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The Purification and Properties of Microsomal Palmitoyl-Coenzyme A Synthetase

BY J. BAR-TANA, G. ROSE AND B. SHAPIRO
Department of Biochemistry, The Hebrew University–Hadassah Medical School, Jerusalem, Israel

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The isolation and purification of palmitoyl-CoA synthetase from rat liver microsomes is described. Several methods suitable for enzyme assay are described. The general properties and kinetic parameters of the purified enzyme were determined and are discussed in relationship to microsomal fatty acid activation.

Long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) is a key enzyme in fatty acid metabolism. First studied by Kornberg & Pricer (1953) in guinea-pig liver, this enzyme has been shown to occur also in brain, muscle, intestinal epithelium, adipose tissue and bacteria (Vignais, Gallagher & Zabin, 1958; Pande & Mead, 1968*a*; Ailhaud, Sarda & Desnuelle, 1962; Senior & Isselbacher, 1960; Rose & Shapiro, 1960; Brindley & Hübscher, 1966; Samuel, Estroumza & Ailhaud, 1970). Some conflicting reports have appeared recently on the intracellular distribution of this enzyme (Farstad, Bremer & Norum, 1967; Pande & Mead, 1968*a*). However, with regard to rat liver, jejunum epithelium and heart muscle, the activity seems to be localized mainly in the endoplasmic reticulum (De Jong & Hülsmann, 1970; Lippel, Robinson & Trams, 1970). The reported specificity for the fatty acid substrate covers the wide range of C_5–C_{24} saturated fatty acids, and also unsaturated fatty acids (Kornberg & Pricer, 1953). With regard to the mechanism of fatty acid activation, the role of fatty acyl adenylate as an intermediate of the overall reaction (Berg, 1956; Vignais & Zabin, 1958; Bar-Tana & Shapiro, 1964), as well as the occurrence of possible active and inactive forms of enzyme (Bar-Tana & Shapiro, 1964; Rao & Johnston, 1967), have not been clarified until now, because of the difficulties encountered in the purification of the enzyme. Attempts to isolate the enzyme from mammalian sources have failed mainly because of the difficulty in solubilizing the particulate fraction. The present paper describes the purification of long-chain fatty acyl-CoA synthetase derived from rat liver microsomes. The general properties related to the overall reaction are discussed.

MATERIALS AND METHODS

Materials

ATP (disodium salt), UTP, GTP, ITP, CTP, cysteine, GSH and pantetheine were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Pantetheine was obtained by reduction of pantethine. CoASH, AMP, NADPH, myokinase, lactate dehydrogenase, pyruvate kinase and hexokinase were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Dithiothreitol (Cleland, 1964) and sodium deoxycholate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. 5,5-Dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. Triton X-100 was from Serva, Heidelberg, Germany. Bovine serum albumin fraction V was obtained from Pentex Inc., Kankakee, Ill., U.S.A. Sephadex G-200, Sepharose 6-B, DEAE-Sephadex A-25 and DEAE-Sephadex A-50 were from Pharmacia, Uppsala, Sweden, and hydroxyapatite was from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Pyrophosphatase was prepared by the method of Heppel & Hillmoe (1955). Egg phosphatidylcholine was prepared by the method of Pangborn (1950) and palmitoyl-CoA was prepared from palmitic anhydride by the method of Seubert (1960). Palmitic anhydride was prepared as described by Selinger & Lapidot (1966). 1-^{14}C-labelled fatty acids were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [^{32}P]phosphoric acid was from Nuclear Research Centre, Beersheva, Israel. [γ-^{32}P]ATP was prepared by the method of Penefsky (1967).

Preparation of enzyme

Rat liver microsomal preparation. Rats (this laboratory strain, 100–150g body wt.) were fed *ad libitum*. The animals were decapitated, and the livers were removed and homogenized for 20s in the cold in 4.0vol. of 0.25M-sucrose in a Waring Blendor. The homogenate was centrifuged for 20min at 15000*g* and the supernatant further centrifuged at 100000*g* for 60min. The precipitate obtained was suspended in 2.0ml of water/g of liver and centrifuged again at 100000*g* for 60min. The washed microsomal pellet was resuspended in 0.5ml of water/g of liver, freeze-dried and stored at −15°C. The protein content of these microsomal preparations ranged from 45 to 55% of the dry weight.

Lipid extraction of freeze-dried microsomal preparation. All of the following procedures were carried out at −15°C. A 1.0g portion of freeze-dried microsomal preparation was extracted with 100ml of dry butan-1-ol in a Waring Blendor. The residue obtained on centrifugation at 15000*g* for 10min was treated once more with 100ml of butan-1-ol

followed by 100ml of acetone and finally washed twice with 100ml of ether. The ether-washed residue was dried exhaustively in a stream of N_2 and stored at −15°C. The protein content of this preparation ranged from 65% to 70% of the dry weight ('lipid-depleted microsomal preparation').

Deoxycholate extraction. A 1.0g portion of 'lipid-depleted' microsomal preparation was suspended in 20ml of ice-cold 0.1M-glycylglycine–NaOH buffer, pH 8.0, containing 0.125M-sucrose and 2.5% (w/v) of sodium deoxycholate. The suspension was homogenized in a Potter–Elvehjem-type homogenizer, and the clear solution was kept at 4°C for 10min, after which it was diluted with 6vol. of cold 1.5mM-dithiothreitol and centrifuged at 100000*g* for 60min. The clear supernatant was dialysed overnight at 4°C against 75vol. of 50mM-tris–HCl buffer, pH 7.9, containing 0.1M-sucrose, 1mM-EDTA and 0.25mM-dithiothreitol ('deoxycholate extract').

