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DUAL LOCALIZATION AND PROPERTIES OF ATP-DEPENDENT LONG-CHAIN FATTY ACID ACTIVATION IN RAT LIVER MITOCHONDRIA AND THE CONSEQUENCES FOR FATTY ACID OXIDATION

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SUMMARY

1. Direct evidence is given for the existence of two ATP-dependent palmitoyl-CoA synthesizing enzymes, localized in different compartments of the rat-liver mitochondrion.

2. About 90 % of the total activity of rat liver mitochondria is localized in the mitochondrial outer membrane and about 10 % in the inner membrane-matrix compartment.

3. The two enzyme systems show different apparent K_m's for fatty acid and ATP, and different apparent K_i's for AMP and adenosine.

4. The inner membrane-matrix enzyme, in contrast to the outer membrane and the microsomal enzyme, is strongly inhibited by octanoate.

5. Comparison of the kinetics of the two ATP-dependent long-chain fatty acid-activating enzymes with the kinetics of long-chain fatty acid oxidation shows, that during fatty acid oxidation at low concentrations of palmitate or oleate, in the presence of carnitine, the outer membrane acyl-CoA synthetase is operating. In the absence of carnitine and at high concentrations of long-chain fatty acids the activation reaction occurs in the inner membrane-matrix compartment of the mitochondrion.

6. The long-chain fatty acid concentration needed for half-maximal velocity of fatty acid oxidation by isolated rat liver mitochondria is about 1 μM in the presence of carnitine and 100–200 μM in the absence of carnitine.

INTRODUCTION

The subcellular distribution of the ATP-dependent long-chain acyl-CoA synthetase (acid: CoA ligase (AMP), EC 6.2.1.3) in the rat liver cell has been extensively studied[1–5]. It is generally agreed that the enzyme is localized both in mitochondria and microsomes. In fact we found that mitochondria and microsomes exhibit about equal activities[3,4]. The study of the intramitochondrial localization of long-chain fatty acid activation has obtained much less attention. NORUM *et al.*[6] reported "that the mitochondrial ATP-dependent enzyme most likely is confined to the outer membrane". Also from experiments of VAN DEN BERGH *et al.*[7], it can be concluded that the bulk of

the ATP-dependent long-chain fatty acid activation is localized in the mitochondrial outer membrane.

The data of the present paper support these findings. In addition we are able to demonstrate, by the use of Nagarse (subtilopeptidase A; EC 3.4.4.16), which destroys the greater part of ATP-dependent long-chain fatty acid activation in rat-liver mitochondria[4,8], that the activity remaining after Nagarse treatment is localized in the inner membrane-matrix compartment. Several properties of the two enzyme systems are described.

BREMER[9] and FRITZ[10] have suggested that activated long-chain fatty acids are transported through the mitochondrial inner membrane, which is impermeable for acyl-CoA[11], as acylcarnitines. This is very likely to be the case with heart sarcosomes, but for liver mitochondria an alternative mechanism may well exist. VAN DEN BERGH[12] and ROSSI *et al.*[13] reported carnitine-independent oxidation of long-chain fatty acids by rat liver mitochondria, an observation which is confirmed by the present study. DE JONG AND HÜLSMANN[14] showed that Nagarse treatment does not impair palmitate oxidation by rat liver mitochondria.

From the kinetics of the carnitine-independent long-chain fatty acid oxidation and the dual localization and properties of long-chain fatty acid activation, it is concluded that in the absence of carnitine the fatty acid activation needed for fatty acid oxidation occurs in the inner membrane-matrix compartment of the rat liver mitochondrion.

MATERIALS AND METHODS

Reagents

Nagarse was purchased from Serva Entwicklungslabor, Heidelberg. 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 the albumin by charcoal treatment[15]. After dialysis and Millipore filtration, the albumin was used as a clear, neutral solution. Fatty acid–albumin solutions were prepared by mixing the potassium salt of the fatty acid with albumin in a molar ratio of 7:1. *p*-Dimethylaminobenzylamine (Lot-KP-1065) was purchased from Cyclo Chemical (Division Travenol Laboratories Inc., Los Angeles, Calif.), rotenone from Penick and Co., New York, N.Y. and antimycin A and oligomycin from Sigma.

DL-[*Me*-^{3}H]Carnitine with specific activity of 75 μC/μmole was kindly donated by Dr. J. Bremer from Oslo University, and L-carnitine was a gift from Otsuka Pharmaceutical Factory (Osaka, Japan). The radioactive carnitine was diluted with L-carnitine to a specific activity of 0.05 μC/μmole L-carnitine. Radioactive fatty acids were purchased from the Radiochemical Centre (Amersham, England). All other reagents were prepared exactly as described before[3].

Animals

Male rats of the Wistar strain were used for the experiments (weight 200–250 g). The animals had free access to food and water and were killed by cervical fracture and subsequent bleeding.

