically active holocarboxylase. Both reactions 1 and 2 are mediated by the same enzyme; such enzymes are generically termed holocarboxylase synthetases.

The formation of active pyruvate carboxylase from its apoenzyme and (+)-biotin in the thermophile *Bacillus stearothermophilus* conforms to this general mechanism but, in addition, exhibits an almost complete requirement for acetyl-CoA as an allosteric activator; this activation is antagonized by L-aspartate (Cazzulo, Sundaram & Kornberg,

1970a). It is the main purpose of this paper to examine the nature of the effect exerted by these allosteric modulators on the formation of the active enzyme, and on the thermal denaturation of the apoenzyme and the holoenzyme synthetase.

MATERIALS AND METHODS

Materials. NADH, ATP, AMP, sodium pyruvate, tris base, (+)-biotin, 5,5'-dithiobis-(2-GSH nitrobenzoic acid) and L-aspartic acid were obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; malate dehydrogenase, 5'-AMP (free acid) and CoA were from Boehringer (London) Ltd., London W.5, U.K.; methylmalonic acid and DL-erythro- β -hydroxyaspartic acid (A grade) were from Calbiochem Ltd., London W.1, U.K.; DL- β -methylaspartic acid was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; and maleic acid, succinic acid and dicyclohexylcarbodi-imide were from British Drug Houses Ltd., Poole, Dorset, U.K. D-[carbonyl- ^{14}C]-Biotin of specific radioactivity 58 mCi/mmol was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals used were analytical reagents.

Biotinyladenylate was prepared by the condensation of (+)-biotin and adenosine 5'-phosphoric acid in the presence of dicyclohexylcarbodi-imide (Lane, Rominger, Young & Lynen, 1964b). The product was standardized by colorimetric assay with hydroxylamine and ferric chloride. Biotinylhydroxamate was prepared from (+)-biotin by the method described by Christner, Schlesinger & Coon (1964). Acetyl-CoA was prepared from CoA

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and acetic anhydride by the method of Stadtman (1957) and was measured with hydroxylamine and dithiobis-(2-nitrobenzoic acid) by the procedure of Srere, Brazil & Gonen (1963) as described by Canovas & Kornberg (1966). Pyruvate apocarbonylase and pyruvate holocarbonylase synthetase from *B. stearothermophilus* were prepared as described by Cazzulo, Sundaram, Dilks & Kornberg (1971). The active pyruvate (holo)carbonylase was prepared by the method of Cazzulo, Sundaram & Kornberg (1970b). Protein was determined by the spectrophotometric method of Warburg & Christian (1941).

Enzyme assays. Pyruvate apocarbonylase and holocarbonylase synthetase activities were determined as previously described (Cazzulo *et al.* 1971); any modifications introduced are given in the legends. Pyruvate holocarbonylase was assayed at 45°C by the procedure of Cazzulo *et al.* (1970b).

Methods relating to experiments described in Fig. 6. (a) Holocarbonylase synthesis. The incubation system (0.3 ml) for the synthesis of holoenzyme from apoenzyme differed from the standard system (Cazzulo *et al.* 1971) in not containing acetyl-CoA and in having 0.2 μ mol of CoA (where indicated); 24 μ g (0.016 unit) of synthetase and 38.8 μ g (0.58 unit) of apocarbonylase were used. After incubation at 45°C for the times indicated, the holoenzyme formed was measured as described by Cazzulo *et al.* (1970b) (see Fig. 6a).

(b) Formation of biotinylhydroxamate. The reaction mixture contained, in 0.43 ml: tris-HCl, pH 7.6, 20 μ mol; $MgCl_2$, 1 μ mol; ATP, 0.25 μ mol; (+)-[^{14}C]biotin, 0.023 μ mol (1.33 μ Ci); hydroxylamine hydrochloride (neutralized with KOH), 200 μ mol; and holoenzyme synthetase, 0.16 unit (240 μ g of protein). A second system was supplemented with 0.3 μ mol of CoA. After incubation at 45°C for the times indicated, the mixtures were cooled in ice, 0.35 μ mol of [^{12}C]biotin and 2.5 μ mol of [^{12}C]biotinylhydroxamate (in a volume of 0.2 ml) were added as carriers. The mixtures then received 1.5 ml of ethanol. After centrifugation, 1.8 ml of the supernatant fluid was dried under vacuum. The residue was dissolved in 0.4 ml of a 1:1 (v/v) mixture of ethanol and water and portions (25 μ l) of the concentrates were chromatographed at room temperature on Whatman no. 1 filter paper in butan-1-ol-acetic acid-water (5:1:1, by vol.), to separate (+)-biotin and biotinylhydroxamate. The radioactive spots were located by radioautography; biotinylhydroxamate was detected by the acidic ferric chloride spray (Christner *et al.* 1964). The radioactive areas of the chromatograms were cut out and the radioactivity in them was determined in a Packard 4000 scintillation spectrometer, with 10 ml of toluene-2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene scintillator. The amount of radioactive biotinylhydroxamate formed is expressed as a percentage of the radioactive biotin added to the incubation.

