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REGULATORY CHARACTERISTICS OF PHOSPHO*ENOL*PYRUVATE CARBOXYLASE FROM THE EXTREME THERMOPHILE, *THERMUS AQUATICUS*

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Summary

Phospho*enol*pyruvate carboxylase from the extremely thermophilic bacterium, *Thermus aquaticus* YT-1, exhibits a virtually absolute requirement for acetyl CoA and there is strong positive cooperativity in the interaction of this activator with the enzyme. Several tricarboxylic acid cycle intermediates inhibit the enzyme. These findings suggest an anaplerotic role for the enzyme and an allosteric modulation of its activity by acetyl CoA and tricarboxylic acid cycle intermediates.

Phospho*enol*pyruvate carboxylase (orthophosphate:oxaloacetate carboxylase (phosphorylating), EC 4.1.1.31) and pyruvate carboxylase (pyruvate:carbon-dioxide ligase (ADP-forming), EC 6.4.1.1) fulfil analogous functions in living organisms by synthesizing oxaloacetate from the three-carbon metabolic intermediates, phospho*enol*pyruvate and pyruvate respectively, by carbon dioxide fixation. Phospho*enol*pyruvate carboxylase occurs in a wide variety of plant sources and in bacteria drawn from several genera [1] and pyruvate carboxylase is distributed in bacteria, yeasts, fungi and animal tissues [2]. A moderately thermophilic *Bacillus* employs pyruvate carboxylase rather than phospho*enol*pyruvate carboxylase to generate oxaloacetate from three-carbon metabolite [3]. By interesting contrast, the extremely thermophilic bacterium, *Thermus aquaticus* YT-1, apparently uses phospho*enol*pyruvate carboxylase and not pyruvate carboxylase for oxaloacetate production [4]. We report here some of the regulatory characteristics of the *Thermus* enzyme, the only known thermophilic phospho*enol*pyruvate carboxylase.

The *Thermus* phospho*enol*pyruvate carboxylase was partially purified as follows. A cell-free extract [4] was fractionated on a column of DEAE-cellulose by adsorbing the enzyme in 50 mM Tris-HCl buffer (pH 8) to the ion-exchanger and eluting it with buffer containing a 100—250 mM linear gradient of KCl. The enzyme in the active fractions was precipitated with polyethyleneglycol (PEG 6000) added to a concentration of 12% (w/v). The precipitate, dissolved in 20 mM Tris-HCl buffer (pH 8), was passed through a column of AH-Sepharose and the enzyme was eluted with buffer containing a 100—700 mM linear gradient of KCl. The active fractions were pooled, diluted 5-fold with 10 mM sodium potassium phosphate buffer, pH 6.5, and fractionated on a phosphocellulose column; the enzyme was eluted with buffer containing a 100—400 mM linear gradient of KCl. The active fractions were pooled and concentrated osmotically in a dialysis sac against solid sucrose and the concentrate was dialyzed against 10 mM Tris-HCl, pH 8. Although this preparation, about 180-fold purified over the cell-free extract, was not homogeneous, it was free of pyruvate kinase, lactate dehydrogenase, malate dehydrogenase, citrate synthase and acetyl CoA-hydrolyzing activity. The absence of the last two activities made the preparation suitable for a kinetic study of the activation of the enzyme by acetyl CoA. The standard system for the assay of enzyme activity contained in a final volume of 0.5 ml: 50 μmol Tris-HCl (pH 8); 5 μmol $MgCl_2$; 15 μmol $KHCO_3$; 5 μmol phospho*enol*pyruvate; 0.15 μmol acetyl CoA; 0.065 μmol NADH; 1 I.U. malate dehydrogenase and enzyme. For the assays at 45°C pig heart malate dehydrogenase (Boehringer Corporation) was used but for the assays at 65°C the more thermostable malate dehydrogenase purified from *T. aquaticus* [5] was substituted. All components except enzyme were mixed and preincubated for 2 min at the assay temperature, the reaction was initiated by the addition of enzyme and the rate of decrease in absorbance at 340 nm was monitored. It was observed that acetyl CoA was not degraded during the carboxylation of phospho*enol*pyruvate in this system. This showed that acetyl CoA functioned as an activator and not as a substrate and further confirmed that the enzyme preparation was pure enough for a study of the kinetics of acetyl CoA activation.

