BBA 45901

A COMPARATIVE STUDY OF PALMITOYL-CoA SYNTHETASE ACTIVITY IN RAT-LIVER, HEART AND GUT MITOCHONDRIAL AND MICROSOMAL PREPARATIONS

J. W. DE JONG AND W. C. HÜLSMANN

Department of Biochemistry, Rotterdam Medical School, Dijkzigt Hospital, Rotterdam (The Netherlands)

(Received December 10th, 1969)

SUMMARY

1. With the aid of marker enzymes, mitochondrial and microsomal fractions of rat-liver, heart and jejunum epithelium were characterized. In heart the palmitoyl-CoA synthetase activity was shown to be localized mainly in the sarcosomes; in intestine the microsomes were the site of long-chain acyl-CoA synthesis, while in liver mitochondria and microsomes were about equally active in catalyzing palmitoyl-CoA synthesis.

2. Nagarse treatment almost completely destroyed the cardiac and hepatic palmitoyl-CoA synthetase activity, in contrast to several other membrane-bound enzymes.

INTRODUCTION

In a previous study[1] the relative contribution of the ATP-dependent fatty acid activating enzymes[2,3] [acid:CoA ligase (AMP), EC 6.2.1.2 and EC 6.2.1.3] and the GTP-linked fatty acid activation system[4] was investigated. In sonicated rat-liver mitochondria the "GTP system" had 3–6% of the activity of the "ATP system", when octanoate, palmitate or oleate were used as substrates. We suggested that the "GTP system" was of minor importance in rat-liver mitochondria[1,5,6]. Further research was therefore focused on the ATP-dependent long-chain fatty acyl-CoA synthetase in mitochondrial and microsomal fractions of several tissues.

KORNBERG AND PRICER[3] found in 1953 biosynthesis of long-chain acyl-CoA as well in soluble as in insoluble fractions of guinea pig liver. BORGSTRØM AND WHEELDON[7] reported the occurrence of the enzyme in liver mitochondria and microsomes of the same species. VIGNAIS *et al.*[8] found the highest rate of palmitoyl-CoA production in rat brain in the soluble fraction. Later work revealed that also intestinal preparations from several species contained palmitoyl-CoA synthetase activity. Concerning the subcellular localization in intestinal epithelium contradicting results have appeared[9–12]. Several workers estimated the activation of palmitate in rat liver[1,5,13–19]. A wide range of specific activities in subcellular fractions (mostly mitochondria and microsomes) of liver was reported. Finally GALTON AND FRASER[20] communicated palmitoyl-CoA synthetase activity in adipose tissue homogenates.

There seemed to be a remarkable dearth of reports on long-chain acyl-CoA synthetase in muscle preparations, although fatty acid oxidation is the main source of energy in cardiac muscle, if the concentration of long-chain fatty acids in the coronary blood is sufficiently high. We therefore investigated the ability of rat-heart fractions to synthesize palmitoyl-CoA.

We measured the palmitoyl-CoA synthetase activity in liver, gut and heart by a method developed in our laboratory[14]. This assay, a modification of the method described by FARSTAD *et al.*[13], in which the palmitoyl-CoA formed was trapped with L-carnitine as palmitoylcarnitine, made it possible to avoid the hydroxylamine trap commonly used. While this work was in progress, we discovered the destructive effect of Nagarse (subtilopeptidase A, EC 3.4.4.16) treatment of heart homogenates on the palmitoyl-CoA synthetase activity.

EXPERIMENTAL

Reagents

From Serva Entwicklungslabor, Heidelberg, Nagarse was purchased. Other enzymes and nucleotides were obtained from C.F. Boehringer and Sons, Mannheim. Bovine serum albumin, Fraction V, was supplied by Sigma Chemical Co., St. Louis, Mo., or by Pentex Inc., Kankakee, Ill. Fatty acids were removed from albumin by charcoal treatment[21]. After dialysis and Millipore filtration the albumin was used as a clear, neutral solution. Albumin–fatty acid complexes in a 1:7 molar ratio were prepared by adding to the albumin an adequate amount of recrystallized potassium palmitate in water. Oligomycin and kynuramine were obtained from Sigma Chemical Co., rotenone from Penick and Co., New York, N.Y.

