ACYL-CoA HYDROLASE(S) IN RABBIT MAMMARY GLAND WHICH CONTROL THE CHAIN LENGTH OF FATTY ACIDS SYNTHESISED

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SUMMARY: A factor (Fraction IV) is present in the cytosol of lactating rabbit mammary gland which interacts directly with fatty acid synthetase to terminate chain elongation. This causes the release from the synthetase of unesterified medium-chain fatty acids ($C_{8:0}$-$C_{12:0}$) which are characteristic of rabbit milk. Fraction IV contains acyl-thioester hydrolase(s) which are active towards medium- as well as long-chain acyl-CoA esters. It is proposed that this activity could control chain termination by cleaving medium-chain acyl groups from the acyl carrier protein of fatty acid synthetase.

We have recently described (1) the partial purification from the cytosol of lactating rabbit mammary gland of a factor (Fraction IV) which contains protein and has a molecular weight of 40,000-50,000. It alters the chain length of the fatty acids synthesised by purified fatty acid synthetase from mainly $C_{14:0}$-$C_{16:0}$ to $C_{8:0}$-$C_{12:0}$ acids. These medium-chain acids are characteristic of rabbit milk.

This paper shows that Fraction IV contains an enzyme (or enzymes) which hydrolyses both medium- and long-chain acyl-CoA esters. It is postulated that this enzyme(s) is responsible for the synthesis of medium-chain fatty acids which occurs in the lactating gland *in vivo*.

MATERIALS AND METHODS

We have described the preparation from lactating rabbit mammary gland of (a) subcellular fractions, (b) Fraction IV by Sephadex G-100 chromatography of the cytosol, and (c) purified fatty acid synthetase (1). Acetyl-CoA carboxylase (EC 6.4.1.2) associated with the washed microsomal fraction was inhibited

by incubating with avidin in 50 mM Tris-HCl buffer pH 7.5 containing 0.5 M NaCl at 25°C for 15 min. Excess avidin was removed by washing the fraction with this buffer. Purified rabbit mammary gland acetyl-CoA carboxylase was a gift from Dr. R. Manning.

The system used to test the effects of Fraction IV on the pattern of fatty acids synthesised was as follows. Incubations (1.0 ml) contained 100 mM potassium phosphate buffer pH 7.2, 8 mM $MgCl_2$, 5 mM potassium citrate, 10 mM $KHCO_3$, 5 mM ATP, 0.24 mM NADPH, 1 mM EDTA, 5 mM rac-glycerol 3-phosphate, 50 μM [1-^{14}C]acetyl-CoA (4.4 μCi/μmole), and 0.12 mg purified fatty acid synthetase (50 nmole NADPH oxidised/min) and were for 15 min at 37°C. The additions to the incubation are shown in Table 1. Lipids were analysed as described previously (1).

The acyl-CoA hydrolase activities of Fraction IV (100 μg protein) and of purified fatty acid synthetase (18 μg protein; 614 nmole NADPH oxidised/min per mg protein) were assayed as follows. The [1-^{14}C]acyl-CoA esters were each used separately at 3 μM (0.5-2.1 nCi) i.e. below the critical micellar concentration of palmitoyl-CoA (2). They were incubated, in a volume of 0.25 ml, with 50 mM potassium phosphate buffer pH 7.4 for 30 sec (fatty acid synthetase) or for 1 min (Fraction IV) at 30°C. The reaction rates were uniform for these times. The reaction was stopped with 1.0 ml of Dole's reagent (3) and unreacted acyl-CoA was extracted using the method of Bar-Tana et al. (4) except that the lower phase was washed three times with 0.6 ml portions of n-heptane. Portions of the lower phase were used to determine the radioactivity of the remaining acyl-CoA. The syntheses of the [1-^{14}C] acyl-CoA esters of chain lengths $C_{4:0}$-$C_{16:0}$ were based on the method of Sanchez et al. (5).

