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SUBCELLULAR DISTRIBUTION OF COENZYME A : EVIDENCE FOR A SEPARATE COENZYME A POOL IN PEROXISOMES[1]

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SUMMARY : Subcellular fractions enriched in peroxisomes, that were prepared by differential centrifugation, contained more free and total (free plus esterified) CoA than could be accounted for by mitochondrial contamination. Separation of peroxisomes and mitochondria by isopycnic centrifugation of fractions enriched in peroxisomes resulted in a bimodal distribution of free and total CoA in which both peak fractions coincided with the fractions that contained most of the peroxisomal and mitochondrial marker enzyme activities respectively. These results indicate that peroxisomes have a separate CoA-pool. This CoA appears to be available for the thiolase reaction during peroxisomal β-oxidation of fatty acids but not for the activation of fatty acids by the peroxisomal acyl-CoA synthetase.

β-oxidation of palmitoyl-CoA by purified peroxisomal fractions from rat liver is stimulated by CoA when assayed under hypotonic conditions and in the presence of detergent (1), but not when studied in isotonic media that contain sufficient albumin to counteract the detergent effects of the fatty acyl-CoA substrate (2,3). These observations suggest that like mitochondria, rat liver peroxisomes possess their own CoA stores that are available for the thiolase reaction during β-oxidation. Our present experiments with rats treated with the hypolipidemic drug clofibrate indicate that this is the case. Clofibrate induces the proliferation of hepatic peroxisomes (4), increases the β-oxidizing capacity of peroxisomes 5- 10-fold (1,2) and causes a 3-fold rise in hepatic CoA content (5,6).

MATERIALS AND METHODS

Male Wistar rats weighing 200 g were kept on a regular chow diet containing 0.3 % clofibrate for 14 days. Liver homogenates (25 %, w/v) in 0.25 M

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sucrose containing 0.1 % ethanol and 5 mM dithiothreitol were fractionated as in (7) into a N-fraction, containing the nuclei and part of the mitochondria, a (ν-N) fraction, containing most of the mitochondria, and a λ-, P- and S-fraction mostly enriched in peroxisomes and lysosomes, microsomes and soluble components respectively. The (ν-N) fraction corresponds to Leighton's (8) original ν-fraction minus de Duve's (9) N-fraction. Subfractionation of the λ-fraction was carried out by isopycnic centrifugation in a sucrose gradient containing dextran and ethanol (8) and 5 mM dithiothreitol. The gradient was linear with respect to volume (20 ml) between densities 1.15 and 1.27. 2.5 ml of λ-fraction in 0.25 M sucrose containing peroxisomes from 8.3 g of liver were loaded on top of the gradient and centrifuged for 60 min at 92 000 g av in a M.S.E. SW30 rotor. Fractions were deproteinized with $HClO_4$ and CoA was measured on the neutralized supernatants by the cycling method of Veloso and Veech (10). This method determines the sum of free CoA plus acetyl-CoA. Acetyl-CoA was measured separately by first reacting all of the free CoA in the sample with N-ethylmaleimide. Free CoA was estimated from the differences in cycling rates obtained with and without pretreatment of the samples with N-ethylmaleimide (11). Acid-insoluble long chain acyl-CoAs were determined on the pellets after liberation of CoA by alkaline hydrolysis at pH 12 for 60 min at 55°. Results were always corrected for inhibition of the cycling reactions by the neutralized $HClO_4$ extracts. Throughout the text the term 'total CoA' refers to the sum of free CoA, acetyl-CoA and long chain acyl-CoA. Acid-soluble CoA derivatives other than acetyl-CoA could not be determined accurately in the subcellular fractions. Protein and marker enzymes were determined as in (2,7). Peroxisomal oxidation of [1-^{14}C]-palmitate and [1-^{14}C]-palmitoyl-CoA was measured by following the generation of acid-soluble radioactive oxidation products and H_2O_2 as described in (2,3).