DL-[*Me*-^{3}H]Carnitine was kindly donated by Dr. J. Bremer (Oslo). The radioactive carnitine was diluted with L-carnitine chloride, supplied by Koch–Light Lab. Ltd., Colnbrook, to a specific activity of 0.032 mC/mmole L-carnitine.

Preparations

Male Wistar rats, weighing 200–250 g, used for liver and heart preparations, had free access to food and water. When intestine was needed, rats were treated *per os* one week before use with Metronidazolum [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] (about 4 mg/24 h, for at least 5 days) to get rid of intestinal *Trichomonas vaginalis*[22]. The rats were fasted for about 48 h. All rats were killed by cervical fracture and subsequent bleeding. After removal of the organs care was taken to keep the preparations at 0–4°. Centrifugational g values refer to the bottom of the tube.

Liver was immediately chilled in 0.25 M sucrose–10 mM Tris–HCl (pH 7.4). The liver was weighed, cut into small pieces and washed with sucrose–Tris to remove blood constituents. A 10% (w/v) homogenate was prepared by grinding at low speed in a Potter–Elvehjem homogenizer with a Teflon pestle. The liver homogenate was subsequently centrifuged for 5 min at 900 $\times$ g in a Sorvall RC2-B centrifuge (Rotor SS-34) to remove nuclei, intact liver cells, erythrocytes and debris. The supernatant fluid was taken and centrifuged for 10 min at 5100 $\times$ g. The precipitate of (heavy) mitochondria was suspended in half the original volume of sucrose–Tris and centrifuged for 10 min at 12000 $\times$ g. The fluffy layer was removed from the mitochondrial pellet by gently shaking with small amounts of sucrose–Tris, and the mitochon-

drial fraction was resuspended in sucrose–Tris with the aid of an homogenizer. The 5100 × *g* supernatant fluid was also spun for 10 min at 12000 × *g*. The precipitate, consisting mainly of light mitochondria, was discarded. The supernatant fluid was centrifuged for 50 min at 135000 × *g* in a Beckman L2-65B ultracentrifuge (Rotor SW-27). The microsomal precipitate was washed with small amounts of sucrose–Tris and resuspended in this medium.

From two rat hearts the aortae and auricles were cut away. The hearts were opened, washed with 0.25 M sucrose–10 mM Tris–HCl (pH 7.4) to remove the blood, and weighed. The hearts were placed in 0.25 M sucrose–5 mM ATP–10 mM EDTA (pH 7.4). After chopping with a McIlwain tissue slicer[23], the mince (about 5 % w/v) was subjected to the (hand-operated) homogenizer mentioned above. The mince was magnetically stirred for 30 min and once more subjected to the homogenizer. The heart homogenate was centrifuged for 5 min at 500 × *g* in the Sorvall centrifuge. The supernatant fluid was centrifuged for 10 min at 12000 × *g*. The sarcosomal pellet was washed with a small amount of sucrose–Tris, carefully suspended in sucrose–Tris (about half the original volume) and recentrifuged for 10 min at 12000 × *g*. The sediment was suspended in sucrose–Tris after rinsing the pellet. A heart microsomal fraction was prepared from the first 12000 × *g* supernatant fluid by centrifugation for 50 min at 135000 × *g*.

