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Presence and Features of Fatty Acyl-CoA Binding Activity in Rat Hepatic Peroxisomes

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We studied the fatty acyl-CoA binding activity of rat liver peroxisomes. After subcellular fractionation of rat liver treated with or without clofibrate, a peroxisome proliferator, the binding activity with [1-^{14}C]palmitoyl-CoA was detected in the light mitochondrial fraction in addition to the mitochondrial and cytosol fractions. After Nycodenz centrifugation of the light mitochondrial fraction, the binding activity was detected in peroxisomes. The peroxisomal activity depended on the incubation temperature and peroxisome concentration. The activity also depended on the concentration of 2-mercaptoethanol, and a plateau of activity was unexpectedly found at 2-mercaptoethanol concentrations from 20 to 40 mM. Clofibrate increased the total and specific activity of the fatty acyl-CoA binding of peroxisomes by 7.9 and 2.5 times compared with the control, respectively. In the presence of 20% glycerol at 0°C, approximately 90% of the binding activity was maintained for up to at least 3 wk. After successive treatment with an ultramembrane Amicon YM series, about 70% of the binding activity was detected in the M.W. 30,000–100,000 fraction. When the M.W. 30,000–100,000 fraction was added to the incubation mixture of the peroxisomal fatty acyl-CoA β-oxidation system, a slight increase in the β-oxidation activity was found. 2-Mercaptoethanol (20 mM) significantly activated the fatty acyl-CoA β-oxidation system to 1.4 times control. After gel filtration of the M.W. 30,000–100,000 fraction, the peaks of fatty acyl-CoA binding protein showed broad elution profiles from 45,000 to 75,000. These results suggest that fatty acyl-CoA binding activity can be detected directly in peroxisomes and is increased by peroxisome proliferators. The high binding activity in the presence of higher concentrations of 2-mercaptoethanol indicates the importance of the SH group for binding. The apparent molecular weight of the binding protein may be from 45,000 to 75,000.

Key words: β-oxidation, fatty acyl-CoA binding protein, 2-mercaptoethanol, peroxisome, SCP-2.

Peroxisomes play a catabolic role in the β-oxidation of very-long-chain fatty acids (*1, 2*). These organella also play an anabolic role in the biosynthesis of cholesterol (*3, 4*), bile acids (*5, 6*) and plasmalogen (*7–9*). We have reported that acetyl-CoA derived from peroxisomal β-oxidation is utilized for anabolic metabolism (*10–13*). By using clofibrate, a peroxisome ploliferator, we clarified that acetyl-CoA from peroxisomes is utilized for the biosynthesis of plasmalogen (*12, 13*). In other words, acetyl-CoA from peroxisomal β-oxidation is incorporated into the 1-alkenyl group of ethanolamine plasmalogen (*13*).

Plasmalogen biosynthesis starts with the reaction between acyl-CoA and dihydroxyacetonephosphate (DHAP) to form 1-acyl DHAP. The next step is the replacement of the acyl moiety with fatty alcohol, forming 1-alkyl DHAP, which is then reduced to 1-alkyl glycerophosphate. Biosynthesis up to 1-alkyl glycerophosphate takes place exclusively in the peroxisomes (*7–9*).

We have reported that the fatty alcohol destined to form the 1-alkenyl group of plasmalogen is synthesized as a nascent fatty alcohol within the peroxisomes (*14*). Furthermore, the acetyl-CoA utilized for the synthesis of this nascent fatty alcohol is supplied by peroxisomal β-oxidation (*13*). It has been reported that the peroxisomal chain elongation of acyl-CoA with octanoyl-CoA as the primer forms dodecanoyl-CoA (*15*). We clarified that peroxisomes can utilize dodecanoyl-CoA as a primer, and form mainly hexadecanol, accompanied by chain elongation using acetyl-CoA (*16*). Thus, we estimate that in plasmalogen biosynthesis, peroxisomes degrade ordinary long chain fatty acids to form octanoyl-CoA, and the resulting acetyl-CoA is partly utilized for the chain elongation of octanoyl-CoA, forming dodecanoyl-CoA, and then partly utilized for the biosynthesis of fatty alcohol (hexadecanol) from this dodecanoyl-CoA (*16*). We recently reported that octanoyl-CoA among medium chain fatty acyl-CoAs is mainly generated from the peroxisomal β-oxidation of long chain fatty acids (*17*). Therefore, we supposed that the fatty acyl-CoA binding protein, which catches octanoyl-CoA generated through β-oxidation,

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Abbreviations: ACBP, acyl-CoA binding protein; DHAP, dihydroxyacetone-phosphate; FABP, fatty acid binding protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; nsLTP, nonspecific lipid transfer protein; PBS, phosphate-buffered saline; SCP-2, sterol carrier protein-2; SCP-x, sterol carrier protein-x.

is present in the peroxisomes and provides the octanoyl-CoA for the chain elongation reaction and subsequent fatty-alcohol synthesis.

