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CHAIN LENGTH SPECIFICITIES OF PEROXISOMAL AND MITOCHONDRIAL β-OXIDATION IN RAT LIVER

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The β-oxidation system for fatty acids and the novel fatty acyl-CoA oxidase activity of liver peroxisomes were characterized in terms of assay requirements and of chain length specificity of substrate. Both activities showed a specificity for medium to long-chain length acyl-CoA's (C_{10}-C_{16}), with a well defined peak of activity at C_{12}. The specific activities were similar and, in each case, oleoyl-CoA was a better substrate than stearoyl-CoA. The mitochondrial fatty acyl-CoA dehydrogenase activity showed a broader range (C_4-C_{16}), with the shorter-chain length substrate being the more active and no preference being shown for oleoyl- over stearoyl-CoA. Using palmitoyl-CoA as substrate, the total enzymatic activity for the first step of β-oxidation in the mitochondrion was 7.7-fold greater than for the corresponding step in the peroxisome.

There are now reports on the presence of a fatty acyl-CoA oxidizing system in the peroxisomes of castor bean endosperm (1), Tetrahymena (2), rat liver (3) and yeast (4). In contrast to the mitochondrial fatty acyl-CoA oxidizing system, Cooper and Beevers (1) suggested that the first dehydrogenase in the peroxisomal system transfers its electrons directly to O_2, producing H_2O_2. This novel enzyme, a fatty acyl-CoA oxidase, was first assayed and characterized in Tetrahymena by Hryb and Hogg (2). Later, Osumi and and Hashimoto (5) have characterized this oxidase in rat liver. However, the results of our present work differ, in terms of assay conditions and chain length specificities, from the above authors.

In the present study, using the peroxisomal fraction from rat liver (de Duve's fraction L (6)), we have characterized the oxidase and β-oxidation activities chiefly in terms of their chain length specificities. We have also

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re-examined the chain length specificity of mitochondrial fatty acyl-CoA dehydrogenase activity and found it to be somewhat different from previously published data (7,8). The chain length specificities of the mitochondrial and peroxisomal enzymes show a partial complementarity, with the mitochondrial system being more active towards short-chain substrates and the peroxisomal system being more active with medium to long-chain length. Both peroxisomal activities, however, exhibited a sharp peak at chain length C_{12}.

MATERIALS AND METHODS

Preparation of Particulate Fractions -- The peroxisomal (L) and mitochondrial (M) fractions were prepared from the livers of male albino rats (Sprague-Dawley) according to the procedure of de Duve et al. (6). The rats were fed ad libitum on Purina rat chow, and starved overnight previous to sacrifice. The fatty acyl-CoA oxidase activity was found to be localized in the peroxisomal fraction (L).

Enzyme Assays -- The fatty acyl-CoA oxidase activity was assayed by measuring the acyl-CoA dependent H_2O_2 production using a modification of the method described by Hryb and Hogg (2). The oxidase was assayed at 30°C in 50 mM potassium phosphate buffer, pH 7.6, containing 500 U/ml horseradish peroxidase, 25 mM p-hydroxybenzoic acid, 1.0 mM 4-aminoantipyrine (9), 50 µM FAD, 200 µg/ml BSA†, 0.05% (v/v) Triton X-100, and the particulate preparation (L). The reaction was started by adding the acyl-CoA to a final concentration of 50 µM. The increase in absorbance at 500 nm was monitored in a Gilford 2400 recording spectrophotometer. The micromolar extinction coefficient was calculated to be 5.1 $cm^2/\mu mole$ at pH 7.6.

The fatty acyl-CoA dehydrogenase assay used was a modification of the method described by Hoskins (10). The dehydrogenase was assayed at 30°C in 66 mM potassium phosphate buffer, pH 6.8, containing 66 µM DCPIP, PMS (1.0, 0.50, and 0.25 mM), 1.0 mM KCN, 200 µg/ml BSA, 0.067% (v/v) Triton X-100 and the particulate preparation (M). The reaction was started by adding the acyl-CoA to a final concentration of 50 µM. The decrease in absorbance at 600 nm was monitored. A micromolar extinction coefficient for DCPIP of 19.1 $cm^2/\mu mole$ was used.