From two rats the jejunal portions of each intestine were taken and rinsed with 0.15 M NaCl. According to the method of SJÖSTRAND[24] mucus was removed with 5 % (w/v) Ficoll–0.15 M NaCl, and intestinal epithelial sheets were prepared in 2 % (w/v) Ficoll–0.15 M NaCl–0.2 M sucrose (pH 7.4) and collected by centrifugation. The cell sheets were washed and finally homogenized. Further details of the preparation of the homogenate and the differential centrifugation will be described by IEMHOFF *et al.*[22]. In short the homogenate was centrifuged for 10 min at 1000 × *g*. The sediment was rehomogenized and again centrifuged. The combined 1000 × *g* supernatant fluids were centrifuged for 10 min at 15000 × *g*. The resulting mitochondrial pellet was washed with a small amount of sucrose–Tris, suspended and recentrifuged. The sediment was considered to be the intestinal mitochondrial fraction. The combined 15000 × *g* supernatant fluids were centrifuged for 60 min at 368 000 × *g* in the Beckman centrifuge (Rotor 65). The pellet was rinsed and suspended in sucrose–Tris and was designated the intestinal microsomal fraction.

Carnitine palmitoyltransferase was purified from calf-liver mitochondria as described by FARSTAD *et al.*[13].

Palmitoyl-CoA was synthesized according to SEUBERT[25].

Methods

Enzyme assays were carried out at 37°.

Palmitoyl-CoA synthetase [acid:CoA ligase (AMP), EC 6.2.1.3] activity was determined according to FARSTAD *et al.*[13], as modified by VAN TOL AND HÜLSMANN[14]. Palmitoyl-CoA formed from palmitate, coenzyme A and ATP by mitochondrial or microsomal fractions was converted to palmitoyl[^{3}H]carnitine by added [^{3}H]carnitine and carnitine palmitoyltransferase. The incubation mixture consisted of 1 mM potassium palmitate–0.14 mM albumin, 2 mM ATP, 0.2 mM coenzyme A, 5 mM [^{3}H]carnitine (specific activity, 0.032 mC/mmole L-carnitine), 0.3–0.5 mg purified carnitine palmitoyltransferase (0.04–0.1 U), 5 mM phosphoenolpyruvate, about 1.5 U

pyruvate kinase (EC 2.7.1.40), about 1.5 U adenylate kinase (EC 2.7.4.3), 20 mM KCl, 2 mM $MgCl_2$, 120 mM Tris–HCl buffer, 0.5 mM EDTA and 3 μg oligomycin. The reaction was started by the addition of 0.1–0.3 mg mitochondrial or microsomal protein, usually in 0.1 ml 0.25 M sucrose–10 mM Tris–HCl (pH 7.4). Incubation times up to 10 min were used. The final volume was 1.0 ml and the pH 7.4. The reaction was stopped by addition of HCl, and palmitoyl[^{3}H]carnitine was extracted into *n*-butanol[13]. Aliquots of 0.5 ml were transferred to counting vials. The scintillation mixture consisted of 10 ml toluene containing 50 mg 2,5-diphenyloxazole (Packard) and 3 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (Packard), while 0.4 ml NCS solubilizer (Amersham/Searle Corporation) was added to each counting vial. The counting efficiency for tritium was about 30 % as determined by the channels ratio method.

Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed as described in ref. 26. Liberated inorganic phosphate was determined in deproteinized samples as described by SUMNER[27].

Monoamine oxidase (EC 1.4.3.4) activity was measured according to WEISSBACH *et al.*[28], using kynuramine as the substrate.

Cytochrome *c* oxidase (EC 1.9.3.1) activity was determined by the method of SOTTOCASA *et al.*[29] in which a Clark oxygen electrode was used. In the case of heart sarcosomes, freezing and thawing several times was necessary to obtain maximal activity.

Alkaline phosphatase (EC 3.1.3.1) activity was assayed at pH 10.5 with *p*-nitrophenylphosphate as the substrate[30].

NADPH cytochrome *c* reductase (EC 1.6.2.3) activity was measured in the presence of 0.15 μM rotenone by following the reduction of cytochrome *c* at 550 nm[29].