Sterol carrier protein 2 (SCP-2), alternatively called nonspecific lipid transfer protein (nsLTP), is a small basic protein assumed to participate in the intracellular transport of sterols and other lipids (*18, 19*). SCP-2 has been purified from the cytosolic fraction of liver. The purification of SCP-2 has been monitored by measuring the sterol transfer activity (*20, 21*). By using an antibody against purified SCP-2, this protein has been reported to be primarily present in the peroxisomes (*22–24*). Recently, recombinant SCP-2 has been isolated and its binding with fatty acyl-CoA studied *in vitro* (*25–27*). However, fatty acyl-CoA binding activity has not been detected directly in whole peroxisomes. It is also unknown whether only one kind of binding protein with fatty acyl-CoA is present in peroxisomes.

In order to clarify these points, first, we studied the presence and features of fatty acyl-CoA binding activity in peroxisomes. We determined the binding activity in peroxisomes isolated from rat liver treated with or without clofibrate. The supernatant after centrifugation of the sonicated peroxisomes was incubated with [1-^{14}C]palmitoyl-CoA, and the binding activity was analyzed.

MATERIALS AND METHODS

Materials—[1-^{14}C]palmitoyl-CoA (2.1 GBq/mmol, 57.1 mCi/mmol) and Aquazol 2 were purchased from New England Nuclear (USA). Palmitoyl-CoA, Nycodenz, 2-mercaptoethanol, NAD^+, CoA, dithiothreitol, and molecular weight markers for gel filtration chromatography were obtained from Sigma (USA). Lipidex-1000 was purchased from Packard (USA). Clofibrate and all other reagents were of analytical grade and were purchased from Wako Pure Chemicals (Tokyo). Sephacryl S-200 High Resolution was obtained from Amersham Pharmacia Biotech (Sweden).

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, Tokyo) for at least 7 d prior to use. Then the rats were fed chow containing or not containing 0.25% clofibrate for 14 d. Clofibrate increases peroxisome proliferation as well as fatty acyl-CoA β-oxidation.

Cell Fractionation—Livers were homogenized in 0.25 M sucrose using a Potter-Elvehjem Teflon homogenizer. Ten percent (w/v) liver homogenates were fractionated according to the method of de Duve *et al.* (*28*). Nuclear (N), heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and supernatant (S) fractions were obtained by centrifugation at 600 ×*g* for 10 min, 3,300 ×*g* for 10 min, 12,500 ×*g* for 20 min, and 105,000 ×*g* for 60 min, respectively. Centrifugation at 105,000 ×*g* was carried out in a Hitachi ultracentrifuge (model SCP70H) with an RP65 rotor. Each fraction was washed once at the same centrifugal force and suspended in 0.25 M sucrose.

Preparation of Peroxisomes from Rat Liver—Livers were excised from rats treated with or without clofibrate. Liver homogenates (10%) were prepared in 0.25 M sucrose containing 5 mM Hepes (pH 7.4), 1 mM EDTA, and 1% ethanol (SVEH). The light mitochondrial fraction was prepared according to the method of de Duve *et al.* (*28*) with 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 63,000 ×*g* for 30 min at 4°C in a Hitachi P65-1021N ultracentrifuge using an angular rotor (Hitachi RP50T) (*29*). The precipitate fraction was suspended in about 30 ml of SVEH and centrifuged at 20,000 ×*g* for 30 min for washing. The resulting pellet was suspended in an appropriate volume of SVEH, and used as the peroxisomal preparation in the present experiments.

Assay of Fatty Acyl-CoA Binding Activity—Samples of subcellular and peroxisomal fractions were diluted in 2 ml of phosphate-buffered saline (PBS) containing 0.125 M KCl, 10 mM 2-mercaptoethanol and 1mM EDTA (buffer A). The samples were sonicated five times for 5 s with a Branson Sonifier B-12 at an output of 70 Watts at 0°C, and centrifuged at 10,000 ×*g* for 5 min at 4°C (*24*). The supernatants were appropriately diluted with buffer A, and used as assay samples for fatty acyl-CoA binding activity. Fatty acyl-CoA binding activity was assayed according to the method of Hubbell *et al.* (*30*) with slight modification. Unless otherwise stated, 0.2 ml of the supernatant (in buffer A) after centrifugation was incubated with 0.2 ml of 250 mM Tris HCl buffer (pH 7.4) containing 2.5 mM dithiothreitol in a siliconized microcentrifuge tube (1.5 ml). A blank tube contained buffer A instead of assay sample. After preincubation at 37°C, 0.1 ml of 50 μM [1-^{14}C]palmitoyl-CoA (10,000 dpm) was added. The incubations were carried out at 37°C for 10 min in a shaking water bath. After incubation, the tubes were cooled on ice. Unbound fatty acyl-CoA was removed from the solution by adding 0.15 ml of an ice cold Lipidex-1000/buffer (100 mM Tris HCl buffer contaning 1 mM dithiothreitol) suspension (1:1, v/v) and incubating for 10 min at 0°C. The fatty acyl-CoA binding was calculated from the amount of radioactivity present in the supernatant after centrifugation of the tubes at 10,000 ×*g* for 4 min at 4°C.

