Accumulation of Medium Chain Acyl-CoAs during β-Oxidation of Long Chain Fatty Acid by Isolated Peroxisomes from Rat Liver

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We have reported fatty alcohol synthesis accompanied by chain elongation in liver peroxisomes (*Biochim. Biophys. Acta*, 1346, 38 (1997)). In the present experiment, we studied what kind of acyl-CoA(s) destined to be utilized as primer for fatty alcohol synthesis accumulate(s) during peroxisomal β-oxidation. Peroxisomes were prepared from rat liver treated with clofibrate, a peroxisome proliferator, and incubated with [U-^{14}C]palmitate, in order to investigate acyl-CoAs after β-oxidation. At 1 mM concentration, MgATP activated β-oxidation, but inhibited β-oxidation at concentrations higher than 1 mM. After incubation of peroxisomes with palmitate, various acyl-CoAs were formed. Among medium-chain labelled acyl-CoAs, octanoyl-CoA was mainly detected. These results suggest that octanoyl-CoA accumulates during β-oxidation of palmitate. When peroxisomes were incubated with [9,10-^{3}H]palmitate and [9,10-^{3}H]stearate, among medium-chain acyl-CoAs, octanoyl-CoA and decanoyl-CoA were primarily detected, respectively, suggesting the occurrence of at least 4 cycles of β-oxidation of both fatty acids by peroxisomes.

Key word peroxisome; β-oxidation; fatty acyl-CoA

Peroxisomes play a catabolic role in the β-oxidation of very-long-chain fatty acids[1—3] and poly-unsaturated fatty acids.[4—6] These organella also have an anabolic role in the biosynthesis of cholesterol,[7—10] bile acids[11—13] and plasmalogen.[14—16] We have reported that acetyl-CoA derived from peroxisomal β-oxidation is utilized for anabolic metabolism.[17—20] The activity of the peroxisomal β-oxidation system of rats is greatly increased by treatment with clofibrate, a peroxisome proliferator, while that of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis, is significantly decreased. Under these conditions, acetyl-CoA from peroxisomes is utilized for the biosynthesis of plasmalogen.[19,20] In other words, acetyl-CoA from peroxisomal β-oxidation is incorporated into the 1-alkenyl group of ethanolamine plasmalogen.[20]

In plasmalogen biosynthesis, the first reaction is formation of 1-acyl dihydroxyacetone-phosphate (DHAP) from acyl-CoA and DHAP. The next step is replacement of the acyl moiety with fatty alcohol, forming 1-alkyl DHAP, which is then reduced to 1-alkyl glycerophosphate (GP). After a few steps, 1-alkyl GP finally forms 1-alkenyl 2-acyl glycero-3-phosphoethanolamine, *i.e.*: ethanolamine plasmalogen. Biosynthesis up to 1-alkyl GP takes place exclusively in peroxisomes.[14—16]

We reported that the fatty alcohol destined to form the 1-alkenyl group of plasmalogen is not of extraperoxisomal origin, but is synthesized as nascent fatty alcohol within peroxisomes.[21] Furthermore, acetyl-CoA utilized for the synthesis of this nascent fatty alcohol is supplied by peroxisomal β-oxidation.[20] Horie *et al.*[22] reported that peroxisomal chain elongation of acyl-CoA with octanoyl-CoA as the primer forms dodecanoyl-CoA. Recently, we reported that peroxisomes can utilize dodecanoyl-CoA as a primer, and form mainly hexadecanol, accompanied by chain elongation using acetyl-CoA.[23] Thus, we estimated that in plasmalogen biosynthesis, peroxisomes degrade ordinary long chain fatty acids to form octanoyl-CoA, and the resulting acetyl-CoA is partly utilized for chain elongation of octanoyl-CoA, forming dodecanoyl-CoA, and then partly utilized for biosynthesis of fatty alcohol (hexadecanol) from this dodecanoyl-CoA.[23]

Peroxisomes can oxidize long chain and very long chain fatty acids. In this experiment, we studied what length of carbon-chain acyl-CoA(s), which can be utilized as a primer for fatty alcohol synthesis, would be generated by peroxisomal β-oxidation of long chain fatty acid. After palmitate and stearate were incubated with peroxisomes, acyl-CoAs formed by β-oxidation were analyzed.

MATERIALS AND METHODS

Materials [U-^{14}C]Palmitate (3.7 MBq/mmol, 0.1 mCi/mmol) and Aquazol 2 were purchased from New England Nuclear (U.S.A.). [9,10-^{3}H]Palmitate (3.7 MBq/mmol, 0.1 mCi/mmol) and [9,10-^{3}H]stearate (3.7 MBq/mmol, 0.1 mCi/mmol) were obtained from American Radiolabeled Chemicals Inc. (U.S.A.). Various acyl-CoAs ($C_{2:0}$, $C_{4:0}$, $C_{6:0}$, $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, and $C_{16:0}$), Nycodenz, NAD^+, $NADP^+$, CoA, ATP, dithiothreitol and antimycin A were obtained from Sigma (U.S.A.). Clofibrate, lactate dehydrogenase, pyruvate and all other reagents were of analytical grade and were purchased from Wako Pure Chemicals (Japan).