The peroxisomal β-oxidation assay used was a modification of the methods described by Cooper and Beevers (1) and Lazarow and de Duve (3). The assay was performed at 30°C in 50 mM potassium phosphate buffer, pH 7.4, containing 200 µM NAD+, 100 µM CoA, 50 µM FAD, 1.0 mM KCN, 12 mM DTT, 200 µg/ml BSA, 0.025% (v/v) Triton X-100 and the particulate preparation (L). The reaction was started by adding the acyl-CoA to a final concentration of 50 µM. The increase in absorbance at 340 nm was monitored.

Protein concentration was determined by a biuret method (11) after the protein was precipitated and washed with 0.5N perchloric acid. Crystallized and lyophilized BSA was used as the standard.

Materials -- Horseradish peroxidase, Type II (E.C. 1.11.1.7), FAD, FMN, p-hydroxybenzoic acid, 4-aminoantipyrine, DCPIP, PMS, NAD+, NADP+, CoA, DTT, BSA, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo. Acyl-CoA's and DL-C_{12}-carnitine·chloride were purchased from P-L Biochemicals, Milwaukee, Wis. Sprague-Dawley rats were obtained from Charles River Breeding Labs., Wilmington, Mass. All other chemicals were of analytical reagent grade.

† Abbreviations used are:

BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; DTT, dithiothreitol.

TABLE I. Requirements For Activity Of The Peroxisomal Fatty Acyl-CoA Oxidase Of Rat Liver.

A- 50 μM C_{16}-CoA as substrate.		B- 50 μM C_{12}-CoA as substrate.	
Complete Assay[a] (7.9 nmoles/min/mg.)	100%	Complete Assay[a] (13.3 nmoles/min./mg.)	100%
- enzyme source	0	- C_{12}-CoA, + 100 μM DL-C_{12}-carnitine·Cl	0
- C_{16}-CoA	0	- C_{12}-CoA, + 100 μM CoA, + 100 μM DL-C_{12}-carnitine·Cl	24
- peroxidase	0		
+ 2X peroxidase	100		
- chromogen[b]	0		
- FAD	70		
- FAD, + 75 μM FMN	66		
- BSA	89		
- Triton	99		
+ 1.0 mM NaN_3	90		

a- for assay conditions see Materials and Methods
b- 25 mM p-hydroxybenzoic acid plus 1.0 mM 4-aminoantipyrine

RESULTS AND DISCUSSION

Table I shows that the fatty acyl-CoA oxidase activity is clearly dependent on enzyme source, palmitoyl-CoA, chromogen, and peroxide. The high concentration of catalase in the peroxisome does not interfere with the assay because of the high activity of peroxidase used (2). Adding NaN_3 to inhibit the peroxisomal catalase does not increase the oxidase rate (12). With respect to our previous work on Tetrahymena (2), o-dianisidine could not act as the chromogen in the rat liver assay. Therefore it was replaced with p-hydrobenzoic acid plus 4-aminoantipyrine (9). The omission of BSA from the assay does not alter the activity. However, the assay was not linear for a prolonged (> 2 min) period in the absence of BSA. The omission of Triton did not alter the activity but in its absence there was an optical interference from particle agglutination. The oxidase is an FAD dependent enzyme; FMN could not substitute for FAD. The reaction rate was proportional to the amount of peroxisomal protein added in the range

TABLE II. Characterization Of The Peroxisomal β-Oxidation Of Rat Liver.

