COMPARATIVE ASPECTS OF FATTY ACID ACTIVATION
IN ESCHERICHIA COLI AND CLOSTRIDIUM BUTYRICUM

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1. Introduction

The role of acyl-ACP in the acylation of glycerol-3-phosphate has been recently demonstrated in *E. coli* and *C. butyricum* [1–3].

In *E. coli*, both CoA and ACP derivatives can serve as substrates for this reaction [4], whereas in *C. butyricum* ACP derivatives are used almost exclusively; the biosynthesis of the latter compounds is known to proceed only through the fatty acid cycle [5].

The possible formation of acyl-ACP via fatty acid activation led us to study long chain fatty-acid activating enzyme(s) in both bacteria.

Our results indicate that these enzymes are present in extracts of *E. coli*, but not in these of *C. butyricum*. Induction of these enzymes in *E. coli* occurs when the cells are grown in the presence of oleate, as it was demonstrated for the other enzymes of fatty acid oxidation [6]. ACP cannot replace CoA in the fatty acid activation reaction in either bacteria.

Therefore the difference in the specificity of the acyl donor in both bacteria seems to be related to the ability or lack of ability of the cell to synthesize acyl-CoA derivatives as well as acyl-ACP.

Abbreviations: ACP: acyl-carrier protein; CoA: coenzyme A; Acyl-CoA synthetase: acid: CoA ligase (AMP) (E.C. 6.2.1.3); Thiolase: acetyl-CoA: acetyl-CoA C-acetyl transferase (E.C. 2.3.1.9); Thiol-esterase: palmitoyl-CoA hydrolase (E.C. 3.1.2.2); ACP_{coli} and $ACP_{butyricum}$: acyl carrier protein isolated from *E. coli* and *C. butyricum*, respectively.

2. Material and methods

2.1. *Growth conditions*

Clostridium butyricum (obtained from Dr. H.Goldfine) was grown on glucose at 37°C as described by Broquist and Snell [7]. In biotin-deficient medium, non radioactive or ^3H-labelled oleic acid (10 mg/l) was used. After overnight growth, the cells were washed seven times with 4% bovine serum-albumin in 0.010 M phosphate buffer pH 7.3, then with buffer alone. Negligible radioactivity was found in the last wash when radioactive oleic acid was used in the growth medium.

Escherichia coli K 12 Hfr 139 auxotroph for Thiamine and Pantothenate (obtained from Dr. P.Roy Vagelos) was grown aerobically at 37°C on synthetic medium 63 [8] containing 0.2% of the different carbon sources (see table 1), supplemented with thiamine (0.5 mg/l) and pantothenate (0.5 mg/l). For experiments with oleate, 0.4% Brij 35 was added. Absence of growth was confirmed on Brij 35 [6]. At the end of the log phase, the cells were washed as previously described.

2.2. *Enzyme sources*

The cells were suspended either in 0.010 M phosphate buffer pH 7.3, for acyl-CoA synthetase and thiolesterase assays, or in 0.010 M Tris HCl pH 7.3, containing 0.010 M β-mercaptoethanol, for thiolase determination.

After sonication for 4 × 15 sec at 0°–10°C or breakage of the cells by French Press at 8,000–10,000 psi, the fraction obtained after centrifugation

for 10 min at $300 \times g$ is referred to as homogenate; the supernatant fraction is then obtained by centrifugation for 30 min at $50,000 \times g$.

2.3. Enzymatic assays

Acyl-CoA synthetase: a sensitive assay was developed, based on the insolubility of acyl-CoA in ethyl dioxide. The incubation mixture contained in a final volume of 0.1 ml: 100 mM phosphate buffer, pH 7.6; 4 mM $MgCl_2$; 5 mM ATP; 0.35 mM CoA; 0.7 mM fatty acid. Control experiments without CoA were systematically included. The assay is linear up to 10 min and up to a protein content of 20 γ per assay. After 10 min at 30°C, the reaction is stopped by acidifying with 0.6 ml of 1.5% perchloric acid; the excess fatty acid is eliminated by ether washes (4 × 5 ml).

The aqueous phase, mixed with Bray's solution [9], is used directly for scintillation counting since control experiments have shown that, for acid-insoluble long-chain acyl-CoA, similar results are found after centrifugation and counting of the precipitate. In subsequent experiments, the reaction product was shown to be palmityl-CoA when palmitate was present in the incubation mixture. Thiolase was assayed in the supernatant fraction as described by Stern [10] with slight modifications, and thiolesterase was assayed by using ^{3}H-9,10-palmityl-CoA as substrate. The reaction mixture contained 0.4 mg protein per ml in 100 mM phosphate buffer, pH 7.3 and 0.18 mM ^{3}H-9,10-palmityl-CoA (final volume 0.050 ml). After 10 min at 30°C, 0.5 ml 1.6% perchloric acid was added and the palmitate enzymatically released was extracted with

5 ml petroleum ether; 4 ml were used for scintillation counting in toluene containing PPO (5 g/l) and dimethyl-POPOP (0.3 g/l).

3. Results

As shown in table 1, there is an increase of the specific activity of the thiolase only when oleate is used as carbon source; this has been previously demonstrated by Overath et al. [6].

It is also clear that the specific activity of acyl-CoA synthetase follows a similar pattern. Although activity of the enzyme can be detected on acetate and glucose, a maximum increase in activity occurs only when cells are grown on oleate. Similarly the highest specific activity of thiolesterase is found when oleate is present in the growth medium; no activity is detected when the cells are grown on rich-medium.

