PHOSPHOENOLPYRUVATE CARBOXYLASE OF E. COLI: DISCRIMINATION OF REGULATORY SITES FOR FOUR KINDS OF ALLOSTERIC EFFECTORS BY THE METHOD OF GENETIC DESENSITIZATION

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SUMMARY

The effect of mutational alteration of phosphoenolpyruvate (PEP) carboxylase on sensitivity to four kinds of allosteric effectors was studied. In several mutants the enzyme was differentially and independently desensitized to each of the effectors, indicating the existence of at least four distinct binding sites for the effectors. Mutants, whose enzyme was desensitized not only to one effector but also to two or three effectors in combination were obtained with a high frequency.

The activity of PEP carboxylase (EC 4.1.1.31) of E. coli W* is known to be activated by acetyl-CoA (CoASAc)(1), fructose 1,6-diphosphate (FDP)(2) and free fatty acid (FA) or its CoA derivative (CoASFA)(3), and is inhibited by L-aspartate (Asp)(4,5), reflecting its multiple roles in cellular metabolic regulation. Furthermore, the enzyme is activated by high concentrations (1-3 M) of organic solvents such as dioxane and alcohols (6). Recently we presented the evidence which suggests the identity of the FA-binding domain with the domain for the organic solvent (3).

The enzyme activity is regulated by so many effectors that it is especially interesting to discriminate the allosteric sites on the enzyme for binding with the respective effector. One of the most effective approaches to this problem is to examine whether or not it is possible to obtain an altered enzyme which has lost the sensitivity to a given effector but retains the sensitivity to the other effectors as a consequence of a mutation in the structural gene for the enzyme (7,8,9). This communication describes the

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* The enzyme of strain K12 showed essentially the same properties as that of strain W (unpublished work).

evidence which supports the existence of four distinct allosteric sites on the enzyme and some properties of the mutationally altered enzyme.

METHODS

E. coli K12 was used as a wild strain of the bacteria. PEP-carboxylase-negative mutants (glu-1 and glu-2)(10) of E. coli K12, which require glutamate in addition to glucose as carbon source (11), were used as parent strains for the isolation of the revertants. Reversion was induced by the treatment of the cells with N-methyl-N'-nitro-N-nitrosoguanidine (MNG)(12), and the revertants were selected based on the nutritional requirements mentioned above.

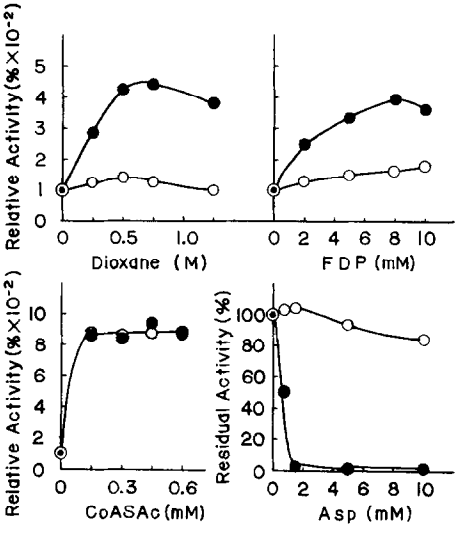

Fig. 1 Influences of the effectors on the activities of PEP carboxylases from the wild strain (—●—) and strain TS2-6 (—○—). The two strains of bacteria were grown for 12-20 hours at 30°C in medium E (13) fortified with casamino acids (1 g/l) and thiamine hydrochloride (10 mg/l). The cells (1 g of wet weight) harvested by centrifugation were suspended in 1 ml of 0.1 M Tris-H_2SO_4 buffer (pH 7.7) containing 3 mM glutathione, and were disrupted with a sonic disintegrator (20 kHz; Ohtake Co.) for 1 min. The supernatant which was obtained by centrifugation of the mixture at 20,000×g for 20 min., was used as the enzyme. The enzyme activity in the presence and absence of each effector was determined by the previously described method (5). The reaction mixture contained the following constituents in μmoles in a total volume of 0.10 ml: PEP, 0.8; $KH^{14}CO_3$ (6.5×10^5 cpm/μmole), 1.0; $MgSO_4 7H_2O$, 1.0; $NADH_2$, 0.2; Tris-H_2SO_4 (pH 8.5), 10.0; 1 I.U. of malate dehydrogenase (EC 1.1.1.37); the enzyme (5-11 μg of protein); and the effector as indicated. The reaction was carried out at 30°C for 5 min. and was terminated by addition of 0.4 ml of 0.05 N HCl. Residual $H^{14}CO_3^-$ was removed with the aid of several pieces of dry ice and then an aliquot of the mixture was counted for its radioactivity with a gas flow counter (Nuclear Chicago).

Specific activities of the wild enzyme and of the TS2-6 enzyme in the absence of the effector were 32.4 and 7.84 mμmoles CO_2 incorporated/min./mg of protein, respectively.

The methods for cultivation of the bacteria and for preparation and assay of the enzymes are described in the legend of Fig. 1.

RESULTS AND DISCUSSION

Genetic desensitization: Some kinetic properties were investigated with an altered enzyme of one of the revertants obtained. Fig. 1 shows the effect of increasing concentrations of each effector on the activity of the enzyme from strain TS2-6 in comparison with that of the wild strain. The wild enzyme was activated maximally about 4-fold by dioxane, FDP or CoASAc under the conditions employed. In contrast, the TS2-6 enzyme was not activated significantly by dioxane or by FDP, though it was activated to the same extent by CoASAc as the wild enzyme. In addition, the TS2-6 enzyme was inhibited only 15% even by 10 mM Asp, whereas the wild enzyme was almost completely inhibited by 2 mM Asp. This slight inhibition of the TS2-6 enzyme was weakened by further addition of CoASAc (not shown in the figure), indicating that heterotropic effect between CoASAc and Asp operated in the TS2-6 enzyme as in the case of the wild enzyme. The alteration of the sensitivity described above was not attributable to any effector contaminating the crude cell-free extract, because when both enzymes from TS2-6 and the wild strain were mixed together, the activities were additive. The molecular weight of the TS2-6 enzyme was found to be about the same as that of the wild enzyme (3.5×10^5) by the method of gel filtration through Sephadex G-200.