Preparation of subcellular fractions

Twice-washed heavy rat liver mitochondria were prepared exactly as described before[3]. Nagarse-treated mitochondria were prepared by stirring a 10% rat liver homogenate in 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4) with 0.2 mg/ml Nagarse for 30 min at 0° before the mitochondria were isolated and washed twice. 12000 × *g* supernatant was isolated by carefully pipetting off the supernatant after centrifuging a 5100 × *g* supernatant of a 10% rat liver homogenate for 10 min at 12000 × g_{max}.

Separation of mitochondrial membranes

The method used to separate the mitochondrial outer membranes from the inner membranes is a modification of the method of PARSONS *et al.*[16] described before[3]. Instead of nine fractions of 3 ml, however, four fractions of 6 ml were collected (see also ref. 17). The remaining content of the gradient tube is not plotted in the figures, but is considered when calculating the recoveries.

Long-chain fatty acid oxidation by isolated rat liver mitochondria

Mitochondria, used for the oxidation experiments, were isolated from a 10% rat liver homogenate, as described before[3], and washed once. O_2 uptake was measured with a Clark "oxygen electrode". The reaction medium contained 32 mM KCl, 4.5 mM $MgCl_2$, 73 mM Tris–HCl, 1 mM EDTA, 1 mM DL-malate, 2.3 mM ATP, 2.3 mM ADP, 8 mM potassium phosphate buffer, 20 mM sucrose and about 3 mg of mitochondrial protein. Variable amounts of fatty acid, complexed to albumin in a molar ratio of 7:1, and 0.5 mM L-carnitine were added as indicated. The reaction volume was 2.2 ml, the temperature 37°, and the pH 7.4.

Enzyme assays

Long-chain acyl-CoA synthetase was measured in three ways. Firstly as a modification of the method of FARSTAD *et al.*[1], described before[3], in the presence of 5 mM KCN (Method A). Secondly by using the same incubation medium as in Method A except that [1-^{14}C]palmitate or [1-^{14}C]oleate was used together with nonradioactive carnitine. After incubation, the radioactive fatty acid, fatty acyl-CoA and acylcarnitine were extracted and separated as described before[18]. The chromatogram was developed with chloroform–methanol–water (77:35:7, by vol.). The radioactive acyl-CoA and acylcarnitine were scraped from the thin-layer plate and transferred to counting vials. After the addition of 10 ml of toluene (containing 5 g of 2,5-diphenyloxazole, 0.3 g of dimethyl-1,4-bis-[5-phenyl-oxazolyl-2]benzene and 30 g of thixotropic gel powder per l), the radioactivity was measured by liquid scintillation counting (Method B). In the third method (C), fatty acid activation was measured as hydroxamate formation, as described by PANDE AND MEAD[2]. The incubation medium was modified and contained: a hydroxylamine–Tris mixture of pH 7.4 (500 mM hydroxylamine and 100 mM Tris), 20 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5–1.0 mM potassium palmitate, 3 μg oligomycine, 5 mM GSH, 0.5 mM CoA, 5 mM ATP, 5 mM phosphoenolpyruvate, 2 units each of pyruvate kinase and adenylate kinase, and mitochondrial protein as indicated. After 1 min preincubation, the reaction was started with the addition of a warm solution of potassium palmitate in water. The reaction volume was 1 ml, the temperature, 37° and the reaction time, 30–60 min. Monoamine oxidase (EC 1.4.3.4) was assayed according to the method of DEITRICH AND ERWIN[19]

with *p*-dimethylaminobenzylamine as a substrate. Other enzymes and protein were measured as described elsewhere[3,17]. All enzyme assays were carried out at 37°.

Where indicated, mitochondria and submitochondrial fractions were treated with ultrasonic vibration before incubation (Branson S-75 sonifier; 3 min at 20 kHz). The temperature was maintained between 0 and 4° by cooling in an ice–salt bath.

RESULTS AND DISCUSSION

Intramitochondrial localization of ATP-dependent long-chain acyl-CoA synthetase in untreated mitochondria

In Fig. 1 the distribution of ATP-dependent long-chain acyl-CoA synthetase and the distribution of marker enzymes of submitochondrial fractions are shown. The figures represent one typical experiment out of a series of three. As marker enzymes for the inner and outer membranes, cytochrome *c* oxidase (EC 1.9.3.1) and rotenone-insensitive NADH–cytochrome *c* reductase are used, respectively[20,21] (*cf.* also refs. 3 and 17). After swelling in 20 mM phosphate buffer, the mitochondria are brought on a continuous sucrose density gradient and centrifuged for 2 h in the Beckman ultracen-

