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Evidence that the medium-chain acyltransferase of lactating-goat mammary-gland fatty acid synthetase is identical with the acetyl/malonyltransferase

Jan MIKKELSEN,* Peter HØJRUP,† Hans F. HANSEN,‡ Jan Krogh HANSEN* and Jens KNUDSEN*
**Institute of Biochemistry and †Institute of Molecular Biology, University of Odense, DK-5230 Odense M, Denmark, and ‡Laboratory of Molecular Endocrinology 6321, University of Copenhagen Department of Clinical Chemistry, Rigshospitalet, DK-2200 Copenhagen N, Denmark*

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Competitive binding experiments with malonyl-CoA and [1-^{14}C]acetyl-CoA, [1-^{14}C]butyryl-CoA or [1-^{14}C]decanoyl-CoA indicate that all these substrates are transferred to lactating-goat mammary-gland fatty acid synthetase by the same transferase. Isolation and determination of the amino acid sequence of [1-^{14}C]decanoyl-labelled CNBr-cleavage peptide from the decanoyltransferase site showed that this transferase is identical with the acetyl/malonyltransferase.

The reaction mechanisms involved in the individual reactions catalysed by different fatty acid synthetases are in principle similar, i.e. they are independent of the different structural organization of the synthetase complex in plants, bacteria, yeast and vertebrates (Bloch & Vance, 1977). However, there appear to be differences in the loading and terminating reactions.

With the yeast fatty acid synthetase the terminating reaction involves a transfer of the long-chain acyl group from the enzyme to CoA to form acyl-CoA products, and there are also separate transferases for loading acetyl and malonyl groups on to the enzyme (Bloch & Vance, 1977). The terminating transferase of this enzyme has been shown to be identical with the malonyl-loading transferase (Engeser *et al.*, 1979).

With fatty acid synthetase isolated from mammalian tissues the terminating reaction can be either a thioester hydrolase or an acyltransferase reaction. Short-chain (C_4 and C_6) fatty acid synthesis is terminated by a transferase reaction, probably the acetyl/malonyl-loading transferase (Hansen & Knudsen, 1980). Medium-chain fatty acid synthesis in mammary gland of non-ruminants is terminated by a separate specific thioester hydrolase (Kudsen *et al.*, 1976; Libertini & Smith, 1978). Medium-chain fatty acid synthesis in ruminant mammary gland is terminated by a transferase reaction of the fatty acid synthetase, forming acyl-CoA products (Knudsen & Grunnet, 1982).

The formation of an acyl *O*-ester intermediate at the active site of the loading transferase has been shown to be the initial step in the catalysis of fatty acid synthesis by mammalian fatty acid synthetase (McCarthy & Hardie, 1982).

Kinetic evidence and sequence determination of the peptides isolated from the active site of the acetyl/malonyltransferase from both ruminant and non-ruminant mammary-gland synthetases strongly indicate that these two substrates are transferred to the enzyme by the same transferase in these fatty acid synthetases (Stern *et al.*, 1982; McCarthy *et al.*, 1984; Mikkelsen *et al.*, 1985). However, mammalian non-ruminant fatty acid synthetases can only effectively use acetyl-CoA and butyryl-CoA as primers (Bloch & Vance, 1977; Knudsen & Grunnet, 1980). By contrast, ruminant mammary-gland synthetase is able to use efficiently acyl-CoA esters with up to 12 carbon atoms in the acyl chain as primers (Knudsen & Grunnet, 1980).

In the present paper we investigate whether the medium-chain fatty acyl-loading/terminating transferase activity of lactating-goat mammary-gland fatty acid synthetase is catalysed by the acetyl/malonyl-loading transferase or a separate transferase.

Materials and methods

Materials

[1-^{14}C]Acetyl-CoA was synthesized enzymically as described by Hansen *et al.* (1984), [2-^{14}C]-malonyl-CoA was synthesized as described by Rutkovski & Jaworski (1978), and [1-^{14}C]butyryl-CoA and [1-^{14}C]decanoyl-CoA were synthesized as described by Marshal & Knudsen (1977). [2-

^{14}C]Malonate and ^{14}C-labelled fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Mops and dithiothreitol were from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals were of analytical grade.

