Amino acid sequence around the active-site serine residue in the acyltransferase domain of goat mammary fatty acid synthetase

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Goat mammary fatty acid synthetase was labelled in the acyltransferase domain by formation of *O*-ester intermediates by incubation with [1-¹⁴C]acetyl-CoA and [2-¹⁴C]malonyl-CoA. Tryptic-digest and CNBr-cleavage peptides were isolated and purified by high-performance reverse-phase and ion-exchange liquid chromatography. The sequences of the malonyl- and acetyl-labelled peptides were shown to be identical. The results confirm the hypothesis that both acetyl and malonyl groups are transferred to the mammalian fatty acid synthetase complex by the same transferase. The sequence is compared with those of other fatty acid synthetase transferases.

The reaction mechanisms catalysed by different fatty acid synthetases are in principle similar, regardless of the different structural organization of the synthetase complex in plants, bacteria, yeast and vertebrates (Bloch & Vance, 1977). However, differences appear in the loading and terminating reactions.

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

In mammals the fatty acid synthetase terminating reaction can be either a thioester hydrolase or an acyltransferase reaction. Short-chain (C₄ and C₆) fatty acid synthesis is terminated by a transferase reaction, probably the acetyl/malonyl-loading transferase (Hansen & Knudsen, 1980). Medium-chain fatty acid synthesis in non-ruminant mammary gland is terminated by a separate specific thioester hydrolase (Knudsen *et al.*, 1976; Libertini & Smith, 1978). Medium-chain fatty acid synthesis in ruminants is terminated by a transferase reaction forming acyl-CoA products (Knudsen & Grunnet, 1982).

Kinetic evidence and sequence determination of short peptides from the active site of the malonyl- and acetyl-transferase from mammary-gland synthetase strongly indicate that these two substrates are transferred to the enzyme by the same trans-

ferase in mammalian fatty acid synthetase (Stern *et al.*, 1982; McCarthy *et al.*, 1984). However, mammalian non-ruminant fatty acid synthetases can only effectively use acetyl-CoA and butyryl-CoA as primers (Bloch & Vance, 1977; Knudsen & Grunnet, 1980). In contrast, ruminant mammary synthetase is able to efficiently use acyl-CoA with up to C₁₂ chain as primer (Knudsen & Grunnet, 1980).

These observations indicate that the ruminant enzyme contains a different loading transferase or an additional medium-chain transferase capable of both loading and unloading.

The present paper reports the isolation and sequencing of CNBr-cleavage fragments of [1-¹⁴C]acetyl- and [2-¹⁴C]malonyl-labelled goat mammary-gland fatty acid synthetase.

Materials and methods

Materials

[1-¹⁴C]Acetyl-CoA was synthesized enzymically as described by Hansen *et al.* (1984), and [2-¹⁴C]malonyl-CoA was synthesized as described by Rutkovski & Jaworski (1978). [1-¹⁴C]Acetate and [2-¹⁴C]malonate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Propan-2-ol (Chrom A.R. grade) was from Mallinckrodt, Paris, KY, U.S.A. Mops and dithiothreitol were from Sigma Chemical Co., St., Louis, MO, U.S.A. Sephadex G-25 and FPLC Mono S cation-exchange resin were from Pharmacia Fine Chemi-

cals, Uppsala, Sweden. Lichrosorb RP18 was either synthesized as described by Berendsen (1978, 1980) or from E. Merck, Darmstadt, West Germany. Wydac RP18 (30nm pore size) was from the Separation Group, Hesperia, CA, U.S.A. All other chemicals were of analytical grade from E. Merck. Trypsin for subdigestion was a gift from Novo, Bagsvaerd, Denmark.

Purification of fatty acid synthetase

Fatty acid synthetase was isolated from goat mammary gland essentially as described by Knudsen (1972), except that the calcium phosphate-gel-absorption step was omitted and that 2% (v/v) cow serum was added to the homogenization buffer. The enzyme was stored (20–30mg/ml) in 0.2M-potassium phosphate buffer, pH7.0, containing 10mM-dithiothreitol, 1.0mM-EDTA and 10% (v/v) glycerol at -80°C .

