Biochimica et Biophysica Acta, 357 (1974) 14–23

BBA 46777

THE EFFECT OF FASTING ON THE ACYLATION OF CARNITINE AND GLYCEROPHOSPHATE IN RAT LIVER SUBCELLULAR FRACTIONS*

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(Received January 16th, 1974)

SUMMARY

1. The effect of a 48 h fast on the acylation rates of carnitine and glycerophosphate by isolated liver mitochondria and microsomes is measured, in the presence of ATP and CoASH, with palmitate (complexed to albumin) as the acyl donor.

2. During fasting the acylation rate of carnitine increases 2–3 fold in the microsomal fraction and by a small percentage in the mitochondria. It is not inhibited by palmitoyl-CoA at concentrations up to 0.1 mM.

3. During fasting the acylation rate of glycerophosphate decreases by 33 % in isolated mitochondria and by 13 % in microsomes. High concentrations of palmitoyl-CoA (above 0.02 mM) inhibit the reaction.

4. The significance of these results for the partition of long-chain fatty acids between the esterification and oxidation pathways is discussed.

INTRODUCTION

Some years ago we reported the existence of extramitochondrial palmitoyl-CoA: L-carnitine *O*-palmitoyltransferase (trivial name carnitine palmitoyltransferase; EC 2.3.1.21) in rat liver [1], in addition to the well known carnitine palmitoyltransferase located in the inner mitochondrial membrane [2–4]. The extramitochondrial enzyme sediments in the microsomal fraction and its activity is increased in fasted rats [1]. In vivo [5, 6] as well as in vitro studies, both in the perfused liver [7, 8] and in isolated mitochondria [9, 10], indicate that active ketogenesis during fasting depends on the acylation of carnitine by carnitine palmitoyltransferase.

The increase in the activity of carnitine palmitoyltransferase in fasted rat liver was first reported by Norum [11]. Aas and Daae [12] measured the activities of carnitine palmitoyltransferase and acyl-CoA: *sn*-glycerol-3-phosphate *O*-acyltransferase (trivial name glycerophosphate acyltransferase; EC 2.3.1.15) in crude rat

* Part of this work was presented at the 9th International Congress of Biochemistry, Stockholm, 1973.

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liver homogenates and found these enzymatic activities to vary in opposite directions during fasting. These findings suggest a regulation of fatty acid metabolism at the level of the acylations of glycerophosphate and carnitine. Glycerophosphate acyltransferase is located at the endoplasmic reticulum and the mitochondrial outer membrane [13], that is outside the permeability barrier for acyl-CoA [14, 15] (the mitochondrial inner membrane [16–18]). The bulk of the long-chain acid: CoA ligase(AMP-forming)(trivial name acyl-CoA synthetase; EC 6.2.1.3) of the rat liver cell has the same intracellular localization [2, 19–22]. It follows that any competition for long-chain acyl-CoA between carnitine and glycerophosphate, by way of the acylating enzymes, has to take place outside the mitochondrial inner membrane. The relative activities of the esterification and oxidation pathways of fatty acid metabolism in rat liver are known to be affected by the nutritional state of the animal [8, 23]. Therefore we determined the effect of fasting on the acylation rates of carnitine and glycerophosphate in isolated microsomes as well as mitochondria. Evidence has been presented that only a very small part of the mitochondrial carnitine palmitoyltransferase is able to react with extramitochondrial acyl-CoA in intact mitochondria [24–27]. Therefore, the extramitochondrial activity becomes increasingly important in a possible competition for acyl-CoA between the acylation of carnitine and glycerophosphate. Palmitate is a good acyl donor for the acylation of glycerophosphate in mitochondria and microsomes isolated from rat liver [28] and was used in all experiments.

The results presented in this paper, in addition to giving new evidence in favor of the existence of extramitochondrial carnitine palmitoyltransferase, suggest that the regulation of the partition of long-chain fatty acids between the oxidation and the esterification pathways could very well occur at the level of the acylation of carnitine and glycerophosphate by extramitochondrial acyl-CoA. The biggest relative effect of fasting was observed on the activity of microsomal carnitine palmitoyltransferase, which was increased 2–3-fold.