Fatty acid activation according to chain length is shown in fig. 1. The specific activities are markedly enhanced when the cells are transferred from glucose to oleate containing growth medium. This increase appears with all fatty acids tested, the highest level being obtained with hexanoic acid. However, low rates of activation do occur with octanoate, decanoate and tetradecanoate.

Table 2 compares results obtained between E. coli and C. butyricum homogenates. No activity is observed in C. butyricum, either with CoA or homogeneous $ACP_{butyricum}$.

In the case of E. coli, a complete dependence on

Table 1
Induction of the enzymes of fatty acid metabolism in E. coli.

Carbon source	Acyl-CoA synthetase (mμmoles/min/mg)	Thiolase (μmoles/min/mg)	Thiolesterase (mμmoles/min/mg)
Oleate I	0.88	0.66	43.0
Oleate II	0.65	0.015	18.5
Glucose	0.1	–	2
Glycerol	–	–	6.5
Succinate	–	–	7.7
Acetate	0.25	–	8.5
Rich medium (ref. 11)	–	–	–

Enzyme determinations were performed as described in section 2 of the text. Protein content per assay: Acyl-CoA synthetase: 15 γ; thiolase: 4 to 80 γ; thiolesterase: 20 γ.
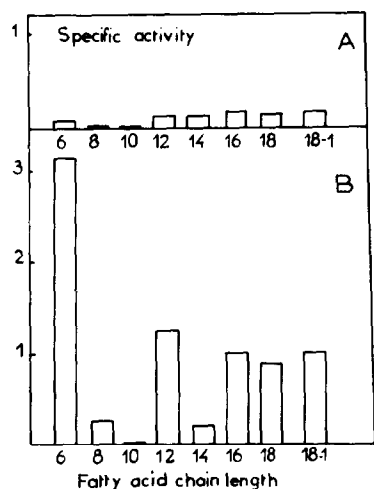
Fig. 1. Induction of fatty acid activating enzyme(s) according to chain length. Carbon sources: A: glucose; B: oleate. C_6 to C_{18}: straight chain saturated fatty acids, and C_{18-1}: 9,10-cis-octadecenoic acid. All fatty acids were present at 0.7 mM concentrations. Other conditions are described in section 2 of the text. 20 γ of proteins were used per assay. Specific activities are expressed in mμmoles acyl-CoA formed/min/mg of protein.

Table 2
Comparison of CoA and ACP in palmitate activation.

Incubation mixture	Palmityl-CoA formed (mμmoles/min/mg)	
	E. coli	C. butyricum *
Complete	1.0	0.128
Complete zero time control	0.1	0.194
Complete, boiled enzyme	0.16	0.135
Complete, ATP omitted	0.2	
Complete, CoA omitted	0.18	0.125
Complete, ACP$_{E.coli}$ (or ACP$_{C.butyricum}$) replacing CoA †	0	0

CoA and ACPs were present at 50 mM concentrations. 25 γ of proteins per assay.

* No thiolesterase activity could be detected in C. butyricum extracts.

† Non-enzymatic binding of palmitate on ACP was found to occur and to be independent of the presence of ATP. Therefore zero time control experiments (complete medium) are subtracted in these two assays.

CoA and ATP is observed. Pure ACP$_{coli}$ cannot replace CoA in this reaction. Similar results are obtained with a 60-fold purified acyl-CoA synthetase preparation *.

4. Discussion

The induction of fatty-acid activating enzyme(s) in *E. coli* occurs under the same conditions as the induction of the last enzyme involved in fatty acid oxidation. Therefore, acyl-CoA synthetase appears to be the initiating enzyme in this process. It is not yet possible to decide whether or not there are distinct fatty acid activating enzymes specific for fatty acids of different chain length, since the ratios of enzyme activities do not change during the acyl-CoA synthetase purification procedures.

The role of thiolesterase is not clear; maximum induction is obtained when the cells are grown on oleate. However, significant levels are present when cells are grown on other media, except for rich medium. It is conceivable that its induction is related to the levels of Krebs cycle intermediaries. We assumed that thiolesterase participates in the control of fatty acyl-CoA concentrations in the cells as suggested by Barnes and Wakil [12], since thiolesterase specific activity follows the pattern of acyl-CoA synthetase specific activity when net induction is observed with oleate.

It is clear from the results shown in table 2, that acyl-ACP cannot be formed directly via fatty acid activation. In the case of *C. butyricum* appropriate controls showed that 3% of ^{3}H-labeled oleic acid present in the medium is incorporated by the cells into complex lipids, whereas 7% remained as free fatty acids; it is not yet possible to decide whether or not CoA derivatives are synthetized in vivo in *C. butyricum*. However, we assumed that the absence of synthesis of acyl-CoA observed *in vitro* is related to their very low activity as acyl donor in glycerol-3-phosphate acylation. Therefore acyl-ACP as end product of fatty acid biosynthesis would be used preferentially in this latter process.

In *E. coli*, on the contrary, the cells seem capable of forming acyl-CoAs through fatty acid activation

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* D.Samuel and G.Ailhaud, unpublished experiments.

and acyl-ACPs via the fatty acid biosynthetic cycle; thus the enzymatic system involved in the esterification of glycerol-3-phosphate does not show any marked specificity for either derivatives.

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