MECHANISM OF PHOSPHOLIPID BIOSYNTHESIS IN ESCHERICHIA COLI: ACYL-COA SYNTHETASE IS NOT REQUIRED FOR THE INCORPORATION OF INTRACELLULAR FREE FATTY ACIDS INTO PHOSPHOLIPID

Tapas K. Ray and John E. Cronan, Jr.
Department of Molecular Biophysics and Biochemistry
Yale University School of Medicine
New Haven, Connecticut 06510

Received January 19,1976

Summary

Previous results have shown that acyl-CoA synthetase is required both for the incorporation of exogenous fatty acids into the phospholipids of E. coli and for the transport of fatty acids into the cell. We have demonstrated that acyl-CoA synthetase is not required for the incorporation of intracellular free fatty acids into phospholipid. This finding indicates that the role of this enzyme in the incorporation of exogenously supplied fatty acids is primarily at the level of fatty acid transport.

Introduction

Overath and coworkers (1) showed that fadD mutants of E. coli (which lack acyl-CoA synthetase activity) are unable to incorporate exogenously supplied fatty acids into phospholipid. This result has been widely interpreted as showing that exogenous fatty acids are transferred into phospholipid from acyl-CoA thioesters. Fatty acyl chains synthesized de novo are incorporated normally into phospholipid by fadD mutants (1). This finding is consistent with these acids being transferred directly from the acyl carrier protein (ACP) molecule on which they were synthesized. The notion that exogenous and endogenous fatty acids are incorporated into phospholipid from different thioesters has been invoked to explain various observations concerning the relA gene control of phospholipid synthesis (2) and various findings on phospholipid fatty acid composition (3,4). Phosphatidic acid synthesis as assayed in vitro proceeds with either acyl-CoA or acyl-ACP as acyl donor (2,5-7).

However, the interpretation of the original results of Overath et al. (1) has become less clear due to more recent studies from the same laboratory.

Klein and coworkers (8) reported that fadD$^-$ strains are unable to accumulate exogenous fatty acids. This result suggests that the failure of fadD$^-$ strains to incorporate fatty acids into phospholipid may be simply due to an inability to transport the acids into the cell. Therefore, the behavior of these strains may not give any information concerning the nature of the acyl intermediate through which exogenous acids are incorporated into phospholipid.

In this paper we show that fadD$^-$ strains incorporate intracellular free fatty acids into phospholipid normally. This result indicates that acyl-CoA synthetase functions in the incorporation of exogenous fatty acids primarily at the level of fatty acid transport.

Materials and Methods

Bacterial Strains. All strains are derivatives of E. coli K-12. Strain TR3 is an E. coli strain with lesions in the gpsA and fadD genes. K27 is a fadD$^-$ strain derived from strain Ymel by Overath and coworkers (1) who refer to the strain as old88. Strain BB20-14, isolated by Bell (9), is defective in the biosynthetic sn-glycerol 3-phosphate dehydrogenase, the defect owing to a lesion in the gpsA gene (10). Strain BB20-14 is also HfrC, phoA$^-$, and glpD$^-$. Strain TR2 (gpsA$^-$, thyA$^-$) was derived from the BB20-14 by treatment with trimethoprin (11).

Strain TR3 was constructed as follows: strain K27 (mel$^-$, fadD$^-$) was mated with the HfrC strain, X340 (proB$^-$, metB$^-$), according to the published procedure (10) and mel$^+$, pro$^+$ recombinants were selected. A strain which inherited metB$^-$ as well as mel$^+$ was mated with the male strain, TR2 (gpsA$^-$, glpD$^-$, thyA$^-$) and thy$^+$ met$^+$ recombinants were selected. A recombinant, called TR3, which inherited gpsA$^-$ and glpD$^-$ in addition to met$^+$, was purified and used in the physiological experiments.

Media and Growth Conditions. Cultures were grown at 37° in a standard medium (12) consisting of medium E supplemented with 0.4% sodium succinate and 0.05% casein hydrolysate (vitamin free from Nutritional Biochemicals). When required, rac-glycerol 3-phosphate was added to the standard medium to a final concentration of 0.02%. Starvation for glycerol 3-phosphate was achieved by a membrane filtration and washing as described by Cronan et al. (12). The removal of [^{14}C]-acetate from the media was accomplished by a similar procedure. After resuspension of the cells, non-radioactive acetate (50 mM) was added to the medium to dilute out any residual radioactive acetate.