RESULTS AND DISCUSSION

In the presence of Fraction IV, purified fatty acid synthetase synthesises medium-chain fatty acids from [1-^{14}C]acetyl-CoA (Table 1, expt. 1). The triglyceride fraction is enriched with these acids. In this experiment, rate-limiting amounts of malonyl-CoA are generated via the acetyl-CoA carboxylase associated with the microsomal fraction. Essentially similar results are obtained when rate-limiting amounts of purified acetyl-CoA carboxylase are used in the presence of avidin-treated microsomal protein (expt. 2). In expt. 2, the proportion of $C_{8:0}$-$C_{12:0}$ acids synthesised is

Table 1. The effects of Fraction IV on the pattern of fatty acids synthesised from [1-^{14}C]acetyl-CoA by fatty acid synthetase in the presence and absence of added malonyl-CoA and microsomes

Experiment	Lipid analysed *	Mole % fatty acids synthesised							Total nmole acetyl-CoA incorporated
		4:0	6:0	8:0	10:0	12:0	14:0	16:0	
1	TG	16	8	17	41	13	4	1	17
	TFA	35	12	16	26	8	2	1	27
2	TG	12	5	25	42	10	4	1	8
	TFA	38	8	19	24	7	3	1	21
3	TG	16	7	0	1	1	38	37	20
	TFA	29	6	0	0	1	28	36	45
4	TG	26	5	3	17	9	12	28	8
	TFA	50	4	2	8	4	11	21	17
5	TG	54	12	6	18	6	2	2	2
	TFA	69	10	5	10	4	2	0	5
6	TG	-	-	-	-	-	-	-	-
	TFA	60	7	7	16	6	4	1	21
7	TG	-	-	-	-	-	-	-	-
	TFA	34	5	5	25	14	13	4	29

* TG = triglyceride; TFA = total fatty acids

Additions were: Expt.1, microsomal protein (2.5 mg) and Fraction IV (0.34 mg protein); Expt.2, avidin-treated microsomal protein (0.42 mg), purified acetyl-CoA carboxylase (22 µg protein; 11.5 nmole malonyl-CoA formed/min) and Fraction IV (1.02 mg protein); Expt.3, as Expt.2 but omitting Fraction IV; Expt.4, avidin-treated microsomal protein (0.74 mg), 60 µM malonyl-CoA added at the start of the incubation, and Fraction IV (1.44 mg protein); Expt.5, avidin-treated microsomal protein (2.22 mg), 40 µM acetyl-CoA was used, and malonyl-CoA was added at a constant rate of 5 nmole/min throughout the 12 min incubation period, and Fraction IV (0.86 mg protein); Expt.6, as Expt.5 but omitting avidin-treated microsomal protein; Expt.7, as Expt.2 but omitting avidin-treated microsomal protein.

increased as the amount of Fraction IV is increased to 1.02 mg protein. In the absence of Fraction IV (expt. 3), no medium-chain acids are formed.

The optimum concentration of malonyl-CoA for the synthesis of long-chain fatty acids by fatty acid synthetase is 60 μM (6). Expt. 4 shows that there is a dramatic decrease in the proportion of medium-chain acids formed when 60 μM malonyl-CoA is added at the start of the reaction. However, when a low and constant concentration of malonyl-CoA is added over a 12 min period (expt. 5), medium-chain acids are formed. This also occurs in the absence of microsomal protein as shown in expts. 6 and 7 where rate-limiting amounts of malonyl-CoA are added at a constant rate or are generated from purified acetyl-CoA carboxylase. This latter result shows that Fraction IV exerts its effect even when the synthesised acids are not esterified into glycerides.

We conclude from these results that, in the presence of different rate-limiting amounts of malonyl-CoA, Fraction IV interacts directly with fatty acid synthetase to terminate chain elongation. This causes the release from the synthetase of unesterified medium-chain acids. The effect of Fraction IV is not dependent on the subsequent esterification of these fatty acids into glycerides. We therefore tested whether Fraction IV contains acyl-thioesterase activity. Acyl-CoA esters of varying chain lengths were used as model substrates to test the specificity of the hydrolase(s) of both Fraction IV and of purified fatty acid synthetase. The results obtained (Fig. 1a) show that the synthetase will specifically hydrolyse $C_{14:0}$ and $C_{16:0}$ acyl-CoA esters. This agrees with the results of Smith and Abraham (7) who used purified synthetase from lactating rat mammary gland.