MATERIALS AND METHODS

Materials

sn-[1(3)-^{3}H]Glycero-3-phosphate was prepared enzymatically as described by Smith and Hübscher [29] using [1(3)-^{3}H]glycerol. L-[*Me*-^{3}H]carnitine was prepared according to Stokke and Bremer [30] and kindly donated by Drs P. H. E. Groot and H. R. Scholte. Radioactive glycerol and methyliodide were purchased from the Radiochemical Centre, Amersham, England. L-carnitine and L-palmitoylcarnitine were donated by Dr I. Kizawa from Otsuka Pharmaceutical Co., Osaka, Japan and all enzymes were purchased from Boehringer and Sons, Mannheim, Germany.

Complexes of palmitate and defatted bovine serum albumin (molar ratio 7 : 1) were prepared exactly as described before [21]. All other reagents used in the experiments have been described before [1].

Animals

Male rats of the Wistar strain were used for all experiments. The animals had free access to food and water (except in the fasting experiments where only water was supplied) and were killed by cervical dislocation. The average body weight decreased

from 258 to 228 g during a 48 h fast; the average liver weight decreased from 10.3 to 6.4 g. There was no drastic change in the overall hepatic protein concentration and in the amounts of cytosolic, mitochondrial and microsomal protein per gram of liver.

Isolation of subcellular fractions

Heavy rat liver mitochondria (M) and microsomes (P) were isolated as described before [1], except that the mitochondria were washed once. The mutual contamination of the fractions was 11–13 % as judged by the activities of cytochrome *c* oxidase (EC 1.9.3.1) and NADPH-cytochrome *c* reductase (EC 1.6.2.4). The contamination was the same if fed or fasted rats were used. Where indicated the isolated fractions were suspended in water instead of 0.25 M sucrose. The subcellular fractions were used within 30 min after isolation.

Assay procedures for the acylation of glycerophosphate and carnitine

The incubations were carried out for 5 min at 37 °C and pH 7.4 in a final volume of 1 ml. The substrate of the acylation reaction (palmitoyl-CoA) was formed during a 5 min preincubation of the enzyme fractions (mitochondria or microsomes) with palmitate (complexed to albumin), ATP and CoASH in the presence of an ATP regenerating system (see below). The reaction was started after this preincubation by the addition of radioactive glycerophosphate or carnitine. As the activity of acyl-CoA synthetase in the subcellular fractions is much higher than the activity of the subsequent acylations (cf. ref. 1) this acyl-CoA feeding system will not only generate palmitoyl-CoA during the preincubation, but also replenish any palmitoyl-CoA used in the acylation reactions or hydrolyzed by palmitoyl-CoA hydrolase (EC 3.1.2.2.).

The assay medium consisted of 100 mM Tris–HCl, 20 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 5 mM ATP, 5 mM phosphoenolpyruvate, 0.5 units of pyruvate kinase (EC 2.7.1.40), 1.5 units of adenylate kinase (EC 2.7.4.3), 0.5 mM potassium palmitate, 0.07 mM bovine serum albumin, 2 mM GSH, 5 mM KCN and variable amounts of CoASH and carnitine or glycerophosphate, as indicated. Where indicated palmitoyl-CoA was added instead of CoASH and the preincubation was omitted. Sometimes a simplified assay medium was used consisting of 100 mM Tris–HCl (pH 7.4), 20 mM KCl, 0.5 mM EDTA, 2 mM GSH and variable amounts of palmitoyl-CoA. The reactions were stopped by the addition of concentrated HCl and the radioactive products were specifically extracted with *n*-butanol [13, 19]. Further analysis of the reaction products by thin-layer chromatography (now using ^{14}C as the label) gave essentially the same results as published by Daae and Bremer [13]. The activities were proportional with the protein concentration up to 0.2 mg of mitochondrial and up to 0.4 mg of microsomal protein.

Sonication or repeated cycles of freezing and thawing did not affect the activities measured in the mitochondrial fraction and caused inhibition of the microsomal carnitine palmitoyltransferase. Therefore the isolated subcellular fractions were used without further treatment. All other enzymes were assayed as described before [1, 21].

Protein determinations

The protein content of the subcellular fractions was determined by the method

of Lowry et al. [31]. Specific activities of enzymatic reactions are expressed as munits/mg of mitochondrial or microsomal protein.

