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Regulation of phosphoenolpyruvate carboxylase activity in *Escherichia coli*

In recent years, phosphoenolpyruvate carboxylase (EC 4.1.1.31) has been shown to catalyze the key reaction in Enterobacteriaceae[1-3], supporting the biosynthetic activity of the tricarboxylic acid cycle by replenishing oxaloacetate. As to the mechanism for controlling this enzyme activity, it was shown by us and by other groups that the enzyme is activated by CoASAc and inhibited by aspartate in two kinds of bacteria, *Escherichia coli*[4-6] and *Salmonella typhimurium*[7]. The enzyme from the former organism was also inhibited by fumarate and malate[6]. The effects of this activator and these inhibitors on the enzyme activity appeared instantaneously and were reversible.

In this report, evidence is presented which characterizes aspartate as an allosteric inhibitor of phosphoenolpyruvate carboxylase in *E. coli*, and some properties of this enzyme are described.

From sonic extracts of *E. coli* W grown on a glucose–salts medium, the enzyme was purified by high-speed centrifugation, protamine sulfate treatment, ammonium sulfate fractionation and calcium phosphate gel treatment. The highest purification obtained was about 45-fold. The enzyme preparation catalyzed the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate stoichiometrically and was free from citrate synthetase (EC 4.1.3.7). The assay mixture contained PEP, $KH^{14}CO_3$, $MgCl_2$, CoASAc, NADH, malate dehydrogenase (EC 1.1.1.37) and the enzyme preparation. The enzyme activity was measured by counting the total radioactivity fixed, which exclusively existed in malate.

The possibility that the inhibitor, *e.g.* aspartate, fumarate or malate, exerts its action as the analogue of oxaloacetate seems to be improbable because oxaloacetate shows only a slight inhibition compared with these inhibitors. For example, 5 mM oxaloacetate caused 8% inhibition under the reaction conditions where 5 mM aspartate caused 90% inhibition. The assay method for this experiment was similar to that of PALACIÁN, DE TORRONTEGUI AND LOSADA[8].

The enzyme reactions in the presence of various effectors were investigated kinetically. As shown in Fig. 1, the shape of the curve representing the relationship between rate and PEP concentration was converted from a normal hyperbola to an S-shaped curve by the addition of aspartate. Because of this abnormal inhibition by aspartate, aspartate does not seem to act as a simple competitive inhibitor with the substrate or the product, though the inhibition is gradually relieved on increasing the concentration of PEP. The substrate co-operativity which is reflected in the S-shaped curve is known to be characteristic of many allosteric enzymes[9]. In addition, such a co-operativity is revealed or increased by the presence of allosteric inhibitors. Therefore, in our system too, aspartate was supposed to exert its inhibitory action on the enzyme activity by occupying an allosteric regulatory site. On the other hand, the decrease in the concentration of CoASAc which is the allosteric activator of this enzyme[5] converted the normal hyperbola to the slightly S-shaped curve in the absence of aspartate (Fig. 1). However, when CoASAc was completely removed, this co-operativity disappeared again in accordance with the result by CÁNOVAS AND KORNBERG[5]. It seems to us that the substrate co-operativity appears only in a limited range of CoASAc concentration.

Abbrevation: PEP, phosphoenolpyruvate.

