THE SUBMITOCHONDRIAL DISTRIBUTION OF ACID:COA LIGASE (AMP) AND PALMITYL-COA:CARNITINE PALMITYLTRANSFERASE IN RAT LIVER MITOCHONDRIA

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Recently a method for the separation of the outer and inner mitochondrial membranes has been worked out in the Wenner-Green Institute, Stockholm, Sweden (Sottocasa et al 1966). We have used this method for the study of the intramitochondrial localization of acyl-CoA synthetase (acid:CoA ligase AMP, EC 6.2.1.3) and carnitine palmityltransferase (palmityl-CoA:carnitine palmityltransferase, EC 2.3.1...) in relation to mitochondrial marker enzymes. Results are presented which indicate that acyl-CoA synthetase is confined to the outer mitochondrial membrane, carnitine palmityltransferase and β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) to the inner mitochondrial membrane, and glutamate dehydrogenase (EC 1.4.1.3) to the matrix of the mitochondrion.

MATERIALS AND METHODS

DL-CH_3-^3H-carnitine was prepared as previuosly described (Bremer and Norum 1966). Carnitine palmityltransferase was prepared from calf liver mitochondria and assayed by an isotope exchange method (Norum 1964). The activity of the acyl-CoA synthetase was assayed by incubating the test material with ATP, CoA and

palmitate in the presence of a vast excess of carnitine palmityltransferase and radioactive carnitine, thus trapping the activated palmitate as palmityl-L-CH_3-^3H-carnitine (Farstad, Bremer and Norum 1966). The β-hydroxybutyrate dehydrogenase and the glutamate dehydrogenase were assayed by measuring the rate of reduction of NAD. The activity of glucose-6-phosphatase (EC 3.1.3.8) was measured by the liberation of phosphate from glucose-6-phosphate.

A heavy mitochondrial fraction was isolated from livers of female Wistar rats weighing about 200 g (de Duve et al 1955). The mitochondria were separated into an "outer membrane fraction" and an "inner membrane fraction" according to Sottocasa et al (1966) with minor modifications. Mitochondria were swelled in ice-cold 10 mM Tris-HCl buffer (pH 7.5) for 10 min, then contracted by the addition of an equal volume of 1.15 M sucrose containing 2 mM ATP and 2 mM $MgCl_2$. After at least 10 min on ice, an aliquot of this suspension was treated with ultrasonic vibration (Branson Sonifier, 30 sec at 2.5 A). Samples (3 ml) of the not-sonified and of the sonified mitochondrial suspensions were layered over 1.3 M sucrose (2.5 ml) in centrifuge tubes of the Spinco SW 39 rotor, and centrifuged for 3 hours at 39000 rev/min. Sottocasa et al (1966) have presented electronmicroscopic evidence for the following fractionation of the mitochondria by this method: Solubilized matrix is found in the upper sucrose layer. The outer membranes are concentrated at the interphase between the two sucrose solutions. This fraction is contaminated with solubilized matrix. The inner mitochondrial membranes, in part with their matrix, form the pellet in the bottom of the tube.

The content of the tubes was divided in three fractions: the 0.4 M sucrose supernatant (S), the interphase (I) and the pellet (P) by carefull suction from the top with a pipette with a bent tip.

RESULTS AND DISCUSSION

Table I shows the results from a representative experi-

Table I

Distribution of acyl-CoA synthetase and carnitine palmityltransferase in relation to marker enzymes in mitochondrial subfractions of rat liver.

Enzyme	Units per g of liver			Relative values (M) = 100 per cent			Recovery
	(E)	(M)		(S)	(I)	(P)	
A-CoA S	1.3	0.18	Nonson.	14.3	11.6	56.8	82.7
			Sonif.	11.7	31.1	4.9	47.4
Carn PT	3.3	2.3	Nonson.	1.7	5.6	94.5	101.8
			Sonif.	9.5	9.4	84.5	103.4
β-OH-B DH	13.3	7.6	Nonson.	0	2.7	94.1	96.8
			Sonif.	0.9	4.5	83.6	89.0
Glut DH	13.0	8.8	Nonson.	4.1	4.7	79.5	88.3
			Sonif.	27.0	8.1	58.1	93.2
G-6-P'ase	15.0	0.3	Nonson.	22.3	67.1	12.9	102.5
			Sonif.	28.0	22.4	42.0	92.0
Protein	155	23	Nonson.	8.8	7.7	76.6	93.1
			Sonif.	18.6	10.0	60.7	89.3

The enzymes were assayed as described in the text. Abbreviations: β-OH-B DH, β-hydroxybutyrate dehydrogenase; Glut DH, glutamate dehydrogenase; Carn PT, carnitine palmityltransferase; A-CoA S, acyl-CoA synthetase; G-6-P'ase, glucose-6-phosphatase; Nonson., nonsonified; Sonif., sonified.
The absolute values for the cytoplasmic extract (E) and for the heavy mitochondrial fraction (M) are given as units per g wet weight of liver, and protein as mg per g wet weight of liver. The relative values for the supernatant (S), the interphase (I) and the pellet (P) are given as per cent of the activities in the heavy mitochondrial fraction (M). The nonson. series are from mitochondrial suspensions centrifuges without preceding sonication. The sonified series were treated as described in the text.

ment. The activities for the cytoplasmic extract (E), and for the heavy mitochondrial fraction (M), are given in units per g fresh liver. The activities for the different mitochondrial subfractions are given as per cent of the untreated (M)-fraction. Both β-hydroxy butyrate dehydrogenase, glutamate dehydrogenase and carnitine palmityltransferase are enzymes exclusively confined to the mitochondria (Beaufay et al 1959, Norum and Bremer 1966). From the absolute values in Table I it can be deduced that about two third of the mitochondria in the cytoplasmic extract was recovered in the mitochondrial fraction.