Ammonium sulphate fractionation. To 100ml of the above extract 4.5g of $(NH_4)_2SO_4$ was added slowly, the pH being kept at 8.2–8.3. The supernatant obtained by centrifuging the suspension at 15000*g* for 20min was acidified slowly to pH 7.4 with 1M-HCl. To the clear supernatant obtained after centrifugation of the suspension at 15000*g* for 20min was added 25g of $(NH_4)_2SO_4$, the pH being maintained at 7.4. The residue obtained after centrifugation was suspended in 25ml of 0.4M-KCl in '20% (v/v) glycerol buffer', composed of 20% (v/v) glycerol, 50mM-tris–HCl buffer, pH 7.9 (at 4°C), 0.025% (w/v) deoxycholate, 1mM-EDTA and 0.25mM-dithiothreitol and was dialysed against 100vol. of 0.4M-KCl in 20% (v/v) glycerol buffer. The dialysed clear solution was stored at −15°C ('ammonium sulphate fraction').

DEAE-Sephadex fractionation. The ammonium sulphate fraction was diluted with 0.4M-KCl in 20% (v/v) glycerol buffer to give a protein concentration of 15mg/ml, and mixed for 30min with a suspension of DEAE-Sephadex A-50 previously equilibrated with the same buffer. The proportion of anion-exchanger to protein was kept at 0.25ml bed volume of resin/mg of protein. The suspension was filtered on a Buchner funnel and the residue washed with the same buffer. The filtrate and washing were then diluted with 20% (v/v) glycerol buffer to a final concentration of 0.2M-KCl and applied on a column of DEAE-Sephadex A-25 previously equilibrated with 0.2M-KCl in 20% (v/v) glycerol buffer. The activity was eluted by means of 0.2M-KCl in 20% (v/v) glycerol buffer ('DEAE-Sephadex fraction').

Hydroxyapatite fractionation. The DEAE-Sephadex fraction was applied on a column of hydroxyapatite equilibrated with 0.2M-KCl in glycerol buffer. The amount of hydroxyapatite used was kept at 20mg/mg of protein. The column was packed with a 1:1 (w/w) mixture of hydroxyapatite and cellulose. Activity was eluted stepwise, by washing successively with 0.05, 0.1, 0.2, 0.4 and 0.5M-K_2HPO_4 in 3.5% (w/v) glycerol buffer. The fractions with the highest specific activity obtained by the 0.2–0.5M-K_2HPO_4 elution steps were dialysed against 3.5% (v/v) glycerol buffer and concentrated by precipitation with 40% (w/v) $(NH_4)_2SO_4$ ('hydroxyapatite fraction').

Gel filtration. The hydroxyapatite fraction was applied in a minimal volume on a column (150cm × 2.5cm) of Sephadex G-200 previously equilibrated with 3.5% (v/v) glycerol buffer. The enzyme activity was eluted by ascending chromatography with 3.5% (v/v) glycerol buffer as the eluent. The fractions containing the highest specific activity were pooled, dialysed against 20% (v/v) glycerol buffer and stored at −15°C ('purified enzyme fraction').

Methods of enzyme determination

CoASH disappearance. The reaction mixture contained 150mM-tris–HCl buffer, pH 7.4, 0.25mg of Triton X-100, 2mM-EDTA, 50mM-$MgCl_2$, 20mM-ATP, 200μM-potassium palmitate, 300μM-CoASH and 5–15μg of enzyme protein in a total volume of 0.25ml. Incubations were carried out for 10min at 37°C and terminated by the addition of 0.75ml of 0.5mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1M-potassium phosphate buffer, pH 8.0. Addition of enzyme at the termination of the reaction served as a blank. The decrease in E_{413} was measured in a Gilford spectrophotometer. The molar extinction coefficient given by CoASH under these conditions was assumed to be $1.36 \times 10^4 cm^{-1}$ (Ellman, 1958).

Palmitoyl-CoA formation from [1-^{14}C]*palmitate.* The reaction mixture contained 150mM-tris–HCl buffer, pH 7.4, 0.25mg of Triton X-100, 2mM-EDTA, 50mM-$MgCl_2$, 20mM-ATP, 200μM-potassium [1-^{14}C]palmitate (1μCi/μmol), 600μM-CoASH and 2–10μg of enzyme protein in a total volume of 0.25ml. Incubations were carried out for 10min at 37°C and terminated by the addition of 1ml of Dole reagent (Dole, 1956), 0.35ml of water and 0.6ml of heptane. The contents were mixed and centrifuged, and the heptane layer was removed. The lower phase was washed five times with 0.6ml portions of heptane. All washings were discarded and 0.4ml of the lower phase was assayed for radioactivity. In blank experiments the enzyme was added at the end of the incubation.

Palmitate formation from [1-^{14}C]*palmitoyl-CoA.* The reaction mixture contained 150mM-tris–HCl buffer, pH 7.4, 0.25mg of Triton X-100, 2mM-EDTA, 20mM-$MgCl_2$, 25mM-AMP, 4.0mM-$Na_4P_2O_7$ and 2mM-[1-^{14}C]-palmitoyl-CoA (1μCi/μmol) in a total volume of 0.25ml. Incubations were carried out at 37°C with shaking for 20min. The reaction was terminated by the addition of 1ml of Dole reagent, 0.35ml of water and 0.6ml of heptane and mixing. After centrifugation the upper heptane layer containing the radioactive palmitic acid was separated and the lower phase washed once with 0.6ml of heptane containing a few nanomoles of palmitic acid carrier. After centrifugation the heptane layer was added to the original heptane supernatant and a portion was assayed for radioactivity. AMP was omitted in the blank experiments.