Labelling of fatty acid synthetase

Fatty acid synthetase that had been stored in 0.25M-potassium phosphate buffer, pH7.0, containing 10mM-dithiothreitol, 1.0mM-EDTA and 10% glycerol was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (240g/l) and redissolved to a concentration of more than 10mg/ml in 100mM-Mops/NaOH buffer, pH7.0, containing 1.0mM-dithiothreitol and 10% glycerol and dialysed against the same buffer at room temperature.

The dialysed synthetase was diluted to 5mg/ml with ice-cold 100mM-Mops buffer, pH7.0, containing 1.0mM-EDTA and cooled to 0°C on ice. The $[1-^{14}\text{C}]$ acetyl-CoA (specific radioactivity 4.0–15.0Ci/mol) or $[2-^{14}\text{C}]$ malonyl-CoA (specific radioactivity 3.5–15.5Ci/mol) was added to a final concentration of 200 μM , and the sample was mixed by shaking for 1s. Two 20 μl portions were removed as quickly as possible for determination of total binding, and the reaction was stopped immediately by addition, per ml of incubation mixture, of 2ml of 0.45M-Tris/HCl buffer, pH8.3, containing 14.5M-urea and solid urea to make the final concentration of urea 8M.

Reduction, carboxymethylation and CNBr cleavage

The substrate-labelled enzyme was reduced under N_2 with either mercaptoethanol (Chestfield *et al.*, 1963) or tributylphosphine (Rüegg & Rudinger, 1977). The reduced enzyme was carboxymethylated by incubation with iodoacetamide for 90min under N_2 .

The added amount of iodoacetamide was adjusted to be equal to the calculated amount of thiol groups or thiol groups plus tributylphosphine in the incubation.

The reduced and carboxymethylated enzyme was diluted with 3vol. of water and precipitated

with an equal volume of ice-cold 50% (w/v) trichloroacetic acid. The precipitate was washed with 10% trichloroacetic acid and suspended in 70% (v/v) formic acid (1.0ml/5mg of enzyme). Then 5mol of tryptophan/mol of methionine in the enzyme and 1mg of CNBr/mg of enzyme were added. The tube was flushed with N_2 , closed and incubated in the dark with vigorous shaking for 16h at room temperature.

The advantage of using tributylphosphine over mercaptoethanol for the reduction was that it prevented loss of thioester-bound substrates, which was extensive when mercaptoethanol was used for reduction.

Digestion of reduced and carboxymethylated fatty acid synthetase and purified CNBr-cleavage peptide with trypsin

The trichloroacetic acid-precipitated enzyme was washed with 0.1M-ammonium acetate buffer, pH7.5, and suspended in the same buffer (4mg/ml) containing 1.0mM- CaCl_2 and 0.2mg of 1-chloro-4-phenyl-3-tosylamidobutan-2-one ('TPCK')-treated trypsin/ml. The digestion was carried out on a shaking bath at 37°C for 48h with one additional addition of trypsin after 24h.

Pure $[1-^{14}\text{C}]$ acetyl-labelled CNBr-cleavage peptide (15nmol) was incubated with 9 μg of trypsin in 200 μl of 0.1M- NH_4HCO_3 buffer, pH8.3, containing 1.0mM- CaCl_2 at 37°C for 4h with continuous agitation.

Purification of CNBr-cleavage fragment

The CNBr-treated solution was centrifuged and injected (3ml portions) directly on a stainless-steel high-performance chromatography column (25cm $\times$ 0.6cm) packed with Lichrosorb RP18 (10 μm particles). The column was equilibrated with 30% buffer B (0.1% trifluoroacetic acid in propan-2-ol) and 70% buffer A (0.1% trifluoroacetic acid in water) with a flow rate of 1ml/min. The fragments were eluted by running the column isocratically for 15min and then with a gradient of buffer B from 30% to 80% with an increment of 0.8%/min. The absorbance of the eluate was monitored continuously at 220nm, and radioactivity was determined in 50 μl portions of 1.5ml fractions by liquid-scintillation counting.

