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# Transacylation as a chain-termination mechanism in fatty acid synthesis by mammalian fatty acid synthetase

## Synthesis of medium-chain-length ($C_8$–$C_{12}$) acyl-CoA esters by goat mammary-gland fatty acid synthetase

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1. Ruminant mammary-gland fatty acid synthetases can, in contrast with non-ruminant mammary enzymes, synthesize medium-chain fatty acids. 2. Medium-chain fatty acids are only synthesized in the presence of a fatty acid-removing system such as albumin, β-lactoglobulin or methylated cyclodextrin. 3. The short- and medium-chain fatty acids synthesized were released as acyl-CoA esters from the fatty acid synthetase.

Medium-chain fatty acid synthesis in rat and rabbit mammary glands is caused by the presence of a specific medium-chain terminating enzyme in the cytosol in these tissues (Knudsen *et al.*, 1976; Libertini & Smith, 1978). A similar enzyme is not present in goat mammary gland, although octanoic acid, decanoic acid and dodecanoic acid amount to 20mol% of the fatty acids synthesized in this tissue (Grunnet & Knudsen, 1979*a*). In contrast, goat mammary-gland fatty acid synthetase is by itself able to synthesize medium-chain fatty acids in the presence of the microsomal fraction and substrates for triacylglycerol synthesis (Grunnet & Knudsen, 1979*b*). Goat mammary-gland fatty acid synthetase exhibits both medium-chain thioesterase (Grunnet & Knudsen, 1978) and transacylase activity (Knudsen & Grunnet, 1980). We have also shown that medium-chain fatty acids synthesized *de novo* can be incorporated directly into triacylglycerol without the need of an activation step (Grunnet & Knudsen, 1981).

The present paper investigates the nature of the medium-chain product and the effect of fatty acid and acyl-CoA binding factors on medium-chain fatty acid synthesis by goat mammary-gland fatty acid synthetase.

### Materials and methods

#### *Materials*

NADPH, dithiothreitol, β-lactoglobulin, α-cyclodextrin and bovine serum albumin (fraction V, fatty acid-poor) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. CoA was obtained from Boehringer-Mannheim, Mannheim, Germany. [1-$^{14}$C]Acetyl-CoA was synthesized as described by Stadtman (1957) and malonyl-CoA was synthesized by the method of Eggere & Lynen (1962). α-Cyclodextrin was methylated with dimethyl sulphate in a 1:1 mixture of BaO and $Ba(OH)_2$ to afford 2,6-di-*O*-methyl-α-cyclodextrin (Kuhn & Trischmann, 1963; Casu *et al.*, 1968).

#### *Methods*

Lactating goats, 2–3 weeks *post partum*, were used. Subcellular fractionation of mammary gland and purification of fatty acid synthetase were carried out as described by Knudsen (1972). Fatty acid synthetase was incubated in 0.1 M-potassium phosphate buffer, pH 7.0, which contained 40 μM-[1-$^{14}$C]acetyl-CoA, 1 mM-EDTA and 240 μM-NADPH. Malonyl-CoA was infused continuously during the incubation by using a model 971 Harvard infusion pump fitted with eight 1.0ml syringes. The infusion tube was fixed near the bottom of the incubation vessel, which was shaken in a rotary motion in a Buchler Evapomix at 37°C.

#### *Incorporation of [1-$^{14}$C]acetate into non-esterified and esterified fatty acids*

NaOH (5 M) (1 ml/ml of incubation) was added to half of each incubation to stop the reaction. Total fatty acid synthesis was measured, and the pattern of fatty acids synthesized was determined by radio-g.l.c. (Knudsen *et al.*, 1981).

The other half of the incubation was stopped by addition of 80 μl of 15 M-$H_2SO_4$, 600 μg of each saturated even-numbered $C_4$–$C_{18}$ fatty acid was added as a carrier and the mixture was extracted three times with 2ml of diethyl ether. Both the pooled diethyl ether extract (non-esterified fatty



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acids) and the aqueous phase (esterified fatty acids) were analysed for [1-$^{14}$C]acetate incorporation into individual fatty acids as described above.