Measurement of radioactivity

The radioactive samples were dissolved in Insta-gel (Packard Instrument Co.) and counted in a Packard Tri-Carb liquid scintillation spectrometer Model 3380.

RESULTS

In Table I the acylation of 2.5 mM L-carnitine is compared using different assay conditions. It can be seen that the addition of palmitoyl-CoA instead of CoASH to the complete assay mixture (see Materials and Methods) does not change the rate of palmitoylcarnitine production in either mitochondria or microsomes. It can be concluded therefore that the generation of palmitoyl-CoA from palmitate and CoASH during the preincubation is quantitative and gives optimal rates of palmitoylcarnitine formation. If the simplified assay mixture is used (in which no fatty acid activation can occur; see Materials and Methods) the acylation rate of carnitine by 90 μM palmitoyl-CoA is suboptimal. At low palmitoyl-CoA concentrations (9 μM) a continuous regeneration of palmitoyl-CoA during the incubation is necessary to measure optimal rates of palmitoylcarnitine formation. It is concluded that the complete assay medium is necessary to measure optimal activities of carnitine palmitoyltransferase. The high specific activity of acyl-CoA synthetase in both isolated mitochondria and microsomes (70–80 munits/mg at 37 °C; see refs 1 and 21) provides a quantitative acylation of the limiting amounts of CoASH (up to 0.1 mM) during

TABLE I

ACYLATION OF L-CARNITINE IN RAT LIVER FRACTIONS USING DIFFERENT ASSAY CONDITIONS

For details of the complete and simplified assay mixtures see Materials and Methods. In this experiment the enzyme fractions (isolated from the liver of a rat fasted for 24 h) were suspended in water and the KCN was omitted from the assay medium. If palmitoyl-CoA was added, the reaction was started without preincubation. A representative experiment out of a series of three is shown. The values given are the average of duplicate measurements using 0.1 and 0.2 mg of mitochondrial or 0.2 and 0.4 mg of microsomal protein. The activities are given as nmoles of palmitoylcarnitine formed per mg of protein/min. The [L-carnitine] was 2.5 mM. The addition of 0.07 mM bovine serum albumin to the simplified assay mixture did not increase the measured activities.

Assay mixture	Carnitine palmitoyltransferase activity (munits/mg)	
	Mitochondria	Microsomes
Complete, 9 μM CoASH, 5 min preincubation	14.8	5.0
Complete, 90 μM CoASH, 5 min preincubation	26.3	10.1
Complete, 9 μM palmitoyl-CoA	17.1	5.5
Complete, 90 μM palmitoyl-CoA	25.2	9.0
Simplified mixture, 9 μM palmitoyl-CoA	2.3	1.5
Simplified mixture, 90 μM palmitoyl-CoA	12.5	4.3