Analysis of Radioactive Lipids. The incorporation of [1-^{14}C]-acetate into lipid was analysed as described previously (12). Free fatty acids were separated from phospholipids on thin layer chromatograms by a two step development procedure (12) and the appropriate areas of silica gel were counted in Aquasol scintillator. The fractions were identified by cochromatography of the authentic lipid standards.

Uptake of exogenous fatty acids. The uptake of radioactive fatty acids into growing cultures was assayed exactly as described by Klein et al. (8).

Estimation of total free fatty acids accumulated during starvation. TR3 cells were grown in 1 l of the standard medium containing 0.02% sn-glycerol 3-phosphate

Table I

Uptake of exogenous fatty acids by mutant and wild type strains

Strain	Fatty acid	Uptake of free fatty acids (nmoles/mg protein/hr)
Experiment I		
TR3	oleate	6.0
Ymel	oleate	80.0
K27*	oleate	7.0
Experiment II		
TR3	palmitate	8.0
Ymel	palmitate	83.5
K27	palmitate	10.0
Experiment III		
TR3	palmitate	4.5
TR3	oleate	1.4
TR3	oleate	2.8
Experiment IV		
Ymel	oleate	85.6
K27	oleate	4.5

*The culture of K27 used in the experiments presented in this paper and in the construction of strain TR3 was obtained from the Coli Genetic Stock Center, Yale University. Following the completion of this work, another sample of K27 was obtained directly from Dr. Overath. This isolate had no detectable acyl-CoA synthetase activity in agreement with the results of Overath et al. (1) and thus the culture of strain K27 we used seems to have been a partial revertant.

to about 2.5-3.0 x 10^8/ml. The cells were collected by centrifugation, washed once with medium E and resuspended in 1 l of the standard medium. The resuspended cells were then incubated with shaking at 37°. After growth stopped (90 min), the cells were incubated for another 60 min during which time fatty acids accumulated. The suspension was then centrifuged and the lipids were extracted from the packed cells according to the procedure of Ames (13). Free fatty acids were separated from phospholipid by thin layer chromatography and quantitatively estimated by microtitration with 0.005 N NaOH using cresol red

Table II

Acyl-CoA synthetase activity of mutant and wild type strains

The standard assay mixture (16) contained 380 mM Tris-HCl pH 7.4, 8 mM $MgCl_2$, 10 mM ATP, 0.5 mM dithiothreitol, 50 µM CoA SH, 30 µM [^{14}C] palmitic acid (specific activity 1000 cpm/nmole), 1 mg/ml Triton X-100 and 10 µg crude enzyme protein (15) in a final volume of 0.1 ml. The assays were run at 30° for 10 min. The reaction was stopped by adding 500 µg bovine serum albumin and 0.6 ml of 2% perchloric acid. The precipitate was washed 5-6 times with ether to remove unreacted fatty acid. The precipitate was finally dissolved in mild alkali and counted in Aquasol scintillator solution.

Strains	Acyl-CoA synthetase activity[a] U[b]/mg protein
TR3	0.31 ± 0.05
K27	0.45 ± 0.04
Ymel	4.50 ± 0.12

[a]The results are the averages of three experiments ± S.E.

[b]A unit of enzyme activity is 1 nmole of fatty acid incorporated per min.

indicator as described by Ray et al. (14). The recovery of fatty acid was monitored by an internal standard of radioactive oleic acid and was found to be >95%.

Results and Discussion

Characterization of strain TR3. Strain TR3 was unable to grow on oleate as the sole carbon source. However the strain grew well on other carbon sources such as acetate, succinate, or glucose (the strain is *glpD*$^-$ which precludes growth on glycerol or G3P[1]). Strain TR3 therefore has a growth phenotype similar to the original acyl-CoA synthetase mutants of Overath et al. (1).

Klein and coworkers (8) showed that acyl-CoA synthetase is required for fatty acid transport. We have, therefore, assayed strain TR3 and find it is defective in the transport of exogenously supplied fatty acids (Table I).

[1]Abbreviation used is G3P for *sn*-glycerol 3-phosphate.

Strain TR3 accumulates fatty acids (either palmitate or oleate) at <10% of the rate of the wild type strain (Ymel). Similar values were found when incorporation into either whole cells or into purified phospholipid was assayed. The rate of uptake we observed in the wild type strain agrees very well with that reported by Klein et al. (8). These in vivo results therefore suggest that strain TR3 is very deficient in acyl-CoA synthetase activity.