*Incorporation of [1-$^{14}$C]acetate into acyl-CoA esters and protein-bound fatty acids*

Incubations to be analysed for [1-$^{14}$C]acetate incorporation into acyl-CoA esters and protein-bound fatty acids were stopped by addition of 50 $\mu$l of 70% $HClO_4$. The non-esterified fatty acids were extracted with diethyl ether and analysed by radio-g.l.c. as described above. Acyl-CoA carriers, 10–30 nmol of the saturated even-numbered acyl-CoA esters ($C_4$–$C_{16}$) were added to the diethyl ether-extracted perchloric acid-treated incubations and pH was adjusted to 7.0 with 5 M-KOH. The precipitated proteins and potassium perchlorate were removed by centrifugation, washed twice with 0.5 ml of water and analysed for total fatty acids as described above. The washings were combined with the neutralized supernatant and the mixture was analysed for [1-$^{14}$C]acetate incorporation into individual acyl-CoA esters by high-pressure liquid chromatography as described below. The recovery of [1-$^{14}$C]decanoyl-CoA taken through the extraction procedure was 92%.

*Reverse-phase high-pressure liquid chromatography of $C_4$–$C_{16}$ acyl-CoA esters*

Acyl-CoA esters ($C_4$–$C_{16}$) were separated on a stainless-steel column (25 cm × 0.41 cm) packed with Lichrosorp RP18 (5 $\mu$M). The eluent consisted of a mixture of 45 mM-tetrabutylammonium phosphate, pH 5.5, and methanol and the flow was 1.5 ml/min. The elution was initiated with 63% (v/v) methanol for 15 min, followed by 68% (v/v) methanol for 3 min, and finally a gradient (0.4%/min) to 80% (v/v) methanol. The elution was continued with this concentration for 30 min. The eluate from the column was collected in 1.5 ml fractions and the radioactivity was determined. The total amount of acyl-CoA esters synthesized was calculated from the amount found in the individual peaks.

*Protein determination*

Proteins were precipitated with 15% (w/v) trichloroacetic acid and measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

## Results

Increasing the amount of albumin added to fatty acid synthetase from lactating-goat mammary gland gradually increased the proportion of $C_{10:0}$ fatty acid synthesized (Table 1). With 5.2 mg of albumin/ml of incubation, the proportion of $C_{10:0}$ fatty acid synthesized was greater than that of $C_{12:0}$ fatty acids. With 10.4 mg of albumin/ml present, the proportion of $C_{10:0}$ fatty acid synthesized was four times greater than that of $C_{12:0}$ fatty acid.

Rate-limiting concentrations of malonyl-CoA had to be used to obtain these effects by high concentrations of albumin. The addition of all of the malonyl-CoA at the start of the incubation strongly suppressed the effect of albumin on $C_{10:0}$ fatty acid synthesis (results not shown).

The observed effect of high malonyl-CoA concentrations is in full accordance with the model proposed by Sumper *et al.* (1969) for chain

Table 1. *The effect of albumin and β-lactoglobulin on medium-chain fatty acid synthesis by lactating goat mammary-gland fatty acid synthetase*

The incubation system is described in the Materials and methods section. The incubation contained 100 $\mu$g of fatty acid synthetase (specific activity 980 nmol of NADPH oxidized/min per mg of protein) and 40 $\mu$M-[1-$^{14}$C]acetyl-CoA (sp. radioactivity 2.6 mCi/mol), in a total volume of 0.5 ml. Albumin or β-lactoglobulin were added as shown. Malonyl-CoA was infused at a rate of 2 nmol/min. Incubation time was 15 min. The values for the radioactivity distribution in fatty acids are means of two determinations. The values for total amount of fatty acids synthesized are means ± half the difference between duplicates.

| Protein added (mg) | Distribution of radioactivity in fatty acids (%) | | | | | | | Total acetate incorporated from [1-$^{14}$C]acetyl-CoA (nmol) |
|---|---|---|---|---|---|---|---|---|
| | $C_{4:0}$ | $C_{6:0}$ | $C_{8:0}$ | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | |
| Albumin | | | | | | | | |
| 0 | 35 | 7 | — | 2 | 3 | 34 | 19 | 4.1 ± 0.1 |
| 0.52 | 33 | 6 | 2 | 1 | 14 | 29 | 15 | 4.4 ± 0.3 |
| 1.04 | 25 | 8 | 1 | 9 | 14 | 28 | 15 | 5.7 ± 0.2 |
| 2.6 | 28 | 6 | 2 | 15 | 11 | 24 | 14 | 5.4 ± 0.5 |
| 5.2 | 43 | 12 | 3 | 21 | 5 | 8 | 8 | 4.6 ± 0.5 |
| β-Lactoglobulin | | | | | | | | |
| 2.5 | 37 | 5 | — | 11 | 12 | 25 | 10 | 4.9 ± 0.4 |
| 5.0 | 36 | 7 | 1 | 14 | 9 | 24 | 9 | 4.9 ± 0.3 |

elongation and termination by fatty acid synthetase. High concentrations of malonyl-CoA favour, according to this model, long-chain fatty acid synthesis.