Carboxylesterase (EC 3.1.1.1) activity was measured manometrically as described by HÜLSMANN[31] (see also refs. 32 and 33). Glyceroltributyrate was used as the substrate. 10^{-6} M eserine was included in the incubation medium to inhibit the cholinesterase (EC 3.1.1.8)[33].

Carnitine palmitoyltransferase activity was assayed according to NORUM[34] with palmitoyl-CoA as the substrate. Protein was determined by the biuret method as described by JACOBS *et al.*[35].

RESULTS AND DISCUSSION

Liver

Table I shows the specific activity of the long-chain acyl-CoA synthetase in liver mitochondrial and microsomal preparations together with the activity of some enzymes from which the cross-contamination can be calculated. From the distribution of these marker enzymes it can be concluded that no gross amounts of mitochondrial inner membranes (marker: cytochrome *c* oxidase[36]) or outer membranes (marker: monoamine oxidase[36,37]) pollute the microsomal fraction. Comparatively more microsomes contaminate the mitochondrial fraction. It can be seen from Table I that the microsomal marker enzymes glucose-6-phosphatase[38], NADPH cytochrome *c* reductase[29,39] and carboxylesterase (*cf.* the review given by HAYASE AND TAPPEL[40]) in the mitochondrial fraction of liver have between 12 and 18 % of the specific activities of these enzymes in the microsomal fraction. DE DUVE *et al.*[38], FARSTAD *et al.*[13] and

VAN TOL AND HÜLSMANN[14] reported that the heavy *plus* light mitochondrial fraction had about the same protein content as the microsomal fraction. From the specific activities of palmitoyl-CoA synthetase in Table I it can be concluded that the long-chain acyl-CoA synthetase activity in rat-liver mitochondria and microsomes is approximately the same.

From refs. 1 and 13–17 it can be seen that quite different specific activities are obtained by various investigators for the palmitoyl-CoA synthetase in rat-liver mitochondria and microsomes. These discrepancies may be due to: (1) hormonal and dietary fluctuations, (2) the lability of acyl-CoA synthetase, as has been noticed by BORGSTRØM AND WHEELDON[7], and/or (3) different fractionation methods and activity determinations*.

The dual localization of palmitoyl-CoA synthetase agrees with the concept of the liver as an organ which consumes fatty acids by oxidative processes in the mitochondria, in addition to producing triglycerides and phosphatides mainly in the microsomes. After their synthesis in the microsomes the bulk of the lipids is passed into the systemic circulation.

Heart

Rat heart yields a relatively small amount of microsomes on homogenization (Table I)[32,41]. The rat-liver microsomal markers NADPH cytochrome *c* reductase[29,39] and carboxylesterase[32,40] exhibit also in heart the highest specific activity in the expected microsomal fraction (see Table I and ref. 32). From the specific activities of cytochrome *c* oxidase, a marker enzyme for heart sarcosomes[32,42], and the aforementioned microsomal markers in the sarcosomal and microsomal fractions, it can be concluded that a mutual contamination of 20–25 % exists. From Table I it can be seen that the specific activity of the palmitoyl-CoA synthetase is somewhat higher in the microsomal fraction. Hence it can be concluded that heart microsomes are able to activate palmitate. Their contribution to the total fatty acid activation of heart cells, however, is small, < 15 % of the mitochondrial activation.

Heart muscle, contrary to liver, mainly consumes fatty acids as substrates. In order to maintain an appropriate ATP level for contraction, acyl-CoA is broken down in the sarcosomes. The finding of a palmitoyl-CoA synthetase in the sarcosomes is therefore not unexpected. That heart microsomes do not contribute very much to fatty acid activation is in agreement with the function of the organ. The organ mainly generates mechanical work and does not, unlike liver, produce large amounts of substrates for the benefit of other organs.