The fractions from the performic acid-stable radioactivity-containing peaks were pooled individually and freeze-dried. The freeze-dried residue was dissolved in 0.25M-sodium formate buffer, pH3.8, containing 8M-urea and 10% (v/v) propan-2-ol and loaded on a Pharmacia FPLC Mono S cation-exchange column equilibrated with the same buffer. The adsorbed peptides were eluted by running the column for 20min isocratically with 96% loading buffer and 4% 1.0M-LiCl in loading

buffer (buffer B) followed by increasing buffer B at 1.0%/min to 24% buffer B and then at 2%/min to 65% buffer B.

Peptide content and radioactivity in the eluate were monitored as described above.

The fractions from the radioactivity-containing peaks were pooled, and the urea was removed on a short (5 cm $\times$ 0.4 cm) Lichrosorb RP18 column. Up to 10 ml of the pooled fractions from the radioactive peak was loaded in 2 ml portions on to the column equilibrated with 20% 0.1% trifluoroacetic acid in propan-2-ol (buffer B) and 80% 0.1% propan-2-ol in water (buffer A). The peptides were eluted with a gradient of 1.0% buffer B/min to 60% buffer B.

The fractions from the radioactivity-containing peak from the above short RP18 column were freeze-dried and redissolved in 0.1% trifluoroacetic acid in 20% propan-2-ol in water and further purified on a Wydac RP18 (30 nm pore size) column (12 cm $\times$ 0.8 cm) by using the same gradient as described above for the short RP18 column to 46% buffer B.

Determination of stability towards performic acid oxidation

Peptides labelled with [1- 14 C]acetate through a serine ester bond were distinguished from thioesters through their stability to performic acid oxidation. A 100 μ l portion of the pooled fractions from a radioactive peak was evaporated to dryness and incubated in 200 μ l of formic acid solution containing 10% (v/v) of 35% (v/v) H₂O₂ overnight at 0°C. For [1- 14 C]acetyl-labelled peptides, released [1- 14 C]acetate was removed by addition of 500 μ l of acetic acid and evaporated to dryness. The dry residue was dissolved in 200 μ l of formic acid and added to 4 ml of Hydrocount and its radioactivity counted by liquid scintillation.

For [2- 14 C]malonyl-labelled peptides the performic acid reaction mixture was evaporated to dryness, and the dry residue was dissolved in 10 mM-Tris/HCl buffer, pH 8.5, and loaded on a cellulose thin-layer plate (Merck 5552). The plate was developed in butanol/acetic acid/water (5:3:2, by vol.) and scanned for radioactivity on a Radioactive TLC scanner.

Purification of tryptic-digest peptides

The reaction mixture from the tryptic digestion of the acetyl-labelled enzyme was run on a Sephadex G-25 column (2.5 cm $\times$ 90 cm) equilibrated with 0.1 M-ammonium acetate buffer, pH 7.5. The peptides were eluted with a flow rate of 25 ml/min, and the eluate was collected in 10 ml fractions. The radioactivity in the performic acid-stable peak was pooled and freeze-dried.

The freeze-dried residue was dissolved in 25 mM-

ammonium acetate buffer, pH 6.6, and loaded on a Lichrosorb RP18 (10 μ m particles) column (25 cm $\times$ 0.4 cm) equilibrated with 8% (v/v) ethanol in 25 mM-ammonium acetate buffer, pH 6.6. The peptides were eluted with gradient (0.2%/min) of 20% ammonium acetate buffer in ethanol. The radioactivity-containing peak was freeze-dried and rechromatographed on the same column equilibrated with 0.1% trifluoroacetic acid in 8% propan-2-ol in water and eluted with a gradient (0.2%/min) of 0.1% trifluoroacetic acid in propan-2-ol.

The reaction mixture from the tryptic digest of the [1- 14 C]acetyl-labelled CNBr-cleavage peptide was loaded directly on a Nucleosil RP18 (10 μ m particle size) column (25 cm $\times$ 0.4 cm) equilibrated with 0.1% trifluoroacetic acid containing 5% propan-2-ol. The peptides were eluted with a gradient (1.5%/min) of propan-2-ol containing 0.1% trifluoroacetic acid.

Mass spectrometry

The purified [1- 14 C]acetyl-labelled tryptic peptide was deuterioacetylated and *NO*-permethylated as described previously (Roepstorff, 1978). Electron-impact mass spectra were obtained on a Finnigan MAT 311 A mass spectrometer by direct introduction of the sample. The electron energy was 70 eV and the resolution 1000.

