ACTIVATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE OF *ESCHERICHIA COLI* BY FREE FATTY ACIDS OR THEIR COENZYME A DERIVATIVES

K. Izui, T. Yoshinaga, M. Morikawa and H. Katsuki

Department of Chemistry, Faculty of Science

Kyoto University, Kyoto, Japan

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SUMMARY: Phosphoenolpyruvate carboxylase of *Escherichia coli* was found to be remarkably activated by free fatty acid, such as laurate and oleate, and by its CoA derivative. The regulatory site of the enzyme for binding with these compounds was discriminated from the site for acetyl-CoA (one of the allosteric activators), on the basis of the isolation of the mutant having the altered phosphoenolpyruvate carboxylase which is almost insensitive to these compounds but still sensitive to acetyl-CoA. Physiological significance of this activation was also discussed.

Phosphoenolpyruvate carboxylase (PEP carboxylase, EC 4.1.1.31) of Enterobacteriaceae is an allosteric enzyme, which is activated by acetyl-CoA (1,2) and by fructose 1,6-diphosphate (3,4) and inhibited by L-aspartate (2,5,6), fumarate (5,6) and L-malate (5,6). As a curious property of this enzyme, the activity is remarkably enhanced by high concentrations of various organic solvents such as dioxane, alkylcellosolves, alcohols and dimethylsulfoxide (7,8). During the course of our investigation on the mechanism of this activation with the *E. coli* enzyme, we found that long-chain alcohols are also powerful activators at much lower concentrations: *i.e.*, 0.3 mM n-heptyl alcohol gave a 5-fold activation, while 1,000 mM ethanol was required for the same extent of activation. This observation led us to the discovery of the activation by long-chain free fatty acids (FAs) and their CoA derivatives (FA-CoA) which are presumed to be normal metabo-

TABLE I

Activation of PEP Carboxylase by Free FAs.

Addition	Concentration (mM)	Tween 80 (0.006%)	Activity (cpm fixed/min)
None		-	1,420
"		+	1,450
Caprylate	1.0	-	1,620
"	3.0	-	2,270
"	10.0	-	6,250
Laurate	0.2	-	12,330
"	1.0	-	15,280
"	1.0	+	15,540
Myristate	0.02	-	4,470
"	0.2	-	5,220
"	1.0	+	4,930
Palmitate	0.002	-	1,540
"	0.02	-	1,980
"	0.2	-	1,400
"	1.0	+	1,250
Stearate	0.002	-	1,480
"	0.02	-	1,490
"	0.2	-	1,400
"	1.0	+	920
Oleate	0.2	-	14,170
"	1.0	+	220
None, minus PEP		-	40
Laurate, minus PEP	1.0	-	60

Partially purified PEP carboxylase of E. coli W cells was prepared as described previously (4,13). The assay mixture contained the following constituents in μmoles in 0.10 ml: potassium PEP, 0.4; $MgSO_4$, 1.0; $KH^{14}CO_3$ (6×10^5 cpm/μmole), 1.0; $NADH_2$, 0.1; Tris-H_2SO_4 buffer (pH 8.5), 10; 1 I.U. of malate dehydrogenase (EC 1.1.1.37); the enzyme (3 μg of protein) and free FA as indicated. The reaction was carried out at 30°C for 5 min, and terminated by adding 0.40 ml of 0.05 N HCl. The radioactivity

incorporated into malate was measured. The activity is indicated as total radioactivity incorporated per min under the assay conditions. For the preparation of FA solution, 0.5 ml of ether solution of FA was mixed with 10 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.5) containing or not containing Tween 80 and the mixture was homogenized with a Thermo-Mixer (Thermonics Co. Ltd. Tokyo) under reduced pressure until evaporation of ether. Because of salt formation of FA with Mg^{2+}, the clear reaction mixture was not obtained and the concentration given in the table is for the maximum possible concentration to which the enzyme was subjected. It was confirmed in a separate experiment that the effects of these FAs on malate dehydrogenase, which is the other enzyme component of the assay system, were negligibly small.

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lites in the cells. Although a number of enzymes have been reported to be inhibited by free FA (9-12), it seems that no soluble enzyme has ever been described which is activated by free FA.