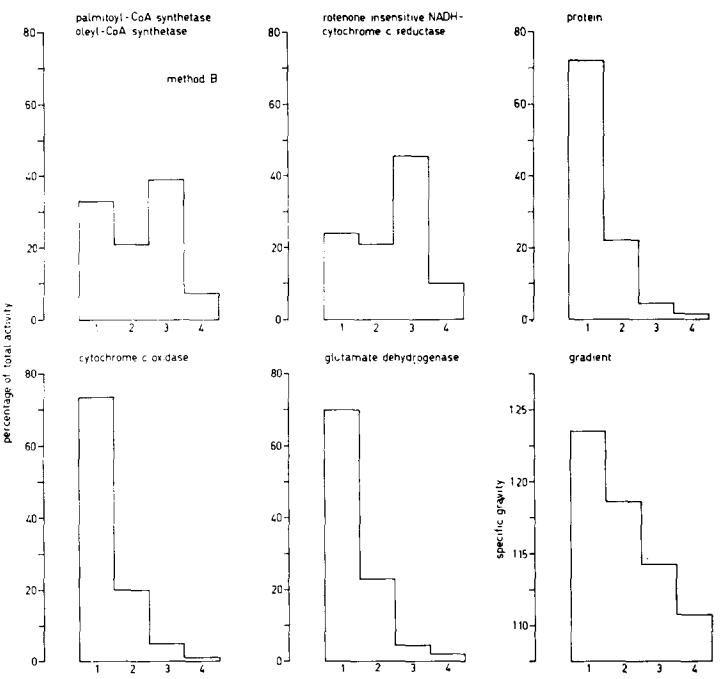

Fig. 1. The distribution of ATP-dependent long-chain acyl-CoA synthetase and some marker enzymes in submitochondrial fractions isolated from twice-washed rat liver mitochondria. After swelling in 20 mM phosphate buffer (pH 7.2) containing 0.02% of bovine serum albumin, the outer and inner membranes were separated in a continuous sucrose density gradient. The gradient was fractionated and the enzymes were assayed as described in MATERIALS AND METHODS. Mitochondria and submitochondrial fractions were treated with ultrasonic vibration before incubation (see MATERIALS AND METHODS). The percentage of the total activity in Fractions 1–4 is plotted against the fraction number. The recovery of the activities of the different enzymes in the gradient fractions varied between 87 and 104%. For specific activities in twice-washed rat liver mitochondria and further details of the enzyme assays, see Table I and refs. 3 and 17.

trifuge at 131000 × g_{max} (rotor SW-27). For details of the gradient fractionation, see MATERIALS AND METHODS. As can be seen from Fig. 1 this method yields a good separation of outer membranes and inner membranes. In these experiments the soluble matrix enzyme glutamate dehydrogenase (EC 1.4.1.2)[22] has the same distribution as the inner membrane enzyme cytochrome *c* oxidase. This indicates that the fractionation procedure is so mild that the inner membrane remains intact during the fractionation. More than half of the total activity of rotenone-insensitive NADH–cytochrome *c* reductase is found in Fractions 3 and 4. In these fractions the percentages of cytochrome *c* oxidase and glutamate dehydrogenase are very low, so that it can be concluded that the outer membrane fractions are very little contaminated with inner membrane-matrix enzymes. Palmitoyl-CoA synthetase and oleyl-CoA synthetase have exactly the same distribution and both very much like the outer membrane marker enzyme rotenone-insensitive NADH–cytochrome *c* reductase, except that a reproducible and significantly higher percentage of the total activity is recovered in the inner membrane-matrix fraction (Fraction 1). This indicates that the bulk of the ATP-dependent long-chain acyl-CoA synthetase is localized in the mitochondrial outer membrane, but it suggests also that some activity is present in the inner membrane-matrix fraction. From studies of DE JONG AND HÜLSMANN[4] and PANDE AND BLANCHAER[8] it is evident that Nagarse (a subtilopeptidase A) can very effectively destroy ATP-dependent long-chain acyl-CoA synthetase. The synthetase is destroyed for more than 90 %. In rat liver mitochondria a small percentage of the acyl-CoA synthetase is resistant to Nagarse (Table I, *cf.* ref. 14). This resistant part of the enzyme is probably not accessible to Nagarse. This, together with the information obtained from the gradient experiments (Fig. 1), led us to think of the possibility that the ATP-dependent long-chain acyl-CoA synthetase of rat liver mitochondria could have a double localization. We therefore decided to investigate this further and to perform the same experiments as illustrated in Fig. 1 with Nagarse-treated mitochondria.

Intramitochondrial localization of ATP-dependent long-chain acyl-CoA synthetase in Nagarse treated mitochondria

Table I shows the effect of Nagarse on the marker enzymes used and on the ATP-dependent long-chain acyl-CoA synthetase. Whereas cytochrome *c* oxidase is totally resistant, the outer membrane marker enzyme rotenone-insensitive NADH–cytochrome *c* reductase is about 35 % destroyed by the Nagarse treatment (see MATERIALS AND METHODS). Another outer membrane marker enzyme, monoamine oxidase, is totally resistant to Nagarse treatment[4,8]. Monoamine oxidase and rotenone-insensitive NADH–cytochrome *c* reductase have exactly the same distribution, whether mitochondria are treated with Nagarse prior to fractionation or not. Also Nagarse treatment neither changes cytochrome *c* oxidase nor glutamate dehydrogenase distribution patterns after fractionation (*cf.* Fig. 1 and Fig. 2). Therefore it can be concluded that Nagarse treatment has no effect on the localization of the marker enzymes used. However, Nagarse treatment has a very clear effect on the distribution pattern of the ATP-dependent long-chain acyl-CoA synthetase. Without Nagarse treatment of mitochondria, about 90 % of the total activity is localized in the outer membranes (see Fig. 1), but after Nagarse treatment there appears an opposite picture: about 90 % of the total activity is localized in the inner membrane-matrix fraction (see Fig. 2). We conclude from these experiments that the ATP-dependent long-chain fatty acid