β-Lactoglobulin, a specific milk protein present in ruminant milk, has, like albumin, a high binding affinity for fatty acids. β-Lactoglobulin also increased the synthesis of medium-chain fatty acids by goat mammary synthetase when incubated with rate-limiting amounts of malonyl-CoA (Table 1). About four times more β-lactoglobulin than albumin was required to obtain the same effect on $C_{10:0}$ fatty acid synthesis. This difference closely resembles the binding affinities of these two proteins for long-chain fatty acids (Spector *et al.*, 1969; Spector & Fletcher, 1970).

When the same amount of albumin (10.5 mg/ml) was added to fatty acid synthetases from lactating-rat, rabbit, guinea-pig or mouse mammary gland, there was no corresponding increase in the proportion of medium-chain fatty acids synthesized (Table 2). Hence the effect is only observed with ruminant mammary synthetase.

In an attempt to investigate the nature of products from the fatty acid synthetase, it became clear that it was impossible to recover small amounts of either non-esterified fatty acids or acyl-CoA esters initially bound to defatted bovine serum albumin, even though large amounts of cold carrier were used. To overcome this problem we tested other known acyl-CoA- and fatty-acid-binding compounds. Cyclodextrins like 2,6-di-*O*-methyl-α-cyclodextrin

Table 2. *The effect of albumin on medium-chain fatty acid synthesis by lactating sheep, cow, goat, rabbit, rat, mouse and guinea-pig mammary-gland fatty acid synthetases*

The incubation conditions were as described in Table 1. The specific activities (nmol of NADPH oxidized/min per mg of protein) of the fatty acid synthetases were: sheep, 1231; goat, 980; cow, 443; rabbit, 993; rat, 1347; mouse, 2892; and guinea-pig, 311. Albumin was added as indicated. Incubation time was 15 min. The values for the radioactivity distribution in fatty acids synthesized are means of two determinations. The values for the total amount of fatty acids are means ± half the difference between duplicates.

| Source of lactating-mammary-gland fatty acid synthetase | Presence of albumin (5.2 mg) | Distribution of radioactivity in fatty acids (%) | | | | | | | Total acetate incorporated from [1-$^{14}$C]acetyl-CoA (nmol) |
|---|---|---|---|---|---|---|---|---|---|
| | | $C_{4:0}$ | $C_{6:0}$ | $C_{8:0}$ | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | |
| Sheep | – | 37 | 2 | — | — | 6 | 40 | 15 | 3.8 ± 0.2 |
| | + | 42 | 10 | 2 | 17 | 6 | 17 | 6 | 4.3 ± 0.2 |
| Cow | – | 34 | 2 | 1 | 3 | — | 38 | 22 | 4.9 ± 0.1 |
| | + | 23 | 7 | 5 | 13 | 14 | 23 | 15 | 5.8 ± 0.1 |
| Goat | – | 26 | 3 | — | 3 | 6 | 38 | 24 | 5.0 ± 0.1 |
| | + | 26 | 8 | 6 | 23 | 8 | 17 | 12 | 6.3 ± 0.1 |
| Rabbit | – | 43 | — | — | — | — | 37 | 20 | 3.9 ± 0.3 |
| | + | 39 | 9 | — | — | — | 36 | 16 | 4.2 ± 0.3 |
| Rat | – | 24 | 6 | — | 2 | 11 | 44 | 13 | 4.4 ± 0.1 |
| | + | 21 | 5 | 3 | 4 | 5 | 43 | 19 | 7.5 ± 0.1 |
| Mouse | – | 37 | 8 | — | — | — | 22 | 33 | 4.3 ± 0.0 |
| | + | 34 | 5 | — | — | — | 22 | 39 | 5.1 ± 0.1 |
| Guinea-pig | – | 33 | 8 | — | — | — | 30 | 29 | 4.9 ± 0.1 |
| | + | 41 | 9 | 1 | 1 | — | 26 | 22 | 5.6 ± 0.2 |

Table 3. *Effect of 2,6-di-O-methyl-α-cyclodextrin on the composition of fatty acids synthesized by fatty acid synthetase from lactating goat mammary gland*