The present communication describes the activation of the enzyme by free FA and FA-CoA, and discusses its physiological significance.

Table I shows the effects of various free FAs on the PEP carboxylase activity. As can be seen from the table, free saturated FAs activated the enzyme to various extents at the concentration of 1 mM and the degree of activation increased with the increase in the chain length of FA up to laurate which showed the maximum (10-fold) activation. Further increase in the chain length of FA showed a tendency to decrease the extent of activation. Stearate showed no activation at the concentration of 0.2 mM.

Fig. 1 shows the effect of increasing concentrations of laurate and oleate on the enzyme activity. The activity was increased by increasing the concentration of laurate. In the case of oleate, on the other hand, the activation reached a maximum at 0.2 mM and then decreased with increasing concentrations of it. The concentrations required for the half-maximum activation, $A_{0.5}$ values, of laurate and oleate were 0.12 mM and

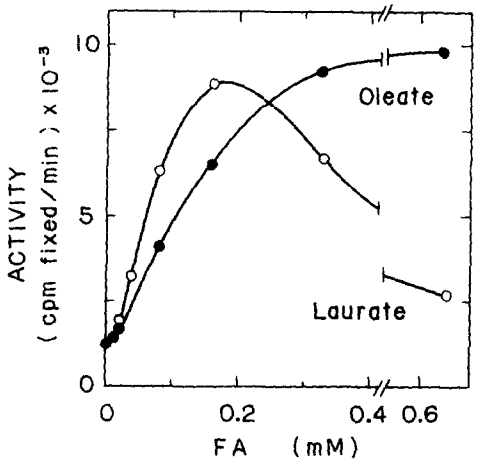

Fig. 1. Effect of increasing concentrations of laurate and oleate on the PEP carboxylase activity. The reaction conditions were the same as described in Table I. When the effect of oleate was examined, 0.01% Tween 80 was used as dispersing agent, but not used in the case of laurate.

0.06 mM, respectively. The values for the saturated acids with shorter chain length than laurate were much larger than that for laurate though not given in figures.

As in the case of free FAs, lauryl-CoA and oleyl-CoA showed a powerful activation (10-fold). Unlike to palmitate, palmityl-CoA showed a 2-fold activation (Fig. 2). $A_{0.5}$ values of lauryl-CoA and oleyl-CoA were 0.04 mM and 0.06 mM, respectively. It is interesting to note that the enzyme affinity for lauryl-CoA is 3 times as high as that for free laurate.

In order to investigate the nature of activation by lauryl-CoA, the activation kinetics was followed by the spectrophotometric method (1). As a result of the experiments, the activation occurred without lag and it was released reversibly by the addition of bovine serum albumin (15). The enzyme affinity for PEP was increased by the addition of lauryl-CoA, and $A_{0.5}$ of lauryl-CoA was decreased by fructose 1,6-diphosphate and in-

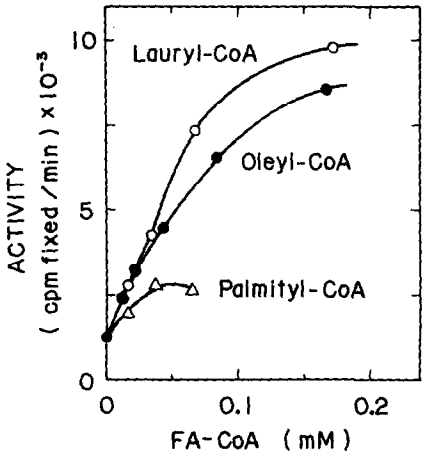

Fig. 2. Effect of increasing concentrations of lauryl-, oleyl- and palmityl-CoA on the PEP carboxylase activity. The reaction conditions were the same as described in Table I. Tween 80 was not used. Lauryl- and oleyl-CoA were synthesized according to the method of Goldman and Vagelos (14). Palmityl-CoA was purchased from Sigma Chemicals Co. Before the use of FA-CoA solution, it was acidified with 0.1 N HCl and shaken with ether to remove free FA and then neutralized with 0.1 N NaOH. The effects of these FA-CoA on malate dehydrogenase, which is the other enzyme component of the assay system, were negligibly small under the conditions employed.