Fig. 1. Relationship between acetyl-CoA concentration and rate of holocarbonylase formation at different fixed concentrations of L-aspartate. The formation of active enzyme from 0.58 unit (38.8 μ g) of the apoenzyme, in the presence of biotin, Mg^{2+} , ATP and 0.01 unit of holoenzyme synthetase (8.8 μ g of protein) was measured as described by Cazzulo *et al.* (1971). $\circ$, Without aspartate; Δ , 3.3 mM-aspartate; $\square$, 5 mM-aspartate; $\bullet$, 10 mM-aspartate (acetyl-CoA scale for these four curves is on the lower abscissa); $\blacktriangle$, 10 mM-aspartate (acetyl-CoA scale on the upper abscissa).

tion system. A correction was made for the small amount of radioactive material that was present in the [¹⁴C]-biotin sample and could not be separated from biotinyl-hydroxamate by chromatography (see Fig. 6b).

RESULTS

Effect of acetyl-CoA and L-aspartate on pyruvate carboxylase formation. Under the conditions of assay used, there was virtually no formation of active pyruvate carboxylase from the apoenzyme and (+)-biotin unless acetyl-CoA was also added. However, the effect exerted by this activator was complex: as Fig. 1 shows, the rate of holoenzyme formation exhibited sigmoid kinetics when plotted against the concentration of acetyl-CoA, and this sigmoidicity became more obvious when increasing concentrations of the antagonist L-aspartate were also included in the system. This effect of L-aspartate in raising the threshold concentration of acetyl-CoA required for the formation of the active enzyme is also evident from the results summarized in Table 1: whereas only 2.34 μM-acetyl-CoA was required to elicit half the maximal rate of holoenzyme formation in the absence of L-aspartate, 398 μM-acetyl-CoA was required for this rate of holoenzyme formation to be observed in the presence of 10mM-L-aspartate. Similarly, the concentrations of L-aspartate required to inhibit holoenzyme formation to a given extent increased as the concentration of acetyl-CoA added was raised (Fig. 2). Thus, in the presence of 30 μM-acetyl-CoA, the addition of 3.7mM-L-aspartate produced 50% inhibition of the rate of holoenzyme formation, whereas nearly three times this concentration of aspartate was required to produce this effect if 300 μM-acetyl-CoA was also present (Table 2). These results show the allosteric and mutually antagonistic nature of the action of these effectors. However, although the Hill coefficients (apparent *n* values) calculated from the results shown in Figs. 1 and 2, and presented in Tables 1 and 2, confirm the allosteric nature of the observed modulations, it is apparent that the coefficient for one effector remained constant even when the concentration of the other effector was varied. These quantitative effects of acetyl-CoA and L-aspartate on the rate of formation of the holoenzyme are strikingly similar to the effects they exert on the activity of this enzyme (Cazzulo *et al.* 1970b). To establish unequivocally that these effects were specifically allosteric and not merely due to a possible stabilization of the apoenzyme or the synthetase, the action of acetyl-CoA and L-aspartate was studied in the temperature range 30–55°C; the effects observed at 55 or 45°C were closely similar to those at 30°C, a temperature at which the two proteins are stable.

Table 1. Concentrations of acetyl-CoA required to elicit half-maximal rates of holoenzyme formation ([A]_{0.5}), and apparent *n* values, at different concentrations of L-aspartate

These values were obtained from the results shown in Fig. 1; the apparent *n* values were calculated from Hill plots.