Other methods. P_i was determined by the method of Parvin & Smith (1969). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) with dry albumin as a standard or by the method of Warburg & Christian (1941). Measurements of radioactivity were carried out by scintillation counting in toluene–ethanol (2:1, v/v) scintillation fluid. Polyacrylamide-gel electrophoresis was carried out as described by Clarke (1964) with tris–glycine buffer, pH 8.4, and gels of 7.5% (w/v) polyacrylamide containing 0.3% of cross-linker. Gels were stained as described by Chrambach, Reisfeld, Wyckoff & Zaccari (1967).

RESULTS

Isolation and purification of enzyme. Lipid depletion of the microsomal preparation is necessary for the following procedure to yield any fractionation of the enzyme. The lipid-depleted microsomal preparation is stable at −15°C for at least 2 months and serves as a suitable starting material for the preparation of purified enzyme (Table 1). As lipid depletion in aqueous medium causes complete inactivation, only freeze-dried microsomal preparation may be subjected to this procedure under strictly anhydrous conditions. Extraction of lipid-depleted microsomal preparation by deoxycholate is very efficient, with almost complete solubilization of the microsomal proteins. The pellet obtained by centrifugation of the deoxycholate extract is enriched in ribonucleoprotein and is devoid of activity. After extraction by deoxycholate, extensive dialysis is carried out to prevent enzyme inactivation caused by the presence of a high concentration of sodium deoxycholate (0.4%, w/v) in the extraction medium. However, a certain minimum concentration of sodium deoxycholate (0.025%, w/v) was maintained throughout the purification procedure, as no activity could be eluted from the anion-exchanger resin (DEAE-Sephadex) in its total absence from the eluent buffer even at high salt concentrations (up to 1.1 M) and at pH values between 7.4 and 10.5. The presence of deoxycholate in the eluent buffer of the hydroxyapatite and Sephadex G-200 fractionation steps is not essential for the elution of the enzyme fraction. However, attempts to separate the detergent from the enzyme fraction by these two purification procedures were unsuccessful.

The elution profile of the purified enzyme fraction subjected to gel filtration through Sephadex G-200 is shown in Fig. 1. Preliminary molecular-weight determination of the purified enzyme fraction by gel filtration through Sepharose 6-B gave a value of 250000±25000. The determined molecular weight is presumably exaggerated owing to the presence of an unknown amount of detergent bound to enzyme.

On polyacrylamide-gel disc electrophoresis palmitoyl-CoA synthetase activity was found only with the protein band remaining at the start of the column. The purified protein fraction could not be introduced into the column electrophoretically, even by lowering the gel concentration to 2% or by varying the cross-linker concentration from 2 to 25% of the total gel concentration.

The purified enzyme fraction was free of adenosine triphosphatase and inorganic pyrophosphatase when assayed by the procedures described in the

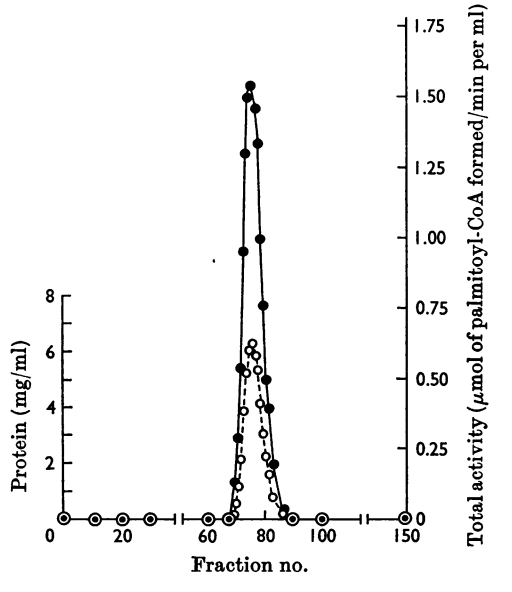

Fig. 1. Sephadex G-200 elution profile of palmitoyl-CoA synthetase. Gel filtration was carried out in a column (150 cm × 2.5 cm) at 4°C, with 20% (v/v) glycerol buffer as the eluent. Fractions (5.0 ml) were collected and analysed for protein and total enzyme activity as described in the Materials and Methods section. ●, Total activity; ○, protein.

Table 1. *Purification of palmitoyl-CoA synthetase*

Activity was assayed by [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

	Total protein (mg)	Sp. activity (nmol of palmitoyl CoA formed/min per mg of protein)	Total activity (μmol of palmitoyl-CoA formed/min)
Freeze-dried microsomal preparation	4400	17.7	78.5
Lipid-depleted microsomal preparation	4300	17.7	76.0
Deoxycholate extract	4200	18.0	76.0
Ammonium sulphate fraction	2250	35	78.5
DEAE-Sephadex fraction	650	107	69.5
Hydroxyapatite fraction	210	171	36
Sephadex G-200 fraction	91	250	23