We have confirmed these in vivo results by direct assay of acyl-CoA synthetase activity in vitro (Table II). In good agreement with the whole cell data we find that strain TR3 has only about 7% of the normal level of acyl-CoA synthetase activity.

Metabolism of intracellular free fatty acids in strain TR3. Cronan and coworkers (12) showed that a $fadD^+$ strain defective in β-oxidation ($fadE^-$) accumulated intracellular free fatty acids when phospholipid synthesis was blocked by glycerol starvation of a glycerol auxotroph. Upon restoration of phospholipid synthesis (readdition of glycerol), a rapid incorporation of the free fatty acid into phospholipid was observed. This system therefore seemed ideal to study the role of acyl-CoA synthetase in the incorporation of intracellular free fatty acids into phospholipid.

Strain TR3 was starved for G3P and labeled with [^{14}C]-acetate during the time interval (1 hr) of free fatty acid accumulation. A direct determination of the amount of intracellular free fatty accumulated showed that 165 ± 18 nmoles (3 determinations) of free fatty acid (per mg of total cellular protein) had been accumulated. The [^{14}C]-acetate was then removed by filtration and the cells were resuspended in the presence of G3P (to allow the resumption of phospholipid synthesis) (Fig. 1). Following the addition of G3P to the starved cultures, the radioactivity in the free fatty acid fraction declined very rapidly and was transferred to the phospholipid fraction. Therefore, essentially all the free fatty acid was incorporated into phospholipid (Fig. 1). The rate of incorporation was very similar to that observed by Cronan et al. (12) in a β-oxidation negative strain which has normal acyl-CoA synthetase activity (8).

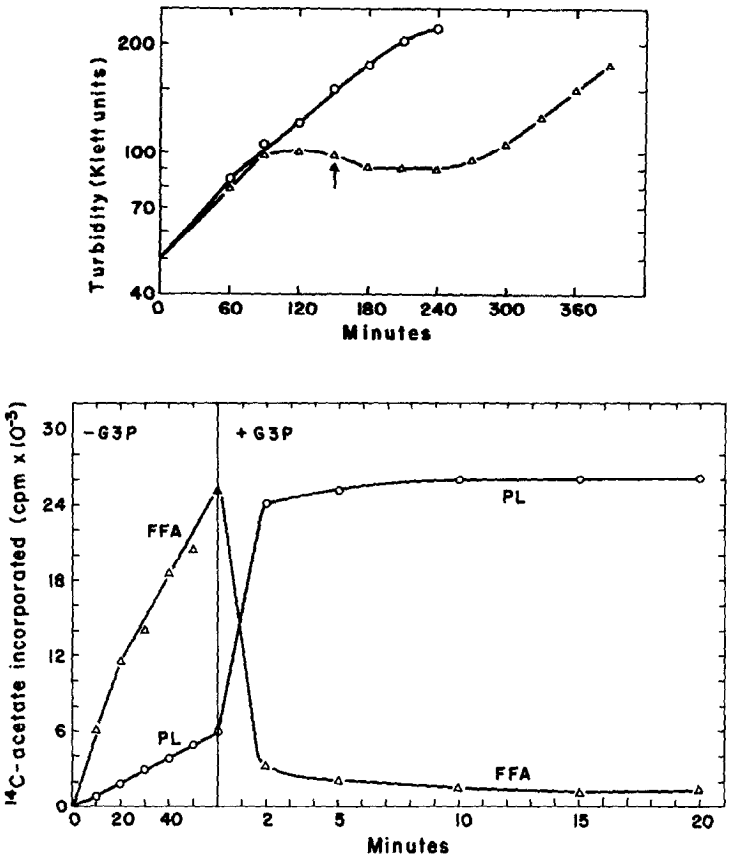

Figure 1. Strain TR3 was starved for G3P at t=0. After 10 min of starvation, the culture was supplemented with Na[1-^{14}C] acetate (10 μCi/ml, 45 mCi/mmole) and incubated for 60 min. The culture was then filtered to remove the [^{14}C]-acetate and the cells were resuspended in the standard medium supplemented with G3P and K acetate (50 mM) and incubated further. The upper panel shows the growth of strain TR3 during these maniuplations. The lower panel shows the radioactive lipid composition of the culture. The arrow in the upper panel and the veritcal line in the lower panel denote the time of addition of G3P.

