Rat mammary-gland fatty acid synthase

A simple purification procedure and stoichiometry of CoA ester binding

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A simple procedure was devised which allows purification of rat lactating-mammary-gland fatty acid synthase to a high degree of purity, with recoveries of activity exceeding 50%. Over 50mg of enzyme was isolated from 60g of mammary tissue. The specific activity of the purified enzyme was about 2.5 μ mol of NADPH oxidized/min per mg of protein at 37°C. The enzyme appeared homogeneous by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and by immunodiffusion analysis. Each mol (M_r 480000) of the enzyme bound 3 mol of acetyl and 3–4 mol of malonyl groups when the binding experiments were performed at 0°C for 30 s. The presence of NADPH did not influence the binding stoichiometry for these acyl-CoA derivatives. Approx. 2 mol of taurine was found per mol of the performic acid-oxidized enzyme, suggesting that there were 2 mol of 4'-phosphopantetheine in the native enzyme. Rat mammary-gland fatty acid synthase required free CoA for activity.

Fatty acid synthases have been purified from various sources, including bacterial (Wood *et al.*, 1978; Kawaguchi & Okuda, 1977), yeast (Lynen, 1969) and animal (Smith & Abraham, 1970; Stoops *et al.*, 1975; Nepokroeff *et al.*, 1975; Muesing & Porter, 1975; Dils & Carey, 1975; Paskin & Mayer, 1976; Buckner & Kolattukudy, 1976; Hardie & Cohen, 1978; Strom *et al.*, 1979). In order to obtain purified fatty acid synthase, most purification schemes employ complex methods such as ion-exchange chromatography with gradient elutions, adsorption and elution from calcium phosphate gels etc., with poor recoveries and variable elution profiles (Smith & Abraham, 1975). Buckner & Kolattukudy (1976) devised a simple method for the purification of fatty acid synthase from the uropygial gland of goose, an organ whose major function is synthesis of lipids. However, this method was not applicable to the purification of synthase from other tissues.

During purification of acetyl-CoA carboxylase from rat lactating mammary gland, we observed that fatty acid synthase always accompanied the carboxylase through many of the purification steps (Ahmad *et al.*, 1978). Complete removal of fatty acid synthase from acetyl-CoA carboxylase proved somewhat difficult. However, most of the fatty acid synthase could be readily separated from the

carboxylase by careful $(\text{NH}_4)_2\text{SO}_4$ fractionation of the protein precipitates containing these enzymic activities. The synthase could then be purified to apparent homogeneity by an additional step. These results are reported here. In addition, evidence for the purity of such preparations, stoichiometry of CoA ester binding, pantetheine content of the enzyme and the effect of free CoA on enzymic activity are described.

Materials and methods

CoA, acetyl-CoA, malonyl-CoA and NADPH were purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. All reagents for SDS/polyacrylamide-gel electrophoresis and Bio-Gel A-1.5m (100–200 mesh) were products of Bio-Rad Laboratories, Richmond, CA, U.S.A. [$1\text{-}^{14}\text{C}$]Acetyl-CoA (58 μ Ci/mol), [$2\text{-}^{14}\text{C}$]malonyl-CoA (47 Ci/mol) and Omnifluor were from New England Nuclear Corp., Boston, MA, U.S.A. Sigma Chemical Co., St. Louis, MO, U.S.A., was the source for bovine serum albumin (essentially fatty acid-free) and dithiothreitol. Phosphotransacetylase (EC 2.3.1.8) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Liquiscint was a product of National Diagnostics, Somerville, NJ, U.S.A. All other chemicals were of the highest purity commercially available.

Abbreviation used: SDS, sodium dodecyl sulphate.

Gel electrophoresis

The method of Weber & Osborn (1969) was used to monitor the purity of the enzyme preparations and to determine molecular weights. Protein samples were denatured in SDS + urea + β -mercaptoethanol and then electrophoresed in 4% (w/v) polyacrylamide gel columns. The molecular weight was estimated by interpolation of the relative mobility of the unknown to a curve constructed from cross-linked bovine serum albumin and catalase as described previously (Ahmad *et al.*, 1978).