Amicon Ultramembrane Treatment of the Binding Protein—The supernatant of the peroxisomal fraction sonication mixture was filtered through an Amicon ultramembrane YM100 (M.W. >100,000). The ultramembrane was washed several times with buffer A, and the filtrate was treated with Amicon YM30 (M.W. 30,000–100,000). After washing the membrane with buffer A, the filtrate was then treated with Amicon YM10 (M.W. 10,000–30,000). Finally the filtrate was treated with Amicon YM3 (M.W. 3000–10,000)). The solution trapped on each membrane was concentrated, and the binding activity and protein content in the solution were assayed.

Gel Filtration Chromatography of Fatty Acyl-CoA Binding Protein–The M.W. 30,000–100,000 fraction was concentrated on an ultramembrane Amicon YM30, and the eluant was changed to 20 mM Tris-HCl buffer (pH 7.4) containing 10% glycerol, 1 mM EDTA, 2 mM DTT, and 4 mM 2-mercaptoethanol (buffer B). The sample (0.4 ml) was loaded onto a Sephacryl S-200 High Resolution column (1.5 × 38 cm) equilibrated with buffer B, and chromatography was performed at a flow rate of 9 ml/h. Fractions of 1 ml were collected, and fatty acyl-CoA binding activity with palmitoyl-CoA and UV absorbance were assayed. The molecular mass markers used were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa).

Enzyme and Protein Assays—In order to estimate the purity of the peroxisomal preparation, the activities of catalase, cytochrome *c* oxidase, esterase, and acid phosphatase were determined as markers for peroxisomes, mitochondria, microsomes, and lysosomes, respectively. Catalases were estimated by the method described in our previous report (*31–33*). Cytochrome *c* oxidase was derermined by the method of Wharton and Twagoloff (*33, 34*). Esterase was measured using *o*-nitrophenyl acetate as a substrate according to the method of Beaufay *et al.* (*35*). Acid phosphatase was determined by the method in our previous report (*32*).

Protein was determined by the Lowry method (*36*) using bovine serum albumin as a standard. Since Nycodenz interferes with the determination of protein, it was removed by co-precipitation of the protein with deoxycholate and trichloroacetic acid before determination.

RESULTS

Patterns of Fatty Acyl-CoA Binding Activity in Subcellular Fractions of Liver Homogenate—Figure 1 shows the results of the subcellular fractionation of liver homogenates. The specific activity of cytochrome *c* oxidase in control rats was the highest in the mitochondrial fraction. Peroxisomal enzymes (catalase and the peroxisomal fatty acyl-CoA β-oxidation system) were localized in the light mitochondrial fraction while esterase was localized in the microsomal fraction. Catalase activity in the light mitochodrial fraction was not increased by clofibrate treatment, but the activity of the fatty acyl-CoA β-oxidation system was increased to about 4.4 times the control level. The fatty acyl-CoA binding activity of each fraction was determined at 37°C as described in "MATERIALS AND METHODS." For the binding assay, each fraction was diluted with buffer A (final concentrations of KCl and 2-mercaptoethanol in the incubation mixture: 50 and 4 mM, respectively). The fatty acyl-CoA binding activity was detected in the light mitochondrial fraction in addition to the mitochondrial and cytosol fractions. The activity in the nuclear fraction was possibly caused by unbroken cells and contamination of the mitochondrial fraction. Clofibrate increased the fatty acyl-CoA binding activities in the mitochodrial, light mitochondrial and cytosol fractions to about 2.5, 2.8, and 4.3 times the control levels, respectively.

Fatty Acyl-CoA Binding Activity of Peroxisomes—Peroxisomes were prepared from the light mitochondrial fraction of the liver homogenate by Nycodenz density centrifugation. The peroxisomes were more than 91% pure, as calculated according to Leighton *et al.* (*31*). This peroxisome preparation, which was contaminated by less than 2.7% mitochondria, less than 5.0% microsomes, and less than 1.4% lysosomes (data not shown), was used in the following experiments on fatty acyl-CoA binding. Figure 2 shows the temperature dependency of the fatty acyl-CoA binding activity of the purified peroxisomes. Differences in the activities between 25 and 37°C were hardly detected, but the activity at 4°C was decreased to about 39% of the level at 37°C. In the following experiments, the incubation was carried out at 37°C.