Methods

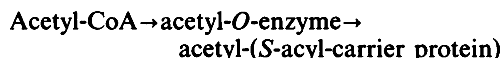
Isolation, purification and sequencing of [1-^{14}C]decanoate-labelled CNBr-cleavage peptide. Goat mammary-gland fatty acid synthetase was labelled, reduced and carboxyamidomethylated and cleaved with CNBr as described for the purification of the acetyl/malonyltransferase active-site CNBr-cleavage peptide (Mikkelsen *et al.*, 1985). [1-^{14}C]Decanoate-labelled peptides containing the label bound through an *O*-ester were identified in the eluates from the reverse-phase h.p.l.c. column by stability towards performic acid oxidation. A portion (100μl) of the fractions that contained radioactivity was evaporated to dryness and incubated for 4h with 200μl of formic acid/35% (v/v) H_2O_2 (9:1, v/v) that had been preincubated for 1h at room temperature. Water (0.5ml) was added and the mixture was extracted with three 2.0ml portions of diethyl ether to remove [1-^{14}C]decanoic acid. The mixture was evaporated to dryness and the dry residue was dissolved in 200μl of formic acid. The dissolved residue was mixed with 4ml of Hydrocount and the radioactivity was determined by liquid-scintillation counting.

Determination of substrate binding to fatty acid synthetase. Fatty acid synthetase (25–30mg/ml) stored in 0.25M-potassium phosphate buffer, pH7.0, containing 1.0mM-EDTA and 1.0mM-dithiothreitol was preincubated at 37°C for 30min before use. Portions of this solution (350μg of fatty acid synthetase) were then diluted with ice-cold 100mM-Mops/NaOH buffer, pH7.0, containing 1.0mM-EDTA, to 325μl. [1-^{14}C]Acetyl-CoA (specific radioactivity 15.2Ci/mol), [1-^{14}C]butyryl-CoA (specific radioactivity 7.5Ci/mol) or [1-^{14}C]decanoyl-CoA (specific radioactivity 18.2Ci/mol) was added in 25μl to give a final concentration of 100μM, together with malonyl-CoA at the concentrations indicated. The sample was mixed by shaking for 1–2s, and two 50μl samples were quickly removed for determination of performic acid-stable binding (i.e. *O*-ester binding). Each sample was mixed with 200μl of formic acid/35% (v/v) H_2O_2 (9:1, v/v) that had been preincubated at 0°C for 1h. The reaction mixture with substrate-loaded enzyme was incubated at 0°C for 4h. With incubations that contained [1-^{14}C]acetyl-CoA, [1-^{14}C]butyryl-CoA and [2-^{14}C]malonyl-CoA the labelled enzyme was precipitated with 2.5ml of ice-cold 10% (w/v) trichloroacetic acid. With incubations that contained [1-^{14}C]decanoyl-CoA water (0.5ml) was added and the mixture was extracted with three 2.0ml portions of diethyl ether to remove free [1-^{14}C]decanoic acid. The sample was evaporated to dryness, and the dry residue was redissolved in 200μl of formic acid and precipitated with 2.5ml of ice-cold 10% (w/v) trichloroacetic acid. The precipitated enzyme was collected on a Millipore RAWP 1.2μm-pore filter and washed three times with 2.5ml of ice-cold 10% trichloroacetic acid. The filter was air-dried and transferred to a liquid-scintillation vial with 10ml of toluene/ethylene glycol monoethyl ether (2:1, v/v) containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene. The counting efficiency was determined to be 73% with fatty acid synthetase carboxyamidomethylated with iodo[1-^{14}C]acetamide. Control incubations contained samples treated the same way as described above except that trichloroacetic acid was added before the substrates.

The extent of disappearance of thioester was monitored in a parallel incubation by measuring the extent of oxidation of [2-^{14}C]malonyl-CoA by h.p.l.c. as described previously (Hansen *et al.*, 1984).

Results and discussion

McCarthy & Hardie (1982) have shown that the sequence of substrate binding (acetyl-CoA) to mammary-gland fatty acid synthetase is as follows:


Acetyl-CoA→acetyl-*O*-enzyme→ acetyl-(*S*-acyl-carrier protein)

These authors also provided evidence that the acetyl-*O*-enzyme represents the acetyltransferase site. Furthermore it has been shown that acetate and malonate are transferred from their respective CoA esters to rabbit and goat mammary-gland fatty acid synthetases by the same transferase (McCarthy *et al.*, 1984; Mikkelsen *et al.*, 1985).

The aim of the present experiment was to investigate whether decanoyltransferase and acetyl/malonyltransferase are identical in goat mammary-gland fatty acid synthetase. We therefore investigated only competitive binding to the enzyme of substrates as *O*-esters.