Antibody production

Antibodies against rat mammary-gland fatty acid synthase were produced in rabbits. The immune serum was processed and then stored as described previously by Ahmad *et al.* (1979).

Assay of fatty acid synthase activity

The fatty acid synthase activity was measured by the procedure of Smith & Abraham (1975), except that the assay temperature was 37°C. A unit of enzyme activity is defined as the oxidation of 1 μ mol of NADPH/min, and specific activity is expressed in units per mg of protein. It is essential to incubate the enzyme with 250 mM-phosphate buffer, pH 6.5, containing 10 mM-dithiothreitol at 37°C for 30 min before performing the assays, especially if the enzyme is in dilute buffers or if it has been stored at low temperature.

Amino acid analysis and determination of taurine content

The 4'-phosphopantetheine content of rat mammary-gland fatty acid synthase was determined by measurement of the taurine generated on hydrolysis of performic acid-treated enzyme. For taurine determination 4–5 mg (8–10 nmol of fatty acid synthase) of the protein was oxidized with 300 μ l of performic acid [5% (v/v) H₂O₂ in 88% (v/v) formic acid] for 3 h at 4°C (Takahashi, 1973). After addition of 30 μ l of 48% (v/v) HBr (Moore, 1963), the contents were evacuated overnight and then subjected to acid hydrolysis as described below.

For amino acid analysis, to 20–40 μ g of fatty acid synthase was added 100 μ l of toluene-*p*-sulphonic acid containing 0.2% 3-(2-aminoethyl)indole (Liu & Chang, 1971). The hydrolysis was performed *in vacuo* at 110°C for 24, 48 and 72 h. The amino acid analyses were performed on a Beckman 120C amino acid analyser.

Binding of acyl-CoA esters to fatty acid synthase

Fatty acid synthase (0.375 mg) was incubated at 0°C for 30 s with either labelled acetyl-CoA or malonyl-CoA in the presence or absence of 0.15 mM-NADPH. The reactions were terminated by the addition of 30 μ l of ice-cold 80% (w/v) trichloroacetic acid. The contents were further kept at 0°C

for 30 min. The precipitated protein was recovered by centrifugation (6000g for 5 min), washed with 5 $\times$ 0.2 ml of ice-cold 20% trichloroacetic acid, and then dissolved in 0.1 ml of 0.1 M-NaOH. Control fatty acid synthase samples treated with trichloroacetic acid before addition of labelled acyl-CoA derivative were run to detect non-specific binding. In addition, labelled methylated bovine serum albumin (0.3 mg) was run in parallel each time; its recovery (usually 75–80%) was used to calculate the recovery of protein in test samples. All determinations were done in duplicate, with agreement within 10%. The binding studies were performed on three different enzyme preparations, with similar results.

Protein and radioactivity determinations

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard ($A_{280}^{1\%}$ 6.5) and by measuring the A_{280} . An absorbance of 0.89 was determined for a 'standard' 1 mg/ml solution of rat mammary-gland fatty acid synthase by Libertini & Smith (1979). The agreement between the method of Lowry *et al.* (1951) and A_{280} absorbance was within 10–11%. The dye-binding method of Bradford (1976), with ovalbumin as a standard, was found to overestimate the amount of protein by a factor of 2 compared with the Lowry and A_{280} assays.

Radioactivity was determined in a Searle Analytic Mark III scintillation spectrometer with Liquiscint as the scintillator. The counting efficiency was 85–90%.

Results

Purification of fatty acid synthase

Most of the steps (steps 1–4 of Table 1) of fatty acid synthase purification are identical with those used in the purification of acetyl-CoA carboxylase from rat lactating mammary gland (Ahmad & Ahmad, 1981). The enzyme emerging from the DEAE-cellulose DE-52 column is precipitated by the addition of a saturated and neutralized solution of (NH₄)₂SO₄ to give 50% saturation. The pellet is collected by centrifugation at 27000g for 15 min and extracted successively with (NH₄)₂SO₄ solutions of 40, 35, 33, 30 and 28% saturation. Most of the synthase activity is found in (NH₄)₂SO₄ solutions of 30 and 33% saturation. The synthase is precipitated by adjusting the (NH₄)₂SO₄ concentrations to 35% saturation. Most of these steps are completed within 24–36 h.