RESULTS AND DISCUSSION

In a first series of experiments the subcellular distribution of marker enzymes and of free CoA plus acetyl-CoA and of long chain acyl-CoA was studied in livers from clofibrate-treated rats (Fig. 1 and Table I, Panel A). In a second series of experiments free CoA and acetyl-CoA were measured separately along with the various marker enzymes (Table I, Panel B). As shown in Figure 1 the subcellular distribution of total CoA most closely resembled that of glutamate dehydrogenase indicating that a large part of the CoA was associated with the mitochondria. From Table I it can be calculated that approximately 60 percent of the total CoA present in the original homogenates was in the free form. The table also shows that the subcellular distribution patterns of free CoA and of total CoA were almost identical. The λ-fraction, mostly enriched in peroxisomes, contained 19.3 $\pm$ 2 percent of the sedimentable[2]

[2]Sedimentable CoA : Sum of CoA present in the sedimentable fractions (N, ν-N, λ, P).

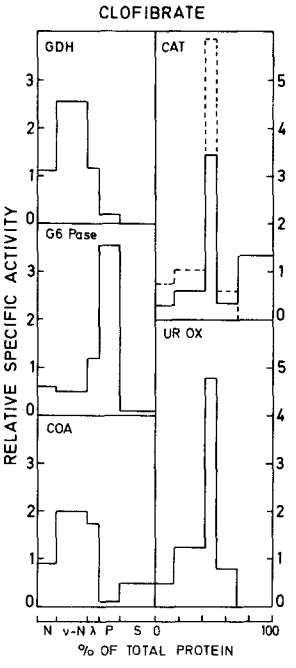

Fig. 1 :
Subcellular distribution of total CoA and of marker enzyme activities after differential centrifugation of liver homogenates from clofibrate-treated rats. Results are expressed as relative specific activity versus cumulative percentage of total recovered protein. Results are means for 3 experiments. Recoveries after fractionation : protein : 101 %; glutamate dehydrogenase (GDH - mitochondria) : 88 %; catalase (CAT - peroxisomes) : 95 %; urate oxidase (UR OX - peroxisomes) : 113 %; glucose-6-phosphatase (G6 Pase - microsomes) : 88 %; CoA : 93 %. Broken line : sedimentable catalase activity.

total CoA and 23.7 $\pm$ 2.4 percent of the sedimentable free CoA. The values for sedimentable (> 99 % of total) glutamate dehydrogenase in the corresponding λ-fractions were only 9.8 $\pm$ 0.6 percent ($P < .025$) and 15.2 $\pm$ 1.7 percent ($P < .05$) suggesting that the λ-fractions contained CoA that was associated with organelles other than mitochondria i.e. peroxisomes. This is also shown in Table II where the percentage of sedimentable CoA associated with each subcellular fraction is compared with the percentage of sedimentable glutamate dehydrogenase present in that fraction. More free as well as total CoA was present per unit of glutamate dehydrogenase in the λ-fraction than in any other fraction. Similar fractionation experiments were carried out with livers from normal rats, but because of the low CoA content of these livers, free CoA was not determined separately. λ-fractions from normal

Table I : Percent distribution (%) and relative specific activity (RSA) of CoA and its derivatives after fractionation of liver homogenates from clofibrate-treated rats. The data are means for two separate groups of 3 experiments each (group A and B). The distribution of total CoA and of the various marker enzymes in livers of group A is also shown in Fig. 1.

Subcellular fraction :		N	ν-N	λ	P	S	Homogenate	Recovery
							nmol/g liver	%
A : free CoA + acetyl-CoA	%	15.5	52.5	16	1.5	14.5	430 ± 64	99
	RSA	.93	2.05	1.71	.09	.45		
long chain acyl-CoA	%	12.5	42.5	20.5	7	17	98 ± 8	66
	RSA	.75	1.65	2.23	.41	.52		
total CoA	%	15.5	51	16.5	1.50	15	528 ± 56	93
	RSA	.90	1.99	1.77	.09	.48		
B : free CoA	%	7	51	18	1.5	22	355 ± 47	99
	RSA	.66	1.90	1.73	.07	.67		
acetyl-CoA	%	9	28	12	5	46	129 ± 30	93
	RSA	.85	1.0	1.15	.25	1.40		
free CoA + acetyl-CoA	%	7.5	45.5	16.5	2.5	28	484 ± 63	97
	RSA	.72	1.69	1.60	.12	.86		