Concn. of L-aspartate present (mM)	[A] _{0.5} for acetyl-CoA (μM)	Apparent <i>n</i> value
0	2.34	2.3
3.3	18.6	2.1
5	55.0	1.9
10	398	1.9

Fig. 2. Relationship between concentration of L-aspartate and rate of holocarboxylase formation at different fixed concentrations of acetyl-CoA. Holoenzyme formation was measured as described under Fig. 1, but with various amounts of L-aspartate, in the presence of ○, 30 μM, △, 75 μM, □, 150 μM, and ●, 300 μM-acetyl-CoA.

Table 2. Concentrations of L-aspartate required to produce 50% inhibition of holoenzyme formation ([I]_{0.5}), and apparent *n* values, at different concentrations of acetyl-CoA

These values were obtained from the results shown in Fig. 2; the apparent *n* values are calculated from Hill plots.

Concn. of acetyl-CoA present (μM)	[I] _{0.5} for L-aspartate (mM)	Apparent <i>n</i> value
30	3.71	4.1
75	5.25	4.0
150	6.92	4.1
300	10.7	3.9

Effects of dicarboxylic acids. L-Aspartate was the most effective inhibitor of holoenzyme formation, as it was of pyruvate carboxylase activity, of a variety of C₄ dicarboxylic acids tested (Table 3). With the exception of β-methylaspartate, the C₄

Table 3. *Effect of dicarboxylic acids on the formation and activity of pyruvate holocarboxylase*

Pyruvate holocarboxylase was formed from 0.58 unit of apoenzyme (38.8 μ g of protein), biotin, Mg^{2+} and ATP, in the presence of 0.01 unit of holoenzyme synthetase (8.8 μ g of protein), 75 μ M-acetyl-CoA and the compounds listed at 10mM; its activity was assayed by the procedure of Cazzulo *et al.* (1971). The effect of the compounds listed, at 10mM, on the activity of the holoenzyme (19.2 μ g of protein, specific activity 7.5), was determined at 45°C as described by Cazzulo *et al.* (1970b).

Addition	Inhibition (%)	
	Holo-carboxylase formation	Holo-carboxylase activity
None	0	0
L-Aspartate	91	83
DL- β -Methylaspartate	13	36
β -erythro-Hydroxy-aspartate	11	5
Maleate	29	0
Methylmalonate	9	9
Succinate	29	12

Fig. 3. Inactivation of apocarboxylase and holoenzyme synthetase at different temperatures. Samples of the apocarboxylase and of the synthetase in 50mM-tris-HCl, pH7.6, containing 1mM-EDTA (protein concentration, 0.2mg/ml) were heated at the temperatures indicated for 10min. They were then cooled rapidly in ice and the activity remaining was assayed; ●, apocarboxylase; □, synthetase.

acids (at 10mM) inhibited holoenzyme synthesis more than they inhibited its activity; indeed, 10mM-maleate produced no significant effect on the latter.

Effect of acetyl-CoA and aspartate on the thermal denaturation of the apocarboxylase and the synthetase. The patterns of thermal denaturation of the apo-

Fig. 4. Effect of acetyl-CoA on the thermal inactivation of apocarboxylase and holoenzyme synthetase. Experimental conditions were similar to those described in Fig. 3, except that acetyl-CoA at the concentrations indicated was added to the samples before heat treatment of the apocarboxylase (●) at 58°C and the synthetase (▲) at 60°C.

enzyme and the synthetase are presented in Fig. 3. Both proteins are reasonably stable when heated for 10min up to 50°C, a temperature appreciably lower than the optimum temperature for the growth of the thermophile. As the temperature was further raised, the proteins began to decay sharply, the decrease in the stability of the apoenzyme being somewhat more rapid than that of the synthetase. The presence of acetyl-CoA significantly retarded the thermal decay of the apoenzyme: as shown in Fig. 4, more than half of this protein was denatured in 10min at 58°C in the absence of acetyl-CoA but, under these conditions, 0.2mM-acetyl-CoA afforded virtually complete protection. Its protective effect on the synthetase, also illustrated in Fig. 4, was appreciably weaker. The synthetase, in this experiment, was incubated at 60°C rather than at 58°C, so that it decayed to approximately the same extent in the absence of acetyl-CoA as did the apoenzyme at 58°C. CoA itself was considerably less effective than was acetyl-CoA in protecting the apoenzyme from thermal denaturation; GSH at a similar concentration had no significant effect. The ability to protect thus appears to be specific to acetyl-CoA and is presumably related to its role of allosteric activator.