TABLE I

EFFECT OF NAGARSE TREATMENT ON MITOCHONDRIAL ENZYME ACTIVITIES

Palmitoyl-CoA synthetase activity was proportional to protein in untreated as well as in Nagarse-treated mitochondria. In Method A about 0.1 mg (untreated mitochondria) or 1–2 mg (Nagarse-treated mitochondria) of protein was incubated for 5–10min with 0.5 mM potassium palmitate, complexed with 0.07 mM bovine serum albumin. In Method C about the same amounts of protein were incubated with 1 mM potassium palmitate for 30–60 min. For preparation of the mitochondria (untreated and Nagarse-treated) and for further details of enzyme assays, see MATERIALS AND METHODS. The means and the standard error of the means of the enzyme activities are given as nmoles of substrate metabolized per mg of protein per min.

Enzyme:	*Palmitoyl-CoA synthetase*				*Rotenone insensitive NADH–cytochrome c reductase*		*Cytochrome c oxidase*	
Number of experiments:	*7*		*5*		*4*		*3*	
	Method A	*Inhibition* (%)	*Method C*	*Inhibition* (%)		*Inhibition* (%)		*Inhibition* (%)
Untreated mitochondria	69.5 ± 14.5	—	77.7 ± 11.0	—	415 ± 98	—	2283 ± 517	—
Nagarse-treated mitochondria	3.5 ± 0.8	95	3.8 ± 0.7	95	268 ± 83	35	2400 ± 533	0

TABLE II

COMPARISON OF SOME KINETIC PROPERTIES OF THREE ATP-DEPENDENT LONG-CHAIN ACYL-CoA SYNTHETASES, LOCALIZED IN DIFFERENT PARTS OF THE RAT LIVER CELL

Palmitoyl-CoA synthetase is measured as described in MATERIALS AND METHODS (Method A). In untreated mitochondria, Nagarse-treated mitochondria and 12000 × *g* supernatant the properties of the fatty acid-activating enzymes, present, respectively, in the outer mitochondrial membrane, the inner mitochondrial membrane-matrix compartment and the microsomes, are estimated (for motivation, see the text). K_m and K_i values must be regarded as apparent and were determined by the Lineweaver–Burk method (see *e.g.* ref. 23 and Fig. 3). Inhibitions by AMP and adenosine were competitive with respect to ATP in all enzyme fractions tested. For preparation of the enzyme fractions and details of the enzyme assay, see MATERIALS AND METHODS. In the measurement of the K_i for AMP, the ATP-regenerating system (phosphoenolpyruvate, adenylate kinase and pyruvate kinase) was omitted from the incubation medium.

Enzyme fraction	*Untreated mitochondria*	*Nagarse-treated mitochondria*	*12000 × g supernatant*
K_m palmitate (mM)	0.05	0.18	0.11
K_m ATP (mM)	0.7	0.2	0.4
K_i AMP (mM)	0.2	0.8	0.2
K_i adenosine (mM)	0.1	0.2	0.1