Animals Male Wistar rats weighing about 200 g were maintained in a light- and temperature-controlled environment and fed Clea chow CE-2 (Nihon Clea Inc., Tokyo, Japan) for at least 7 d prior to use. Then the rats were fed a chow containing 0.25% clofibrate for 14 d. Clofibrate increases peroxisome proliferation as well as fatty acyl-CoA β-oxidation, but decreases HMG-CoA reductase. Augmentative acetyl-CoAs from the increased peroxisomal β-oxidation consequently flows mainly to the 1-alkenyl group of ethanolamine plasmalogens.

Preparation of Peroxisomes from Rat Liver The livers were excised from the rats treated with clofibrate. A 10% liver homogenate was prepared in 0.25 M sucrose containing 5 mM Hepes (pH 7.4), 1 mM EDTA and 1% ethanol (SVEH).

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The light mitochondrial fraction was prepared according to the method of de Duve *et al.*[24] with a slight modification. The light mitochondrial fraction from 10 g of the liver was suspended in SVEH, layered over 25 ml of 27.7% Nycodenz-SVEH medium (d.=1.175) in a 30-ml Hitachi centrifugation tube and centrifuged at 63000×***g*** for 30 min at 4 °C in a Hitachi P65-1021N ultracentrifuge using an angle rotor (Hitachi RP50T).[25] The precipitate fraction was suspended in about 30 ml of SVEH and centrifuged at 20000×***g*** for 30 min for washing. The resulting pellet was suspended in an appropriate amount of SVEH, and this was used as the peroxisomal preparation in the present experiment.

Peroxisomal Incubations and Assay of Peroxisomal β-Oxidation Peroxisomal fractions were incubated using nonsolubilizing (iso-osmotic) incubation conditions. Unless otherwise stated, the incubations (1 ml) contained 130 mM-KCl, 20 mM-Hepes, 0.1 mM-EGTA, 0.5 mM-NAD^+, 0.1 mM-$NADP^+$, 0.4 mM-CoA, 1 mM-MgATP, 0.1 mM-dithiothreitol, 2 units of lactate dehydrogenase/ml, 20 mM-pyruvate, 10 mg of antimycin A/ml and 2 mg of defatted BSA/ml, pH 7.2. The substrate was always [U-^{14}C]palmitate (200000 dpm), which was added as a complex with BSA. The incubations usually contained about 0.05 mg of peroxisomal protein/ml, and were carried out at 37 °C in a shaking water bath.

Peroxisomal β-oxidation of [U-^{14}C]palmitate was measured as acid-soluble radioactivity released after various periods of incubation. An aliquot (100 μl) of the incubation mixtures was usually withdrawn and added to an equivalent volume of ice-cold $HClO_4$ (10% v/v). The radioactivity in the supernatant was measured after a brief centrifugation to remove denatured proteins.[26]

A minor mitochondrial contaminant is contained in the peroxisomal fractions. However, when free palmitate is used as a peroxisomal substrate, the resulting palmitoyl-CoA is essentially unavailable for mitochondrial β-oxidation because carnitine has not been added to the incubation mixture. It is also well established that few, if any, intermediates accumulate during mitochondrial β-oxidation. It is therefore unlikely that a significant portion of the intermediates observed was due to contaminating mitochondrial activity.

Preparation of Fraction Containing Acyl-CoA Esters After various periods of incubation, 900 μl of ice-cold glacial acetic acid was added to the resulting sample, and heptadecanoyl-CoA (40 nmol/ml) was added as an internal standard. Also, 144 μl of saturated $(NH_4)_2SO_4$ was added, and the tube was placed in a boiling-water bath for 2 min, and then allowed to cool to room temperature. Each sample was extracted with 18 ml of diethyl ether and again with 14 ml of diethyl ether to remove free palmitate. An aqueous layer containing acyl-CoAs was extracted for 1 h with 12 ml of methanol/chloroform (2 : 1, v/v) with continuous shaking. After centrifugation (40000×g_{av}·min), the supernatant was retained. The pellet was resuspended in 4.5 ml of methanol/chloroform (2 : 1, v/v) and re-centrifuged. The solvent was reduced from the combined supernatants under N_2 blowing at 60 °C until the volume was about 2 ml. The pH was adjusted to 6—7 by adding 2.0 ml of 1 M-ammonium acetate, and the sample was freeze-dried.

