PROPERTIES OF THE ACTIVATION OF PHOSPHOENOLPYRUVATE CARBOXYLASE FROM *ESCHERICHIA COLI* BY ACYL DERIVATIVES OF COENZYME A*

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1. Introduction

Potent activation by acyl derivatives of coenzyme A, notably acetyl-CoA, is a characteristic feature of the enzymes (pyruvate carboxylase, PEP carboxylase) which catalyze the anaplerotic synthesis of oxalacetate from a three-carbon precursor [1]. In the case of several pyruvate carboxylases previous studies have demonstrated that incubation with reagents, e.g. TNBS**, which react preferentially with amino groups, causes specific inactivation of the acetyl-CoA-dependent catalytic activity [2,3]. The date obtained in these studies suggested that one or more lysyl residues participated in the interaction of pyruvate carboxylase with the allosteric activator [2,3]. We have therefore examined the specificity for activation of PEP carboxylase by certain analogs of acetyl-CoA, and also the sensitivity of the acetyl-CoA-dependent catalytic activity to specific inactivation by TNBS. The data presented here indicate that, despite the metabolic analogy noted above, the molecular structure of the acetyl-CoA binding site on PEP carboxylase appears to differ from that present on pyruvate carboxylase.

2. Materials and methods

PEP carboxylase was prepared from *Escherichia coli B* grown on glycerol as carbon source (Grand Island Biological Co.) by a modification of the procedure of Canovas and Kornberg [4]. The preparations employed in these studies had specific activities in the range 8-10 units/mg. PEP carboxylase was assayed spectrophotometrically in the presence of malate dehydrogenase and NADH [4]. Protein was estimated from the absorbance at 280 nm assuming $\epsilon^{0.1\%}_{280\ nm} = 1.0$. Acetyl-CoA, acetyl-pantetheine, acetyl-3′-dephosphoCoA and acetyl-deaminoCoA were prepared by acetylation of the appropriate sulfhydryl derivative as described by Simon and Shemin [5]. The acetyl thioesters were further purified by chromatography on DEAE-Sephadex A-25(Cl^-) and gel-filtration on Sephadex G-10 as described by Fung [6]. D-pantethine was reduced to the sulfhydryl derivative by treatment with excess $NaBH_4$ prior to acetylation. Dephospho-CoA, deaminoCoA, all other acyl-CoA derivatives and PEP were obtained from PL-Biochemicals Inc. The purity of the acylCoA derivatives was established as described previously [7]. Pantethine, hexanoic acid, octanoic acid and decanoic acid were obtained from Sigma Chemical Co.

3. Results and discussion

Table I summarizes the data obtained when various acyl-derivatives of CoA and related compounds are tested as activators of PEP carboxylase from *E. coli*. The presence of an acyl thioester group appears to be an essential feature since activation is observed in the presence of acetyl-pantetheine but not, as previously noted (4), in the presence of CoASH. Additional groups besides the acyl thioester appear however to be necessary since simple acetyl-thioesters,

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** Abbreviations: The following abbreviations are employed: TNBS, trinitrobenzenesulfonate; FDP, fructose-1,6-diphosphate; PEP, phosphoenolpyruvate.

Table 1
Specificity of activation of PEP carboxylase from *E. coli* by acyl derivatives of coenzyme A

Derivative[a]	Apparent K_A (mM)	n	Relative V_{max}
A. Acetyl-CoA	0.42	2.2	100
B. Acetyl-pantetheine[b]	3.06	1.9	2
Acetyl-3′-dephospho-CoA	0.10	1.9	81
Acetyl-deaminoCoA	0.32	2.0	130
C. Propionyl-CoA	0.84	2.3	104
n-Butyryl-CoA	1.50	2.2	73
n-Valeryl-CoA	1.64	2.2	50
n-Hexanoyl-CoA[c]	1.58	2.0	53
n-Heptanoyl-CoA	1.05	2.3	58
n-Octanoyl-CoA[c]	0.21	2.1	90
n-Nonanyl-CoA	0.11	2.4	120
n-Decanoyl-CoA[c]	0.024	2.5	118

[a]Inactive derivatives - CoASH, succinyl-CoA, glutaryl-CoA, acetyl-mercaptosuccinate, acetyl-mercaptoacetate. [b]Addition of 5′-ADP or 3′,5′-ADP (1 to 10mM) does not enhance the extent of activation by acetyl-pantetheine. [c]No stimulation by Na^+ hexanoate, Na^+ octanoate or Na^+ decanoate was observed when added at concentrations over the range 0.1 to 10mM. The assay system contained, in 0.3 ml, 100mM Tris–Cl pH 8.5, 2.5mM PEP, 5mM Mg^{2+}, 10mM $KHCO_3$, 10μg malate dehydrogenase, 0.1mM NADH and the acyl-CoA over an appropriate concentration range. After equilibration to 25°C the reaction was initiated by addition of 5-20 μg PEP carboxylase (specific activity = 6–8 units/mg) and the initial velocity obtained from the decrease in absorbance at 340nm. The maximal velocity was obtained from a plot of reciprocal velocity versus reciprocal $[\text{acyl-CoA}]^2$ which is linear in all cases at the higher acyl-CoA concentrations.