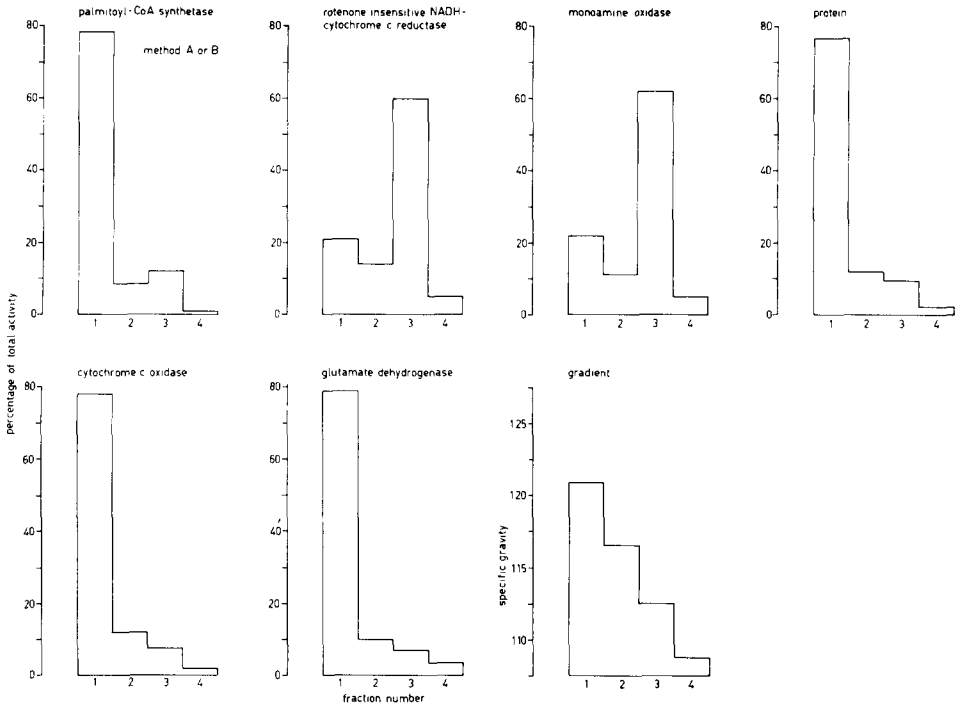

Fig. 2. The distribution of ATP-dependent long-chain acyl-CoA synthetase and some marker enzymes in submitochondrial fractions isolated from twice-washed Nagarse-treated rat liver mitochondria. For separation of mitochondrial membranes, fractionation of the sucrose gradient and enzyme assays, see MATERIALS AND METHODS and the legend to Fig. 1. The percentage of the total activity in Fractions 1–4 is plotted against the fraction number. The recoveries of the activities of the different enzymes in the gradient fractions varied between 91 and 110%. Specific activities in Nagarse-treated twice-washed rat liver mitochondria are given in Table I (see also refs. 3, 4 and 17).

activation in rat liver mitochondria has a dual localization. The enzyme is localized both in the outer membrane and in the inner membrane-matrix compartment.

Kinetics of the ATP-dependent long-chain fatty acid activating enzymes from rat liver

In Table II some properties of the differently localized, ATP-dependent long-chain fatty acid-activating enzymes of the rat liver cell are compared. For the microsomal enzyme we used 12000 × *g* supernatant (see MATERIALS AND METHODS). This supernatant does not contain a significant amount of mitochondria, and the soluble protein of the rat liver cell does not contain long-chain fatty acid activation[1–3]. For estimating the properties of the outer membrane-localized fatty acid activation we used twice-washed, intact, heavy rat liver mitochondria, which when prepared by differential centrifugation are little contaminated with microsomes[3]. Because about 90% of long-chain fatty acid activation resides in the outer membrane, it was not necessary to isolate the mitochondrial outer membranes separately. For the estimation of the properties of the inner membrane-matrix fatty acid-activating activity, Nagarse-treated mitochondria were used after sonication. Sonication enhances this activity under the experimental conditions used, whereas in mitochondria not treated with Nagarse, sonication has no detectable effect on the activity of long-chain fatty acid activation.