The residue was extracted with 2.4 ml of methanol and centrifuged at 3000×***g*** for 30 min, and the supernatant was retained. The pellet was resuspended in 0.8 ml of methanol and re-centrifuged, and the solvent was removed from the combined supernatants under N_2 gas. The residue was dissolved in 5% acetonitrile in 50 mM-KH_2PO_4, pH 5.3 and analyzed by HPLC.[26—28]

HPLC Analysis of Acyl-CoA Esters A Shimadzu Series LA-10 HPLC apparatus (Shimadzu, Japan) with a 5-mm LiChrospher 100 RP-18 column (4×250 mm) (Merck, Germany) was used for HPLC. The column was maintained at 40 °C. All solvents were degassed with helium. The mobile phase was the following gradient of acetonitrile in 50 mM-KH_2PO_4, pH 5.3 : isocratic 5% (v/v), 5 min; isocratic 10%, 0.1 min; linear gradient 10—30%, 9.9 min; linear gradient 30—60%, 40 min; linear gradient 50—5%, 5 min. The mobile phase was pumped at a constant rate of 1.5 ml/min. The column was re-equilibrated for at least 20 min under the starting conditions between analyses to maintain reproducibility of retention times. The ultraviolet absorbance of the column eluate was monitored with an SPD-10A Shimadzu UV spectrophotometric detector at 210 nm. Retention times of acetyl-CoA, butyryl-CoA, hexanoyl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, palmitoyl-CoA, heptadecanoyl-CoA and stearoyl-CoA were 5.03, 10.81, 14.70, 18.76, 23.76, 31.97, 38.90, 44.48, 51.02 and 55.08 min, respectively. Peak fractions corresponding to various acyl-CoAs were collected and concentrated. Radioactivity was measured using an Aloka-LSC 700 scintillation counter (Tokyo), with Aquazol 2 (New England Nuclear, Boston, MA, U.S.A.) as a scintillator.[27,28]

Enzyme and Protein Assays To estimate the purity of the peroxisomal preparation, the activities of catalase, cytochrome-c oxidase, esterase and acid phosphatase were determined as markers of peroxisomes, mitochondria, microsomes and lysosomes, respectively. Catalase was estimated by the method described in our previous report.[29—31] Cytochrome-c oxidase was determined by the method of Wharton and Tzagoloff.[31,32] Esterase was measured using *o*-nitrophenyl acetate as a substrate according to the method of Beaufay *et al.*[33] Acid phosphatase was determined by the method in our previous report.[30]

Protein was determined by the Lowry method[34] using bovine serum albumin as a standard. Since Nycodenz interferes with the determination of protein, it was separated by co-precipitation of the protein with deoxycholate and trichloroacetic acid before determination.

RESULTS

Preparation of Peroxisomes Using Nycodenz Density Centrifugation The liver was excised from a rat treated with clofibrate. Peroxisomes were prepared from the light mitochondrial fraction of liver homogenate by Nycodenz density centrifugation. The result is shown in Table 1.

Peroxisomes were purified approximately 28-fold over liver homogenate and were more than 94% pure, as calculated according to Leighton *et al.*[29] This peroxisome preparation, which was contaminated with less than 4.8% of microsomes and less than 1.2% of mitochondria, was used in the following experiment on fatty alcohol biosynthesis.

Effect of MgATP on Peroxisomal β-Oxidation When peroxisomes were incubated with [U-^{14}C]palmitate, the effects of MgATP on fatty acid β-oxidation were studied. Incu-

Table 1. Purification of Peroxisomes from Rat Liver

	Protein (mg/g liver)	Catalase	Esterase	Cyt. C oxidase	Acid phosphatase
Homogenate	266±122	S.A. 68.3±31.8 (1)	1.11±0.38 (1)	6.06±1.32 (1)	0.019±0.001 (1)
	⟨⟨100%⟩⟩	T.A. 12530±480	407±19	1778±17	7.39±0.45
Purified	1.54±0.36	S.A. 1940±584 (28.4)	0.261±0.009 (0.24)	0.373±0.14 (0.06)	0.0025±0.001 (0.13)
peroxisomes	⟨⟨0.58%⟩⟩	T.A. 2871±1291 ⟨⟨22.9⟩⟩	0.709±0.121 ⟨⟨0.17⟩⟩	0.517±0.194 ⟨⟨0.029⟩⟩	0.003±0.001 ⟨⟨0.041⟩⟩
	Compartmentalization (%)	as peroxisomes: >93.7	as microsomes: <4.80	as mitochondria: <1.23	as lysosomes: <0.26

S.A.: units/mg protein (purity over homogenate), T.A.: units/g liver (recovery from homogenate). Rats were fed a chow containing 0.25% clofibrate for 14 d. The light mitochondrial fraction was prepared in 0.25 M sucrose containing 5 mM HEPES, 1 mM EDTA and 0.1% ethanol, and centrifuged in 27.7% Nycodenz at 63000×***g*** for 30 min, as described in the text. The precipitate was used as the peroxisomal preparation. S.A. and T.A. are specific activity and total activity, respectively.

bation was performed in the presence of 0.25 or 0.4 mM CoA. The result is shown in Fig. 1.

The β-oxidation activity was increased by the addition of MgATP, and was the highest at the MgATP concentration of 1 mM. The activity in the presence of 1 mM MgATP was 16.4 times greater than the control (absence of MgATP). At concentrations of MgATP higher than 1 mM, the activity was decreased, resulting in 27.5% and 7.3% of the highest activity in the presence of 5 mM and 10 mM MgATP, respectively. Osmundsen and collaborators had performed the experiment at 5 and 10 mM MgATP,[26,35] but our data indicated that the reaction might be inhibited by MgATP at these concentrations. Therefore, we performed the experiment using 1 mM MgATP.