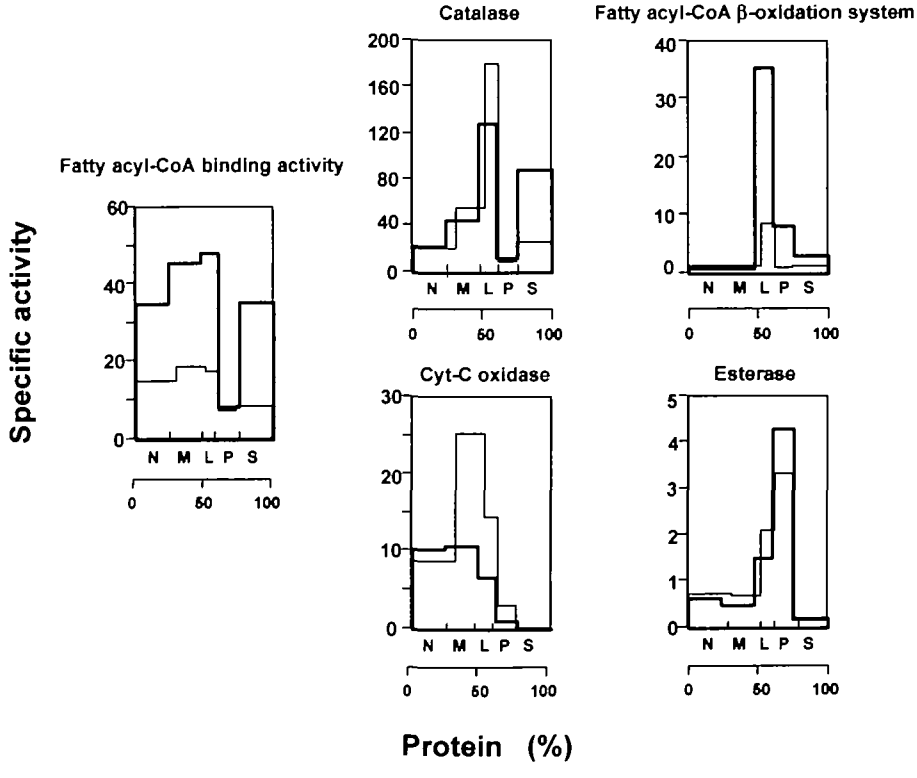

Fig. 1. **Pattern of fatty acyl-CoA binding activity in the subcellular fraction of liver homogenate.** Rats were fed chow, with (——) or without (——) 0.25% clofibrate, for 2 wk. Homogenates were prepared from rat liver, and fractionated according to the method of de Duve. *et al.* (*28*). The ordinate represents the relative specific activity of fatty acyl-CoA binding, and the relative specific activity of the peroxisomal enzymes: catalase, fatty acyl-CoA β-oxidation system, and the relative specific activity of the marker enzymes: esterase (microsomal marker), cytochrome *c* oxidase (mitochondrial marker). The abscissa represents the protein content relative to the total. The fatty acyl-CoA binding activity of each fraction was determined at protein concentrations below 0.04 mg/ml in the assay sample. The specific activity of binding of the light mitochondrial fraction of treated rats was 48.3 (control rats: 17.1) nmol/mg protein. The specific activities of catalase and the fatty acyl-CoA β-oxidation system in the light mitochondrial fraction of treated rats were 128 (control rats: 180) U/mg protein and 35.6 (8.03) U/mg protein, respectively. The specific activities of esterase (microsomal fraction) and cytochrome *c* oxidase (mitochondrial fraction) of treated rats were 4.28 (control rats: 3.34) U/mg protein and 10.7 (25.3) U/mg protein, respectively. Typical data from 3 experiments are shown. N, nuclear fraction; M, mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, cytosolic fraction.

Figure 3 shows the concentration dependency of the binding activity of peroxisomes isolated from rat liver treated with or without clofibrate.

Effect of KCl and 2-Mercaptoethanol on Fatty Acyl-CoA Binding Activity—As described previously, buffer A was used to dilute the assay samples, resulting in the incubation mixtures containing 50 mM KCl and 4 mM 2-mercaptoethanol. Unexpectedly, when PBS buffer was used for dilution, only a small amount of binding activity was detected. Therefore, the effects of KCl and 2-mercaptoethanol on the binding activity were studied. When the KCl concentration was decreased from 50 to 5 mM, the binding activity did not change. However, a decrease in 2-mercaptoethanol from 4 to 0.4 mM resulted in a reduction of the binding activity to about 25% (Fig. 4a). This result indicates the 2-mercaptoethanol dependency of the binding activity.

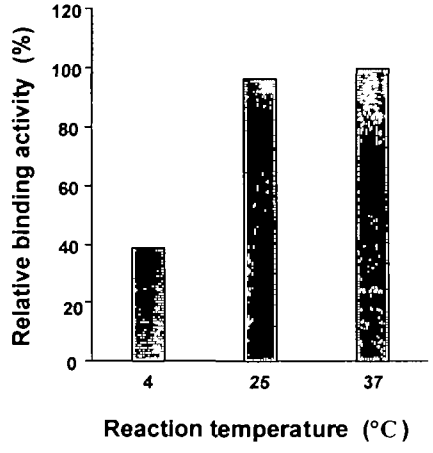

Fig. 2. **Temperature dependency of the fatty acyl-CoA binding activity of peroxisomes.** Peroxisomes were prepared by Nycodenz centrifugation from the light mitochondrial fraction of rat liver treated with clofibrate. The supernatant after centrifugation of the sonicated peroxisomes was incubated with 10 μM [1-^{14}C]palmitate at 4, 25, or 37°C, and the fatty acyl-CoA binding activity was determined. At 37°C, the binding activity was 105 nmol/mg protein.