A - 50 μM C_{16}-CoA as substrate.		B - 50 μM C_{12}-CoA as substrate.	
Complete Assay[a] (15.1 nmoles/min./mg.)	100%	Complete Assay[a] (32 nmoles/min./mg.)	100%
- enzyme source	0	- C_{12}-CoA, + 100 μM DL-C_{12}-carnitine·Cl	73
- C_{16}-CoA	0	- C_{12}-CoA, - CoA, + 100 μM DL-C_{12}-carnitine·Cl	0
- NAD+	0		
- NAD+, + 100 μM NADP+	7		
- CoA	96		
- FAD	78		
- KCN	89		
- DTT	100		
- BSA	102		

a - for assay conditions see Materials and Methods

of 0-0.2 mg/ml. The oxidase has a pH maximum of 8.0-9.0. Substituting DL-C_{12}-carnitine·chloride for the acyl-CoA did not produce a reaction. However, if CoA was also added to the test, a small reaction was observed. This indicates the presence of a medium-chain carnitine acyltransferase in the peroxisome. Lowering the oxygen content of the gas phase from 100% to 1.0% (v/v) decreased the fatty acyl-CoA oxidase activity by only 37%[1].

Table II shows the effect of cofactor omission on the peroxisomal β-oxidation system. The activity was dependent on enzyme source, NAD+ and palmitoyl-CoA. NADP+ could not substitute for NAD+. The omission of CoA, KCN, DTT and BSA had little effect on the activity. Their omissions however, did affect the linearity of the assay. FAD is required for optimal activity. If acyl-CoA is replaced by DL-C_{12}-carnitine·chloride, a high rate of reaction is observed.

The chain length specificities for acyl-CoA substrates (50 μM final concentration) of the peroxisomal fatty acyl-CoA oxidase and the β-oxidation system, as well as the mitochondrial fatty acyl-CoA dehydrogenase (calculated at V_{max} PMS)

[1] Hryb, D.J. and Hogg, J.F., manuscript in preparation.

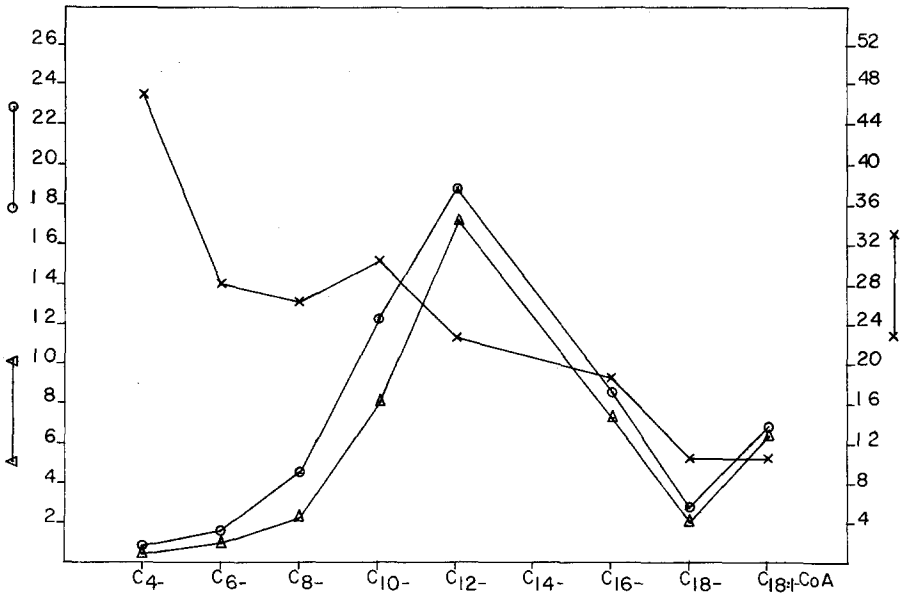

FIGURE 1. Reaction Rate vs Substrate Chain Length. Experimental conditions are described in Materials and Methods. Rates are given in nanomoles/min./ milligram of protein, with the dehydrogenase values being obtained by extrapolation to PMS saturation (10). (Δ——Δ) Peroxisomal fatty acyl-CoA oxidase; (o——o) Peroxisomal β-oxidation; (x——x) Mitochondrial fatty acyl-CoA dehydrogenase.