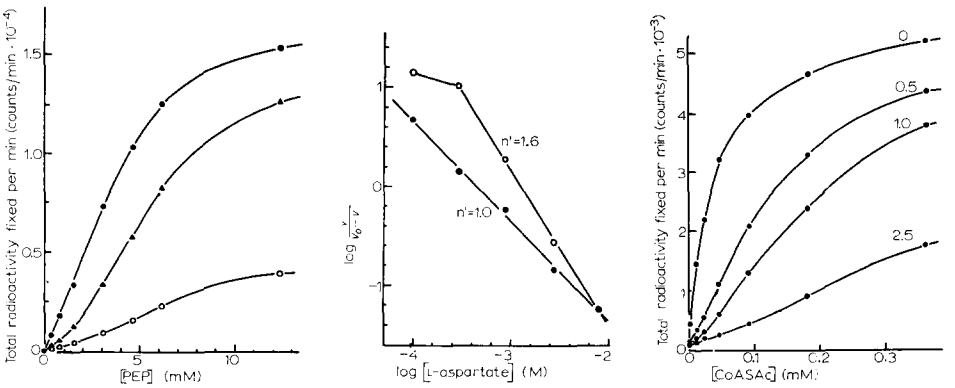

Fig. 1. Effect of PEP concentration on enzyme activity in the presence of various concentrations of CoASAc or aspartate. The reaction mixture contained in a total volume of 0.35 ml: Tris–HCl buffer (pH 8.5), 35 μmoles; $MgCl_2$, 3.5 μmoles; $KH^{14}CO_3$ ($6 \cdot 10^5$ counts/min per μmole), 5 μmoles; NADH, 0.63 μmole; 4 international units of malate dehydrogenase; 57 μg of the enzyme; potassium PEP as indicated. In addition, each reaction mixture contained 0.091 μmole of CoASAc (●—●), 0.009 μmole of CoASAc (○—○), or 0.091 μmole of CoASAc *plus* 0.87 μmole of aspartate (▲—▲), respectively. The mixture was incubated for 5 min at 30°. The radioactivity was measured with a gas-flow counter.

Fig. 2. Effect of aspartate concentration on the enzyme activity in the presence of various concentrations of CoASAc. The relation is represented by an expression of the form[10]:

$$\log \frac{v}{v_0 - v} = \log K' - n' \log [I]$$

The symbols v, v_0 and $[I]$ indicate the rate in the presence and absence of the inhibitor and the concentration of the inhibitor, respectively. Since the equation is formally identical with Hill's empirical relation for the binding of oxygen to haemoglobin, n' corresponds to Hill coefficient. The reaction conditions were the same as those in Fig. 1, except for the pH of the buffer (7.7), the amounts of $MgCl_2$ (1.8 μmoles) and cyclohexylammonium PEP (2.5 μmoles) and the addition of aspartate as indicated. In addition, each reaction mixture contained 0.007 μmole (●—●) or 0.07 μmole (○—○) of CoASAc, respectively. In the latter case half the amount of enzyme was used.

Fig. 3. Effect of CoASAc concentration on the enzyme activity in the presence of various concentrations of aspartate. The reaction conditions were the same as those in Fig. 2 except for the specific radioactivity of $KH^{14}CO_3$ ($1.4 \cdot 10^6$ counts/min per μmole) and the amount of the enzyme, 20 μg. The numbers on the curves refer to the concentrations of aspartate in mM added to the reaction mixture. The radioactivity was measured with a liquid-scintillation counter.

The effects of aspartate and CoASAc concentrations on the enzyme activity were studied and the results are shown in Figs. 2 and 3, respectively. Hill coefficients, which are employed for the quantitative representation of the co-operativity, were calculated for aspartate according to MONOD, CHANGEUX AND JACOB[10], and were found to be 1.0 and 1.6 in the presence of 0.02 mM and 0.2 mM CoASAc, respectively (Fig. 2). In other words, the co-operativity of aspartate can be seen at the high concentration of CoASAc. As shown in Fig. 3, the inhibition by aspartate was gradually relieved by increasing the concentration of CoASAc and the co-operativity of CoASAc was revealed by increasing the concentration of aspartate. It follows that aspartate and CoASAc are antagonistic in their effects.

The action of aspartate as an effector was reflected in its protection of the enzyme against heat inactivation. The enzyme in solution lost 90% of its activity by heating

at 55° for 5 min. The inactivation could be prevented almost completely by the presence of 15 mM aspartate. Fumarate and malate were also effective, whereas oxaloacetate, PEP and CoASAc were not so effective. Therefore, the form of the enzyme bound with the inhibitor seems more heat-stable.

Phosphoenolpyruvate carboxylase from spinach leaves prepared according to the method of BANDURSKI[11] was neither activated nor inhibited by aspartate. Therefore, a special device for controlling the activity is supposed to be inherent in the enzyme from Enterobacteriaceae.

From the facts presented above, aspartate seems to cause an inhibition of the enzyme by occupying the site(s) distinct from the active site.

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Comparative effects, *in vitro*, of various detergents on liver glucose-6-phosphate phosphohydrolase, inorganic pyrophosphate–glucose phosphotransferase, and acid inorganic pyrophosphatase activities

A variety of detergents previously have been demonstrated to affect the hydrolytic activity of classical liver glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) (see, for example, refs. 1–6). Recent studies[7–13] indicate that this enzyme also catalyzes the hydrolysis of PP_i and the transfer of a phosphoryl group from PP_i to the hydroxyl group attached to the number six carbon atom of glucose. Deoxycholate was employed as activating detergent in these studies which were carried out in our laboratory[7–11], while Triton X-100 was employed elsewhere[12,13]. The effects of a variety of other anionic, cationic, and non-ionic detergents on the various hydrolytic