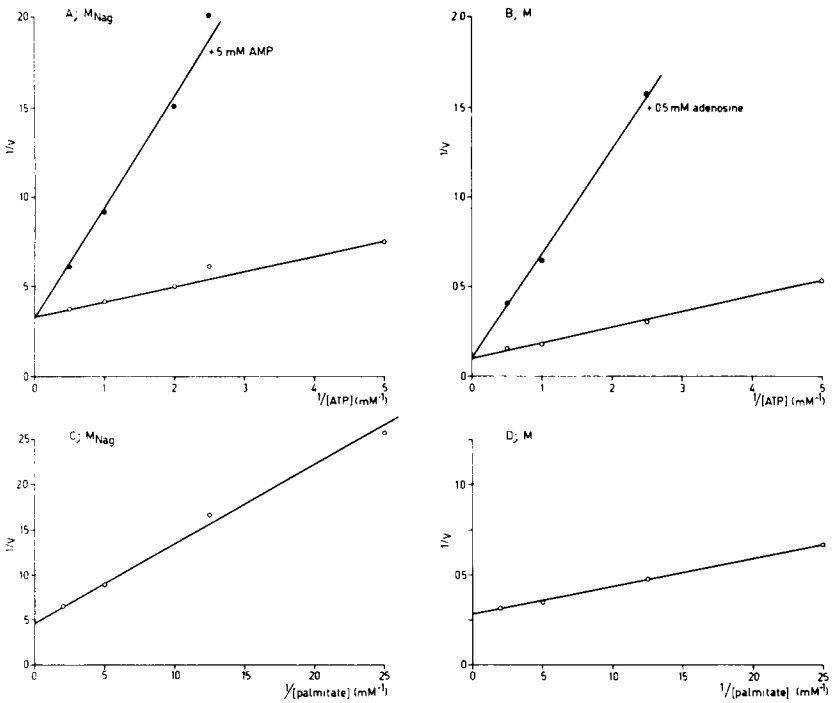

Fig. 3. Kinetic properties of two ATP-dependent long-chain acyl-CoA synthetases, present, respectively, in the outer membrane (B and D) and in the inner membrane-matrix compartment (A and C) of rat liver mitochondria. Palmitoyl-CoA synthetase is measured as described in MATERIALS AND METHODS (Method A). In untreated and Nagarse-treated mitochondria the properties of the fatty acid-activating enzymes, present, respectively, in the mitochondrial outer membrane and the mitochondrial inner membrane-matrix compartment, are estimated (for motivation see the text). K_m and K_i values derived from these figures (see Table II) must be regarded as apparent values. Lineweaver–Burk plots (see *e.g.* ref. 23) are given for the substrates palmitate (C and D) and ATP (A and B) in untreated and Nagarse-treated mitochondria. It can be seen that the inhibitions of AMP in Nagarse-treated mitochondria and of adenosine in untreated mitochondria are of the competitive type with respect to ATP. The enzyme activities (v) are given as μmoles of substrate metabolised per mg of protein per h.

As can be seen from Fig. 2, in Nagarse-treated mitochondria the bulk of the fatty acid activation is localized in the inner membrane-matrix compartment. The values given in Table II are derived from Lineweaver–Burk plots (*cf.* ref. 23) of which some are shown in Fig. 3. The inhibitions by AMP[2,14] and by adenosine[14] are both of the competitive type with respect to ATP in the microsomal enzyme (12000 × g supernatant) as well as in the two mitochondrial enzyme systems (untreated and Nagarse-treated mitochondria). With respect to these inhibitions, the microsomal and the outer membrane enzyme respond in the same way, that is they have a low apparent K_i for AMP and adenosine when compared to the inner membrane-matrix enzyme system. In addition to this characteristically low K_i for AMP inhibition, there is another important characteristic: the apparent K_m for ATP is relatively high for the microsomal enzyme and even higher for the outer membrane fatty acid-activating enzyme. This means that a lowering of the phosphorylation state in the compartment outside the mitochondrial inner membrane could result in inhibition of fatty acid activation. The

TABLE III

INHIBITION OF THREE ATP-DEPENDENT LONG-CHAIN ACYL-CoA SYNTHETASES, LOCALIZED IN DIFFERENT PARTS OF THE RAT LIVER CELL BY SHORT- AND MEDIUM-CHAIN FATTY ACIDS

For preparation of the enzyme fractions and details of the palmitoyl-CoA synthetase assay (Method C), see MATERIALS AND METHODS and the legend to Table I. In the 12000 × *g* supernatant, untreated and Nagarse-treated mitochondria the properties of the palmitate activating enzymes, present respectively, in the microsomes, the mitochondrial outer membrane and the mitochondrial inner membrane-matrix compartment, are estimated (for motivation, see the text). The means and (if possible) the standard error of the means of the palmitoyl-CoA synthetase activities are given.

Additions	*Number of experiments*	*Palmitoylhydroxamate formation (nmoles/mg of protein per min)*					
		12000 × g supernatant	*Inhibition (%)*	*Untreated mitochondria*	*Inhibition (%)*	*Nagarse-treated mitochondria*	*Inhibition (%)*
None	4	43.3 ± 7.7	—	79.2 ± 12.5	—	4.0 ± 0.8	—
0.5 mM sodium octanoate	4	38.3 ± 6.0	12	67.2 ± 10.0	15	1.3 ± 0.2	68
5 mM sodium butyrate	2	44.5	0	80.0	0	2.0	50
10 mM sodium propionate	2	43.5	0	80.8	0	2.5	38

phosphorylation state in the inner membrane *plus* matrix fraction is much lower than that outside the inner membrane[24,25]. Moreover WALTER AND STUCKI[26] showed that the intramitochondrial AMP level during fatty acid oxidation is elevated about 5 times above the control level. Since the K_m for ATP of fatty acid activation in the inner membrane-matrix compartment is lower and the K_i for AMP is higher, when compared to fatty acid activation outside the mitochondrial inner membrane, fatty