The negative modifier, L-aspartate, also stabilized the apoenzyme against thermal denaturation, though not as effectively as did acetyl-CoA. In the absence of either effector, the half-life of the apocarboxylase at 58°C was 9min (Fig. 5). This was prolonged to 27min by the addition of 10mM-L-aspartate; however, in the presence of only 0.1mM-acetyl-CoA, the apoenzyme retained half of its activity at 58°C for more than 60min. L-Aspartate

Fig. 5. Time-course of inactivation of apocarboxylase at 58°C in the presence and absence of allosteric modifiers. The apocarboxylase was held at 58°C, for different periods of time, as described in Fig. 3, after the addition of acetyl-CoA or L-aspartate as indicated; the residual apocarboxylase was then assayed. ▲, No additions; □, 0.1 mM-acetyl-CoA; ●, 10 mM-L-aspartate.

Table 4. Requirements for pyruvate holocarboxylase formation with biotinyladenylate as biotin donor

The formation of pyruvate holocarboxylase from 53 μ g of the apoenzyme, in the presence of 75 μ g of holoenzyme synthetase and acetyl-CoA, but with (+)-biotin, Mg^{2+} and ATP replaced by 0.067 μ mol of biotinyl-5'-AMP, was measured as described by Cazzulo *et al.* (1971). Omissions and additions are indicated below. The amount of holoenzyme formed in the complete system was 0.173 unit; this was defined as 100% for the present purpose.

System	Pyruvate holocarboxylase formed (% of complete)
Complete	100
EDTA (3 μ mol) added	110
Biotinyladenylate omitted; ATP (0.125 μ mol), $MgCl_2$ (0.5 μ mol) and biotin (0.07 μ mol) added	125
L-Aspartate (3 μ mol) added	50
Biotinyladenylate omitted; biotin (0.07 μ mol) and AMP (0.05 μ mol) added	1
Biotinyladenylate omitted	1
Acetyl-CoA omitted	6
Pyruvate apocarboxylase omitted	0
Synthetase omitted	0

had no protective effect on the synthetase even at 55°C.

Biotinyl adenylate as intermediate in holoenzyme formation. Synthetic biotinyl-5'-AMP (biotinyl-

Table 5. Biotinylhydroxamate synthesis mediated by pyruvate holocarboxylase synthetase

The amounts of [^{14}C]biotinylhydroxamate formed when 0.24 unit of holoenzyme synthetase (0.88 mg of protein) was incubated for 30 min at 45°C with [^{14}C]biotin, ATP, Mg^{2+} and hydroxylamine, but in the absence of CoA, in a total volume of 0.42 ml, were determined as described in the legend to Fig. 6(b). Omissions and additions are indicated below.

System	[^{14}C]Biotinylhydroxamate synthesized (nmol)
Complete	11
L-Aspartate (2 μ mol) added	9
$MgCl_2$ omitted, EDTA (2 μ mol) added	0
ATP omitted	0
Synthetase omitted	0

adenylate) can replace biotin and ATP in the enzymic conversion of the apo- into the holo-carboxylase (Cazzulo *et al.* 1970a). As shown in Table 4, a mixture of biotin and AMP did not substitute for biotinyladenylate in this process, nor was the holoenzyme formed if either the apoenzyme or the holoenzyme synthetase was omitted. The formation of active carboxylase with the adenylate as biotin donor no longer required Mg^{2+} to be present. However, acetyl-CoA was still required and the reaction was still inhibited by L-aspartate; the kinetics of these interactions were similar to those observed with (+)-biotin, Mg^{2+} and ATP as substrates (Cazzulo *et al.* 1970a).

To determine whether these allosteric modulators acted on the enzymic formation of biotinyl-5'-AMP (reaction 1), as well as on its interaction with the apoprotein (reaction 2), reaction (1) was studied in the absence of added apoprotein: under these conditions, [^{14}C]biotin was incubated with hydroxylamine, Mg^{2+} , the holoenzyme synthetase and ATP, the [^{14}C]biotinyladenylate formed being trapped and measured as [^{14}C]biotinylhydroxamate. As shown in Table 5, the enzymic formation of biotinyl-5'-AMP, thus assayed, occurred only when ATP and the holoenzyme synthetase were present; unlike the synthetase-mediated interaction of biotinyl-5'-AMP and apoenzyme (Table 4), the synthetase-mediated formation of the adenylate did not occur in the absence of Mg^{2+} . Also in contrast with the further reaction of biotinyladenylate, its enzymic formation was not significantly inhibited by L-aspartate. The results of Table 5 were obtained with incubation mixtures devoid of acetyl-CoA, which already shows that this effector does not play an essential role in the formation of biotinyl-5'-AMP. Since hydroxylamine had to be present throughout the incubation period,