The incubation system is described in the Materials and methods section. The incubations contained fatty acid synthetase (232 μg; specific activity 902 nmol of NADPH oxidized/min per mg of protein), and 2,6-di-*O*-methyl-α-cyclodextrin as indicated in a volume of 1.0 ml. Malonyl-CoA was infused at the rate of 1.75 nmol/min. Incubation time was 15 min. The values for the distribution of radioactivity in fatty acids are means of two determinations. Values for total fatty acids synthesized are means ± half the difference between duplicates.

| 2,6-Di-*O*-methyl-α-cyclodextrin added (mg) | Distribution of radioactivity in fatty acids (%) | | | | | | | Total acetate incorporated from [1-$^{14}$C]acetyl-CoA (nmol) |
|---|---|---|---|---|---|---|---|---|
| | $C_{4:0}$ | $C_{6:0}$ | $C_{8:0}$ | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | |
| 0 | 35 | 12 | 4 | 5 | 11 | 28 | 5 | 3.55 ± 0.05 |
| 1.17 | 30 | 9 | 8 | 20 | 13 | 14 | 6 | 4.48 ± 0.06 |
| 2.34 | 33 | 12 | 13 | 24 | 7 | 7 | 4 | 4.66 ± 0.12 |
| 4.68 | 32 | 15 | 16 | 25 | 5 | 5 | 2 | 4.10 ± 0.01 |

Table 4. *Products synthesized by goat mammary-gland fatty acid synthetase in the presence of 2,6-di-O-methyl-α-cyclodextrin*

The incubation conditions were as described in Table 3. The amount of added 2,6-di-*O*-methyl-α-cyclodextrin was 2.34 mg/ml. Incubation time was 15 min. Values for nmol of fatty acid synthesized are means of two determinations. Values for acetate incorporated are means ± half the difference between duplicates.

| Product | Fatty acids synthesized (nmol) | | | | | | | Total acetate incorporated from [1-$^{14}$C]acyl-CoA (nmol) |
|---|---|---|---|---|---|---|---|---|
| | $C_{4:0}$ | $C_{6:0}$ | $C_{8:0}$ | $C_{10:0}$ | $C_{12:0}$ | $C_{14:0}$ | $C_{16:0}$ | |
| (I) Total fatty acids | 1.04 | 0.50 | 0.43 | 0.86 | 0.21 | 0.38 | 0.16 | 3.58 ± 0.02 |
| Esterified fatty acids | 0.96 | 0.39 | 0.34 | 0.85 | 0.10 | — | — | 2.64 ± 0.02 |
| Non-esterified fatty acids | 0.11 | 0.09 | 0.08 | 0.05 | 0.12 | 0.38 | 0.13 | 0.96 ± 0.04 |
| (II) Total fatty acids | 0.99 | 0.51 | 0.42 | 0.82 | 0.15 | 0.40 | 0.19 | 3.48 ± 0.15 |
| Fatty acids in $HClO_4$-precipitated material | 0.35 | 0.13 | 0.15 | 0.26 | — | — | — | 0.89 ± 0.13 |
| Acyl-CoA esters | 0.63 | 0.16 | 0.17 | 0.36 | 0.04 | — | — | 1.36 ± 0.12 |
| Non-esterified fatty acids | 0.12 | 0.07 | 0.03 | — | 0.09 | 0.49 | 0.21 | 1.01 ± 0.07 |

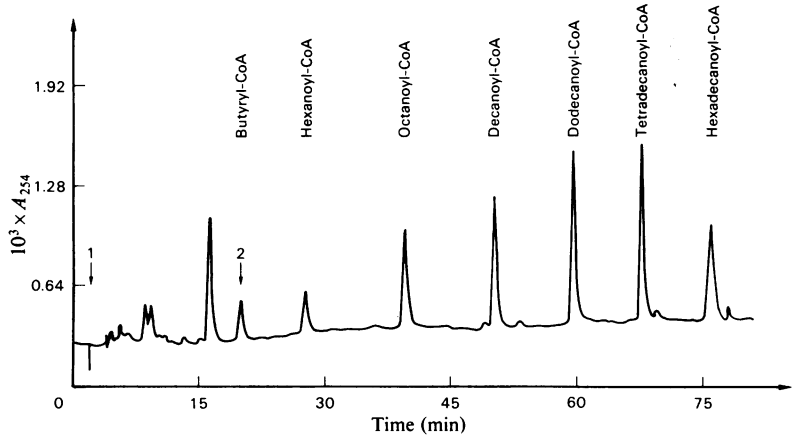


Fig. 1. *Separation of acyl-CoA esters by reverse-phase high-pressure liquid chromatography*

Details of separation conditions are given in the Materials and methods section. Injections and the start of gradient are indicated with arrows 1 and 2 respectively.

have been shown to stimulate fatty acid synthesis in *Mycobacterium smegmatis* by complexing with the acyl-CoA esters synthesized by the fatty acid synthetase from this organism (Machida *et al.*, 1973).