Bartlett *et al.* used 0.2 mM CoA in the β-oxidation reaction.[26] When we compared the effect of 0.2 mM and 0.4 mM CoA on β-oxidation activity, the activity at 0.4 mM was 2.23 times that at 0.2 mM (Fig. 1). Therefore, we used 0.4 mM CoA.

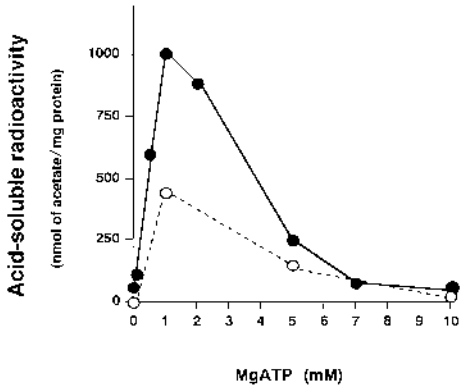

Fig. 1. Effect of MgATP on Peroxisomal β-Oxidation Using [U-^{14}C]-Palmitate

Isolated peroxisomal fractions were incubated with 200 μM [U-^{14}C]palmitate in the presence of 0.2 mM (○) or 0.4 mM CoA (●). At 20 min, samples of the incubations (100 μl) were removed for measurement of acid-soluble radioactivity as described in the text. The incubations contained 0.05 mg of peroxisomal protein/ml. Typical data of 3 experiments are shown.

Effects of Incubation Time on the β-Oxidation Activity of [U-^{14}C]Palmitate and on the Pattern of Acyl-CoA Intermediates We studied the time course of the β-oxidation activity of peroxisomes using [U-^{14}C]palmitate as a substrate. The reaction was performed in the presence or absence of pyruvate and lactate dehydrogenase, which are regeneration systems of NAD^+. The result is shown in Fig. 2. β-Oxidation activity was increased for at least 30 min.

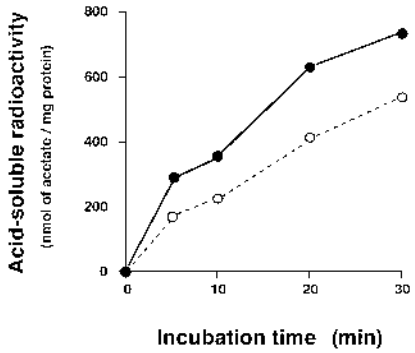

Fig. 2. Time Course of Peroxisomal β-Oxidation Using [U-^{14}C]Palmitate as a Substrate

Isolated peroxisomal fractions were incubated with 200 μM [U-^{14}C]palmitate, in the presence (●) or absence (○) of 20 mM-pyruvate plus 2 units of lactate dehydrogenase/ml. At the time points indicated, samples of the incubations (100 μl) were removed for measurement of acid-soluble radioactivity. The incubation mixture contained 0.05 mg of peroxisomal protein/ml.

The activity in the presence of pyruvate and lactate dehydrogenase was increased approximately 1.5 fold compared to that in the absence of pyruvate and lactate dehydrogenase. These results were consistent with those of Bartlett *et al.*[26] Namely, β-oxidation reaction might have been performed smoothly, since NAD^+ was regenerated from NADH, which is a by-product of β-oxidation, by the lactate dehydrogenase reaction.

Typical patterns of acyl-CoA esters synthesized from β-oxidation of [U-^{14}C]palmitate are shown in Fig. 3; acetyl-CoA is not shown in the Figures. After incubation for 5 min, the radioactivity of palmitoyl-CoA was low. Therefore, a small amount of added palmitate seemed to have changed to CoA ester. However, acyl-CoAs were detected, the chain lengths of which are shorter than that of palmitoyl-CoA. Thus, a small amount of palmitoyl-CoA seems to be synthesized and further oxidized. After incubation for 10 min, the amounts of palmitoyl-CoA and acetyl-CoA were each increased about 5.7 times as compared with those after 5 min. In other words, palmitate seems to be sufficiently changed to CoA ester and oxidized, forming a great deal of acetyl-CoA. After incubation for 30 min, palmitoyl-CoA was decreased and acetyl-CoA was increased. This means that the reaction was developing further with time. As shown in Fig. 3, acyl-CoAs of various chain lengths were detected after β-oxidation, but among medium-chain acyl-CoAs, mainly octanoyl-CoA was synthesized after 20 min of the incubation. The reaction did not stop at the chain length of octanoyl-CoA, and acyl-CoAs of shorter chain length were also detected. However, the amount of octanoyl-CoA was increased with time.