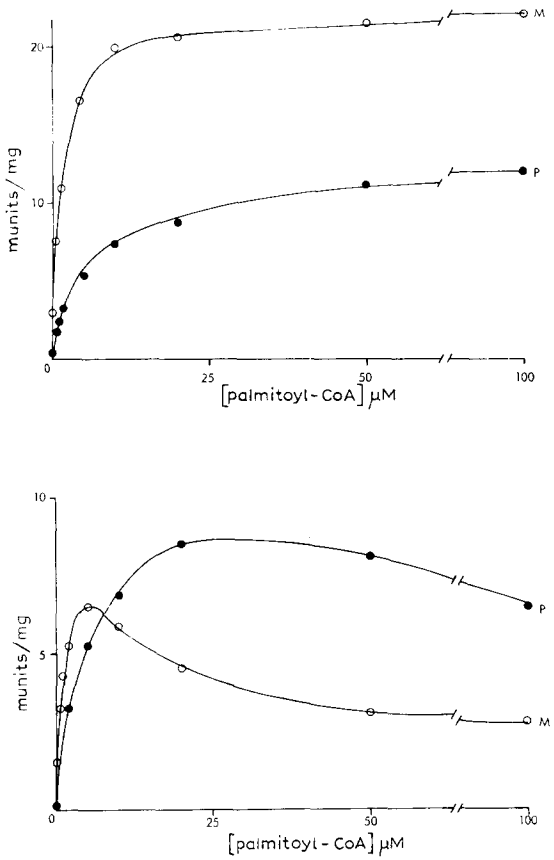

Fig. 1. The influence of the [palmitoyl-CoA] on the acylation of 0.25 mM L-carnitine (upper figure) and 3.1 mM L-glycero-3-phosphate (lower figure) by mitochondria (M) and microsomes (P) isolated from the liver of a rat fasted for 48 h. The activities, which are a measure for the enzymatic activity of carnitine palmitoyltransferase and of glycerophosphate acyltransferase respectively (see text), are the average of duplicate values. For details of the isolation of the subcellular fractions and the assay procedure see Materials and Methods.

the 5 min preincubation. This was checked by autoradiography using [^{14}C]palmitate (cf. ref. 1) and is also supported by the experiments shown in Table I. Both low (9 μM) and high (90 μM) concentrations of CoASH are quantitatively converted to palmitoyl-CoA during the preincubation. Furthermore, the complete assay mixture provides a constant palmitoyl-CoA concentration during the actual incubation with radioactive carnitine or glycerophosphate.

Fig. 1 (upper part) shows the acylation of 0.25 mM L-carnitine, a concentration close to the intracellular content [32–35], as a function of the palmitoyl-CoA concentration in mitochondria and microsomes isolated from fasted rat liver. 0.5 mM palmitate (complexed with albumin), ATP and CoASH provide the formation of palmitoyl-CoA during a 5 min preincubation (see above). It can be seen from this figure that the concentration of palmitoyl-CoA, at which half maximal activity is measured, is much lower in the mitochondria (2 μM) than in the microsomes (7 μM).

TABLE II

THE ACYLATION OF CARNITINE AND GLYCEROPHOSPHATE BY SUBCELLULAR FRACTIONS ISOLATED FROM LIVERS OF FED AND FASTED RATS (48 h)

The acylation rates of carnitine and glycerophosphate measured in isolated mitochondria (M) and microsomes (P) are given per mg of protein together with the standard deviations. The number of experiments, each run in duplicate, is given in brackets. The acylation rate of carnitine, which is a measure for the activity of carnitine palmitoyltransferase (see text) was measured with 0.25 mM L-carnitine and 0.1 mM palmitoyl-CoA. The acylation rate of glycerophosphate, which is a measure for glycerophosphate acyltransferase activity (see text) was measured with 3.1 mM L-glycerophosphate and 5 μM or 20 μM palmitoyl-CoA in the mitochondria and microsomes respectively (cf. Fig. 1). For further details of the assay conditions and the isolation of the subcellular fractions see Materials and Methods.

Enzyme activities (munits/mg)	Fed rats		Fasted rats	
	(M)	(P)	(M)	(P)
Carnitine palmitoyltransferase	16.7±3.9 (6)	6.4±1.7 (6)	18.7±3.1* (4)	14.0±3.9** (6)
Glycerophosphate acyltransferase	9.7±2.4 (4)	11.1±4.5 (5)	6.5±2.1*** (4)	9.7±3.9* (5)

* Probability (P) that the difference with the value in fed rats is caused by chance alone $0.10 < P < 0.20$

** $0 < P < 0.01$

*** $0.05 < P < 0.10$

The K_m for CoASH of the acyl-CoA synthetase reaction, measured under comparable conditions, is about 20 μM in mitochondria as well as microsomes (unpublished observation; see also ref. 19). This indicates that the carnitine palmitoyltransferase reaction is rate limiting in this system, even if the concentration of CoASH is below 20 μM. If the system is saturated with palmitoyl-CoA (100 μM) the specific activity of carnitine palmitoyltransferase in the microsomes is more than 60 % of the mitochondrial value. The maximal activities, measured with 100 μM palmitoyl-CoA, are summarized in Table II. It shows that the specific activity of carnitine palmitoyltransferase increases 2–3-fold during fasting. The activity in the mitochondria shows a 12 % increase, which is, however, hardly significant statistically (cf. refs 1 and 11).