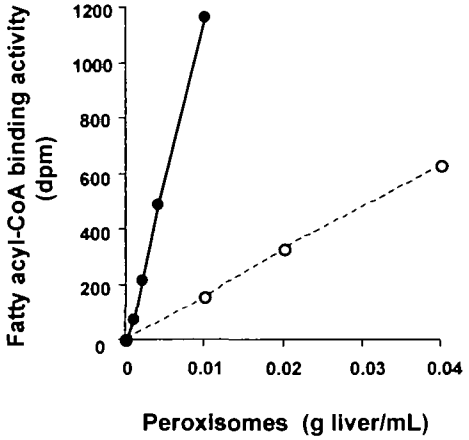

Fig. 3. **Peroxisomal concentration dependency of fatty acyl-CoA binding activity.** Peroxisomes were prepared from the light mitochondrial fraction of rat liver treated (●) or not treated (○) with clofibrate. The supernatant of the sonicated peroxisomes was incubated with 10 μM [1-^{14}C]palmitate, and the fatty acyl-CoA binding activity was determined.

Figure 4b shows that the binding activity was increased in proportion to the concentration of 2-mercaptoethanol. Unexpectedly, a plateau of activity was seen between 20 and 40 mM 2-mercaptoethanol. The activity in 24 mM 2-mercaptoethanol was 3.1 times that in 4 mM 2-mercaptoethanol. Dithiothreitol (20 mM) also increased the binding activity, but the effect was approximately 59% that of 20 mM 2-mercaptoethanol (data not shown).

In the presence of 24 mM 2-mercaptoethanol, egg white lysozyme, which is not a fatty acyl-CoA binding protein, revealed only a small amount of nonspecific binding with fatty acyl-CoA independent of lysozyme concentration, but the peroxisomal preparation clearly bound fatty acyl-CoA

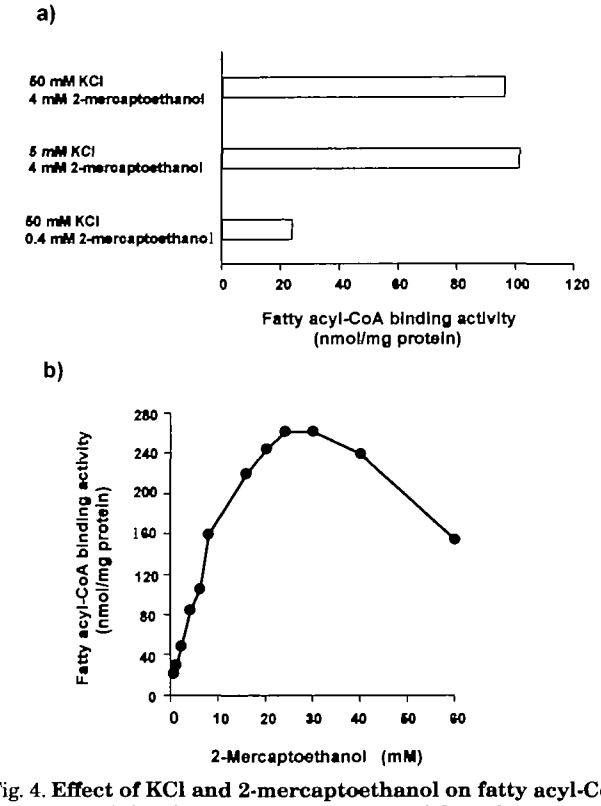

Fig. 4. **Effect of KCl and 2-mercaptoethanol on fatty acyl-CoA binding activity.** Peroxisomes were prepared from the light mitochondrial fraction of rat liver treated with clofibrate. (a) The binding activity was measured in the presence of KCl (50 or 5 mM) and 2-mercaptoethanol (4 or 0.4 mM) in the incubation mixture. At concentrations of 50 mM KCl plus 4 mM 2-mercaptoethanol, the binding activity of the peroxisomes was 96.3 nmol/mg protein. (b) The binding activity was determined in the presence of 50 mM KCl and various concentrations of 2-mercaptoethanol. At 24 mM 2-mercaptoethanol, the binding activity of the peroxisomes was 263 nmol/mg protein.

TABLE I. **Effect of clofibrate on the fatty acyl-CoA binding activity of rat hepatic peroxisomes.**

	Fatty acyl-CoA binding activity			
	nmol/g liver	(T/C)	nmol/mg protein	(T/C)
Control (C)	27.6 ± 16.5	(1)	111 ± 20	(1)
Clofibrate (T)	219 ± 84	(7.9*)	263 ± 40	(2.5*)

Peroxisomes were isolated from rat liver treated with or without clofibrate. The binding activity of the peroxisomes was assayed in the presence of 24 mM 2-mercaptoethanol. Data are means ± SD of 3 experiments. * indicates a significant difference ($p < 0.005$).

(data not shown). This indicates that the peroxisomal binding with fatty acyl-CoA may be specific, even at high concentrations of 2-mercaptoethanol. Thereafter we determined the binding activity in the presence of 24 mM 2-mercaptoethanol.

Effect of Clofibrate on the Fatty Acyl-CoA Binding Activity of Rat Hepatic Peroxisomes—Table I shows the binding activity of peroxisomes isolated from rat liver treated with or without clofibrate. Clofobrate increased the total and specific activities to 7.9 and 2.5 times the control values in the presence of 24 mM 2-mercaptoethanol, respectively.