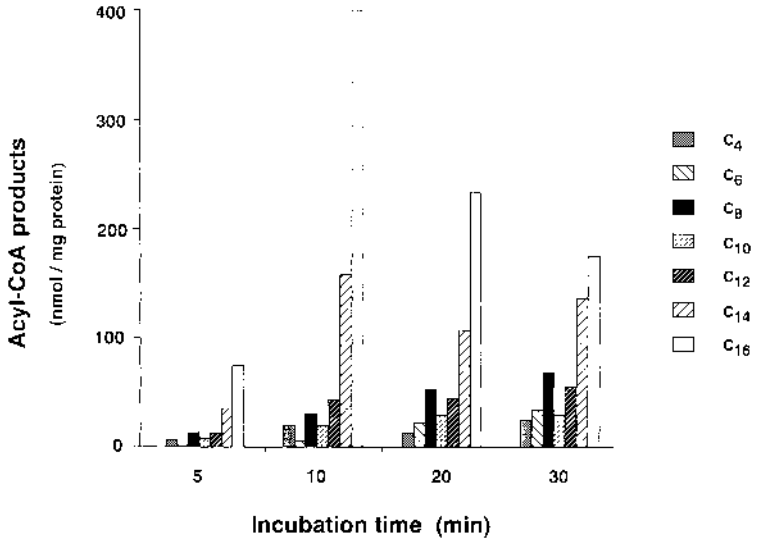

Fig. 3. Formations of [U-^{14}C]Acyl-CoA Esters after Incubation with Peroxisomes

Peroxisomal fractions were incubated with 200 μM [U-^{14}C]palmitate, as described in the legend to Fig. 2. At the time points indicated, samples (900 μl) were mixed with 900 μl of ice-cold glacial acetic acid. The acyl-CoA esters were subsequently extracted and chromatographed. The resulting data are presented as histograms, for incubations in the presence of 10 mM-pyruvate plus 2 units of lactate dehydrogenase/ml. The identities of the various peaks of radioactivity are indicated: C_{16}, [^{14}C]palmitoyl-CoA; C_{14}, [^{14}C]tetradecanoyl-CoA; C_{12}, [^{14}C]dodecanoyl-CoA; C_{10}, [^{14}C]decanoyl-CoA; C_8, [^{14}C]octanoyl-CoA; C_6, [^{14}C]hexanoyl-CoA; C_4, [^{14}C]butyryl-CoA; C_2, [^{14}C]acetyl-CoA. The numbers in parentheses at the top of the column represent content of acyl-CoA products.

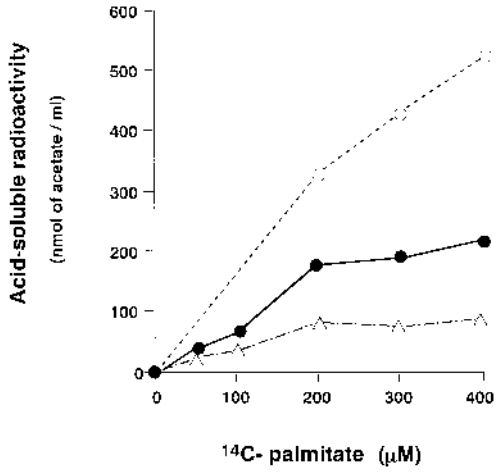

Fig. 4. Effects of Various Concentrations of [U-^{14}C]Palmitate Used as a Substrate for Peroxisomal β-Oxidation

Peroxisomal fractions were incubated with various concentrations of [U-^{14}C]palmitate. The incubations contained 0.025 mg (△), 0.05 mg (●) or 0.1 mg (○) of peroxisomal protein/ml. After 20 min of incubation, the samples were quenched for measurement of acid-soluble radioactivity.

Effect of the Concentration of [U-^{14}C]Palmitate on the Pattern of Acyl-CoA Intermediates β-Oxidation activity after incubation with various concentrations of [U-^{14}C]palmitate is shown in Fig. 4. The reaction was performed with 3 concentrations (0.025, 0.05, and 0.1 mg/ml) of peroxisomal protein. With low concentrations of the protein (0.025 and 0.05 mg/ml), β-oxidation activity was increased in proportion to the concentration of the substrate. The activity approximately plateaued at a substrate concentration of 200 μM and above. However, with high concentrations of peroxisomal protein (0.1 mg/ml), the activity was increased depending on the substrate concentration up to at least 400 μM. This indicates that with low concentrations of peroxisomal protein, substrate concentrations of 200 μM and above are sufficient for peroxisomal β-oxidation, but high concentrations of peroxisomes are not saturated by such substrate concentrations.

Patterns of acyl-CoAs synthesized with low concentrations of the protein are shown in Fig. 5. Various acyl-CoAs were synthesized. Among medium chain acyl-CoAs, octanoyl-CoA was the main one detected at a substrate concentration of 200 μM and above (Figs. 5a, b). Therefore, when [U-^{14}C]palmitate was used as a substrate, among medium-chain acyl-CoAs, octanoyl-CoA is synthesized by peroxisomal β-oxidation, in the presence of sufficient substrate.

Comparison of β-Oxidation Activity and the Pattern of Acyl-CoA Intermediates between [9,10-^{3}H]Palmitate and [9,10-^{3}H]Stearate In the body, stearate as well as palmitate is present, and so we were interested in whether the β-oxidation products of stearate differ from those of palmitate.