Fig. 6. Effect of CoA on (a) holocarboxylase synthesis and (b) the formation of biotinylhydroxamate. ●, ○, CoA added; ▲, △, no CoA added. See the Materials and Methods section for the experimental procedure.

to cause adequate amounts of the labelled product to be formed and trapped, the possible stimulatory role of acetyl-CoA could not be determined directly. However, free CoA was found to mimic the action of acetyl-CoA, albeit less effectively, in promoting the conversion of apo- into holo-carboxylase (Fig. 6a): about 25% as much active enzyme was formed in the presence of 0.3–0.6 mM-CoA as was formed in the presence of similar concentrations of acetyl-CoA. It was thus possible to test the effect of relatively high concentrations of CoA on the enzymic synthesis of biotinyl-5'-AMP; as shown in Fig. 6(b), virtually identical rates of [¹⁴C]biotinylhydroxamate formation were observed in the absence and in the presence of CoA. It thus appears that, of the two reactions mediated by the holoenzyme synthetase, the formation of biotinyl-5'-AMP (reaction 1) is not affected by the allosteric modulators, whereas the enzymic interaction of biotinyl-5'-AMP with the apoenzyme (reaction 2) exhibits the same sensitivity to acetyl-CoA and L-aspartate as is observed when (+)-biotin and ATP are the reactants in the overall conversion. It may thus be concluded that the target for the allosteric modulators is the apoprotein rather than the holoenzyme synthetase.

DISCUSSION

The results presented in this paper provide evidence for the allosteric nature of the action of acetyl-CoA and L-aspartate on the formation of pyruvate carboxylase from its apoenzyme, and

indicate the site at which these agents affect this conversion. They also support the occurrence, in the thermophilic bacillus here studied, of the two-step mechanism of holoenzyme synthesis established for other biotin-dependent carboxylases (Siegel *et al.* 1963; Lane *et al.* 1964b; Höpner & Knappe, 1965). In particular, it has been shown that the apoenzyme isolated from the thermophile is converted into the holoenzyme in the presence of the holoenzyme synthetase and either (+)-biotin and ATP or synthetic biotinyl-5'-AMP: acetyl-CoA is required for this process and its action is antagonized by L-aspartate. This almost complete dependence of holoenzyme formation on acetyl-CoA appears to be unique to the thermophile system, although some stimulation has been observed also with a similar system isolated from *Saccharomyces cerevisiae* (Sundaram, Cazzulo & Kornberg, 1971).

Studies of the enzymic formation of biotinyl-5'-AMP, by trapping the product as biotinylhydroxamate, suggested that the adenylate remained bound to the holoenzyme synthetase in the conversion of apo- into holo-carboxylase and was released only in the presence of the appropriate acceptor: little biotinylhydroxamate was formed if hydroxylamine was added after the incubation of holoenzyme synthetase, (+)-biotin, Mg²⁺ and ATP. The enzymic formation of biotinyl-5'-AMP was not affected by the allosteric agents that affected the interaction of biotinyl-5'-AMP and the apoenzyme.

This indication that acetyl-CoA and L-aspartate affect the conformation of the apoprotein, and thus affect its ability to accept biotin from biotinyl-5'-AMP, is supported by two main findings. First, acetyl-CoA and L-aspartate protect the apoenzyme against thermal denaturation more effectively than they protect the holoenzyme synthetase (Fig. 4). Secondly, the kinetic parameters of the action of these effectors on holocarboxylase formation are strikingly similar to those on holocarboxylase activity (Cazzulo *et al.* 1970b). Both cases provide strong evidence for co-operative interactions; in both cases, the changes observed (particularly the constancy of the Hill coefficient for one allosteric modulator as the concentration of the other was varied) were similar but appear to be at variance with the predictions of the model proposed by Monod, Wyman & Changeux (1965). The close similarity in the fractionation properties, electrophoretic mobility and molecular size of the apoprotein (Cazzulo *et al.* 1971) and the holoenzyme (Cazzulo *et al.* 1970b) further strengthens the belief that, in the thermophile, acetyl-CoA interacts with the pyruvate apo- and holo-carboxylase and that this interaction is antagonized by L-aspartate.

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