The sensitivities of other several altered PEP carboxylases to each effector were examined in a similar manner to the procedure of Fig. 1. The results obtained at a given concentration of each effector are shown in Fig. 2. On four axes starting from the original point are plotted relative enzymatic activities in the presence of each effector to the activity in the absence of the effector. The shape of quadrilateral which is obtained by connecting these four points shows the mode of response of each enzyme to the four effectors. For example, the TS2-73 enzyme was neither activated by dioxane and FDP nor inhibited by Asp (Fig. 2b) unlike the wild enzyme (Fig.

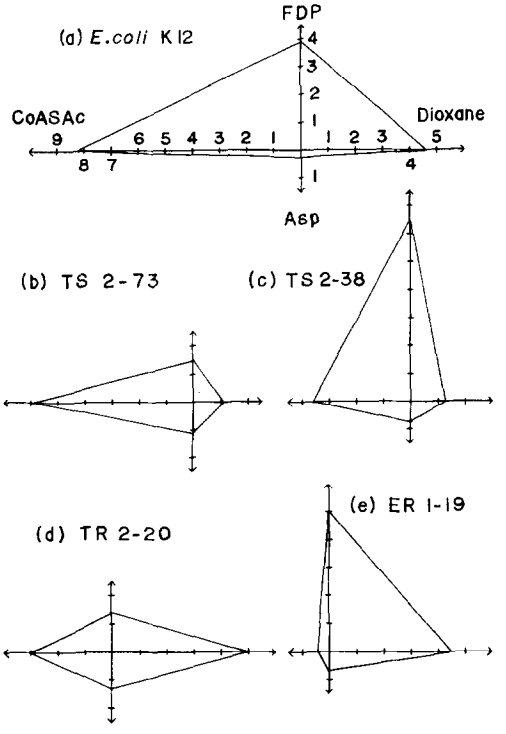

Fig. 2 Influences of the effectors on the activities of PEP carboxylases from wild strain and several mutants of E. coli.

The methods for cultivation of bacteria and for preparation and assay of the enzymes are described in the legend of Fig. 1 except for the concentration of the effector. Final concentration of each effector was as follows: CoASAc, 0.3 mM; FDP, 5 mM; dioxane, 0.6 M; Asp, 1.5 mM.

Specific activities of these enzymes in the absence of the effector (μmoles CO_2 incorporated/min./mg of protein) were as follows: (a) 29.2, (b) 5.13, (c) 0.07, (d) 0.17, (e) 0.47.

2a) but was activated 5.9-fold by CoASAc. The TS2-38 enzyme was desensitized to dioxane and Asp but responded to FDP and, to a lesser extent, to CoASAc (Fig. 2c). The enzymes of TR2-20 and ER1-19 also showed characteristic responses to the four effectors, and they were activated by Asp and inhibited by CoASAc, respectively (Figs. 2d and 2e).

The facts that the sensitivities of the enzymes to the three activators were changed to various extents and independently from each other owing to mutation strongly suggest the existence of three distinct allosteric sites for the activators on the wild enzyme. Furthermore, the fact that the sensitivity to an organic solvent such as dioxane and alcohols was lost without

any change in the sensitivity to other effectors seems to provide a clue to the mode of activation by high concentrations (0.5-2.0 M) of organic solvent. It might be said that the organic solvent exerts its action through direct interaction with the enzyme at its own limited domain but not through changing the physicochemical properties of the medium surrounding the enzyme molecule.

As in the case of the desensitization to the activators, many revertants having PEP carboxylases desensitized to Asp but retaining the enzyme activities were obtained (Fig. 2). These results, together with some evidence reported previously (2), suggest more conclusively that the site for binding with Asp is distinct from the active site.

TABLE I Sensitivities of the Mutationally Altered Enzymes to the Effectors

Desensitized to[1)]				Number of Strains
Dioxane	FDP	CoASAc	Asp	
+				1
	+			1
		+		1
			+	2
+	+			1
+		+		
+			+	2
	+	+		
	+		+	
		+	+	
+	+	+		2
+		+	+	
+	+		+	1
	+	+	+	
+	+	+	+	

1) The criterion of "desensitization" is described in the text and the assay conditions were the same as in Fig. 2. (+) represents "desensitization to the corresponding effector."

Multiple desensitization to the effectors: In Table I is shown a classification of the mutationally altered enzymes according to their sensitivities to the effectors, and the number of strains which belong to each class. When sensitivity of the enzyme of a mutant to a given effector

(degree of activation or inhibition) is less than one-third of that of the wild enzyme, it is defined as "the enzyme desensitized to the given effector." As seen from the table, it is of great interest to note that many mutationally altered enzymes were obtained which lost the sensitivity to two or three effectors in combination. This "multiple desensitization" may be attributable either to the simultaneous destruction of two to three binding sites for the effectors or to the destruction of the common mechanism which is involved in the transmission of regulatory signals from the effectors to the active site. In order to decide the alternatives, studies on kinetics and binding of the effectors with the enzyme are now in progress in our laboratory.

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