Time courses of peroxisomal β-oxidation of [9,10-^{3}H]palmitate and [9,10-^{3}H]stearate are shown in Fig. 6. Very little difference in β-oxidation was detected between palmitate and stearate.

Patterns of acyl-CoAs intermediates are shown in Fig. 7. In the case of palmitate, palmitoyl-CoA was already synthesized after 10 min, and the reaction continued. The amount of palmitoyl-CoA after 20 min of incubation was reduced by half after 10 min, the resulting various acyl-CoAs were synthesized, and among the medium chain acyl-CoAs, mainly octanoyl-CoA was detected (Fig. 7a). Since only 9 and 10 carbon atoms sites were radiolabeled, radioactivity of butanoyl- and hexanoyl-CoA could not be detected. In the case of stearate, on the other hand, one cycle of β-oxidation was already performed after 10 min, so that the palmitoyl-CoA content was greater than that of stearoyl-CoA. After 20 min, the palmitoyl-CoA was further oxidized, and various acyl-CoAs were synthesized. Decanoyl-CoA content was greaer than that of dodecanoyl-CoA and tetradecanoyl-CoA (Fig. 7b). Since we used [9,10-^{3}H]stearate, radioactivity of butyryl-, hexanoyl- and octanoyl-CoA could not be detected.

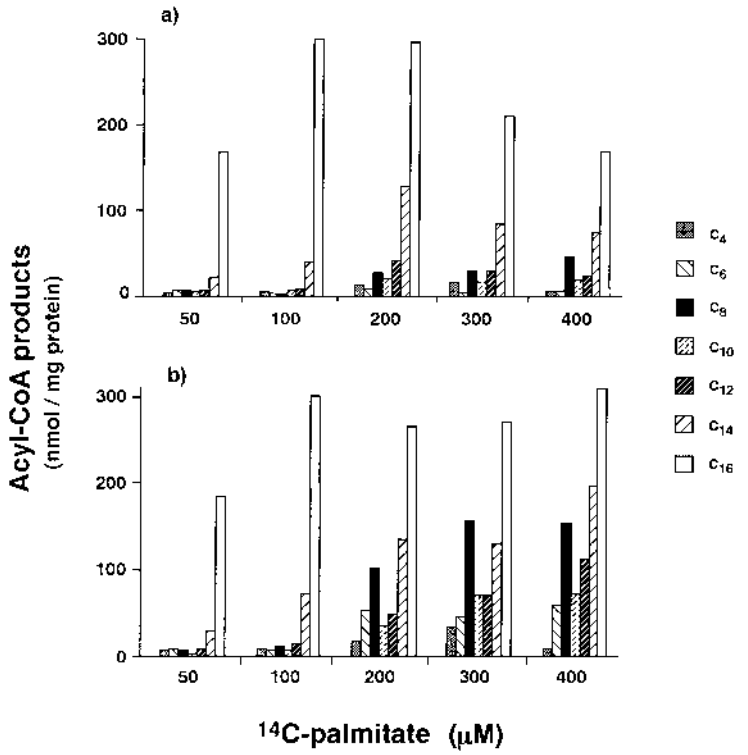

Fig. 5. Effects of Various Concentrations of [U-^{14}C]Palmitate on Acyl-CoA Esters Generated after Incubation with Peroxisomes

Peroxisomes were incubated with various concentrations of [U-^{14}C]palmitate, as described in the legend to Fig. 4. The incubations contained 0.025 mg (a) or 0.05 mg (b) of peroxisomal protein/ml. After 20 min of incubation, the samples were quenched for HPLC analysis of [^{14}C]acyl-CoA esters. The resulting data are presented as histograms.

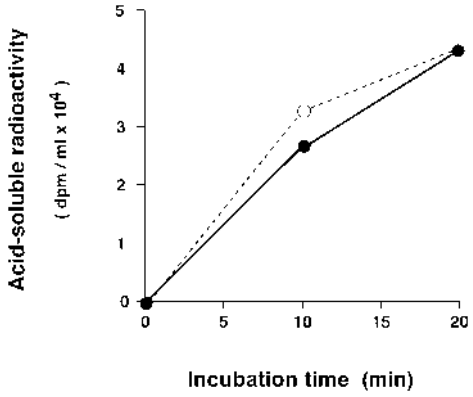

Fig. 6. Time Course of β-Oxidation Using [9,10-^{3}H]Palmitate and [9,10-^{3}H]Stearate as Substrates

Isolated peroxisomal fractions were incubated with 200 μM [9,10-^{3}H]palmitate (●) or [9,10-^{3}H]stearate (○). At the time points indicated, samples of the incubations (100 μl) were removed for measurement of acid-soluble radioactivity. The incubations contained 0.07 mg of peroxisomal protein/ml.