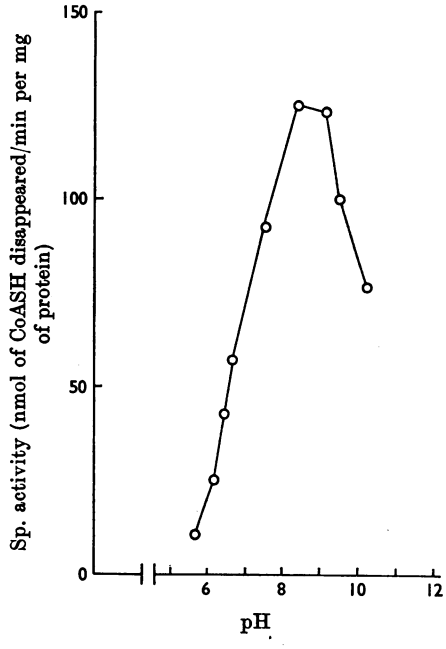

Fig. 2. pH–activity curve of palmitoyl-CoA synthetase. The reaction mixture contained: 77mM-tris–HCl buffer or phosphate buffer, 1mg of Triton X-100/ml, 2mM-EDTA, 25mM-$MgCl_2$, 10mM-ATP, 200μM-potassium palmitate and 185μM-CoASH. Activity was assayed by measuring CoASH disappearance as described in the Materials and Methods section.

Materials and Methods section with protein concentrations in the range specified for measuring fatty acid activation. With palmitoyl-CoA as substrate, palmitoyl-CoA hydrolase activity was shown to contaminate even the most purified enzyme fraction.

The enzyme fractions obtained by the procedure described are relatively stable at protein concentrations greater than 5mg/ml at −15°C in the presence of 20% (v/v) glycerol buffer. Glycerol and dithiothreitol are necessary for stability. Activity declines progressively on storage at 4°C. Thus purification of the enzyme after deoxycholate extraction is accompanied by a progressive decline of enzyme specific activity. Specific-activity values shown in Table 1 are therefore minimal values, which could be improved by speeding up the purification procedure.

General properties: the forward reaction. The pH–activity curve obtained with the purified enzyme fraction with ATP, CoASH and palmitate as substrates is shown in Fig. 2. The curve obtained by the determination of CoASH disappearance resembles the curve describing the [1-^{14}C]palmitoyl-CoA formation. The substrate requirements for the activation system are illustrated in Table 2(*a*). Mg^{2+} and the presence of detergent are necessary for activity. EDTA is added to eliminate non-enzymatic ATP-dependent CoASH disappearance; this effect of ATP was due presumably to contamination with metal ions, which catalyse the oxidation of

Table 2. *Substrate requirements for palmitoyl-CoA synthetase*

(*a*) Forward reaction: activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation. (*b*) Reverse reaction: activity assayed by measuring [1-^{14}C]palmitate formation. Details are given in the Materials and Methods section.

System	Activity (%)
(*a*) Full system	100
−ATP	0
−CoA	0
−$MgCl_2$	0
−Triton X-100	12
−Enzyme	0
+Pyrophosphatase	100
(*b*) Full system	100
−AMP	0
−PP_i	0
−$MgCl_2$	0
−Triton X-100	0
−Enzyme	0
+5,5′-Dithiobis-(2-nitrobenzoic acid) (20μM)	100
+Hexokinase+glucose	85

Table 3. *Substrate specificity of palmitoyl-CoA synthetase*

The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 50mM-$MgCl_2$, 200μM-potassium palmitate, 15mM-ATP or analogue nucleotide and 780μM-CoASH or thiol analogue. Activity was assayed by measuring [1-^{14}C]-palmitoyl-CoA formation as described in the Materials and Methods section. (*a*) Nucleotide analogues; (*b*) thiol analogues.

Substrate	Sp. activity (nmol of palmitoyl-CoA formed/min per mg of protein)
(*a*) ATP	215
GTP	0.0
UTP	33.2
CTP	0.0
ITP	0.0
None	0.0
(*b*) CoASH	235
Pantetheine	0.0
GSH	0.0
Cysteine	0.0
None	0.0

the CoASH thiol group (Harrison, Gray & De Cloux, 1969). With the purified enzyme strict specificity towards ATP and CoASH is observed (Table 3). Specificity towards the fatty acid substrate covers the range of C_{12}–C_{18} saturated as well as unsaturated fatty acids (Table 4). It should be noted that the $V_{max.}$ values described in Table 4 were obtained at saturation concentrations of all other reactants.

The activation of fatty acid requires the presence of Triton X-100 (Fig. 3*a*). Although albumin can replace Triton X-100 it is rather less effective (Fig. 3*b*). The activation of fatty acid is inhibited at high albumin concentrations, presumably because albumin competes with the enzyme for the fatty acid substrate. Egg phosphatidylcholine serves as well as Triton X-100, although when mixed together they interfere with each other (Table 5). Similarly the inhibition observed at high albumin concentrations is not relieved in the presence of Triton X-100. It should be pointed out that Triton X-100 is not required for fatty acid activation catalysed by intact, freeze-dried or lipid-depleted microsomal preparations.

A protein factor isolated from the supernatant fraction by the procedure of Farstad (1967) and capable of activating microsomal palmitoyl-CoA synthetase under specified conditions caused no such activation of the purified enzyme fraction under incubation conditions described in the Materials and Methods section (CoASH disappearance). It remains to be determined whether the difference observed is due to differences in incubation conditions (Aas, 1969; Farstad, 1967).

General properties: the reverse reaction. The substrate requirements for the system catalysing the backward reaction are listed in Table 2(*b*). The enzyme is not inhibited by 5,5′-dithiobis-(2-nitrobenzoic acid) at the concentration specified with palmitoyl-CoA as substrate. Determination of the backward reaction by measuring [1-^{14}C]palmitate derived from [1-^{14}C]palmitoyl-CoA yields the same results as CoASH appearance in the presence of 5,5′-dithiobis-(2-nitrobenzoic acid). The activity of the backward reaction can be followed continuously by measuring E_{413} in the presence of 5–20 μM-5,5′-dithiobis-(2-nitrobenzoic acid). Triton X-100 is required for activity by both assay systems.