By contrast, Fraction IV will hydrolyse medium- as well as long-chain acyl-CoA esters (Fig. 1b). Preliminary experiments indicate that Fraction IV is even more specific towards medium-chain acyl-CoA esters when higher substrate concentrations are used. Fraction IV might therefore contain acyl-thioester hydrolase activity which could specifically release medium-chain fatty acids from the acyl carrier protein of fatty acid synthetase. This

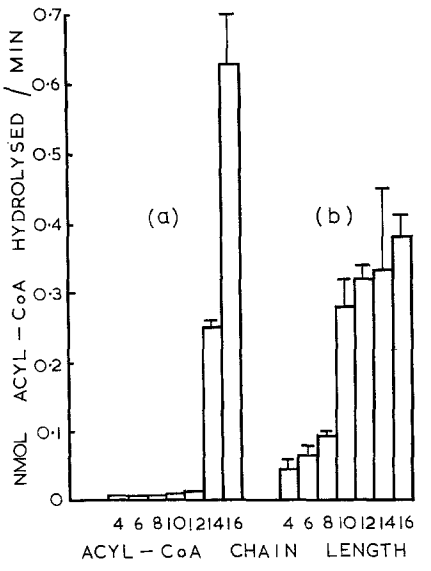

Fig. 1. Specificity towards acyl-CoA esters shown by (a) purified fatty acid synthetase, and (b) by Fraction IV. The assay conditions used are described in the Materials and Methods section. The bars show the limits of error in two experiments using fatty acid synthetase and in three experiments using Fraction IV.

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would decrease the amount of medium-chain acyl groups available for further elongation on the synthetase, and would explain the predominant synthesis of medium-chain acids by the lactating gland *in vivo* (8) and *in vitro* (9).

Long-chain fatty acyl-CoA hydrolases (EC 3.1.2.2) are widely distributed in mammalian tissues (10, 11). Though more active towards long-chain esters, they show a wide chain-length specificity. It is not known whether they act on acyl carrier protein derivatives, and there is no direct evidence for their role in the control of chain termination. E.coli contain two long-chain acyl-CoA hydrolases. One is inhibited by diisopropyl fluorophosphate; it is specific for $C_{12:0}$-$C_{18:0}$ acyl-CoA esters and also hydrolyses palmitoyl-acyl carrier protein (12). It has been implicated in chain termination leading to the formation of long-chain fatty acids. The second enzyme is not affected by the inhibitor and shows a wide chain-length specificity. However, it is more active towards long-chain acyl-CoA esters and again hydrolyses

palmitoyl-acyl carrier protein (13, 14). It has not been implicated in the control of chain termination.

The specificity shown by Fraction IV may be altered by further purification. This will show whether it contains a single enzyme with a broad specificity, or two acyl-thioesterases which are specific for medium- and for long-chain acyl-thioesters respectively. The relative activities of the hydrolase(s) towards acyl-CoA and acyl-acyl carrier protein esters of different chain lengths also needs investigating.

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REFERENCES

1. Knudsen, J., and Dils, R. (1975) Biochem. Biophys. Res. Comm. 63, 780-785.
2. Barden, R.E., and Cleland, W.W. (1969) J. Biol. Chem. 244, 3677-3684.
3. Dole, V.P. (1956) J. Clin. Invest. 35, 150-154.
4. Bar-Tana, J., Rose, G., and Shapiro, B. (1971) Biochem. J. 122, 353-362.
5. Sanchez, M., Nicholls, D.G., and Brindley, D.N. (1973) Biochem. J. 132, 697-706.
6. Carey, E.M., and Dils, R. (1970) Biochim. Biophys. Acta 210, 388-399.
7. Smith, S., and Abraham, S. (1971) J. Biol. Chem. 246, 2537-2542.
8. Carey, E.M., and Dils, R. (1972) Biochem. J. 126, 1005-1007.
9. Strong, C.R., and Dils, R. (1972) Comp. Biochem. Physiol. 43B, 643-652.
10. Kurooka, S., Hosoki, K., and Yoshimura, Y. (1972) J. Biochem. 71, 625-634.
11. Anderson, A.D., and Erwin, V.G. (1971) J. Neurochem. 18, 1179-1186.
12. Barnes, E.M., and Wakil, S.J. (1968) J. Biol. Chem. 243, 2955-2962.
13. Barnes, E.M., Swindell, A.C., and Wakil, S.J. (1970) J. Biol. Chem. 245, 3122-3128.
14. Bonner, W.M., and Bloch, K. (1972) J. Biol. Chem. 247, 3123-3133.