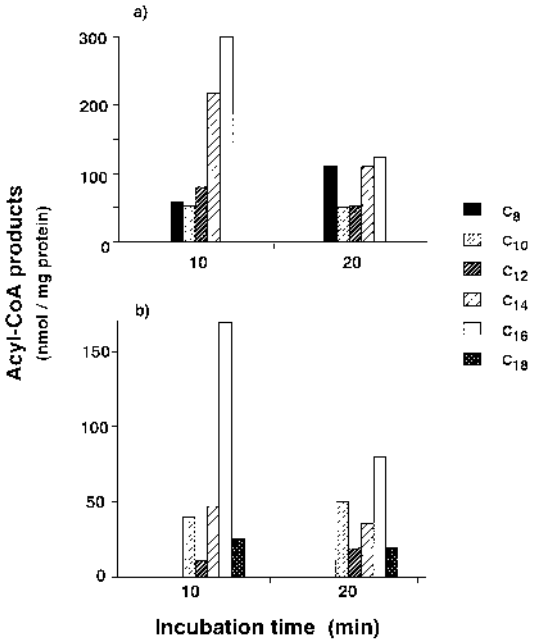

Fig. 7. Acyl-CoA Esters Produced Using [9,10-^{3}H]Palmitate and [9,10-^{3}H]Stearate as Substrates

Peroxisomal fractions were incubated with 200 μM [9,10-^{3}H]palmitate (a) or [9,10-^{3}H]stearate (b), as described in the legend to Fig. 6. After 10 and 20 min of incubation, the samples were quenched for HPLC analysis of [9,10-^{3}H]acyl-CoA esters. The resulting data are presented as histograms. The identities of the various peaks of radioactivity are indicated: C_{18}, [^{3}H]stearoyl-CoA; C_{16}, [^{3}H]palmitoyl-CoA; C_{14}, [^{3}H]tetradecanoyl-CoA; C_{12}, [^{3}H]dodecanoyl-CoA; C_{10}, [^{3}H]decanoyl-CoA; C_8, [^{3}H]octanoyl-CoA; C_2, [^{3}H]acetyl-CoA.

Peroxisomal β-oxidation acitivity was studied using various concentrations (200 and 300 μM) of [9,10-^{3}H]palmitate and [9,10-^{3}H]stearate. Hardly any difference in β-oxidation activity between palmitate and stearate was detected, even though the concentrations of both substrates were changed (data not shown).

Patterns of acyl-CoA intermediates show that a relatively high number of octanoyl-CoAs were synthesized when peroxisomes were incubated with 300 μM palmitate (Fig. 8a). In the case of stearate, various acyl-CoAs formed, and among medium chain acyl-CoAs, decanoyl-CoA was the main one

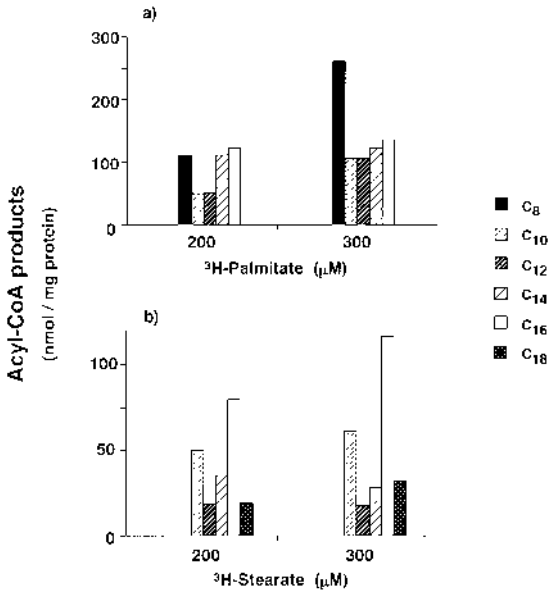

Fig. 8. Influence of Concentrations of [9,10-^{3}H]Palmitate and [9,10-^{3}H]-Stearate on Acyl-CoA Products

Isolated peroxisomal fractions were incubated with 200 μM and 300 μM of [9,10-^{3}H]palmitate (a) or [9,10-^{3}H]stearate (b). The incubations contained 0.07 mg of peroxisomal protein/ml. After 20 min of incubation, the samples were quenched for HPLC analysis on [9,10-^{3}H]acyl-CoA esters.

synthesized (Fig. 8b). In other words, although various acyl-CoAs formed depending on the concentration of the added substrate, among medium chain acyl-CoAs, octanoyl-CoA and decanoyl-CoA were mainly detected using palmitate and stearate as substrates, respectively. We do not deny the possibility that the reaction continues and shorter-chain acyl-CoAs form. However, peroxisomes are reported to prefer longer chain length fatty acid as a substrate, rather than 6 carbon atoms.[35,36] Therefore, even if the reaction proceeds, few acyl-CoAs with chains shorter than hexanoyl-CoA may be detected.

DISCUSSION

We studied what kind of acyl-CoA(s) destined to be utilized as a primer for fatty alcohol synthesis is synthesized by peroxisomal β-oxidation of long-chain fatty acids.