The enzyme at this stage (specific activity ≤ 1.5) is purified further as follows. It is dissolved in 0.25 M-potassium phosphate buffer, pH 6.5, containing 1 mM-EDTA, 1 mM-dithiothreitol and 5% (v/v) glycerol. Usually 50–60 mg (2.5 ml) of the

Table 1. *Purification of fatty acid synthase from rat lactating mammary gland*

Mammary tissue (approx. 100 g) obtained from seven rats served as the source. Similar results were obtained with five different preparations.

Fraction	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
1. Crude extract	170	506	2669	0.189	1	100
2. $(\text{NH}_4)_2\text{SO}_4$ precipitation (0–50% satn.)	54	462	1139	0.406	2.1	91
3. Eluate from DEAE-cellulose DE-52	143	491	786	0.62	3.2	97
4. $(\text{NH}_4)_2\text{SO}_4$ fractionation						
(A) 33% satn.	1.6	184	95	1.93	10.2	36
(B) 30% satn.	1.2	102	65	1.56	8.25	20
5. Bio-Gel A-1.5m*	28.0	100	37	2.6	13.7	
6. $(\text{NH}_4)_2\text{SO}_4$ precipitation (0–35% satn.)	1.5	86	33	2.6	13.2	

* Only a portion (in this case enzyme from step 4B) of the enzyme units recovered in step 4 was chromatographed on the Bio-Gel column. The recovery of units applied to the Bio-Gel column is 90–100%. Therefore the overall recovery of enzymic activity from crude extract at step 4 and beyond is >50%.

Fig. 1. *Purification of fatty acid synthase on a Bio-Gel A-1.5m column*

The enzyme from step 4B (Table 1) was chromatographed on a Bio-Gel A-1.5m column as described in the Results section. ●, Protein (A_{280}); △, specific activity.

For storage the enzyme is dissolved in 0.25M-potassium phosphate buffer, pH 7.0, containing 10mM-dithiothreitol and 20% glycerol. Small portions (0.2–0.3 ml; 50–100mg/ml) are quick-frozen in an acetone/solid- CO_2 bath and then stored at -70°C . Under these conditions the enzyme is stable for 6–9 months.

Purity of fatty acid synthase

(a) *SDS/polyacrylamide-gel electrophoresis*. The purity of several preparations of fatty acid synthase of specific activity >2 was examined. In all these cases the protein applied to the gel columns migrated predominantly (>95%) as a single protein band with M_r 240 000–260 000 (Ahmad *et al.*, 1978).

(b) *Ouchterlony double-diffusion analysis*. When enzyme preparations at various stages of purity were subjected to Ouchterlony double-diffusion analysis (Ouchterlony & Nilsson, 1978) by using antibodies elicited against the highly purified rat mammary-gland enzyme, a single connecting band of precipitation was observed, suggesting purity of the enzyme used to elicit antibodies in the rabbit. The M_r of the polypeptides arising from the dissociation of rat mammary-gland fatty acid synthase is similar to that of the polypeptides constituting rat mammary-gland acetyl-CoA carboxylase (Ahmad *et al.*, 1978). However, in independent experiments no cross-reactivity could be detected when anti-(fatty acid synthase) serum was subjected to immunodiffusion analysis against acetyl-CoA carboxylase.