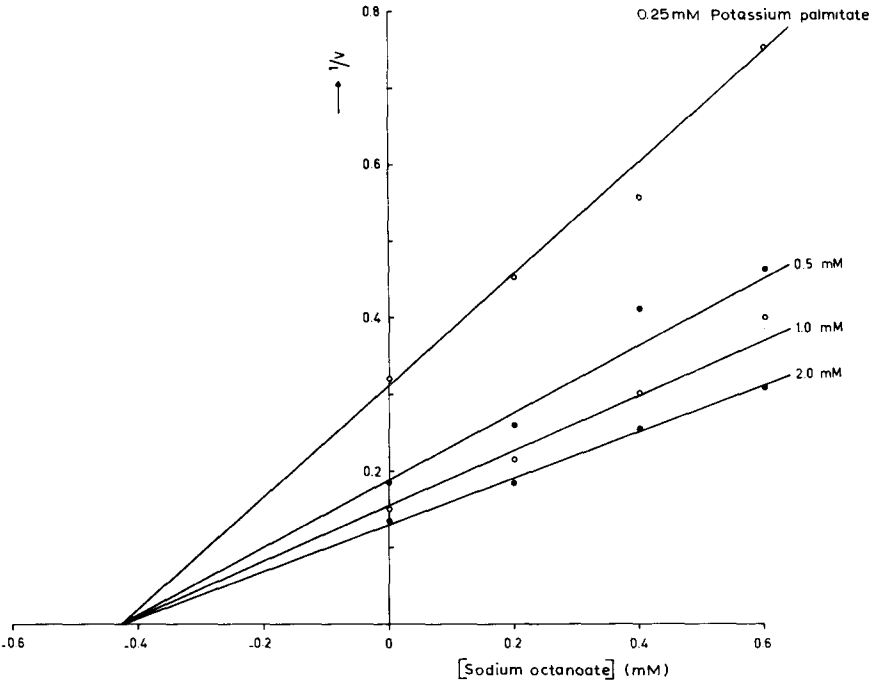

Fig. 4. Inhibition of ATP-dependent long-chain acyl-CoA synthetase by octanoate in Nagarse-treated twice-washed rat liver mitochondria. Palmitoyl-CoA synthetase was measured as described in MATERIALS AND METHODS (Method C). For preparation of Nagarse-treated mitochondria and further details of the enzyme assay see MATERIALS AND METHODS and the legend to Table I. The reciprocal value of the enzyme activity (v in nmoles of palmitate metabolised per mg of protein per min) is plotted against the octanoate concentration (Dixon plot, see *e.g.* ref. 23). As can be seen from the figure, the inhibition by octanoate is probably noncompetitive with respect to palmitate. The K_i for octanoate is 0.4 mM (compare Table III).

acid activation and oxidation can proceed under state 3 conditions (see Fig. 5). This is in agreement with the observation of DE JONG AND HÜLSMANN[14], who showed that in isolated rat liver mitochondria the rate of palmitate oxidation in state 3 is even higher than in state 4.

There is also an appreciable difference in the apparent K_m for palmitate (complexed to albumin) between the outer membrane enzyme and the inner membrane-matrix enzyme system. The inner membrane-matrix enzyme requires a 3–4 times higher palmitate concentration for half-maximal activity than the outer membrane enzyme.

From Table III and Fig. 4 it can be seen that the inner membrane-matrix ATP-dependent long-chain fatty acid activation is inhibited by 67 % with 0.5 mM octano-

ate. In these experiments fatty acid activation was measured as an ATP-dependent hydroxamate formation (see MATERIALS AND METHODS). 5 mM butyrate and 10 mM propionate, under the same conditions, inhibit 50 and 38 %, respectively. As can be seen from Fig. 4 the octanoate inhibition of the inner membrane-matrix enzyme is probably of a noncompetitive type with respect to palmitate. This, together with the relatively weak inhibitions by butyrate and propionate, suggests that the enzyme activating palmitate in the inner membrane-matrix compartment is different from the medium- and short-chain ATP-dependent fatty acid-activating enzymes reported by AAS AND BREMER[27] to be localized in the matrix of rat liver mitochondria. More experiments, however, are needed to differentiate definitely between different enzymes activating fatty acids of various chain lengths localized in the inner membrane and/or matrix of rat liver mitochondria. This study, mainly focussed on long-chain fatty acid activation, shows that there are two ATP-dependent long-chain activating enzyme systems in rat liver mitochondria, one in the outer membrane fraction and one in the inner membrane *plus* matrix fraction (*cf.* ref. 14).

Kinetics of carnitine-dependent and carnitine-independent long-chain fatty acid oxidation by isolated rat liver mitochondria

Fig. 5 shows the influence of the long-chain fatty acid concentration on the O_2 uptake by isolated rat liver mitochondria. The conditions used are optimal for O_2 uptake. It can be seen that in the presence of carnitine a normal Lineweaver–Burk plot is obtained. Oleate and palmitate give exactly the same oxidation rates. The apparent maximal Q_{O_2} in the presence of carnitine is 105 and the apparent K_m for long-chain fatty acid is 1 μM. Without added carnitine the apparent maximal Q_{O_2} is higher with palmitate than with oleate (133 and 100, respectively) and the apparent K_m for fatty acid is 100 μM for palmitate and 200 μM for oleate. The differences between the kinetics of palmitate and oleate oxidation are possibly due to a higher binding affinity of the bovine serum albumin used for oleate when compared to palmitate. As can be seen