livers contained 9.0 ± .7 percent of the sedimentable total CoA and 6.2 ± 1.3 percent of the sedimentable glutamate dehydrogenase activity ($P < .10$, $n = 3$). Table III shows the content of soluble (S-fraction) and sedimentable total CoA in livers from normal and clofibrate-treated rats. In agreement with data obtained by others (5,6), there was an approx. 3-fold increase in hepatic CoA-content after clofibrate treatment. The table also shows estimations of total mitochondrial and peroxisomal CoA content, based on the amounts of CoA and the percentages of mitochondrial (glutamate dehydrogenase) and peroxisomal (sedimentable catalase) marker enzymes recovered in the mitochondrial (ν-N) and peroxisomal (λ) fractions. Also indicated are the estimated total CoA concentrations in the cytosol, mitochondrial matrix and peroxisomes. The estimations are based on : a cytosolic water space of 310 µl per g liver (12); a mitochondrial matrix water space of 0.8 µl per mg mitochondrial protein (13), a mitochondrial protein content of 20 % of total hepatic protein (8) (184 ± 14 mg/g liver in the present experiments) and a mitochondrial volume increase of 33 % after clofibrate treatment (14). The peroxisomal water space was calculated from a peroxisomal protein content of 2.53 % of total hepatic protein (8), a protein weight per unit of dry peroxisomal

Table II : Ratio of percentages of sedimentable CoA and sedimentable glutamate dehydrogenase (GDH) present in subcellular fractions. The data for total CoA are taken from group A of Table I; those for free CoA grom group B of Table I.

Subcellular fraction :	N	ν-N	λ	P
	% Sed.CoA : % Sed.GDH			
Free CoA	.88	.95	1.56	.50
Total CoA	.95	.91	1.77	.68

weight of 0.8 (15), a peroxisomal dry weight to wet weight ratio of 0.26 (15), a hydrate water volume for peroxisomal protein of 0.8 µl/mg (which was subtracted) and a 500 % peroxisomal volume increase after clofibrate treatment (14) It is obvious that the above calculations are only meaningful when applied to total CoA since the time-consuming homogenization and fractionation procedures do not allow to draw conclusions about the *in vivo* partioning of CoA between the free and various esterified forms. In addition, it should be emphasized that these calculations are to be considered as rough indications rather than as accurate estimations, which are not feasable with the currently available techniques.

Conclusive evidence for the presence of CoA in peroxisomes was obtained when λ-fractions of livers from clofibrate-treated animals were subfractionated by isopycnic centrifugation (Fig. 2). The figure shows a bimodal distribution of free CoA in which both peak fractions (4 and 9) coincide with the fractions that contain most catalase and glutamate dehydrogenase

Table III : Content and concentration of total CoA in cytosol, mitochondrial matrix and peroxisomes from rat liver. For calculations see text.

	Control (n = 3)		Clofibrate (n = 3)		Control	Clofibrate
	CoA-content, nmols/g liver				CoA-concentration, mM	
Soluble, cytosol	33.2 ± 4.2		74.2 ± 15.1		.106	.238
Sedimentable	118.3 ± 4.9	(100 %)	416.1 ± 33.9	(100 %)		
Mitochondrial	142.6	146.6 (124 %)	378.1	448.3 (108 %)	4.82	9.62
Peroxisomal	4.0		70.2		.316	.920

