

Possible involvement of NADPH-cytochrome P450 reductase and cytochrome b_5 on β -ketostearoyl-CoA reduction in microsomal fatty acid chain elongation supported by NADPH

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The chain elongation products from $[2-^{14}\text{C}]$ malonyl-CoA and palmitoyl-CoA by rat liver microsomes initiated by NADPH were found mainly to be 18:1 and 18:0. When anti-NADPH-cytochrome P450 reductase or anti-cytochrome b_5 immunoglobulin G were included in this system, not only was the overall chain elongation significantly suppressed, but also a new radioactive product, which was identified as 2-heptadecanone derived from an intermediate β -ketostearate, is accumulated depending upon the amounts of IgG added. These results suggest that NADPH-cytochrome P450 reductase and cytochrome b_5 participate in the conversion from β -ketostearoyl-CoA to β -hydroxystearoyl-CoA, which is the first reductive step of the microsomal chain elongating system.

<i>Immunochemical evidence</i>	<i>Cytochrome b_5</i>	<i>F_{pT}</i>	<i>Microsome</i>	<i>Fatty acid</i>
	<i>Chain elongation</i>			

1. INTRODUCTION

Liver microsomes catalyze both desaturation and chain elongation of fatty acids. Attempts to isolate desaturase have shown it to be a multienzyme system containing microsomal electron-transfer components [1,2]. In contrast to the desaturase system, relatively little is known about the enzymes involved in the chain elongation of fatty acids [3].

Microsomal electron-transport components, NADPH-cytochrome P450 reductase (F_{pT}) and cytochrome b_5 (cyt. b_5), participate in the fatty acid chain elongation initiated by NADPH [4]. However, the question of which reductive steps participate in the microsomal chain-elongating system remains unresolved. These studies were done to clarify this problem using antibodies against purified F_{pT} and cyt. b_5 .

2. EXPERIMENTAL

2.1. Materials

The following materials were obtained from commercial sources: $[2-^{14}\text{C}]$ malonyl-CoA (48.9 mCi/mmol) from New England Nuclear; NADPH from Kyowa Hakko; malonyl-CoA, palmitoyl-CoA, bovine serum albumin, cytochrome c (horse heart) and trypsin from Sigma; DEAE-cellulose (DE 52) and precoated thin-layer plate (LK6DF) from Whatman; Sephadex G-100 and 2',5'-ADP-Sepharose from Pharmacia; Triton X-100 from Wako; fat-free diet from Oriental Kobo. All other chemicals were of analytical grade.

2.2. Preparation of microsomes

Liver microsomes were prepared from refed fasted rats as in [5]. The microsomes were washed once and suspended in 0.25 M sucrose at 13–20 mg protein/ml.

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Table 1

Effect of rabbit anti-cyt. b_5 and anti- F_{PT} IgG on condensation reaction and NADPH-initiated fatty acid chain elongation

IgG (mg IgG/mg Mc)		Condensation (%)	Activity (%)	Elongation		
				Composition (%)		
				Fatty acids	β -Ketostearate	2-Hepta-decanone
Preimmune	0	100	100	93.4	3.0	3.6
	1	104.6	107	94.8	0.3	5.0
	5	103.5	126	95.6	0	4.4
	10	104.5	118.3	93.4	0	6.7
	20	88.1	128.9	91.1	0	8.7
Anti-cyt. b_5	1	82.4	80.0	94.1	n.d. ^a	5.9
	5	102.4	58.0	88.4	n.d.	11.6
	10	97.8	58.2	76.4	n.d.	23.6
Anti- F_{PT}	1	114.2	113.6	91.8	1.1	7.2
	5	140.6	124.8	78.1	3.1	18.9
	10	115.3	91.7	78.2	1.2	20.6
	20	104.7	56.3	70.1	1.6	28.3

^a n.d., not detected

2.3. Enzyme assays

Microsomal overall chain elongation and condensation reactions were measured by the incorporation of [2-¹⁴C]malonyl-CoA (1.2 mCi/mmol) into fatty acids in the presence of palmitoyl-CoA by the assay conditions in [6]. To separate the radioactive reaction products, the organic solvent extract was applied on a thin-layer plate and developed with petroleum ether/ethyl ether/acetic acid (70:30:1, by vol.) [7]. The fractions corresponding to authentic β -ketostearic acid, 2-heptadecanone and fatty acids were scraped into scintillation vials, and 10 ml of Toluene-based mixture were added. Radioactivity was determined using an Aloka liquid scintillation spectrometer. Further identification of the reaction products was achieved by radio gas-liquid chromatography after methylation with diazomethane.

To examine the effects of antibodies on chain elongation and condensation reactions, IgG was added to the microsomes, preincubated at 30°C for 10 min and then centrifuged at $77000 \times g$ for 90 min to remove any unbound antibodies. The resulting microsomal pellets were suspended in

0.25 M sucrose and used for enzyme assay.

NADH- and NADPH-cytochrome c reductase activity were determined as in [8].

2.4. Preparation of anti-cyt. b_5 and anti- F_{PT} antibodies

Cytochrome b_5 was purified to homogeneity from rat liver microsomes as in [9]. NADPH-cytochrome P450 reductase (F_{PT}) was purified from rat liver microsomes as in [10] followed by use of 2',5'-ADP-Sepharose affinity chromatography [11]. Purity of the final preparation was confirmed by SDS disc gel electrophoresis [15].