Stoicheiometry. The stoicheiometry obtained by following simultaneously disappearance of palmitic

Table 4. *Relative specificity for the fatty acid substrate of palmitoyl-CoA synthetase*

The reaction mixture contained: 150 mM-tris–HCl buffer, pH 7.4, 1 mg of Triton X-100/ml, 2 mM-EDTA, 50 mM-$MgCl_2$, 20 mM-ATP, 530 μM-CoASH. Activity was assayed by measuring [1-^{14}C]acyl-CoA formation as described in the Materials and Methods section. $V_{max.}$ values were derived from Lineweaver–Burk plots of the respective saturation curves for each of the fatty acid substrate.

Fatty acid substrate	$V_{max.}$ (nmol of acyl-CoA formed/min per mg of protein)
Butyrate	4
Octanoate	6
Laurate	140
Myristate	200
Palmitate	300
Stearate	278
Linoleate	190
Oleate	94
Lignocerate	5

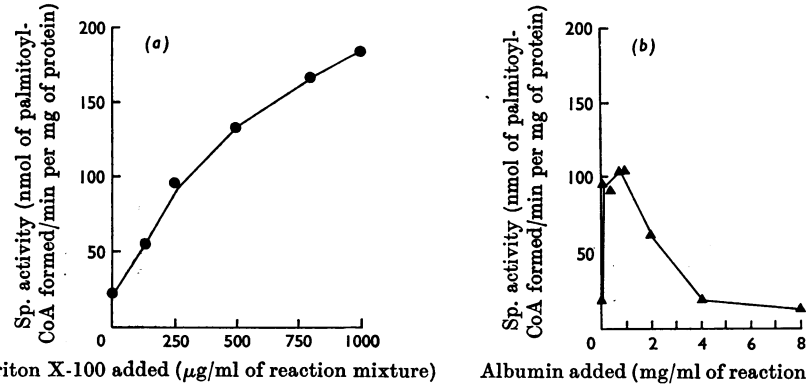

Fig. 3. Triton X-100 (*a*) and albumin (*b*) titration curves of palmitoyl-CoA synthetase. The reaction mixture contained: 150 mM-tris–HCl buffer, pH 7.4, 1 mg of Triton X-100/ml, 2 mM-EDTA, 50 mM-$MgCl_2$, 20 mM-ATP, 200 μM-potassium palmitate, 600 μM-CoASH and 100 μg of enzyme protein/ml. Triton X-100 or albumin was added as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

Table 5. *Effect of Triton, phosphatidylcholine and albumin on palmitoyl-CoA formation*

The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 2mM-EDTA, 50mM-$MgCl_2$, 20mM-ATP, 260μM-potassium palmitate, 400μM-CoASH and additions as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

Additions (per ml of reaction mixture)	Sp. activity (nmol of palmitoyl-CoA formed/min per mg of protein)
None	15.5
Triton X-100 (1mg)	154.0
Phosphatidylcholine (1.5μmol)	184.0
Phosphatidylcholine (3.0μmol)	145.0
Triton X-100 (1mg)+phosphatidylcholine (0.75μmol)	139.0
Triton X-100 (1mg)+phosphatidylcholine (1.5μmol)	122.5
Triton X-100 (1mg)+phosphatidylcholine (3.0μmol)	94.5
Albumin (24mg)	4.6
Albumin (48mg)	0.93
Triton X-100 (1mg)+albumin (24mg)	12.0

Table 6. *Stoicheiometry of the overall reaction of palmitoyl-CoA synthetase*

CoASH disappearance was measured as described in the Materials and Methods section. AMP appearance was measured by coupling the activation reaction to the myokinase–pyruvate kinase–lactate dehydrogenase system (Adam, 1963). PP_i appearance was determined by carrying out the activation reaction in the presence of pyrophosphatase; P_i was determined as described in the Materials and Methods section. Palmitate disappearance was determined by the decrease in [1-^{14}C]palmitate in Dole's (1956) heptane layer by using the assay system for [1-^{14}C]palmitoyl-CoA formation described in the Materials and Methods section. Palmitoyl-CoA appearance was determined as described in the Materials and Methods section. ATP disappearance was measured by using [γ-^{32}P]ATP for the activation reaction in the presence of pyrophosphatase; the separation of [^{32}P]P_i from [γ-^{32}P]-ATP was carried out as described by Pullman (1967). Values obtained in separate experiments are given in the table below.

Product appearance / Substrate disappearance	Ratio
AMP/CoA	1.13, 0.86, 0.92
PP_i/CoA	1.02, 1.06
Palmitoyl-CoA/ATP	1.03, 1.02
Palmitoyl-CoA/palmitate	0.99

acid and CoASH together with appearance of palmitoyl-CoA, AMP and PP_i in the presence of ATP (Table 6) conforms to the following reaction scheme:

$$\text{ATP} + \text{palmitate} + \text{CoA} \rightleftharpoons \text{palmitoyl-CoA} + \text{AMP} + PP_i$$

In the light of the observed balance, contamination of the purified enzyme fraction by non-relevant enzymes acting on the substrates concerned seems to be negligible.