Fig. 1 also shows (lower part) the effect of palmitoyl-CoA concentration on the acylation rate of 3.1 mM glycerophosphate in mitochondria and microsomes isolated from fasted rat liver. The only difference between the experiments shown in the upper part of Fig. 1 is the replacement of carnitine by glycerophosphate. For reasons discussed above in connection with the assay of carnitine palmitoyltransferase it is concluded that also in this system the activation of palmitate is not rate limiting for the acylation of glycerophosphate. Lysophosphatidic acid and phosphatidic acid are the main products (see Materials and Methods and ref. 13) and because of the reported positional specificity and acyl group preferability [36–39] it is concluded that the formation of butanol extractable radioactivity gives an accurate measurement of the activity of glycerophosphate acyltransferase in this system. It can be seen that high levels of palmitoyl-CoA (0.1 mM), which give optimal activities of carnitine palmitoyltransferase (Fig. 1), inhibit the activity of glycerophosphate acyltransferase by 57 %

TABLE III

EFFECT OF [PALMITOYL-CoA] AND [L-CARNITINE] ON THE ACYLATION OF CARNITINE BY FRACTIONS ISOLATED FROM LIVERS OF FED AND FASTED RATS (48 h)

The [palmitoyl-CoA] at which half of the maximal activity is observed was estimated from double reciprocal plots of the ascending part of curves as shown in Fig. 1, using 0.25 mM L-carnitine. The K_m for L-carnitine was estimated by the same method using 100 μM palmitoyl-CoA. For the isolation of mitochondria (M) and microsomes (P) and further details of the assay procedure see Materials and Methods. Where possible standard deviations are given, if not the value is the average of 2 experiments. Corrections were made for the amount of endogenous CoA in the subcellular fractions.

	Fed rats		Fasted rats	
	(M)	(P)	(M)	(P)
[Palmitoyl-CoA] at which half maximal activity is measured (μM)	1.7±0.5	7.1±2.2*	1.8	7.6
K_m for L-carnitine (mM)	0.17±0.11	0.18±0.08	0.21	0.19±0.05

* Probability that the difference with the value in (M) is caused by chance alone is <0.01.

and 24 % in the mitochondria and microsomes respectively. The maximal activities, measured with 5 or 20 μM palmitoyl-CoA in the mitochondria and microsomes respectively, are again summarized in Table II. During a 48 h fast the specific activity of glycerophosphate acyltransferase goes down by 33 % in the mitochondria and by 13 % in the microsomes. The decrease in the microsomal fraction is however hardly significant statistically.

From the ascending part of saturation curves of the type shown in Fig. 1 the concentration of palmitoyl-CoA, at which half maximal activity is observed, was estimated from double reciprocal plots. These values are summarized in Tables III and IV for the acylation of carnitine and glycerophosphate respectively. The apparent

TABLE IV

EFFECT OF [PALMITOYL-CoA] AND [GLYCEROPHOSPHATE] ON THE ACYLATION OF GLYCEROPHOSPHATE BY FRACTIONS ISOLATED FROM LIVERS OF FED AND FASTED RATS (48 h)

The [palmitoyl-CoA] at which the half maximal activity is observed was estimated from double reciprocal plots of the ascending part of curves as shown in Fig. 1, using 3.1 mM L-glycero-3-phosphate. The K_m for glycerophosphate was estimated by the same method using 5 or 20 μM palmitoyl-CoA if measuring the activity of the mitochondria (M) or microsomes (P) respectively. Corrections were made for the amount of endogenous CoA in the subcellular fractions. Where possible standard deviations are given, if not the value is the average of 2 experiments. For the isolation of the subcellular fractions and further details of the assay procedure see Materials and Methods.

	Fed rats		Fasted rats	
	(M)	(P)	(M)	(P)
[Palmitoyl-CoA] at which half maximal activity is measured (μM)	1.6±0.8	4.4	1.4	4.8
K_m for L-glycero-3-phosphate (mM)	0.5±0.3	1.7±0.9	0.3	1.7±1.1

K_m for carnitine or glycerophosphate is shown in the same tables. The K_m values were determined in comparable experiments using a fixed concentration of palmitoyl-CoA and variable amounts of carnitine or glycerophosphate (for details see the legends to Tables III and IV).