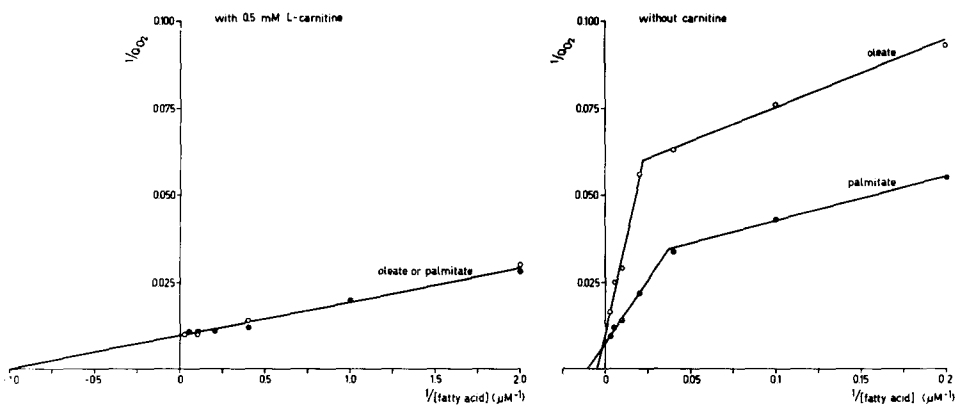

Fig. 5. Kinetics of carnitine-dependent and carnitine-independent long-chain fatty acid oxidation by isolated rat liver mitochondria. O_2 uptake was measured with a Clark "oxygen electrode". For details of the incubation conditions see MATERIALS AND METHODS. The reciprocal value of the Q_{O_2} (μl O_2 metabolized per mg protein per h) is plotted against the reciprocal value of the fatty acid concentration. The Q_{O_2} values shown in this Lineweaver–Burk plot are corrected for O_2 uptake in the absence of added fatty acid (about 40 μl O_2/mg protein per h). The fatty acids used were complexed to albumin in a molar ratio of 7:1.

from Fig. 5 the Lineweaver–Burk plot obtained in the absence of carnitine does not give one straight line in the range of fatty acid concentrations used (5–400 μM). If the fatty acid concentration is below about 40 μM (this is the range shown in the presence of carnitine), the Q_{O_2} is very low. Above about 40 μM there is a sharp cut off in the Lineweaver–Burk plot and the higher concentrations are oxidized rapidly. The sharp cut off indicates that a new enzyme reaction has become rate-limiting in the oxidation proces. Under the conditions used, the oxidative phosphorylation is not limited (ADP and P_i are present). Therefore the increased rate of O_2 consumption at higher concentrations of fatty acid cannot be due to uncoupling effects. In fact, under the conditions used, the mitochondria show respiratory control with palmitate at concentrations up to 0.4 mM (not shown; see also ref. 14). It is very likely then, that at higher fatty acid concentrations the fatty acid is activated by a different activating enzyme. The palmitoyl-CoA synthetase present in the mitochondrial outer membrane has an apparent K_m for fatty acid of 0.05 mM and the palmitoyl-CoA synthetase present in the inner membrane-matrix compartment requires 0.18 mM palmitate for half-maximal activity (see Table II). The latter K_m is of the same order of magnitude as the K_m for long-chain fatty acid in fatty acid oxidation in the absence of carnitine. Therefore we conclude that in the absence of carnitine if the fatty acid concentration is relatively high, the long-chain fatty acids are activated by the ATP-dependent long-chain acyl-CoA synthetase present in the inner membrane-matrix compartment of rat liver mitochondria. If palmitate is oxidized completely to CO_2 and H_2O, the measured activity of the palmitate activation in the inner membrane-matrix compartment, about 3.7 nmoles/mg protein per min (Table I), can theoretically result in a Q_{O_2} for palmitate oxidation at 37° of about 110. This agrees very well with the observed oxidation rates of 100–133 (see Fig. 5).

In the presence of carnitine, the fatty acids needed for fatty acid oxidation can be activated by acyl-CoA synthetase localized outside the barrier for acyl-CoA as well as by acyl-CoA synthetase localized inside this barrier. In the presence of low concentrations of fatty acid (below 40 μM) only the outer membrane enzyme can be operative because of its low K_m for fatty acid and its high activity. As can be seen from Fig. 5 the oxidation of long-chain fatty acid in the presence of carnitine shows an apparent K_m for fatty acid of 1 μM. With this fatty acid concentration the long-chain acyl-CoA synthetase localized in the outer membrane can only reach less than 5 % of its maximal activity (the apparent K_m for palmitate is 50 μM). Because of the very high activity of the ATP-dependent long-chain acyl-CoA synthetase localized in the outer membrane of rat liver mitochondria (Table I), enough acyl-CoA can still be synthesized for optimal fatty acid oxidation in the presence of carnitine at very low levels of long-chain fatty acid.

How much the ATP-dependent long-chain fatty acid activation localized in the inner membrane-matrix compartment *in vivo*, that is in the presence of carnitine[28], contributes to the total amount of activated fatty acid required for fatty acid oxidation and ketone body production has to await further experimentation. That carnitine plays a role in the interaction of fatty acid oxidation and gluconeogenesis is well established[29] and may be demonstrated by its beneficial effect in hypoglycin-induced vomiting sickness[30]. ENTMAN AND BRESSLER[30] showed that after hypoglycin intoxication, long-chain fatty acid oxidation was hampered and that carnitine administration restored palmitate oxidation to normal levels. Administration of carnitine to hypo-

glycin-treated mice prevented both the depression of palmitate oxidation and the hypoglycemia (see also refs. 31 and 32).

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