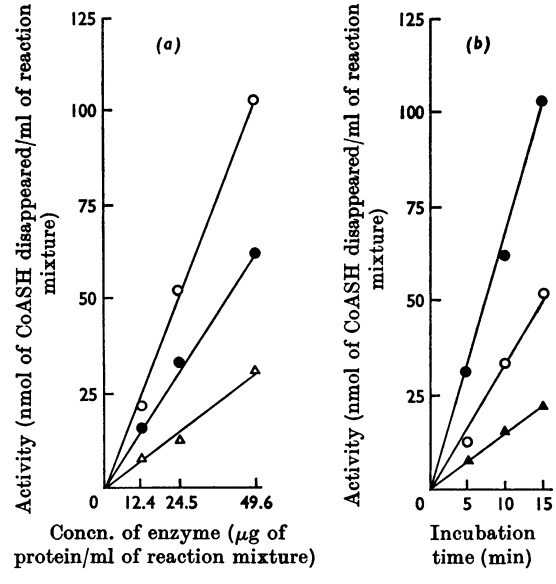

Fig. 4. Enzyme concentration–time curves for the forward reaction of palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 25mM-$MgCl_2$, 10mM-ATP, 200μM-potassium palmitate and 185μM-CoASH. Activity was assayed by measuring CoASH disappearance as described in the Materials and Methods section. (*a*) Enzyme concentration curve: the indicated concentrations of enzyme were incubated for 5min (△), 10min (●) and 15min (○). (*b*) Time curve: the stated concentrations of enzyme (▲, 12.4μg of protein/ml; ○, 24.8μg of protein/ml; ●, 49.6μg of protein/ml) were incubated for the times indicated.

Kinetic parameters. The rates of the forward and the reverse reactions are linear functions of the enzyme concentration up to 15min in the presence of 50μg of enzyme protein/ml of reaction mixture for the

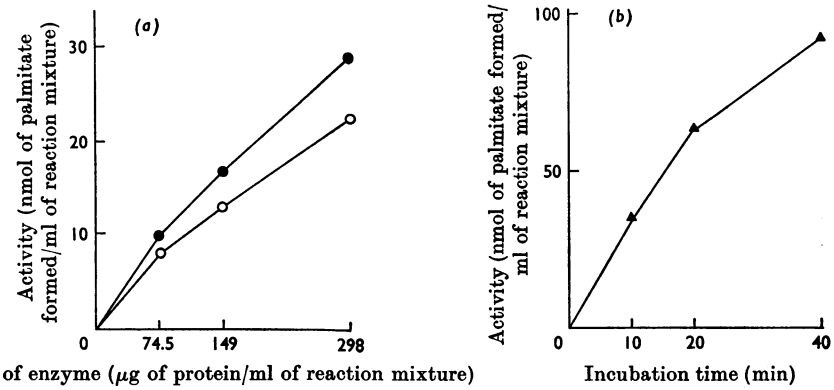

Fig. 5. Enzyme concentration–time curve for the reverse reaction of palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 20mM-$MgCl_2$, 25mM-AMP, 4.0mM-sodium pyrophosphate and [1-^{14}C]palmitoyl-CoA as stated. Activity was assayed by measuring [1-^{14}C]palmitate appearance as described in the Materials and Methods section. (*a*) Enzyme curve: the indicated concentrations of enzyme were incubated with 0.95mM-palmitoyl-CoA as substrate for 30min (○) and 60min (●). (*b*) Time curve: enzyme (280μg of protein/ml of reaction mixture) was incubated with 1.83mM-palmitoyl-CoA for the times indicated.

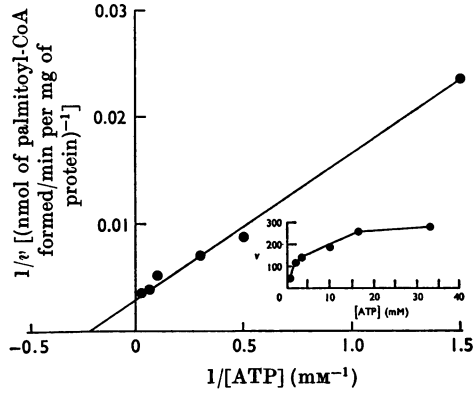

Fig. 6. ATP saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 50mM-$MgCl_2$, 200μM-potassium palmitate, 666μM-CoASH and ATP as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

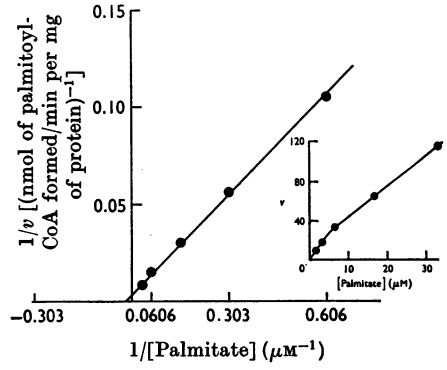

Fig. 7. Palmitate saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 50mM-$MgCl_2$, 20mM-ATP, 530μM-CoASH and potassium palmitate as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

forward reaction and up to 20min with 280μg of enzyme protein/ml of reaction mixture for the backward reaction (Figs. 4 and 5). Under these conditions the palmitoyl-CoA hydrolase activity does not interfere with measurement of the forward reaction. However, because of the high concentration of enzyme and palmitoyl-CoA used with the assay system for the reverse reaction, pronounced palmitoyl-CoA hydrolase activity is present and a suitable blank that lacks AMP has to be used.