The *Thermus* phospho*enol*pyruvate carboxylase was optimally active at pH 8.15, had an absolute requirement for Mg^{2+} (K_m = 0.95 mM at 45°C) and a requirement for HCO_3^- (K_m approx. 2.1 mM). K_m for phospho*enol*-pyruvate was 0.63 mM at 45°C and about 4-fold higher at 65°C; at either temperature the Hill coefficient was approximately 1. The molecular weight of the enzyme, estimated by gel filtration, was approx. 325 000.

As shown in Fig. 1 there was a virtually absolute requirement for acetyl CoA and the plot of reaction velocity against acetyl CoA concentration was distinctly sigmoid. The Hill coefficient (h) deduced from these data was 3.3 and the acetyl CoA concentration required to elicit half-maximal activation ($A_{0.5}$) was 100 μM at 45°C. When the carboxylation reaction was performed at 65°C, the plot of reaction velocity against the activator concentration was still sigmoid and the values of $A_{0.5}$ and h (72 μM and 2.5 respectively) were rather similar to those at 45°C. Experiments in which the reaction velocity was measured at 45°C as a function of phospho*enol*-

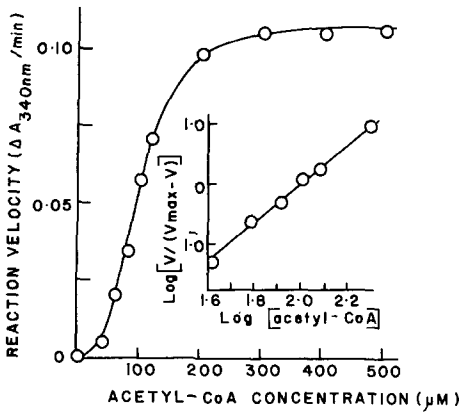

Fig. 1. Dependence of the activity of *T. aquaticus* phospho*enol*pyruvate carboxylase on acetyl CoA. Enzyme activity was measured at 45° C in the standard system with acetyl CoA at the concentrations indicated. The inset presents a Hill plot of the data.

TABLE I

MODULATION OF *T. AQUATICUS* PHOSPHO*ENOL*PYRUVATE (PEP) CARBOXYLASE ACTIVITY BY VARIOUS COMPOUNDS

Enzyme activity was determined at 45° C in three assay systems containing the concentrations of PEP and acetyl CoA ((CoASAc) shown with the addition of compounds at 10 mM concentration as indicated; in other respects the assay system was the standard system. The control reaction rate was the rate obtained without the addition of 'compound tested'.

Compound tested	Reaction rate (% of control)		
	10 mM PEP + 300 μM CoASAc	1 mM PEP + 300 μM CoASAc	10 mM PEP + 50 μM CoASAc
L-Aspartate	96.5	87.6	72.9
Succinate	98.5	49.5	10.0
α-Ketoglutarate	76.4	40.0	31.5
Fumarate	48.6	50.5	31.5
Citrate	75.0	8.5	7.9
Citrate + Mg^{2+} (30 mM)	76.0	75.3	75.2
AMP	98.6	98.0	97.9
ADP	98.6	102.0	98.5
ATP	37.5	37.9	5.2
ATP + Mg^{2+} (30 mM)	96.4	94.8	97.4

pyruvate concentration at various fixed concentrations of acetyl CoA showed that acetyl CoA increased the V approx. 10-fold in the concentration range 50—300 μM and decreased the K_m for phospho*enol*pyruvate approx. 2-fold. Several acyl derivatives of CoA (*n*-butyryl, *n*-hexanoyl and *n*-octanoyl) and free CoA were unable to substitute for acetyl CoA and when present along with acetyl CoA had little effect on the reaction velocity.

Phospho*enol*pyruvate carboxylase from several mesophilic sources is inhibited by L-aspartate and/or by some of the intermediates of the tricarboxylic acid cycle. A few are activated by fructose 1,6-bisphosphate as well as by acetyl CoA [1]. The results presented in Table I show that, of a number of compounds tested, succinate, α-ketoglutarate, fumarate and ATP were the most potent inhibitors of the *Thermus* enzyme, the extent of

inhibition in most cases being dependent on the concentrations of phospho*enol*pyruvate and of acetyl CoA. L-Aspartate was only weakly inhibitory. The finding that inhibition by citrate could be largely counteracted by the addition of a further 30 mM Mg^{2+} suggests that the action of citrate might have been due to chelation with Mg^{2+}, which is an absolute requirement for the enzyme reaction. However, the observation that the extent of citrate inhibition was dependent on the level of phospho*enol*pyruvate and of acetyl CoA may mean that citrate was also an inhibitor like some of the other tricarboxylic acid cycle intermediates. The reversal of ATP inhibition by Mg^{2+} indicates that free ATP, and not the complex with Mg^{2+}, caused the inhibition, a finding also made with pyruvate carboxylase [6]. Fructose bisphosphate was not an activator of *Thermus* phospho*enol*pyruvate carboxylase.