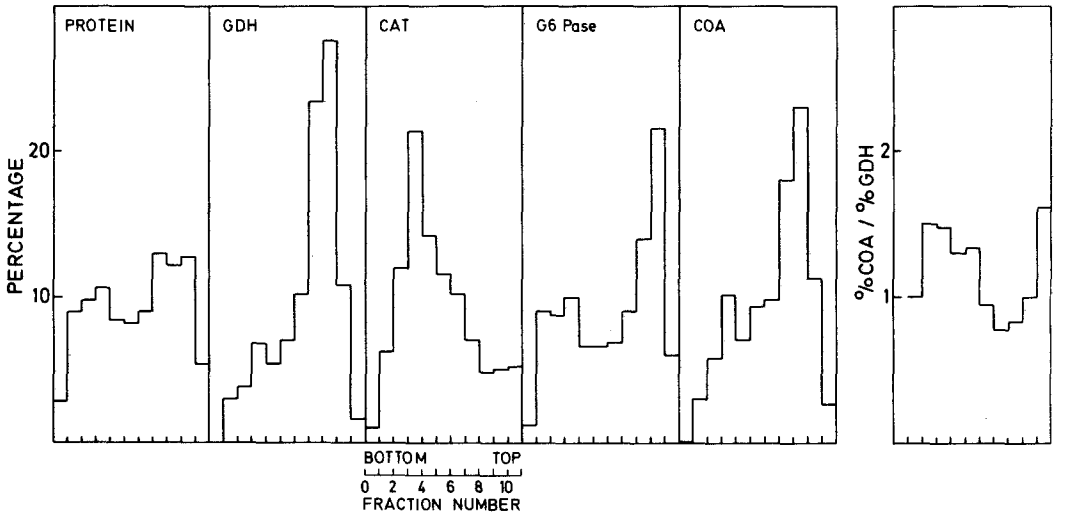

Fig. 2 :
Separation of peroxisomes and mitochondria from livers of clofibrate-treated rats by isopycnic centrifugation. A λ-fraction prepared by differential centrifugation was centrifuged through a linear sucrose gradient between densities 1.15 and 1.27. Results are expressed as the percentage of total recovered activities or amount (protein and CoA) present in each fraction versus cumulative fraction volume. For abbreviations : see Fig. 1; CoA : free CoA. Recoveries for total gradient : protein : 103 %; GDH : 112 %; CAT : 96 %; G6 Pase : 96 %; CoA : 119 %.

activity respectively. Aspecific adsorption of soluble CoA to peroxisomes was ruled out by experiments in which tritiated CoA was added to homogenates or λ-fractions that were subsequently fractionated by differential and/or isopycnic centrifugation. Total CoA also followed a bimodal distribution that was almost identical to that of free CoA (data not shown). The presence of free CoA in peroxisomes after differential and isopycnic centrifugation indicates that peroxisomes contain their own CoA stores but also that the peroxisomal membrane is impermeable for CoA.

The demonstration of a separate CoA-pool in peroxisomes raises the question as to how this CoA, that appears to be continuously used in the thiolase reaction to form acetyl-CoA, is recycled in order to allow the continuing oxidation of fatty acid. Since peroxisomes contain carnitine acetyltransferase (16,17), the intraperoxisomal conversion of acetyl-CoA into acetylcarnitine seems a plausible mechanism. In support of such mechanism are our

previous observations that the bulk of acetyl units is in the form of acetyl-carnitine when peroxisomal palmitoyl-CoA oxidation is assayed in the presence of carnitine (3).

Shindo and Hashimoto (18) and Krisans et al. (19) provided evidence for the presence of some acyl-CoA synthetase activity in peroxisomes. We therefore wondered whether the peroxisomal CoA pool might also be available for the activation of fatty acids. When [1-^{14}C]-palmitate oxidation by λ-fractions from livers of clofibrate-treated rats was studied at a palmitate : albumin ratio of 1.67 (2,3) in the presence of 0.5 mM CoA and 4 mM ATP, 87 nmols of [1-^{14}C]-palmitate per min per g liver entered the oxidation pathway (calculated from the production of acid-soluble labelled oxidation products) and 279 nmols of H_2O_2 per min per g liver were formed (flux through the first dehydrogenation step of peroxisomal β-oxidation; n = 2). The corresponding values for [1-^{14}C]-palmitoyl-CoA oxidation were 117 nmols of [1-^{14}C]-palmitoyl-CoA per min per g and 283 nmols of H_2O_2 per min per g. In the absence of CoA [1-^{14}C]-palmitoyl-CoA oxidation was not altered but only 5 nmols of [1-^{14}C]-palmitate per g entered oxidation and only 11 nmols of H_2O_2 were formed. These observations strongly suggest that the peroxisomal CoA-pool is not available for fatty acid activation and that, if significant acyl-CoA synthetase activity is present in peroxisomes the enzyme may be associated with the outer aspect of the peroxisomal membrane.

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