Addition of 2,6-di-*O*-methyl-α-cyclodextrin to goat mammary-gland fatty acid synthetase resulted in a similar change in the pattern of fatty acids synthesized to that observed with bovine serum albumin (Table 3). Furthermore it was shown that non-esterified fatty acids could be completely recovered from solutions containing 2,6-di-*O*-methyl-α-cyclodextrin, and that this compound did not interfere with the separation of acyl-CoA esters on the reverse-phase liquid-chromatography column (results not shown).

When 2,6-di-*O*-methyl-α-cyclodextrin was added to the incubation, almost all the short- and medium-chain fatty acids synthesized *de novo* were recovered in the esterified fraction (Table 4).

In the presence of 2.34 mg of methylated cyclodextrin, about 81% of the medium-chain ($C_8$, $C_{10}$ and $C_{12}$) product was esterified, whereas all the long-chain products were released as non-esterified fatty acids (Table 4, I). In the esterified fraction, about 40% was bound in the precipitated protein fraction. The remaining fatty acids were present as acyl-CoA esters (Table 4, II). The results in Tables 3 and 4 are typical of at least three independent experiments. A typical chromatogram for separation of acyl-CoA esters is shown in Fig. 1.

## Discussion

The present results show that ruminant mammary fatty acid synthetases, in contrast with

non-ruminant enzymes, are able to terminate fatty acid synthesis at medium-chain length ($C_8$–$C_{12}$). This ability to synthesize medium-chain fatty acids is only expressed in the presence of an acyl-CoA ester-removing system such as bovine serum albumin, β-lactoglobulin and 2,6-di-*O*-methyl-α-cyclodextrin. The medium-chain fatty acids synthesized in the presence of 2,6-di-*O*-methyl-α-cyclodextrin were released as acyl-CoA esters, which shows that the termination mechanism involved is a transacylation. The high proportion of protein-bound medium-chain fatty acids in the product probably represents enzyme-bound intermediates. The fatty acid synthetase concentration was 0.47 μM, and the amount of protein-bound product synthesized was 0.89 nmol, which indicates that 2 mol of intermediate are bound/mol of fatty acid synthetase. The small amounts of non-esterified fatty acids synthesized are most probably caused by the low medium-chain acylthioesterase activity inherent in the goat mammary synthetase (Grunnet & Knudsen, 1978).

The enzyme activities in the goat mammary-gland fatty acid synthetase, responsible for the termination at medium chain length, are unknown. We have previously shown that the loading transacylase in both cow and goat mammary-gland fatty acid synthetase exhibits high activity towards medium-chain acyl-CoA esters (Knudsen & Grunnet, 1980). We therefore suggest that this enzyme is also involved in the termination of medium-chain fatty acid synthesis by analogy with the malonyl- and palmitoyl-transacylase in yeast fatty acid synthetase (Engeser *et al.*, 1979). However, the final solution of this problem will have to await identification of the amino acid sequence around the active sites for the two enzyme activities.

A similar change in the pattern of fatty acids synthesized, which is facilitated by the removal of acyl-CoA esters of specific chain length from the fatty acid synthetase, has been shown for *Mycobacterium smegmatis*. In this system a polysaccharide facilitates the removal of inhibitory products from the fatty acid synthetase complex, and thereby stimulates fatty acid synthesis *de novo*. This results in an increased synthesis of $C_{24}$ and $C_{26}$ fatty acyl-CoA esters compared with that of palmitoyl-CoA (Wood *et al.*, 1977).

We have recently shown that fatty acids synthesized *de novo* by goat mammary synthetase can be directly incorporated into triacylglycerol without the involvement of an intermediate activation step. The present results indicate that the mechanism involves two steps: (1) the production of an acyl-CoA ester attached to the synthetase; and (2) the utilization of the CoA ester by the microsomal fraction. It is not known whether the mechanism also involves a specific medium-chain acyl-CoA ester binding factor. The presence of such a factor exclusively in mammary gland could explain the synthesis of these acids in mammary gland only. However, this point will have to await further clarification. The close link between fatty acid and triacylglycerol synthesis, where the synthesis of a specific fatty acid is dependent on its incorporation into triacylglycerols, may explain the constant proportion of short- and medium-chain fatty acids in milk fat (Morrison, 1970).

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