A more detailed study of the inhibition by succinate (Fig. 2) showed that this inhibitor and the activator, acetyl CoA, were mutually antagonistic in their action. Moreover the fact that succinate inhibition could be virtually completely overcome at sufficiently high concentrations of phospho*enol*pyruvate and acetyl CoA (Table I) suggests that succinate interfered with the interaction of the enzyme with phospho*enol*pyruvate as well.

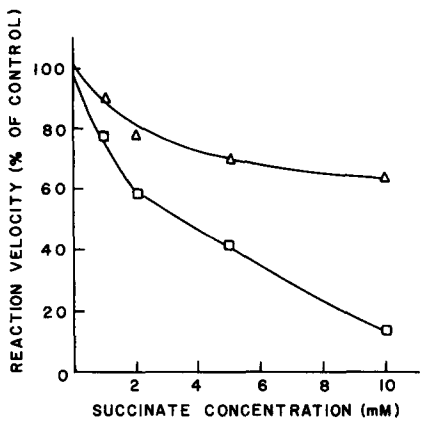

Fig. 2. Antagonism between acetyl CoA and succinate in the carboxylation of phospho*enol*pyruvate catalyzed by *T. aquaticus* phospho*enol*pyruvate carboxylase. Enzyme activity was assayed at 45°C at various succinate concentrations with different fixed concentrations of acetyl CoA. Acetyl CoA concentrations were: □, 50 μM; △, 200 μM. The control reaction velocity was the velocity observed without the addition of succinate.

The *Thermus* phospho*enol*pyruvate carboxylase thus resembles its well-characterized mesophilic counterparts from *Escherichia coli* and *Salmonella typhimurium* in metal requirement, molecular size and activation by acetyl CoA. However, it also possesses some interestingly distinctive characteristics. The strong positive cooperativity evident in the kinetics of activation by acetyl CoA (Fig. 1) has not been reported in the other systems. This cooperativity is manifested at 45 and 65°C and it exemplifies a great advantage that thermophile enzymes offer over cognate mesophile enzymes in that they can be used to study the interactions between proteins and ligands over a wide temperature range that includes elevated temperatures

at which most proteins from mesophiles would be unstable. The lack of activation by fructose bisphosphate and the inability of other acyl derivatives of CoA to substitute for acetyl CoA appear to be other features of the *Thermus* carboxylase not shared by the *E. coli* and *S. typhimurium* enzymes. It has been suggested [1] that phospho*enol*pyruvate carboxylases can be placed in three groups depending on the pattern of their regulation. One of these groups contains the acetyl CoA-activated enzymes, which are all from bacterial sources. Several of these carboxylases are also activated by fructose bisphosphate and all but one are inhibited by L-aspartate. The *Thermus* enzyme can be placed in this category by virtue of its acetyl CoA activation despite the lack of activation by fructose bisphosphate and weak inhibition by L-aspartate. Activation by acetyl CoA and inhibition by aspartate can be rationalized in terms of an anaplerotic role for phospho*enol*pyruvate carboxylase [1]. Inhibition by intermediates of the tricarboxylic acid cycle, which is a property of the *Thermus* and some other phospho*enol*pyruvate carboxylases, can, like the aspartate inhibition, be interpreted as feedback inhibition serving the same metabolic function.

Phospho*enol*pyruvate carboxylase and pyruvate carboxylase, which may be considered to be cognate enzymes in a metabolic sense, exhibit remarkably similar patterns of modulation of enzyme activity [1]. The regulation of pyruvate carboxylase positively by acetyl CoA and negatively by L-aspartate is thought to be allosteric with the activator and the inhibitor binding at different sites on the enzyme [7]. The kinetics of activation of *Thermus* phospho*enol*pyruvate carboxylase by acetyl CoA (Fig. 1) are consistent with a similar mechanism of regulation. The relatively high $A_{0.5}$ value for acetyl CoA should facilitate the efficient modulation of enzyme activity in vivo by changes in the concentration of this activator over a wide range. A point of particular interest about this enzyme is that it is one of the very few large, regulatory enzymes to be investigated from extremely thermophilic bacteria. Its regulation suggests that enzymes in these organisms can be modulated allosterically. This is especially significant in view of Brock's [8] speculation as to whether proteins in thermophilic organisms might be structurally too rigid to undergo allosteric transitions.

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