Amino acid sequence determination

One automatic sequence determination was carried out on a Beckman 890 C sequencer with a 0.25 M-Quadrol programme as described in Petersen *et al.* (1980).

The other automatic sequence determinations were performed on an Applied Biosystems 470 A protein sequencer with chemical and programme (MHTFAI) as recommended by the manufacturer.

Amino acid phenylthiohydantoin derivatives were identified on a reverse-phase h.p.l.c. system, with a Spherisorb 5 ODs 2 column and an ethanol gradient system on a Hewlett-Packard 1084 B liquid chromatograph (Sottrup-Jensen *et al.*, 1980).

Determination of amino acid composition

The peptides were hydrolysed at 110°C for 24, 48 and 72 h in 6 M-HCl containing 0.1% (v/v) phenol in evacuated glass ampoules. The amino acid analysis was carried out on a Beckman 121 B analyser.

The recovery of serine was calculated by extrapolation to time zero, and for isoleucine the values for 72 h hydrolysis were used.

The amount of [1- 14 C]acetyl-labelled CNBr-cleavage peptide hydrolysed was calculated from the radioactivity content of the peptide and the specific radioactivity of [1- 14 C]acetyl-CoA used

for labelling. The recovery of amino acids was 90%, based on the amount of peptide hydrolysed.

Results and discussion

The small tryptic-digest peptide isolated containing the ^{14}C -labelled acetyl group was found to have a blocked *N*-terminal, as neither the dansyl chloride reaction nor automated spinning-cup sequencing yielded any result.

The mass spectra showed that the peptide fraction contained three peptides. By fractional vaporization of the sample (Roepstorff, 1978), the spectrum of the major component was obtained. The *N*-terminal amino acid was *N*-acetylserine, as demonstrated by peaks at m/z 126, 130 and 158. This indicates that the *N*-terminus was already acetylated before the formation of derivatives, in which reactions trideuteroacetyl groups were introduced. The following partial sequence could be identified from the mass spectrum: acetyl-Ser-Leu-Gly-Glu-Val, based on peaks at m/z 158, 285, 356, 513 and 626 respectively. The occurrence of an *N*-terminal *N*-acetylserine is indicative of the serine being the active *O*-acetylserine, which during

tryptic cleavage adjacent to the serine has made an *O*- to *N*-acyl shift (Josefsson, 1963).

In order to isolate a larger peptide containing the active site, acetyl-labelled fatty acid synthetase was degraded with CNBr, and the resulting ^{14}C -labelled peptides were purified as described in the Materials and methods section.

The results from purification of acetyl-loaded performic acid-stable CNBr-cleavage peptide are shown in Figs. 1, 2, 3 and 4. The results show the advantage of combining different reverse-phase columns and ion-exchange columns for purification of CNBr-cleavage peptides. The results also show that a short reverse-phase column can be used successfully for concentrating dilute peptide samples and simultaneously remove a denaturant such as urea. $[2\text{-}^{14}\text{C}]$ Malonyl-labelled peptides behaved similarly to $[1\text{-}^{14}\text{C}]$ acetyl-labelled peptides through the whole purification procedure.

Automated spinning-cup sequencing resulted in the sequence shown in Fig. 5.

The yield of amino acid phenylthiohydantoin derivatives fell drastically between step 9 and 10. The reason for this decrease in yield is not known, but it might be due to an *O*- to *N*-acyl shift of the acetyl group from *O*-acetylserine in position 11 to

Fig. 1. Separation of CNBr-cleavage fragments of $[1\text{-}^{14}\text{C}]$ acetyl-labelled goat mammary fatty acid synthetase by reverse-phase h.p.l.c.

Buffer A was 0.1% trifluoroacetic acid in water, and buffer B was 0.1% trifluoroacetic acid in propan-2-ol. The column (0.6 cm $\times$ 25 cm) was packed with Lichrosorb RP18 (10 μm particles) and eluted at a flow rate of 1.0 ml/min. The performic acid stable fractions are marked with an arrow. For further details see the Materials and methods section. —, A_{220} ; histogram, radioactivity; —, concn. of buffer B in gradient.

even more pronounced with the malonyl-labelled peptide.