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creased by acetyl-CoA or aspartate. The inhibition by aspartate was gradually released with increasing concentrations of lauryl-CoA and the presence of aspartate increased the cooperativity of lauryl-CoA. The detailed results will be presented elsewhere.

The site of the enzyme for binding with free FA or FA-CoA seems to be different from the site for acetyl-CoA. The isolation of a mutant possessing the altered PEP carboxylase which had lost the sensitivity to laurate, lauryl-CoA and organic solvent such as dioxane, in combination, but retained the sensitivity to acetyl-CoA, supports the view mentioned above (Table II). Although a weak activation by palmityl-CoA was first reported by Cooper and Benedict (17) with pyruvate carboxylase (EC 6.4.1.1) of yeast, the fact of similar activation by acetyl-CoA and CoA in their case rather suggests the identity of the site for these three compounds.

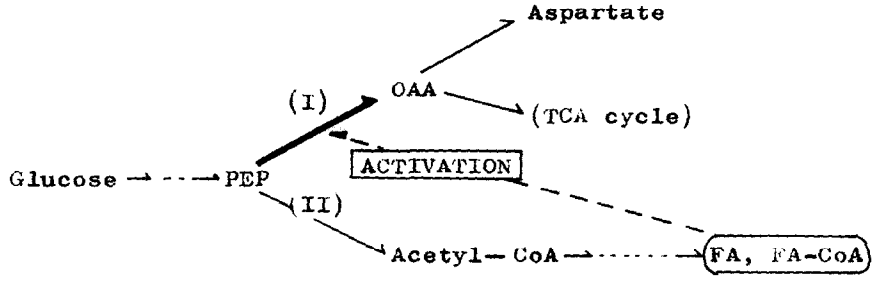
TABLE II

Effect of Laurate and Lauryl-CoA on the Activity of Mutationally Altered PEP Carboxylase.

Addition	Activity (cpm fixed/min)	
	Wild	TS 2-6
None	1,300	360
Acetyl-CoA (0.3 mM)	10,790	4,000
Dioxane (0.5 M)	9,730	670
Laurate (1 mM)	6,840	750
Lauryl-CoA (0.07 mM)	6,700	620

The reaction conditions were the same as described in Table I. The strain TS 2-6 which possesses the mutationally altered PEP carboxylase was obtained as a revertant from PEP carboxylase-negative mutant (PPC-2, isolated by Glansdorff (16)). Crude cell-free extract (50 µg of protein) of the TS 2-6 cells was used as the enzyme.

The results obtained above strongly suggest that free FA or FA-CoA is the third physiological activator of PEP carboxylase of *E. coli*. As to its physiological function, the following mechanism is supposed to be operative in the control of this enzyme activity. The flow of PEP produced in glycolysis is branched mainly into two pathways for the production of oxaloacetate (OAA) (I) and acetyl-CoA (II), a part of which is utilized for FA synthesis. When the demand for FAs is supplied enough in the



cell ——this must be reflected in high levels of FA or FA-CoA——, these compounds channels the metabolic flow of PEP mainly into the supply of OAA (I) through a mechanism of this activation. Thus if glucose and FAs are available to the organism as carbon source, this channeling may presumably be useful for cellular economy, because acetyl-CoA is abundantly supplied through β-oxidation of FA. In this regards, it is interesting that pyruvate kinase (EC 2.7.1.40) has been reported to be inhibited by FA or FA-CoA in rat liver (10) and Arthrobacter crystallopoietes (12). If this is to be the case with E. coli, this channeling may be more effectively accomplished.

Recently it has been reported by numerous workers that free FA or FA-CoA takes a role in metabolic regulation by inhibiting some enzyme activities (9-12). However, as is well known, long-chain FA or FA-CoA is a strong surfactant and consequently a doubt is thrown by other workers on its physiological meaning in metabolic regulation (18). If FA or FA-CoA participates in metabolic regulation as effector, the allosteric activation of enzyme by these compounds must be found in living organism. In fact, this activation of PEP carboxylase of E. coli reported here seems to be the first case. Details of the activation is now under investigation.

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