From the data in Fig. 1, the rate of incorporation of intracellular fatty acids into phospholipid can be calculated to be 32 nmoles of fatty acid incorporated per min per mg of cellular protein. This rate is therefore 200 times greater than the rate that exogenous fatty acids are incorporated into phospholipid by the same strain and 20-fold faster than the rate of exogenous fatty acid incorporation by Ymel, the wild type strain (Table I). Therefore, the incorporation of intracellular free fatty acid into phospholipid is unaffected by the loss of >90% of the cellular acyl-CoA synthetase activity (Fig. 1),

whereas, the rate of incorporation of exogenously added fatty acids into phospholipid is directly dependent on acyl-CoA synthetase activity (Table I). These results allow several conclusions.

First, our results coupled with those of Klein et al. (8) indicate that acyl-CoA synthetase is required for the transport of exogenous fatty acids in E. coli. Once the fatty acids are located intracellularly, this enzyme is not required for incorporation of the acids into phospholipid (Fig. 1). It might be argued that the residual acyl-CoA synthetase activity remaining in the mutant might suffice for the incorporation of intracellular but not extracellular fatty acids. However, since the rate of the former process is 200-fold greater than the rate of the latter, this hypothesis seems untenable. Therefore, the original results of Overath et al. (1) cannot be cited as an indication that exogenously added fatty acids are donated to phospholipid as acyl-CoA thioesters.

Second, our results suggest that intracellular free fatty acids are not transferred as acyl-CoA thioesters either directly to lipid or to another intermediate. Therefore, we believe a renewed effort to detect an acyl-ACP synthetase activity in E. coli is warranted. Samuel and Ailhaud (15) reported unsuccessful attempts to find an enzymatic activity which activated palmitic acid with ACP. However, there are several reasons these workers may have missed observing this enzyme. For instance, E. coli has several thioesterase activities (17-19; Ray, Nunn and Cronan, unpublished) active on acyl-ACP which could have negated the assay through destruction of the product.

Acknowledgments

We thank Dr. Peter Overath for his kind gift of strain K27. This work was supported by NIH research grant AI10186 and Career Development Award GM 70,411.

References

1. Overath, P., G. Pauli and H.U. Schairer (1969) Eur. J. Biochem. 7, 554-574.
2. Leuking, D.R. and H. Goldfine (1975) J. Biol. Chem. 250, 4911-4917.
3. Silbert, D.F., F. Ruch and P.R. Vagelos (1968) J. Bacteriol. 95, 1658-1665.
4. Silbert, D.F. (1970) Biochemistry 9, 3631-3640.
5. Ray, T.K., J.E. Cronan, Jr., R.D. Mavis and P.R. Vagelos (1970) J. Biol. Chem. 245, 6442-6448.

6. van den Bosch, H. and P.R. Vagelos (1970) Biochim. Biophys. Acta 218, 233-248.
7. Ray, T.K. and J.E. Cronan, Jr. (1975) J. Biol. Chem. 250, 8422-8427.
8. Klein, K., R. Steinberg, B. Frithen and P. Overath (1971) Eur. J. Biochem. 19, 442-450.
9. Bell, R.M. (1973) J. Bacteriol. 117, 1065-1076.
10. Cronan, J.E., Jr. and R.M. Bell (1974) J. Bacteriol. 118, 598-605.
11. Dubnau, E. and W. Maas (1968) J. Bacteriol. 95, 531-539.
12. Cronan, J.E., Jr., L.J. Weisberg and R.G. Allen (1975) J. Biol. Chem. 250, 5835-5840.
13. Ames, G.F. (1968) J. Bacteriol. 95, 833-843.
14. Ray, T.K., V.P. Skipski, M. Barclay, E. Essner and F.M. Archibald (1969) J. Biol. Chem. 244, 5528-5536.
15. Samuel, D. and G. Ailhaud (1969) F.E.B.S. Letters 2, 213-216.
16. Polokoff, M.A. and R.M. Bell (1975) J. Lipid Res. 16, 397-402.
17. Barnes, E.M., Jr. and S.J. Wakil (1968) J. Biol. Chem. 243, 2955-2962.
18. Barnes, E.M., Jr., A.C. Swindell and S.J. Wakil (1970) J. Biol. Chem. 245, 3122-3128.
19. Bonner, W.M. and K. Bloch (1972) J. Biol. Chem. 247, 3123-3133.