The serine in position 11 was for acetyl-labelled peptide and malonyl-labelled peptide identified as the phenylthiohydantoin derivative of normal serine and not as that of D-acylserine. Furthermore, no radioactivity was released in this step. This indicates that the *O*- to *N*-acyl shift is quantitative. The sequencing is continued on unlabelled peptide, which is present either due to copurification of labelled and unlabelled peptide or due to loss of the *O*-acyl group during the Edman degradation.

Table 1. Amino acid composition of the [$1\text{-}^{14}\text{C}$]acetyl-labelled CNBr-cleavage peptide and the leading sequence of this peptide obtained from a tryptic digest of the [$1\text{-}^{14}\text{C}$]acetyl-labelled CNBr-cleavage peptide

For experimental details see the Materials and methods section.

Amino acid	Amino acid composition (mol of residue/mol)	
	CNBr-cleavage peptide (estimated)	Leading tryptic peptide
Asp	4	1.0
Thr	—	0.1
Ser	4	0.1
Glu	9	0.1
Pro	4	1.0
Gly	10	2.9
Ala	10	<0.1
Val	2	<0.1
Cys	5	—
Met	—	—
Ile	8	1.9
Leu	4	1.0
Tyr	3	<0.1
Phe	—	<0.1
Lys	1	—
His	2	0.9
Arg	3	0.9
No. of residues	69	10

On the background of the above result it can be concluded that the acetyl- and malonyl-transferase sites are most probably identical in goat mammary fatty acid synthetase.

A comparison of the sequence found with published sequences of the acetyltransferase and the malonyl/palmitoyltransferase of yeast and the acetyl/malonyltransferase from rabbit fatty acid synthetase shows that the sequences close around the active site are very similar (Table 2).

Although the available sequences from previous work shown in Table 2 are too short to allow a conclusion about evolutionary or mechanistic relationship, some interesting similarities are apparent.

The goat mammary-gland acetyl/malonyltransferase sequence is identical with the rabbit acetyl/malonyltransferase sequence for the first five amino acid residues after the serine, and the yeast malonyl/palmitoyltransferase only differs from them in one amino acid residue.

The similarities between the yeast malonyl/palmitoyltransferase and the rabbit acetyl/malonyltransferase are interesting because the yeast enzyme catalyses palmitoyl transfer whereas the rabbit enzyme is only able to transfer short acyl chains ($\text{C}_4\text{--}\text{C}_6$) (Knudsen & Grunnet, 1980).

This indicates that the similarity in the transferases close to the active site is essential for the catalytic functions and that specificity of the enzyme resides somewhere else in the polypeptide chain. If this is true, caution should be taken in using short sequences around the active site for predicting evolutionary relationships.

The goat mammary-gland fatty acid synthetase is also able to transfer medium-chain ($\text{C}_4\text{--}\text{C}_6$) from CoA to the enzyme (Knudsen & Grunnet, 1980). It is not as yet known if this activity is catalysed by the malonyl/acetyltransferase.

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Table 2. Comparison of the amino acid sequences around the active-site residue of some fatty acid synthetase transferases. The serine residue at which the acyl-enzyme intermediate forms is shown in capital letters. Identical residues are boxed. Abbreviations: FAS, fatty acid synthetase. The amino acid in parenthesis is only tentatively identified.

Transferase	Active-site sequence	Reference
Yeast FAS malonyl/palmitoyltransferase	Ala-Gly-His-SER-Leu-Gly-Glu-Tyr-Ala-Ala	Engeser <i>et al.</i> (1979)
Yeast FAS acetyltransferase	Thr-Gly-His-SER-Gln-Gly-Leu-Val-Thr-Ala	From cDNA sequences (E. Schweizer, personal communication)
Rabbit FAS malonyl/acetyltransferase	SER-Leu-Gly-glu-Val-Ala	McCarthy <i>et al.</i> (1984)
Goat FAS malonyl/acetyltransferase	Ile-Gly-His-SER-Leu-Gly-Glu-Val-Ala-(Arg)	Present paper

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