The result from competitive binding experiments shows that malonyl-CoA at a concentration of 400μM (i.e. 4-fold above the concentration of [1-^{14}C]acetyl-CoA, [1-^{14}C]butyryl-CoA and [1-^{14}C]decanoyl-CoA) almost completely inhibits the binding of all three primers (Fig. 1). At equal concentrations (100μM) of substrate and competitor, substrate binding is decreased by 78–86%, indicating that goat mammary-gland fatty acid synthetase acyltransferase binds malonate from malonyl-CoA with a higher affinity than shown by

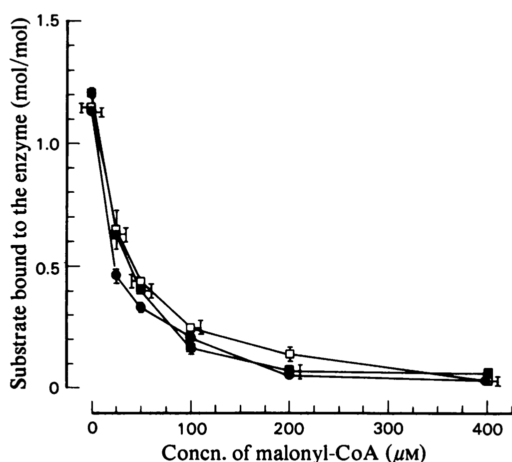


Fig. 1. *Performic acid-stable binding of [1-^{14}C]acetate, [1-^{14}C]butyrate and [1-^{14}C]decanoate to goat mammary-gland fatty acid synthetase*

Fatty acid synthetase (350μg; specific activity 1490nmol of NADPH oxidized/min per mg) was incubated for 1–2s at 0°C in 0.35ml of 100mM-Mops/NaOH buffer, pH7.0, containing 1.0mM-EDTA and 100μM-[1-^{14}C]acetyl-CoA (●) or -[1-^{14}C]butyryl-CoA (□) or -[1-^{14}C]decanoyl-CoA (■) in the presence of increasing concentrations of malonyl-CoA. For experimental details see the Materials and methods section. The values are means of duplicates, the bars represent ±half the difference between duplicate incubations. The results are typical for incubations with three different preparations of fatty acid synthetase.

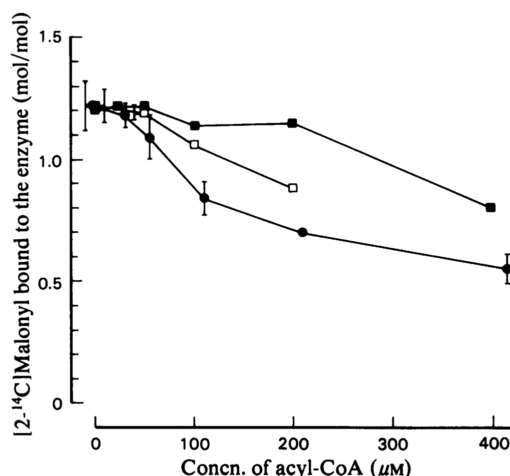


Fig. 2. *Performic acid-stable binding of [2-^{14}C]malonate to goat mammary-gland fatty acid synthetase*

Fatty acid synthetase (350μg; specific activity 1490nmol of NADPH oxidized/min per mg) was incubated for 1s at 0°C in 0.35ml of 100mM-Mops/NaOH buffer, pH7.0, containing 1.0mM-EDTA and 100μM-[1-^{14}C]malonyl-CoA in the presence of increasing concentrations of acetyl-CoA (●), butyryl-CoA (□) or decanoyl-CoA (■). For experimental details see the Materials and methods section. The values are means of duplicates, bars show ±half the difference between duplicate incubations. The results are typical for incubations with at least three different preparations of fatty acid synthetase.

the three primers tested. Similar differences in relative binding affinities were obtained in experiments where fatty acid synthetase was incubated with 100μM-[2-^{14}C]malonyl-CoA in the presence of various concentrations of unlabelled acetyl-CoA, butyryl-CoA and decanoyl-CoA. At equal concentrations of [2-^{14}C]malonyl-CoA and the competing unlabelled primers, the [2-^{14}C]malonate binding was decreased by only 1%, 13% and 31% in the presence of decanoyl-CoA, butyryl-CoA and acetyl-CoA respectively (Fig. 2).

The affinity of the loading transferase for different substrates has not previously been studied directly. Indirect studies with chicken liver fatty acid synthetase show that malonyl-CoA is the preferred substrate (Soulie *et al.*, 1984). Measurements of K_m values for acetyl-CoA and malonyl-CoA of lactating-rabbit, -guinea-pig and -cow mammary-gland fatty acid synthetases all show that the K_m for malonyl-CoA is higher than that for acetyl-CoA (Carey & Dils, 1970; Strong & Dils, 1972; Miatra & Kumar, 1974). Only lactating-rat mammary-gland fatty acid synthetase has a K_m value for malonyl-CoA lower than that for acetyl-CoA (Smith & Abraham, 1970). However, K_m values represent the affinity of the enzyme for a substrate in the overall process of fatty acid synthesis, which includes condensation, reduction and termination reactions, whereas the present results represent affinity for the transferase site only.