The apparent K_A and Hill coefficent (n) were obtained by plotting the data according to the empirical Hill equation:

$$\log_{10} \frac{V}{V_{max}} = n \log_{10} [\text{acyl-CoA}] - \log_{10} K$$

e.g. acetyl-mercaptoacetate, are ineffective (table I, footnote a). Activation by acetyl-pantetheine, although significant, is two orders of magnitude less effective than that observed for derivatives, e.g. acetyl-CoA, which contain an intact nucleotide. The presence of the pyrophosphate bridge appears essential for observation of maximal activation since addition of 5′-ADP or 3′,5′-ADP fails to potentiate the effect of acetyl-pantetheine (table I, footnote b). However the presence of the 3′-phosphate and 6-amino groups of CoA are not essential. Derivatives which lack these groups (acetyl 3′-dephosphoCoA, acetyl deaminoCoA) activate PEP carboxylase as effectively as acetyl-CoA (table I A, B). These observations contrast in several important respects with the activation specificities determined for various pyruvate carboxylases. Typically these latter enzymes are activated weakly by both adenosine-3′, 5′-diphosphate and CoASH, and are inhibited by derivatives in which the nucleotide portion of the CoA molecule is either missing (acetyl-pantetheine) or modified (acetyl 3′-dephosphoCoA, acetyl-deamino-CoA) [6,7]. These contrasting specificities suggest that the molecular structure of the acetyl-CoA binding site on PEP carboxylase differs strikingly from that present on the pyruvate carboxylases.

Further evidence supporting this postulate was obtained from studies in which the extent of activation of PEP carboxylase by various effectors was measured during incubation of the enzyme with 1–2 mM TNBS. As shown in fig. 1 some decrease in the extent of activation by acetyl-CoA is observed under these conditions. However the effect is non-specific since incubation with TNBS also reduces the extent of activation by FDP, GTP and dioxan, and the extent of inhibition by L-aspartate (fig. 1). In the case of FDP the extent of the effect is similar to that observed for acetyl-CoA. It should be noted that incubation with TNBS does not *abolish* the response of PEP carboxylase to any of these effectors. Thus negligible catalytic activity is observed when oxalacetate synthesis is measured in the absence of activator but using the concentration of PEP and Mg^{2+} employed for estimation of activator-dependent catalysis (fig. 1). It seems likely that the effect observed is a secondary consequence of a change in the conformation of the protein which results from modification of residue(s) remote from the catalytic or effector sites. A similar phenomenon is observed when PEP carboxylase is subjected to photoxidation in the presence of Rose Bengal. Hence, in contrast to its effect on various pyruvate carboxylases [2,3], TNBS fails to desensitize PEP carboxylase to activation by acetyl-CoA suggesting that in this latter enzyme reactive lysyl residues do not participate in the enzyme–activator interaction. This observation, taken together with the contrasting response of these two enzymes to acetyl-3′-dephosphoCoA, provides further support for the postulate