Effect of Glycerol on the Stability of Fatty Acyl-CoA Binding Activity—Fatty acyl-CoA binding activity was stable in buffer A at 0°C for at least 2 d, but decreased to about 66% after 5 d. Thus, we studied the effect of glycerol on the stability of the binding activity, and the results are shown in Fig. 5. When 10% glycerol was added to buffer A as the stock solution for the binding protein, the activity was insufficiently stable. In the presence of 20% glycerol, approximately 90% of the binding activity was maintained for up to at least 3 wk.

Apparent Molecular Weight of the Binding Protein—The supernatant after centrifugation of the sonication mixture was successively filtered through Amicon ultramembranes as described in "MATERIALS AND METHODS." Figure 6 shows the binding activity of each fraction. About 72% of the total binding activity was found in the M.W. 30,000–100,000 fraction.

Effect of the Binding Protein on the Peroxisomal Fatty Acyl-CoA β-Oxidation System—The M.W. 30,000–100,000 fraction itself did not reveal any fatty acyl-CoA β-oxidation system activity (data not shown). When the M.W. 30,000–100,000 fraction was added to the fatty acyl-CoA β-oxidation system assay mixture of the peroxisomal preparation, a slight increase in the β-oxidation activity was detected. 2-Mercaptoethanol (20 mM) clearly activated the fatty acyl-CoA β-oxidation system by approximately 1.4 times compared with the activity in the absence of 2-mercaptoethanol. When the M.W. 30,000–100,000 fraction together with 20 mM 2-mercaptoethanol was added to the peroxisomal incubation mixture, the β-oxidation activity was significantly increased about 1.5 times compared with the control, but the increase was slight compared with the incubation mixture in which only 2-mercaptoethanol was added (Fig. 7).

Estimation of the Molecular Size of the Native Acyl-CoA Binding Protein—In order to obtain the molecular size of the native acyl-CoA binding protein, gel filtration chromatography of the fatty acyl-CoA binding protein was performed. The M.W. 30,000–100,000 fraction was applied to a Sephacryl S-200 High Resolution column. The UV absorbance of the fractions was mostly under 0.01 (data not shown). When the fatty acyl-CoA binding activity was assayed, the peak containing the binding protein showed a mobility near that of bovine serum albumin (45,000–75000 kDa). However, due to its broad elution profile, a precise estimation of the molecular mass of the native binding protein could not be obtained (Fig. 8).

In order to confirm the specificity of the binding, the effect of unlabeled palmitoyl-CoA and myrystoyl-CoA on

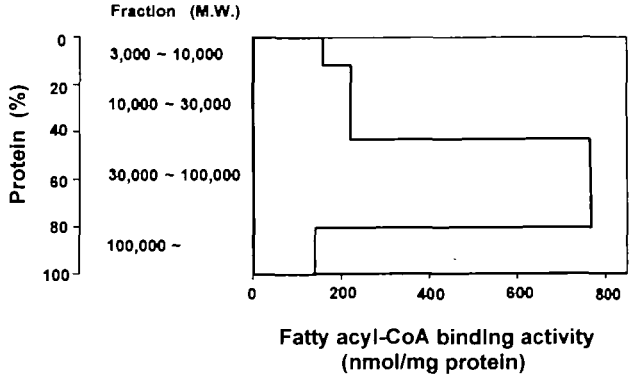

Fig. 6. **Apparent molecular weight of the binding protein.** The supernatant after centrifugation of the sonication mixture was successively filtered through Amicon ultramembranes YM100, YM30, YM10, and YM3 as described in the text. The binding activity of each fraction and the protein content were determined.

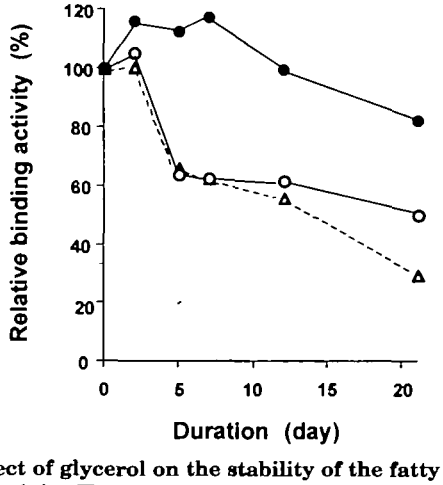

Fig. 5. **Effect of glycerol on the stability of the fatty acyl-CoA binding activity.** The binding protein was stored at 0°C in buffer A with or without glycerol (△, 0% glycerol; ○, 10% glycerol; ●, 20% glycerol). The binding activity was determined in the presence of 24 mM mercaptoethanol. On the first day, the binding activity of the peroxisomes was 208 nmol/mg protein.