were studied (figure 1). The peroxisomal fatty acyl-CoA oxidase shows the ability to oxidize medium to long-chain acyl-CoA's (C_{10}-C_{16}). However, there is a very definite peak of activity at C_{12}. This is in contrast with Osumi and Hashimoto's (5) data on the chain length specificity of the oxidase. Their results show the oxidase to have a broader chain length range (C_{10}-C_{22}), with a peak at C_{16}. However, their oxidase was obtained from rats treated with di-(2-ethylhexyl)phthalate, a compound which causes peroxisomal proliferation and a great increase in the oxidase activity.

The chain length specificity of the peroxisomal β-oxidation system is identical to that of the fatty acyl-CoA oxidase. Their specific activities are also very similar and, in each case, oleoyl-CoA was a better substrate than stearoyl-CoA. Thus these data indicate that the oxidase is the rate-limiting enzyme in the peroxisomal β-oxidation system.

The chain length specificity of the mitochondrial fatty acyl-CoA dehydrogenase shows a broader range (C_4-C_{16}), with a shorter-chain length substrate being the more active and no preference shown for oleoyl- over stearoyl-CoA. The peak activity observed at C_4 is unusual in that it is contrary to previously published data (7,8). Our experiments utilized the same mitochondrial protein and substrate concentration throughout the series, in order that the ratio of substrate to the three fatty acyl-CoA dehydrogenases present in the mitochondria would be the same for all the acyl-CoA's treated. Although our data are in terms of V_{max} PMS, the same chain length specificity pattern was obtained at each PMS level.

If the mitochondrial fatty acyl-CoA dehydrogenase and the peroxisomal fatty acyl-CoA oxidase are taken to be the rate-limiting step in their respective β-oxidation systems, their ratio of activities for the oxidation of palmitoyl-CoA (820 and 106 nmoles/min./g. liver respectively) tells us that the rate of palmitoyl-CoA oxidation in the total mitochondrial fraction is 7.7 times that in the peroxisomal fraction. This ratio is in approximate agreement with the palmitoyl-CoA β-oxidation ratio (mitochondria/peroxisome) of 3.2 obtained by Krahling et al. (13) and of 5.4 by Shindo and Hashimoto (14). However, it is in complete disagreement with Lazarow's claim (15,16) that the bulk of palmitoyl-CoA oxidation in rat liver occurs in the peroxisome. Our results, as well as those of Krahling et al. and of Shindo and Hashimoto were obtained with normal rats, while Lazarow used rats treated with Clofibrate, a hypolipidemic drug which causes peroxisomal proliferation. Shindo and Hashimoto (14) also showed that, if they treated the rats with di-(2-ethylhexyl)phthalate, the ratio changed from 5.4 to 0.67. That is, the ability of the peroxisome to oxidize palmitoyl-CoA increased to the level of the normal mitochondrial rate. On the other hand, Christiansen et al. (17) showed that Clofibrate in the diet also results in a significant stimulation of β-oxidation by the mitochondria, especially for the shorter-chain length fatty acids (below C_{14}).

In conclusion our results indicate that in normal rats the peroxisomal β-oxidation system plays a subservient role to the mitochondrial system in the oxi-

dation of fatty acids by the liver. We submit that for the completion of an oxidation of a medium to long-chain fatty acid initiated in the peroxisome, there must be participation by the mitochondrion. This is clearly shown by their chain length specificities (figure 1). Only the mitochondrion can oxidize fatty acids of less than C_8 chain length, i.e., the mitochondrion must complete an oxidation initiated in the peroxisome. This is also shown to be true by Lazarow's (15) own data where the oxidation of palmitoyl-CoA by a purified peroxisomal fraction gave a stoichiometry of 1:5:5 (C_{16}-CoA: C_2-CoA: NADH).

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