The extent of contamination of the (M)-fraction with microsomes is of importance for the discussion of the localization of the acyl-CoA synthetase. This enzyme has a bimodal localization with about 70 per cent of the activity in the microsomes and about 30 per cent in the mitochondria (Farstad et al 1966). Table I shows, however, that about 15 per cent of the total cellular activity of the acyl-CoA synthetase was recovered in the (M)-fraction, whereas the recovery of the microsomal glucose-6-phosphatase was only 2 per cent. This excludes the possibility that our results on acyl-CoA synthetase are due to microsomal contamination. The acyl-CoA synthetase activity in the (S) and (I) fractions of the not-sonified mitochondria was so low that it might be due to microsomal contamination, as most of the glucose-6-phosphatase activity was found in these fractions. In the sonified specimen, however, most of the synthetase activity was found in the (I) fraction while the glucose-6-phosphatase was lowered in this fraction. Table I reveals that ultrasonic treatment and subsequent centrifugation gave about 50 per cent inactivation of the acyl-CoA synthetase. Even if all inactivation of the enzyme occured in the pellet, the specific activity of the acyl-CoA synthetase in the (I) fraction was much higher than the hypo-

thetic specific activity in the pellet. Thus more synthetase activity was found in the (I) fraction after sonication than could be explained by contamination with microsomes, inner membranes or mitochondrial matrix. It is therefore warranted to conclude that the acyl-CoA synthetase in mitochondria is confined to the outer mitochondrial membrane. Due to the considerable inactivation of the enzyme during the preparation of the sonified fractions, we cannot completely rule out synthetase activity in other mitochondrial subfractions, but our results do not give support to such an idea.

The distribution of the β-hydroxybutyrate dehydrogenase, glutamate dehydrogenase and carnitine palmityltransferase is completely different from that of the acyl-CoA synthetase. Table I shows that the β-hydroxybutyrate dehydrogenase was found mainly in the pellet. Sonication, which effectively strips the swelled and contracted mitochondria for their outer membranes, did not significantly change the distribution of the enzyme. It seems reasonable to conclude that this enzyme is bound to the inner membrane of the mitochondria, and that sonication gives a slight contamination with inner membranes at the interphase. Most of the activity of the glutamate dehydrogenase was also found in the pellet of the not-sonified mitochondria. After the mild sonication used, only about one third of the activity was found in the (S) and (I) fractions. As swelling and contraction alone most likely disrupts the outer membrane, it seems warranted to conclude that glutamate dehydrogenase is confined to the matrix (intercristal space) of the mitochondria. The solubilization of this enzyme thus will be a measure for inner membrane disruption.

The distribution of the carnitine palmityltransferase resembles that of β-hydroxybutyrate dehydrogenase. However, relatively more of the activity was found in the (S) and (I) fractions

both in the sonified and the not-sonified preparations. This is in agreement with the finding that carnitine palmityltransferase is less firmly bound to mitochondrial membranes than is the β-hydroxybutyrate dehydrogenase (Norum and Bremer 1966). Since most of the enzyme activity remains in the pellet also in sonicated mitochondria, it can be assumed that carnitine palmityltransferase is confined to the inner mitochondrial membrane. The results do not, however, completely rule out that some carnitine palmityltransferase activity may be present in the outer membrane.

The distribution of the protein in the different fractions is given in the last row in Table I. The values reveal that more protein was recovered in the supernatant after sonication. This fits well with the partial solubilization of matrix protein and glutamate dehydrogenase.

Other workers have shown that at least part of the fatty acid activation by the ATP dependant enzyme takes place outside the carnitine barrier of the mitochondria (van den Bergh 1966, Yates, Shepherd and Garland 1966, Chappell and Crofts 1964), and that the β-oxidation of fatty acids takes place inside this barrier. Activated fatty acids are transported into the mitochondria as acylcarnitines (Bremer 1962, Fritz 1963). The localization of the carnitine palmityltransferase to the inner mitochondrial membrane, and the ATP-dependant acyl-CoA synthetase to the outer membrane therefore agrees well with previous experimental data. The transferase thus may be looked upon as a sort of "permease" for the activated fatty acids.

There are probably three enzyme systems catalyzing the formation of long-chain acyl-CoA in liver cells. One is microsomal and ATP dependant; the other two are mitochondrial, one ATP dependant and one GTP dependant (Farstad et al 1966, van den Bergh 1966)

In the present investigation we have found that the mitochondrial ATP dependant enzyme most likely is confined to the outer membrane of the mitochondria. If the outer mitochondrial membrane is permeabel for long-chain acyl-CoA, the microsomal and mitochondrial ATP dependant acyl-CoA synthetase will constitute a functional unit in the cell. This may suggest that the microsomal membrane and the outer mitochondrial membrane may be derived from a common source.

The results and conclusions in the present communication differ to a considerable degree from the results and conclusions presented by Allmann, Bachmann and Green (1966). In their studies on the mitochondrial distribution of enzymes, they found both acyl-CoA synthetase, β-hydroxybutyrate dehydrogenase, carnitine palmityltransferase and the fatty acid β-oxidation system associated with the outer membrane of ox heart mitochondria. Our studies are all done with rat liver. It seems unlikely that there should be such a profound organ difference in the organization of the mitochondria. At present we are unable to explain these discrepancies. Most likely they are due to the different fractionation procedures used. It seems pertinent to point out, however, that the results here presented fit well with previous studies of fatty acid oxidation in intact mitochondria.

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