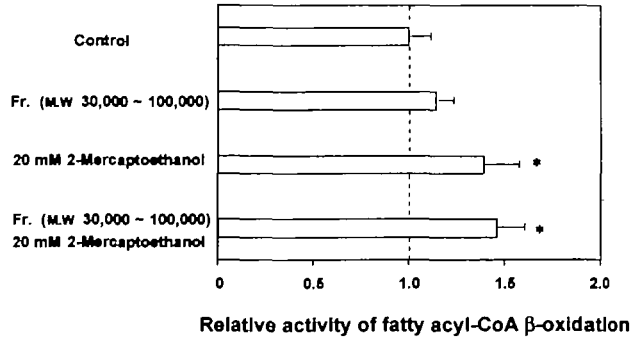

Fig. 7. **Effect of the binding protein and 2-mercaptoethanol on the fatty acyl-CoA β-oxidation system of peroxisomes.** The M.W. 30,000–100,000 fraction (4.5 μg of protein) and/or 2-mercaptoethanol (20 mM) was added to the peroxisomal fatty acyl-CoA β-oxidation system, and the β-oxidation activity was determined. Control activity of the peroxisomal fatty acyl-CoA β-oxidation system was 95.2 u/mg protein. Data are means ± SD of 4 experiments. * indicates a significant difference ($p < 0.01$).

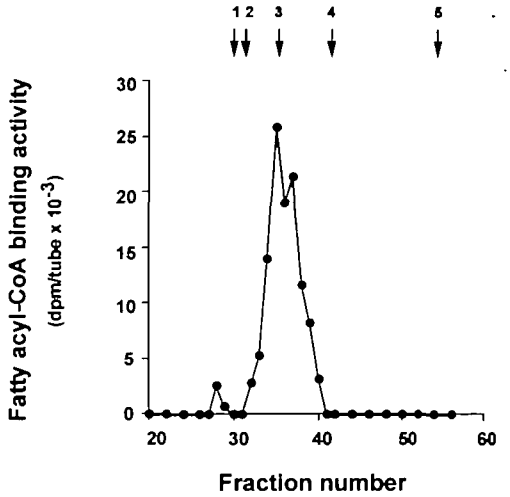

Fig. 8. **Gel filtration chromatography of the fatty acyl-CoA binding protein.** The M.W. 30,000–100,000 fraction was applied to a Sephacryl S-200 High Resolution column (1.5 × 38 cm). Chromatography was performed at a flow rate of 9 ml/h as described in the text. Fractions of 1 ml were collected and assayed for fatty acyl-CoA binding activity with palmitoyl-CoA and by UV absorbance. The positions of molecular mass markers are indicated by arrows: β-amylase (1: 200 kDa), alcohol dehydrogenase (2: 150 kDa), bovine serum albumin (3: 66 kDa), carbonic anhydrase (4: 29 kDa), and cytochrome *c* (5: 12.4 kDa).

the activity of the peak was studied. The activity decreased to 28.3 and 22.8% by the addition of 100 μM palmitoyl-CoA and myrystoyl-CoA, respectively (data not shown).

DISCUSSION

We studied the presence and features of fatty acyl-CoA binding activity in rat liver peroxisomes.

In rat liver treated with or without clofibrate, the fatty acyl-CoA binding activity was detected in the light mitochondrial fraction in addition to the mitochondral and supernatant fractions (Fig. 1). At least three types of nonenzymatic cytosolic proteins [fatty acid binding protein (FABP), acyl-CoA binding protein (ACBP), and SCP-2] that interact with fatty acyl-CoA have been reported. The overall pattern of the relative molar ratio of these proteins (L-FABP/ACBP/SCP-2) reported in liver cytosol appears to be approximately 40:5:1 (*18*). Therefore, in the present study, the binding activity in the supernatant fraction may be caused by FABP, ACBP, and SCP-2, although the relative molar ratio of these proteins was not found. SCP-2 has been reported to be present primarily in peroxisomes (*22–24*). FABP is primarily cytosolic, with the highest concentration near the endoplasmic reticulum, and lower levels localized in the mitochodria and nucleus (*18, 37*). ACBP has been reported to be primarily a cytosolic protein (*18*). From these reports, the binding activity in the light mitochondrial fraction (Fig. 1) may be caused by SCP-2 and/or other binding protein(s). Whether the binding activity shown in the intracellular particulate fractions, other than the light mitochondrial fraction, is dependent on binding protein(s) such as FABP is not yet known.

Sterol carrier protein x (SCP-x) consists of 3-oxoacyl-CoA thiolase and SCP-2 domains (*18, 19*). SCP-2 and SCP-x can be detected in peroxisomes by immunochemical techniques, and SCP-x is found exclusively in peroxisomes (*22–24, 38*). As can be seen from their names, these proteins have long been considered to be carrier proteins of steroids and other lipids. Recently, the binding activity of recombinant SCP-2 with fatty acyl-CoA has been reported *in vitro* (*25–27*). However, no fatty acyl-CoA binding activity has been reported to be detected directly in peroxisomes. In the present experiments, binding activity with [1-^{14}C]palmitoyl-CoA was clearly detected in peroxisomes in the presence of 2-mercaptoethanol (Fig. 3 and Table I).