Initial-velocity rate patterns determined by varying the concentrations of the different substrates participating in the forward reaction are described in Figs. 6–9. Care was taken to maintain the non-variable substrates at saturating concentrations on titration of the variable substrate. Thus the kinetic parameters obtained (Table 7*a*) represent true kinetic values. The optimum ATP/Mg^{2+} concentration ratio was found to be 1:2.5. K_m values for the different fatty acid substrates are

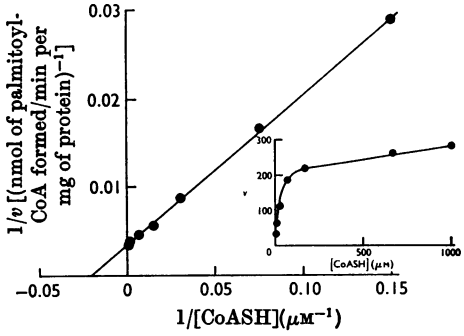

Fig. 8. CoASH saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 50mM-$MgCl_2$, 20mM-ATP, 200μM-potassium palmitate and CoASH as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

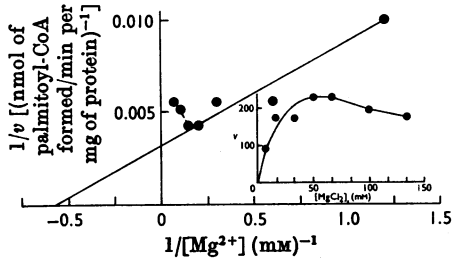

Fig. 9. Mg^{2+} saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 20mM-ATP, 200μM-potassium palmitate, 515μM-CoASH and $MgCl_2$ as stated. Activity was assayed by measuring [1-^{14}C]palmitoyl-CoA formation as described in the Materials and Methods section.

Table 7. *Kinetic parameters of palmitoyl-CoA synthetase*

K_m and $V_{max.}$ values were derived from Lineweaver–Burk plots of the respective saturation curves: (*a*) forward reaction (Figs. 6–9); (*b*) reverse reaction (Figs. 10–12).

(*a*) Substrate	K_m (μM)
ATP	4650
Palmitate	42
CoA	50

$V_{max.}$ = 300nmol/min per mg of protein

(*b*) Substrate	K_m (μM)
Palmitoyl-CoA	2600
AMP	630
Pyrophosphate	890

$V_{max.}$ = 40nmol/min per mg of protein

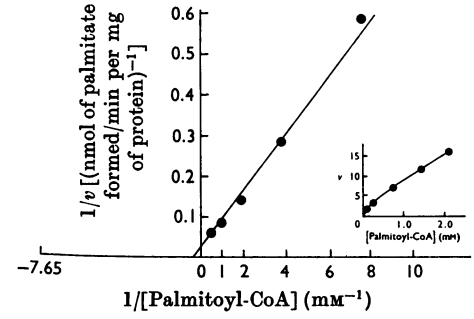

Fig. 10. Palmitoyl-CoA saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 20mM-$MgCl_2$, 25mM-AMP, 4.0mM-sodium pyrophosphate and palmitoyl-CoA as stated. Activity was assayed by measuring [1-^{14}C]palmitate appearance from [1-^{14}C]palmitoyl-CoA as described in the Materials and Methods section.

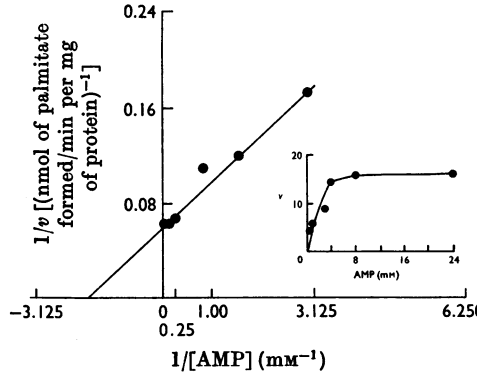

Fig. 11. AMP saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 4.0mM-sodium pyrophosphate, 2.0mM-palmitoyl-CoA and AMP as stated. Activity was assayed by measuring [1-^{14}C]palmitate appearance as described in the Materials and Methods section.

42μM for palmitate, 22μM for myristate, 11μM for laurate, 15μM for stearate and oleate and 25μM for linoleate. The values were obtained with saturating concentrations of the non-variable substrates. Initial-velocity rate patterns determined by varying the concentrations of the substrates participating in the backward reaction are described in Figs. 10–12. Kinetic parameters obtained for PP_i and AMP (Table 7*b*) are apparent kinetic values obtained at a palmitoyl-CoA concentration close to its K_m value. On the other hand PP_i and AMP were maintained

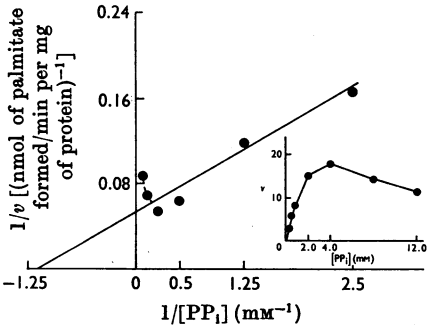

Fig. 12. PP_i saturation curve for palmitoyl-CoA synthetase. The reaction mixture contained: 150mM-tris–HCl buffer, pH 7.4, 1mg of Triton X-100/ml, 2mM-EDTA, 20mM-$MgCl_2$, 25mM-AMP, 2.0mM-palmitoyl-CoA and sodium pyrophosphate as stated. Activity was assayed by measuring [1-^{14}C]palmitate appearance as described in the Materials and Methods section.

at saturating concentrations when palmitoyl-CoA was the variable substrate.