Various acyl-CoAs were formed by peroxisomal β-oxidation of palmitate. Among medium-chain fatty acids, octanoyl-CoA was abundantly formed in the presence of sufficient substrate and after incubation of at least 20 min (Figs. 3, 5, 7, and 8). Bartlett *et al.*[26] reported that dodecanoyl-CoA and tereadecanoyl-CoA were the primary fatty acids formed when peroxisomal β-oxidation was performed at high concentrations of palmitate, a long-chain fatty acid. Their results are not necessarily comparable to ours. In their experiment, 2 cycles and 1 cycle of peroxisomal β-oxidation were performed, so that dodecanoyl-CoA and tereadecanoyl-CoA formed, respectively. They performed β-oxidation reaction in the presence of 5 or 10 mM MgATP.[26,37] In the first reaction of fatty acid β-oxidation, acyl-CoA must be synthesized from free fatty acid, requiring ATP, CoA and Mg^{2+}. However, it is known that high concentrations of Mg^{2+} inhibit thiolase reaction in β-oxidation systems.[1,38] In their data, 3-oxo derivative, a substrate of the thiolase reaction, accumulated. They stated that thiolase reaction was a rate-limiting step of β-oxidation under their conditions. Taken together with the present data, the results of Bartlett *et al.* indicate that high concentrations of MgATP inhibit thiolase reaction, preventing the β-oxidation reaction from proceeding smoothly. Therefore, they detected relatively more long-chain fatty acids than we did in our data. Such obstruction can also be interpreted from the point of view of protein concentration. They used peroxisomal protein concentrations of 0.5 and 0.21 mg/ml. However, we used a concentration of 0.05 mg/ml, which was 1/10—1/4 of their concentrations. In other words, because thiolase reaction was inhibited by a high concentration of MgATP, they required a high concentration of peroxisomal protein.

From the experiment investigating the difference in fatty acid β-oxidation between [9,10-^{3}H]palmitate and [9,10-^{3}H]stearate, it was recognized that at least 4 cycles of β-oxidation from both fatty acids are performed (Figs. 7 and 8). Since only 9 and 10 carbon atom sites were radiolabeled, radioactivity could not be detected, even though more than 4 cycles of β-oxidation had occurred. Therefore, we cannot deny the possibility that the reaction continued to proceed. However, patterns of acyl-CoAs derived from [9,10-^{3}H]palmitate were similar to those from [U-^{14}C]palmitate from the viewpoint of accumulation of octanoyl-CoA (Figs. 3, 5, 7, and 8). Thus, under the present conditions, even if more than 4 cycles of β-oxidation are performed, a small amount of acyl-CoA may form.

Since octanoyl-CoA was abundantly synthesized, 4 cycles of β-oxidation of palmitate may occur. Lazarow[1] reported that when he and his colleagues used 0.034 mg/ml of peroxisomal protein and 10 μM of palmitoyl-CoA in the enzyme reaction of β-oxidation, they calculated 5 cycles of β-oxidation from the amount of formed NADH and acetyl-CoA. This indicates that if there is insufficient substrate for peroxisomes, 5 cycles of the reaction may take place. Reports show that peroxisomal β-oxidation is performed in from 3 to 7 cycles, and cycle numbers change depending on the experimental conditions.[39—41]

Plasmalogen synthesis starts from DHAP, which is formed in the Embden–Meyerhof pathway. Fatty acyl-CoA is reacted with 1-OH group of DHAP, forming 1-acyl DHAP, and then fatty alcohol is replaced by the acyl group. Hajra and colleagues[42,43] reported that peroxisomes participate in a few steps of this pathway. Horie *et al.*[22] reported that the fatty-acid chain elongation reaction in which acetyl-CoA is added to octanoyl-CoA as a primer occurs in peroxisomes and the main product is dodecanoyl-CoA. Also, the enzyme that reduces fatty acid to fatty alcohol has been reported, but this reaction is reversible.[44] In our previous papers, we reported that acetyl-CoA derived from peroxisomal β-oxidation is utilized for phospholipid synthesis, and especially incorporated into the subclass of plasmalogen.[19,20] Nascent fatty alcohol synthesized within peroxisomes is reported to be preferentially utilized for plasmalogen synthesis.[21] Furthermore, we reported that peroxisomes can utilize dodecanoyl-CoA as a primer, and form primarily hexadecanol, accompanied by chain elongation using acetyl-CoA. Since tetradecanoyl-CoA functioned about half as effectively as dodecanoyl-CoA, the possibility that longer-chain fatty acyl-CoAs are directly utilized for fatty alcohol synthesis may be very small or negligi-

ble.[23)]

Therefore, we estimated that in plasmalogen biosynthesis, peroxisomes degrade ordinary long chain fatty acids to form octanoyl-CoA, and the resulting acetyl-CoA is partly utilized for chain elongation of octanoyl-CoA, forming dodecanoyl-CoA, and then is partly utilized for biosynthesis of fatty alcohol from this dodecanoyl-CoA.[23)] Decanoyl-CoA was accumulated after β-oxidation of stearate (Figs. 7 and 8). It is reported that activity of decanoyl-CoA as a primer for fatty-acid chain elongation reaction was approximately 73% of that of octanoyl-CoA.[22)] Thus, decanoyl-CoA may also be useful for chain elongation. The present study suggests that octanoyl-CoA and decanoyl-CoA as primer for chain elongation can be supplied by peroxisomal β-oxidation.

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