Amino acid composition of fatty acid synthase

The amino acid composition of rat mammary gland synthase is given in Table 2. The amino acid composition of rat mammary-gland synthase is remarkably similar to that of the goose enzyme (Buckner & Kolattukudy, 1976).

protein is applied to a Bio-Gel A-1.5m column (1.6 cm $\times$ 84 cm; bed volume 140 ml), previously equilibrated with the above phosphate buffer containing 0.01% (w/v) NaN_3 , and the column is eluted with the same buffer at a flow rate of 7–8 ml/h. Fractions (2.5 ml) are collected at room temperature and monitored for enzyme activity and protein concentration (Fig. 1). Fatty acid synthase emerges as a symmetrical peak and the specific enzyme activity across the peak is constant. Fractions of specific activity >2 are pooled and precipitated in the cold by adding a saturated and neutralized solution of $(\text{NH}_4)_2\text{SO}_4$ to 35% saturation. The specific activity of the precipitated enzyme, when assayed under appropriate conditions, usually exceeds 2.5 units/mg.

Table 2. *Amino acid composition and taurine content of fatty acid synthase*
For details see the text.

	Content (mol%)
Asx	8.8
Thr	4.9
Ser	7.8
Glx	11.8
Pro	5.3
Gly	8.0
Ala	9.2
Cys	1.7†
Val	5.7
Met	1.4†
Ile	3.2
Leu	15.1
Tyr	2.1
Phe	3.0
Lys	3.9
His	2.5
Arg	4.6
Trp	1.1
Taurine	2.1*

* mol/mol of performic acid-oxidized enzyme.

† Determined after performic acid oxidation as cysteine acid and methionine sulphone.

The taurine content of rat mammary-gland synthase is of particular interest here. Two different enzyme preparations gave almost identical values (2.1–2.2 mol of taurine/mol of fatty acid synthase). These results are similar to those previously reported by Buckner & Kolattukudy (1976) and by Arslanian *et al.* (1976) for synthases purified from goose uropygial gland, chicken and rat liver, respectively. However, our results are different from those of Smith & Abraham (1970), who found 1 mol of taurine/mol of rat mammary-gland fatty acid synthase. Since their preparation had low specific activity, it could have contained either apoenzyme or another protein(s) lacking 4'-phosphopanthetheine.

CoA requirement for activity

When the fatty acid synthase reaction was conducted in the presence of phosphotransacetylase reaction components, the oxidation of NADPH was markedly inhibited (Table 3). This activity could be restored on the addition of P_i (results not shown). These results therefore suggest that rat mammary-gland synthase requires free CoA for catalysis, as has been demonstrated for the rat liver enzyme (Linn *et al.*, 1980).

Stoichiometry of acyl-CoA ester binding

After several trials, and by using different methods, a procedure was adopted that gave good

Table 3. *Effect of phosphotransacetylase reaction components on rat mammary-gland fatty acid synthase activity*

Portions (0.01 ml containing 0.21 mg) of enzyme (specific activity = 1.5) were diluted 10-fold in 0.1 M-Tris/HCl buffer, pH 7.5, containing 10 mM- β -mercaptoethanol and incubated at 37°C for 30 min. Samples (0.01 ml) of the diluted enzyme were added to a fatty acid synthase assay mixture (final volume 0.25 ml) containing 100 mM-Tris/HCl buffer, pH 7.5, 0.05 mM-acetyl-CoA, 0.15 mg of bovine serum albumin, 0.14 mM-NADPH, 0.16 mM-malonyl-CoA and, where indicated, 4 or 8 mM-acetyl phosphate and 30 μ g of phosphotransacetylase. Reactions were monitored at 37°C in a Gilford recording spectrophotometer.

Expt. no.	Additions	Activity (nmol/min)
1	None	7.55
2	Acetyl phosphate (8 mM)	7.7
3	Phosphotransacetylase (30 μ g)	5.7
4	Acetyl phosphate (4 mM) + phosphotransacetylase (30 μ g)	1.6
5	Acetyl phosphate (8 mM) + phosphotransacetylase (30 μ g)	0.7

Fig. 2. *Stoichiometry of acyl-CoA ester binding*
The covalent transfer of acetyl (—) and malonyl (---) groups to rat mammary-gland fatty acid synthase was monitored at different concentrations of 14 C-labelled acyl-CoA derivatives. From the amount of radioactivity present in the acid precipitates the number of acyl groups bound to the enzyme was calculated.

recoveries of protein and excellent reproducibility of binding data.