The results in Fig. 1 strongly indicate that decanoate is bound to the same transferase active site as are acetate, butyrate and malonate and that the decanoyltransferase is therefore identical with the acetyl/malonyltransferase.

To obtain further evidence for the identity between the decanoyltransferase and the malonyl/acetyltransferase, the fatty acid synthetase was labelled with [1-^{14}C]decanoate by incubation with [1-^{14}C]decanoyl-CoA. The labelled enzyme was reduced, carboxyamidomethylated and cleaved with CNBr. The CNBr-cleavage peptides labelled with [1-^{14}C]decanoate were separated by reverse-phase h.p.l.c. The transferase active-site peptides that contained [1-^{14}C]decanoate bound through an *O*-ester were identified by their stability towards performic acid oxidation.

The CNBr-cleavage reaction mixture was found to contain only one peptide in which the [1-^{14}C]decanoate was bound through an *O*-ester. This

Peptide	Active-site sequence	Reference
[1-^{14}C]Decanoyl-labelled	(Met)-Gly-Leu-Arg-Pro-Asp^{5}-Gly-Ile-Ile-Gly-^{10} ? -SER-Leu-Gly	Present paper
[1-^{14}C]Acetyl/ [2-^{14}C]malonyl-labelled	(Met)-Gly-Leu-Arg-Pro-Asp^{5}-Gly-Ile-Ile-Gly-His^{10}-SER-Leu-Gly	Mikkelsen *et al.* (1985)

Fig. 3. *Amino acid sequence of [1-^{14}C]decanoyl- and [1-^{14}C]acetyl-labelled CNBr-cleavage peptide from the decanoyl- and acetyl/malonyl-transferase active site of goat mammary-gland fatty acid synthtase*

The serine residue to which the acyl group is attached is shown in capital letters. The amino acid residues in parentheses are only tentatively identified.

peptide was purified to homogenity as described previously for the acetyl/malonyl-active-site CNBr-cleavage peptide. Throughout the whole purification procedure the [1-^{14}C]decanoate-labelled CNBr-cleavage peptide behaved similarly to the [1-^{14}C]acetate/[2-^{14}C]malonate-labelled CNBr-cleavage peptide from the acetyl/malonyl-transferase active site. The only difference observed was that the [1-^{14}C]decanoate-labelled peptide was eluted at a higher concentration of propan-2-ol during reverse-phase h.p.l.c.

The amino acid sequence of the leading part of the [1-^{14}C]decanoate-labelled CNBr-cleavage peptide (Fig. 3) was identical with the leading sequence of CNBr-cleavage peptide from the acetyl/malonyltransferase active site reported by Mikkelsen *et al.* (1985). Amino acid residue 10 was not identified owing to low recovery of the thiohydantoin derivative of the amino acid in this position. The same phenomenon was observed during sequencing of the CNBr-cleavage peptide from the acetyl/malonyltransferase active site. The amount of peptide isolated in the present experiment did not allow subdegradation and identification of this amino acid. However, the complete identity of amino acid residues 1–9 and 11–13 shows without doubt that the two peptides are identical.

The amino acid to which [1-^{14}C]decanoate was bound was identified as serine in position 11 by measuring the release of ^{14}C radioactivity in this step during sequencing. The serine at position 11 was identified as the phenylthiohydantoin derivative of serine and not as the derivative of *O*-decanoylserine. This indicates that some of the [1-^{14}C]decanoate on the serine in position 11 is lost during Edman degradation.

The results in Fig. 3 confirm the results obtained in the competitive binding studies shown in Figs. 1 and 2, and further support the conclusion that the decanoyltransferase is identical with the acetyl/malonyltransferase in goat mammary-gland fatty acid synthetase. The results presented also show that goat mammary-gland fatty acid synthetase contains only one serine decanoyltransferase.

The same transferase is therefore involved in both malonyl- and acetyl-loading and in the termination of fatty acid synthesis at medium-chain length in goat mammary-gland acid synthetase. Yeast fatty acid synthetase also terminates fatty acid synthesis by a transferase reaction resulting in synthesis of acyl-CoA esters, mainly palmitoyl-CoA. However, in this enzyme complex two transferases are involved in substrate loading and termination. Only one of the transferases loads acetate, and a different transferase loads malonate and unloads palmitate (Engeser *et al.*, 1979). The amino acid sequences around the active site of the acetyl-malonyltransferase of rabbit mammary-gland synthetase, the acetyl/decanoyl/malonyl-transferase of goat mammary-gland fatty acid synthetase and the malonyl/palmitoyltransferase of yeast synthetase are identical (Engeser *et al.*, 1979; McCarthy *et al.*, 1984; Mikkelsen *et al.*, 1985). This sequence is therefore only likely to be of importance for catalytic activity, and the sequence determining the transferase specificity must be located at some distance from the domain of the active site.

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