Tables III and IV show that fasting did not affect any of the parameters mentioned significantly. The K_m for carnitine of the carnitine palmitoyltransferase reaction is about 0.19 mM in both mitochondriaand microsomes. The [palmitoyl-CoA] at which half maximal activity is measured however is significantly higher in the microsomal fraction compared to the mitochondria. This difference cannot be explained by the lower amount of protein used in the mitochondrial acylations, since in both mitochondria and microsomes there was a linear relationship between enzymatic activity and amount of protein. It can be seen from Table IV that the K_m for glycerophosphate was rather variable, especially in the microsomes. The reason for this is not clear. It must be noted that the values for the mitochondrial and the microsomal enzyme were not determined in the presence of the same amount of palmitoyl-CoA, as the optimal concentration differed in both fractions (see Fig. 1). Therefore these K_m values for glycerophosphate of the glycerophosphate acyltransferases cannot be compared directly.

DISCUSSION

The present study provides additional evidence in favor of the existence of extramitochondrial carnitine palmitoyltransferase in rat liver [1]. The extramitochondrial acylation of carnitine is increased 2–3-fold in fasting while the increase in the mitochondria is hardly significant statistically. Norum [11] reported activation of carnitine palmitoyltransferase activity in livers from fasted, fat-fed and diabetic rats. Rats treated with thyroxine also have increased hepatic activities of carnitine palmitoyltransferase, with exactly the same distribution between intra- and extramitochondrial activities as fasted rats [40]. So it appears that the extramitochondrial activity is strongly influenced by the hormonal and nutritional state of the animal, while the mitochondrial activity is affected relatively little.

Whereas the microsomal activity of carnitine palmitoyltransferase is more than doubled in fasting, the activity of glycerophosphate acyltransferase is decreased, predominantly in the mitochondria (Table II). The acylation of glycerophosphate is sensitive to inhibition by high concentrations of palmitoyl-CoA, while the acylation of carnitine is not affected under the conditions used (see Fig. 1). The mitochondrial glycerophosphate acylation is more sensitive to inhibition by palmitoyl-CoA than the microsomal activity. Both the intracellular levels of CoASH [32–35] and of long-chain acyl-CoA [33, 41] increase during fasting, so a similar effect could occur in vivo.

The hepatic content of carnitine [32–35] is about the same as the concentration needed for half maximal carnitine acylation in both fed and fasted rat (cf. Table III). No agreement can be found in the literature on the glycerophosphate content of rat liver. As the lowest values are probably the most reliable (see ref. 47) the level in livers from fed rats will be about 0.13 μmoles/gram wet weight [42, 43]. In fasted rats both higher [44] and lower [45] values have been measured (0.05–0.54 μmoles/gram wet weight). As these values are in the same range or lower than the measured apparent K_m for glycerophosphate in the acylation reaction (Table IV) any change in the intracellular content or the K_m could directly influence the acylation rate.

The acylation reactions of carnitine and glycerophosphate are situated metabolically at the branchpoint of fatty acid oxidation (ketogenesis) and lipid synthesis. The percentage of fatty acid metabolized by the liver which is channelled into the pathway of fatty acid oxidation (ketogenesis) is increased in fasted rats [23]. The increase in extramitochondrial carnitine palmitoyltransferase activity may be important in this respect (cf. ref. 46). As the amount of carnitine in the liver cell is limited, acylcarnitine has to be oxidized in order to make possible a continuous formation of acylcarnitine. The oxidation rate will in turn depend on the rate of formation of ADP by energy requiring processes.

While the results described in this paper suggest a regulation of fatty acid metabolism at the level of long-chain acyl-CoA, the author does not want to exclude other points of regulation. The energy requirement of the liver and the substrate availability are important factors determining the partition of fatty acids between oxidation and lipid synthesis. An alternative pathway for glycerolipid synthesis by way of dihydroxyacetone phosphate has been described [47, 48]. The regulation of this pathway remains to be investigated. Monoacylglycerophosphate is an intermediate in the synthesis of both phospholipids and triglycerides and additional regulation at the level of phosphatidic acid is likely [49].

ACKNOWLEDGEMENTS

The author is indebted to Mr T. van Gent for his expert technical assistance and to Ms A. A. E. d'Hamecourt-Broekmans for typewriting the manuscript. Dr W. C. Hülsmann is thanked for this stimulating interest.

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