The transfer of acetyl and malonyl groups to fatty acid synthase was measured at different concentrations of acetyl-CoA and malonyl-CoA. Increasing concentrations of the acyl-CoA derivatives resulted in higher stoichiometry, with the limit stoichiometry approaching about 3 mol each of acetyl and malonyl groups bound per mol of the

enzyme (Fig. 2). These values remained unaffected when the experiments were performed in the presence of NADPH.

Discussion

The simplified procedure described in this paper yielded enzyme of specific activity exceeding the values previously reported for rat mammary-gland synthase (Smith & Abraham, 1975) and for synthases purified from other sources (Dils & Carey, 1975; Muesing & Porter, 1975; Buckner & Kolattukudy, 1976; Paskin & Mayer, 1976; Hardie & Cohen, 1978). In addition, this purification scheme gave the best overall yield (>50%) thus far reported for any of the mammalian synthases. Therefore it is now possible to obtain sizeable amounts of fatty acid synthase of high degree of purity with excellent recoveries in a relatively short period of time from lactating-rat mammary gland.

Several criteria indicate that the purity of rat mammary-gland fatty acid synthase approaches homogeneity. These include: elution behaviour from the Bio-Gel A-1.5 m column, specific activity, results of SDS/polyacrylamide-gel electrophoresis of denatured and reduced protein, 4'-phosphopantetheine content and its behaviour during immunodiffusion experiments.

Various investigators have provided evidence suggesting that the two subunits of mammalian fatty acid synthase may be identical. Owing to the unusually large size of these subunits, techniques other than peptide mapping have been employed to arrive at this conclusion. For example, complete inactivation of the thioesterase activity by phenylmethanesulphonyl fluoride and di-isopropyl fluorophosphate requires incorporation of 2 mol of these compounds per mol of the enzyme (Kumar, 1973; Kolattukudy *et al.*, 1976). Similarly, the results of treating rabbit mammary-gland synthase with elastase and trypsin treatment of rat mammary-gland enzyme labelled with [1,3-¹⁴C]di-isopropyl fluorophosphate have been interpreted to indicate that these two enzymes are homodimers (Guy *et al.*, 1978; Smith & Stern, 1979). Further evidence for the homodimeric nature of these enzymes comes from the recent careful estimates of the 4'-phosphopantetheine content, which approaches 2 mol/mol for the goose, chicken and rat liver enzymes (Buckner & Kolattukudy, 1976; Arslanian *et al.*, 1976). Since each subunit of rat mammary-gland fatty acid synthase appears also to contain a stoichiometric amount of 4'-phosphopantetheine (Table 2), these results provide further support for the identical nature of mammary-gland fatty acid synthase subunits. Besides mammalian enzymes, Wood *et al.* (1978) have provided convincing evidence that the *Mycobacterium smegmatis* fatty

acid synthase is an oligomer of identical, multi-functional polypeptide chains.

The catalytic mechanism of vertebrate fatty acid synthase is unusually complex, since seven different partial reactions are required for the synthesis of a fatty acid (Lynen *et al.*, 1978). The transformation of acetyl- and malonyl-CoA to the end-product is achieved through intermediates which are covalently bound to thiol groups belonging to 4'-phosphopantetheine, the functional group of acyl-carrier protein, and to the cysteine residues of the enzyme. The results of careful binding studies performed at 0°C indicate that three acetyl groups are transferred to rat mammary-gland synthase very rapidly (Fig. 2). A similar stoichiometry for malonyl transfer is indicated, though in this case the number of malonyl groups transferred could approach four. Besides the thiol groups of 4'-phosphopantetheine and cysteine, the hydroxy groups of serine have also been implicated in the catalytic mechanism of mammalian fatty acid synthase (Joshi *et al.*, 1970; Muesing & Porter, 1975). The need for identification of the residues being acylated by acetyl-CoA and malonyl-CoA in rat mammary-gland fatty acid synthase is apparent.

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