DISCUSSION

Attempts to isolate long-chain fatty acyl-CoA synthetase in the past have failed because of the strong adherence of the enzyme to the endoplasmic reticulum. Treatment of the microsomal preparation with detergents such as cholate, deoxycholate or Triton X-100 results in solubilization of the enzyme, but only in the presence of the detergent. Removal of the detergent during purification causes reprecipitation of enzyme activity. Similarly the disruption of the microsomal membrane under alkaline conditions (>pH 10) renders the activity soluble, but further purification of the enzyme is limited to a basic environment with a concomitant loss of enzyme activity. All attempts at neutralization result in reprecipitation of enzyme activity (J. Bar-Tana, G. Rose & B. Shapiro, unpublished work). In the present isolation procedure two steps have been introduced to overcome the problem of reprecipitation: (*a*) removal of lipid components by treatment of the freeze-dried microsomal preparation with organic solvents before dissolution of the particles by detergent; (*b*) maintenance of a certain minimum concentration of the detergent throughout the purification procedure. Under these conditions the lipoprotein content is decreased to a minimum and hydrophobic interactions leading to reprecipitation of membrane constituents are eliminated. This procedure can be applied generally, and may facilitate the isolation of other membrane enzymes that have so far resisted purification.

It was suggested by Pande & Mead (1968*b*) that phospholipid is an 'integral part of the microsomal palmitate activating enzyme', whereas the effect of phospholipid on oleate activation is limited to a detergent-like action. The fact that the relatively drastic lipid-depletion step introduced in the present purification procedure causes no loss in specific enzyme activity makes it seem doubtful that phospholipids exert their effect through lipid–enzyme interaction. The significant increase in protein content/g dry wt. after lipid-depletion of the freeze-dried microsomal preparation makes it unlikely that the extraction of lipid is inefficient under anhydrous conditions. On the other hand catalysis of palmitoyl-CoA synthesis by the purified fraction depends on the presence of Triton X-100, egg phosphatidylcholine or serum albumin. The fact that these different substances can replace each other suggests a non-specific detergent-like function of all of them. However, the possibility that detergents bound to the protein moiety may simulate a lipoprotein complex similar to the original complex presumed to exist in the microsomal preparation cannot be ruled out. Lipid analysis of the purified fraction will clarify the role of detergent and phospholipid in the activation of fatty acids.

The degree of homogeneity of the purified enzyme is open to speculation. Although a single symmetrical elution curve of constant specific activity is obtained on gel filtration, as well as a single band by polyacrylamide-gel disc electrophoresis, the following points should be considered. (*a*) The purification factor is relatively low. However, loss in activity during purification may be caused either by ageing (the enzyme is stable only at −15°C) or by the loss of some cofactor in one of the purification steps. (*b*) The single band obtained by disc electrophoresis stays at the origin, apparently because of the inability of the enzyme to enter the gel. Electrophoretic homogeneity has therefore to be tested in a free medium. (*c*) Appreciable palmitoyl-CoA hydrolase activity was found to be a contaminant of the purified palmitoyl-CoA synthetase. However, the possibility that these two enzyme activities reside in the same protein cannot be dismissed.

The substrate specificity of the purified enzyme fraction is different from that reported for microsomal preparations. In guinea-pig liver (Kornberg & Pricer, 1953) and pig intestinal-mucosa microsomal preparation (Ailhaud *et al.* 1962) the fatty acid-specificity ranged from C_5 to C_{22}, maximal reaction rates being observed with decanoate, laurate or myristate. On the other hand, in both cat and guinea-pig intestinal preparations the highest reaction rate was observed with palmitate (Brindley & Hübscher, 1966). On the basis of differences in the CoASH requirement, two different activation systems have been suggested for rat liver catalysing the activation of long-chain saturated

fatty acids and of long-chain unsaturated fatty acids, i.e. oleate, linoleate, linolenate and arachidonate, respectively (Pande & Mead, 1968*b*). The fatty acid specificity of the purified enzyme fraction reported in the present paper ranges from C_{12} to C_{18}, with negligible activation rates observed beyond this range. Maximum activation rates were observed with palmitate and stearate, consistent with the known fatty acid profiles of rat liver lipids. It should be pointed out that the fatty acid specificity reported here reflects the rates obtained on saturating the enzyme with the fatty acid substrate in each case, thus avoiding differences in solubility or substrate–enzyme interaction. Further, it is noteworthy that the same purified enzyme fraction catalyses the activation of the C_{18} unsaturated fatty acids, thus excluding the necessity for two different activation enzymes as mentioned above. Whether the wide range of specificity towards fatty acid substrates reported for microsomal preparations is due to contamination of the 100000*g* pellet by mitochondrial medium-chain fatty acid synthetase (Bar-Tana, Rose & Shapiro, 1968) or whether it reflects a novel microsomal activation system catalysing activation of C_5–C_{12} fatty acids remains to be elucidated.

With regard to the thiol-group specificity it has been reported (Vignais & Zabin, 1958) that the microsomal enzyme catalyses the formation of palmitoyl-glutathione; the present study shows a strict specificity towards the CoASH thiol group. Similarly a high degree of specificity exists with regard to the adenine nucleotide that participates in the activation of fatty acid. The activation exhibited by UTP is believed to be due to ATP contamination. In this connexion it should be noted that in the activation system isolated from *Escherichia coli* CTP is capable of partially replacing ATP (Samuel *et al.* 1970).

The kinetic parameters obtained with the purified enzyme are in agreement with the respective values for microsomal preparation. With palmitate as the substrate in the microsomal system the K_m values for ATP and CoASH were found to be 4mM and 37μM respectively (Pande & Mead, 1968*b*), corresponding to those of the purified enzyme. The K_m values obtained for the different fatty acid substrates are all of the same order of magnitude, about 10μM.

We hope that the purified enzyme described above will facilitate the elucidation of the mechanism of activation of long-chain fatty acids.

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