Immunization of rabbits and purification of immunoglobulin G (IgG) were done as in [12]. The titer of antiserum was checked by Ouchterlony double immunodiffusion [13]. Anti- F_{PT} IgG inhibited NADPH-cytochrome c reductase activity by 80% at 2 mg IgG/mg microsomal protein, whereas anti-cyt. b_5 IgG inhibited NADH-cytochrome c reductase by 70% at 10 mg IgG/mg microsomal protein.

2.5. Analytical methods

Protein was measured as in [14] with bovine serum albumin as a standard. Polyacrylamide disc gel electrophoresis was done as in [15].

3. RESULTS

3.1. Effects of rabbit anti-cyt. b_5 and anti- F_{pT} IgG on NADPH-initiated microsomal fatty-acid chain elongation and condensation reactions

Preincubation of microsomes with the antibodies suppressed significantly the overall chain elongating reaction in either anti-cyt. b_5 - or anti- F_{pT} IgG (table 1). However, the two antibodies had no effect on the condensation reaction. Separation of the components of the reaction products after saponification revealed an accumulation of 2-heptadecanone, when microsomes were preincubated with the antibodies. 2-Heptadecanone is suggested to be a decarboxylated derivative of an intermediate, β -ketostearate, formed during saponification of the reaction products [7,17]. Here, a further experiment to verify the origin of 2-heptadecanone was done using the initial condensation product, β -ketostearate, which was obtained from the whole reaction system without the addition of NADPH. Results of radio gas-liquid chromatography suggested the sample prepared by saponification and extraction from the condensation product to be 2-heptadecanone (fig.1A), which was subsequently identified positively as such by gas chromatography-mass spectrometry (GC-MC) (not shown).

3.2. Radio gas-liquid chromatography of the reaction products

The reaction products were analyzed in greater detail by radio gas-liquid chromatography in the presence or absence of antibodies, because the fatty acids could not be separated by thin-layer chromatography (section 2.3). In the preimmune control IgG, two major radioactive peaks, the larger 18:1 and the smaller 18:0, were observed as a result of the elongation and subsequent desaturation (fig.1B). However, addition of anti-cyt. b_5 IgG made a remarkable change as compared with that of control IgG. The 18:1 peak disappeared completely and another new radioactive peak clearly appeared, while the major peak was 18:0 (fig.1). The new radioactive peak was identified as

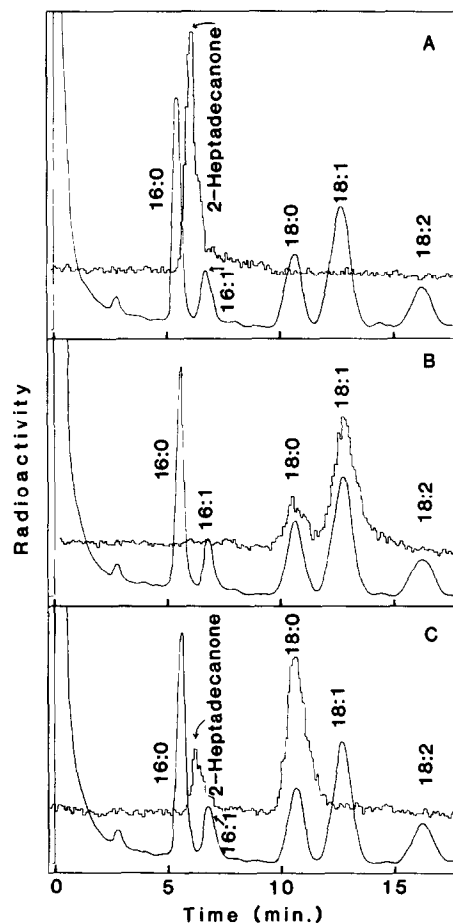


Fig.1. Radio gas-liquid chromatography of the reaction products in the presence or absence of anti-cyt. b_5 antibodies. The microsomes were preincubated with the preimmune or anti-cyt. b_5 IgG as in section 2. The reaction products were methylated by diazomethane after saponification with 10% KOH in methanol for 30 min and applied to a radio gas-liquid chromatography (Hitachi 663) equipped with a 2 m stainless steel column containing 10% EGSSX. The column was programmed to 160–200°C at 5°C/min. (A) Condensation product in the absence of IgG and NADH; (B) chain elongation product in the presence of preimmune IgG; (C) chain elongation product in the presence of anti-cyt. b_5 IgG. The amount of IgG added was 10 mg/mg microsomal protein: (—) gas chromatographic trace; (---) radioactivity trace.

2-heptadecanone by GC-MS as described above (not shown). The ketone accumulation increased depending upon the amount of antibodies added.

4. DISCUSSION

The hepatic microsomal fatty acid elongation reaction requires fatty acyl-CoA as a precursor, malonyl-CoA as a 2-carbon donor and reducing equivalents provided by either NADH or NADPH [16,17]. Recent studies have shown the involvement of cyt. *b*₅ in the transfer from NADH to an elongase enzyme system [18] and of F_{PT} and cyt. *b*₅ in the transfer from NADPH [4]. The NADPH-dependent elongation reaction may occur via two pathways [4]:

- (i) Through cyt. *b*₅ via F_{PT};
- (ii) Directly to the elongase from F_{PT}.

The precise mechanism, however, remains to be resolved. For the elongation of fatty acids by 2-carbon unit, 2 different reductive steps must occur in the process [7]. Up to the present, the reductive steps in which the microsomal electron-transport components participate have been unclear. These investigations revealed an accumulation of 2-heptadecanone derived from β -ketostearate when incubation was done with either anti-cyt. *b*₅ or anti-F_{PT} IgG. This finding suggests that the participation of cyt. *b*₅ and F_{PT} might be in the step of β -ketoacyl-CoA reduction, which is the first reductive step in the chain elongating system.

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