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

Masaki Nagao, Teruo Ishibashi*, Takako Okayasu and Yoh Imai

Department of Biochemistry, Hokkaido University School of Medicine, Sapporo 060, Japan

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The chain elongation products from [2-^{14}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.

Immunochemical evidence *Cytochrome* b_5 *F_{pT}* *Microsome* *Fatty acid Chain elongation*

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.

* To whom correspondence should be addressed

2. EXPERIMENTAL

2.1. *Materials*

The following materials were obtained from commercial sources: [2-^{14}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 (%)	Elongation			
			Activity (%)	Composition (%)		
				Fatty acids	β-Ketostearate	2-Heptadecanone
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-^{14}C]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 × 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

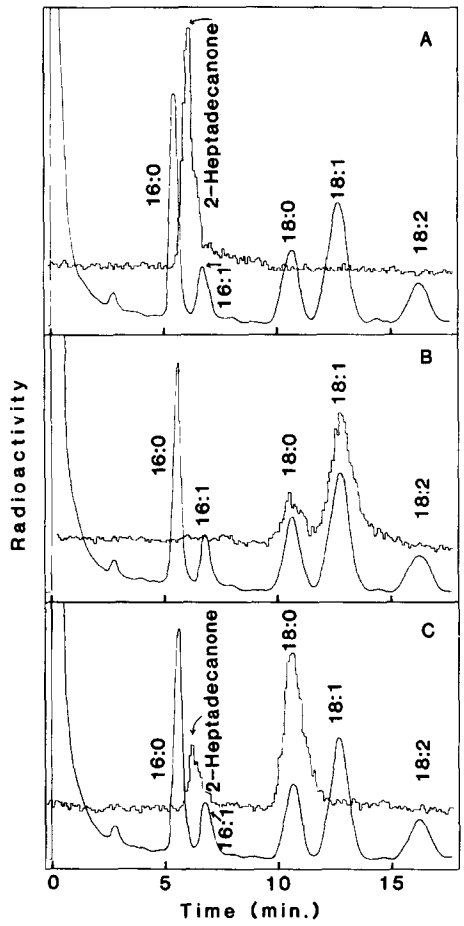

Fig.1. Radio gas–liquid chromatography of the reaction products in the presence or absence of anti-cyt. b_5 antibodies. The microsomes were preincubated with the preimmune or anti-cyt. b_5 IgG as in section 2. The reaction products were methylated by diazomethane after saponification with 10% KOH in 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_{pI} 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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