Intestine

Table I shows the palmitoyl-CoA synthetase activity in mitochondrial and microsomal fractions of rat-intestinal epithelial cells. Cytochrome *c* oxidase has been used as a marker for intestinal mitochondria[9,43]. Carboxylesterase is a microsomal enzyme in rat liver[40] and heart[32]. NADPH cytochrome *c* reductase, which has been used by CLARK *et al.*[43] as a marker of the endoplasmic reticular fraction to differentiate it from membranes of the brush border portion of intestinal cells, shows essentially the same distribution pattern as carboxylesterase (Table I). The mitochondrial frac-

* Using either the carnitine- or the hydroxylamine-trapping method[15], in our hands the high acyl-CoA synthetase activity in the nuclear fraction, as reported by PANDE AND MEAD[15], is absent.

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TABLE I

FATTY ACID ACTIVATION IN RAT-LIVER, HEART AND GUT FRACTIONS

Where indicated the following markers have been used: cytochrome *c* oxidase and monoamine oxidase for the mitochondria; glucose-6-phosphatase, NADPH cytochrome *c* reductase and carboxylesterase for the microsomes. Enzyme activities are given in nmoles/min per mg protein at 37°. Statistics refer to means ± S.D.; *n* = number of experiments.

	Liver				*Heart*				*Gut*			
	n	*Mitochondria*	*n*	*Microsomes*	*n*	*Mitochondria*	*n*	*Microsomes*	*n*	*Mitochondria*	*n*	*Microsomes*
Protein (mg/g tissue, wet wt.)	5	23.8 ± 5.5	4	25.3 ± 10.4	7	10.2 ± 1.8	3	1.48 ± 0.24	3	1.7 ± 0.2	3	2.3 ± 0.7
Palmitoyl-CoA synthetase (Δ palmitoylcarnitine)	6	82.3 ± 20.6	5	77.0 ± 13.7	7	26.8 ± 2.2	3	38.3 ± 4.8	3	10.8 ± 4.6	3	23.1 ± 6.6
Cytochrome *c* oxidase ($-\Delta O_2$)	5	808 ± 309	4	13 ± 16	7	1720 ± 280	3	600 ± 60	3	200 ± 47	3	12.7 ± 9.6
Monoamine oxidase ($-\Delta$ kynuramine)	4	9.7 ± 1.5	3	1.7 ± 0.8		—		—		—		—
Glucose-6-phosphatase (Δ P_i)	4	46 ± 12.8	3	250 ± 12.2		—		—		—		—
NADPH cytochrome *c* reductase (Δ cytochrome *c* reduced)	4	23 ± 8.8	3	182 ± 62	4	8.5 ± 1.0	2	34.2 ± 0.8	3	30 ± 8.3	3	34.2 ± 9.1
Carboxylesterase (Δ CO_2)	3	142 ± 57	3	1120 ± 281	1	65	1	269	3	7670 ± 509	3	11 400 ± 494
Alkaline phosphatase (Δ *p*-nitrophenol)		—		—		—		—	3	3.9 ± 0.8	3	43.5 ± 1.9

tion, when corrected for the apparent microsomal contamination, seems to lack palmitoyl-CoA synthetase. This conclusion is in agreement with the findings of SENIOR AND ISSELBACHER[10,11] and AILHAUD *et al.*[9]. These investigators made use of alkaline phosphatase as a microsomal marker[9,11]. EICHHOLZ AND CRANE[44], however, showed that at least in hamster this enzyme is localized in brush border membranes (see also refs. 45 and 46). The alkaline phosphatase activity in the microsomal fraction (see Table I) results, in part, from contamination with brush border fragments as can be concluded from the sucrase (EC 3.2.1.26) activity[43]. After separation of the brush border fragments from the microsomal fraction, it was shown that in the brush border almost no long-chain acyl-CoA synthetase activity was present (Dr. J. W. O. Van den Berg, personal communication). FORSTNER *et al.*[47] reported a specific activity of synthetase in the brush borders of 1/5 that of the microsomes. From our experiments it can be concluded that in rat-gut palmitoyl-CoA synthetase is mainly or even completely a microsomal enzyme. For preparative reasons intestinal fractions were obtained from fasted rats. SENIOR AND ISSELBACHER[11] stated that fasting did not influence the rat-intestinal palmitoyl-CoA synthetase.