As shown in Fig. 4, the binding activity depends on the concentration of 2-mercaptoethanol, and, unexpectedly, the maximum activity was found at concentrations higher than 20 mM. In the usual assay medium reported for studies of fatty acyl-CoA binding activity, 0 or 1 mM dithiothreitol instead of 2-mercaptoethanol was included (*30, 39, 40*). From the present study, if 2-mercaptoethanol was omitted, the binding activity of the peroxisomes might have been very low. This may be the reason fatty acyl-CoA activity peroxisomes has not been previously reported. The activating effect of 2-mercaptoethanol and dithiothreitol means that the SH group is very important for binding of the protein with fatty acyl-CoA.

Poorthuis *et al.* have reported that nsLTP is stable in 10% glycerol and 5 mM 2-mercaptoethanol (*21*). However, in the present experiments, 10% glycerol could not maintain the binding activity of peroxisomes in the presence of 10 mM 2-mercaptoethanol. The addition of 20% glycerol was necessary to maintain activity (Fig. 5).

The molecular masses of ACBP, FABP, SCP-2, and SCP-x have been reported to be 10, 14–16, 13.2, and 58 kDa, respectively. SCP-2 is generally reported to be present at a 10-fold greater amount than SCP-x (*18*). Therefore, we considered that if the binding protein is SCP-2, the highest activity might be detected in the M.W. 10,000–30,000 fraction. However, as shown in Fig. 6, about 70% of the binding activity was found in the M.W. 30,000–100,000 fraction. After gel filtration of the fraction, the fatty acyl-CoA binding protein peak showed a broad elution profile from 45,000 to 75,000 (Fig. 8). Therefore, it cannot be determined whether the activity in this peak is caused by SCP-x, an aggregate of SCP-2, and/or some other new binding protein(s). Nevertheless, the mobility of the activity peak implies that protein(s) with a molecular mass larger than at least 58 kDa may be present. As shown in Fig. 3 and Table I, the binding activity of peroxisomes is clearly increased by clofibrate treatment. Fujiki *et al.* reported that the amount of SCP-2 not increased by clofibrate at either the protein or mRNA level, whereas the amount of 60-kDa protein (SCP-x) is apparently increased (*41*). Given our results (stability, apparent molecular weight, and clofibrate effect), it seems possible that SCP-x and/or some other new binding protein(s) are present at a level higher than that of SCP-2.

When the M.W. 30,000–100,000 fraction was added to the incubation mixture containing the peroxisomal homogenate for the assay of the fatty acyl-CoA β-oxidation system, a slight increase in β-oxidation activity was found (Fig. 7). The fatty acyl-CoA binding protein was already included within the peroxisomal homogenate, so the further addition of the fatty acyl-CoA binding protein may not have an additional effect on the β-oxidation activity. 2-Mer-

captoethanol (20 mM) clearly activates the fatty acyl-CoA β-oxidation system. This activation is possibly due to an increase in binding activity of the peroxisomal protein with fatty acyl-CoA. In other words, the binding protein may stimulate the binding of β-oxidation system enzymes to fatty acyl-CoA and intermediates, resulting in a smooth β-oxidation reaction. In the case of SCP-2, many functions, such as cellular cholesterol transport, bile acid biosynthesis, steroid genesis and β-oxidation, have been considered (*18, 19, 42, 43*). The high-affinity binding of very-long-chain fatty acyl-CoA esters to the peroxisomal nsLTP (SCP-2) has been reported by Dansen *et al.* (*26*). On the other hand, Wanders *et al.* reported that SCP-x is a peroxisomal branched-chain β-ketothiolase that reacts specifically with 3-oxo-pristanoyl-CoA (*44*). Seedorf *et al.* reported the defective peroxisomal catabolism of branched fatty acyl-CoA in mice lacking SCP-2/SCP-x gene function (*45*). Regardless, the functions of SCP-2 and SCP-x remain to be elucidated. The confirmation and elucidation of the activation mechanism of the β-oxidation shown in the present study require further study.

Recently, we reported that the medium-chain acyl-CoA, which is a primer for the fatty alcohol synthesis involved in plasmalogen synthesis, is not supplied from the outer peroxisomes, but is synthesized in and supplied from the peroxisomes (*14, 17*). Therefore, this nascent fatty alcohol may be utilized in plasmalogen synthesis. We expected that the fatty acyl-CoA binding protein is present in peroxisomes and catches medium chain acyl-CoA, which is formed abundantly after the peroxisomal β-oxidation of long or very long chain fatty acid(s). The protein is thought to promote the smooth use of the caught acyl-CoA in the chain elongation reaction. Regardless, present study suggests that the fatty acyl-CoA binding protein may be present in rat hepatic peroxisomes. The relationship between the peroxisomal fatty acyl-CoA binding protein and plasmalogen synthesis requires further study.

This is the first report to demonstrate the binding activity of rat hepatic peroxisomes with long chain fatty acyl-CoA. In the present study, the fatty acyl-CoA binding activity was detected directly in peroxisomes in the presence of high concentrations of 2-mercaptoethanol, suggesting the importance of the SH group for binding. The binding activity of peroxisomes may be increased by a peroxisome proliferator. The apparent molecular weight of the binding protein may be from 45,000 to 75,000.

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