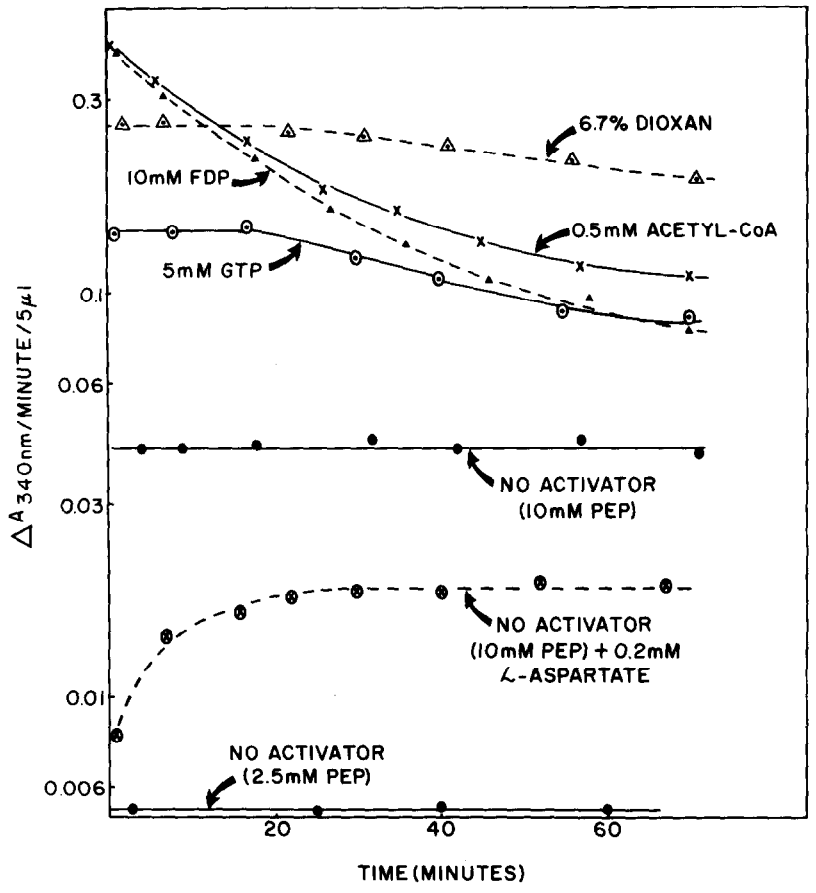

Fig. 1. Effect of incubation with TNBS on the response of PEP carboxylase to various allosteric effectors. The incubation system contained 20 mM NaK phosphate pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2 mM dithioerythritol, 5 μM phenylmethylsulfonyfluoride, 1 mM TNBS and PEP carboxylase (0.54 mg/ml: specific activity = 10.0 units/mg.) in a total volume of 0.2 ml. The incubations were performed at 2°C and were initiated by addition of the enzyme. At the times indicated 5–15 μl aliquots were withdrawn for assay of residual catalytic activity under the various conditions described below. For assay of activator-dependent oxalacetate synthesis the system contained 100 mM Tris–Cl pH 8.5, 2.5 mM PEP, 5 mM Mg^{2+}, 15 mM $KHCO_3$, 10 μg malate dehydrogenase and 0.15 mM NADH in a total volume of 0.3 ml. The activator concentrations employed were acetyl-CoA, 0.5 mM: FDP, 10 mM: dioxan, 6.7%: and GTP, 5 mM. Additional Mg^{2+} (5 mM) was added to the system containing GTP. For assay in the absence of activators, and also in the presence of L-aspartate (0.2 mM) the system contained 100 mM Tris–Cl pH 8.5, 10 mM PEP, 15 mM Mg^{2+}, 15 mM $KHCO_3$, 10 μg malate dehydrogenase and 0.15 mM NADH in a total volume of 0.3 ml. In the figure velocities are expressed as ΔA_{340nm}/min/5 μl.

that in the case of pyruvate carboxylase the lysyl residues which are selectively modified by TNBS interact with the 3′-phosphate of CoA as an essential feature of a productive interaction with this effector.

Table I also defines some features of the specificity with respect to the acyl group which characterize activation of PEP carboxylase by acyl derivatives of CoA. The apparent activator constants (K_A) and maximal velocities observed for homologs of acetyl-CoA become somewhat less favorable as the length of the acyl chain increases from two to six (n-hexanoyl-CoA) carbon atoms. Further increase in the chain length has however the converse effect. Thus for n-decanoyl-CoA the apparent K_A is 20-fold more favorable than that observed for acetyl-CoA while similar maximal velocities are obtained (table I A, C). The Hill

coefficent (n) approximates a value of 2 under these conditions for all derivatives tested and shows no consistent variation with the chain length of the acyl group. Carboxyacyl derivatives of CoA (e.g. succinyl-CoA, glutaryl-CoA) are ineffective as activators of PEP carboxylase when added at concentrations appropriate for observation of activation by the saturated acyl analog (table I, footnote a). Since, in contrast to other reports [8,9], we have been unable to demonstrate activation by n-hexanoate, n-octanoate or n-decanoate (table I, footnote c), the trend to a more favorable apparent K_A observed for the longer chain homologs does not appear attributable to interaction of these derivatives at a site which also accepts the free fatty acid. The acyl specificity as defined in table I appears similar in some respects to that described previously for pyruvate carboxylase purified from *Saccharomyces cerevisiae* [10] and suggests that this region of the activator site on PEP carboxylase may be predominantly hydrophobic.

The differences in the properties of activation of pyruvate carboxylase and PEP carboxylase by acyl derivatives of CoA which have been revealed by these studies are however more striking. Despite the analagous physiological role which may be attributed to this activation [1], it seems clear that the molecular structure of the activator binding sites on the two enzymes are quite dissimilar. The situation appears therefore to represent a case of convergent evolution which is of particular interest since it involves the site for an allosteric effector.

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