The activity found by us, 23 nmoles/min per mg protein at 37°, is higher than that reported by SENIOR AND ISSELBACHER[11] (3–17 nmoles/min per mg protein). RODGERS[48], who used the method of SENIOR AND ISSELBACHER (fatty acyl-hydroxamate formation), measured a specific activity of 97 nmoles/min per mg protein.

In the digestive tract fat from nutrients is attacked by lipases of the succus entericus. Free fatty acids and monoglycerides are subsequently absorbed by the epithelium cells of the small intestine. Evidence has been presented that fatty acids are activated in the intestinal microsomes (Table I, refs. 9 and 10). The acyl-CoA is used for (microsomal) triglyceride and phosphatide synthesis[10–12], after which these

TABLE II

INFLUENCE OF NAGARSE TREATMENT OF RAT-HEART AND LIVER HOMOGENATES ON THE ACTIVITY OF SEVERAL MEMBRANE-BOUND ENZYMES

Mitochondrial and microsomal preparations were isolated from five rat hearts as described under *Preparations*. Before being stirred for 30 min at 0° 5 mg Nagarse were added to one half of the mince. In the case of liver, 15 mg Nagarse were added to one half (50 ml) of a 10% homogenate. Both homogenates were stirred for 30 min at 0° prior to differential centrifugation as described under *Preparations*.

Fraction	*Enzyme*	*Specific activity (nmoles/min per mg protein)*	
		− Nagarse	*+ Nagarse*
Heart			
Mitochondria	Palmitoyl-CoA synthetase	27.5	0.5
	Cytochrome *c* oxidase	1560	1410
Microsomes	Palmitoyl-CoA synthetase	40.0	0.8
	NADPH cytochrome *c* reductase	35.0	2.2
	Carboxylesterase	270	245
Liver			
Mitochondria	Palmitoyl-CoA synthetase	53.3	1.0
	Monoamine oxidase	9.2	10.3
Microsomes	Palmitoyl-CoA synthetase	66.7	0.0
	NADPH cytochrome *c* reductase	267	25
	Glucose-6-phosphatase	250	237

lipids enter the organism mainly *via* the intestinal lymphatics. In contrast to heart cells, the intestinal cells are mainly involved in the production of lipids for other organs. Therefore utilization of lipids for the proper function of intestinal epithelial cells is quantitatively of minor importance. Hence, it is understandable that in the gut the endoplasmic reticulum is the main site of fatty acid activation. This tubular system can function for large molecules or complexes like chylomicrons as a secretory pathway leading to extracellular spaces[49].

In earlier experiments with rat heart, employing the method of CHANCE AND HAGIHARA[50] for preparing sarcosomes (with Nagarse), it was not possible to find significant long-chain acyl-CoA synthesis. The purpose of the use of Nagarse was to obtain more sarcosomal protein. The sarcosomes showed excellent respiratory capacity, good oxidative phosphorylation and respiratory control ratio[51] with various substrates (*cf.* refs. 52–54). However, it can been seen from Table II that Nagarse treatment destroys the fatty acid activating enzyme. Mitochondrial inner membrane and matrix enzymes are probably not accessible to Nagarse. A certain specificity of Nagarse inactivation, which may be useful in localization studies, can be concluded from Table II. In the study of fatty acid oxidation Nagarse treatment could conceivably be used to eliminate the mitochondrial outer membrane and microsomal long-chain fatty acid activating enzyme.

ACKNOWLEDGMENTS

We would like to thank Drs. J. W. O. Van den Berg and W. G. J. Iemhoff for the preparation of intestinal fractions. We are grateful to Miss C. Kalkman for skillful technical assistance.

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