Biochem. J. (1990) 270, 167–173 (Printed in Great Britain)

Effects of thia-substituted fatty acids on mitochondrial and peroxisomal β-oxidation

Studies *in vivo* and *in vitro*

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1. The effects of 3-, 4- and 5-thia-substituted fatty acids on mitochondrial and peroxisomal β-oxidation have been investigated. When the sulphur atom is in the 4-position, the resulting thia-substituted fatty acid becomes a powerful inhibitor of β-oxidation. 2. This inhibition cannot be explained in terms of simple competitive inhibition, a phenomenon which characterizes the inhibitory effects of 3- and 5-thia-substituted fatty acids. The inhibitory sites for 4-thia-substituted fatty acids are most likely to be the acyl-CoA dehydrogenase in mitochondria and the acyl-CoA oxidase in peroxisomes. 3. The inhibitory effect of 4-thia-substituted fatty acids is expressed both *in vitro* and *in vivo*. The effect *in vitro* is instantaneous, with up to 95% inhibition of palmitoylcarnitine oxidation. The effect *in vivo*, in contrast, is dose-dependent and increases with duration of treatment. 4. Pretreatment of rats with a 3-thia-substituted fatty acid rendered mitochondrial β-oxidation less sensitive to inhibition by 4-thia-substituted fatty acids.

INTRODUCTION

Heteroatom-substituted fatty acid derivatives, when administered to rats, have been shown to lower serum concentrations of triacylglycerol and cholesterol (Aarsland *et al.*, 1989) and to induce peroxisomal β-oxidation (Berge *et al.*, 1989*a*). These effects were dependent on the position of the heteroatom (a sulphur atom) in the carbon chain; 2-tetradecylthioacetic acid was a powerful inducer, whereas 3-tetradecylthiopropionic acid was not (Berge *et al.*, 1989*a*). 3-Tetradecylthiopropionic acid was, however, found to cause hepatic lipidosis (Berge *et al.*, 1989*b*).

Substitution of a methylene group in a fatty acid molecule with a sulphur atom prevents β-oxidation beyond the sulphur atom (Lau *et al.*, 1988). The chemical structures of 2-tetradecylthioacetic acid and 3-tetradecylthiopropionic acid suggest that the first compound cannot be β-oxidized, whereas the latter can undergo one cycle of β-oxidation. In view of the different pharmacological effects of the two compounds, we have examined their effects on mitochondrial and peroxisomal β-oxidation both *in vivo* and *in vitro*.

These studies show that 3-tetradecylthiopropionic acid (and its structural analogue 3-octylthiopropionic acid) can cause powerful inhibition of mitochondrial or peroxisomal β-oxidation. A less potent inhibitory effect of 2-tetradecylthioacetic acid (and of its structural analogue 2-nonylthioacetic acid) was found *in vitro*, whereas no inhibitory effect was detectable *in vivo*.

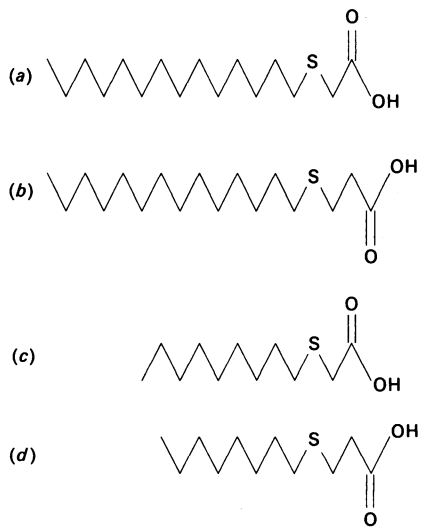
EXPERIMENTAL

Materials

Palmitoyl-CoA, palmitoyl-L-carnitine, CoA (grade 1-L), NAD^+ (grade III), ADP, FAD, rotenone, *cis*-oxalacetic acid (u.v. grade), dithiodipyridine and cytochrome *c* (type III) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. $K_3Fe(CN)_6$ (analytical grade) was purchased from Reidel–De Haen AG, Seelze-Hannover, Germany. 4-*cis*-Decen-1-ol was purchased from ICN Pharmaceuticals, Plainview, NY, U.S.A. Clofibrate and oxalylchloride were obtained from Fluka AG, Buchs, Switzerland. Percoll was purchased from Pharmacia, Uppsala, Sweden. All other reagents were of analytical grade, or of highest purity available. [1-^{14}C]Palmitic acid and [*methyl*-^{3}H]-L-carnitine were purchased from Amersham International, Amersham, Bucks, U.K.

Synthesis of thia-substituted fatty acid analogues

2-Tetradecylthioacetic acid, 3-tetradecylthiopropionic acid, 4-



Scheme 1. Structures of the various thia-substituted fatty acids used in these experiments

(*a*) 2-Tetradecylthioacetic acid; (*b*) 3-tetradecylthiopropionic acid; (*c*) 2-nonylthioacetic acid; (*d*) 3-octylthiopropionic acid.

Abbreviations used: CPT, carnitine palmitoyltransferase; IC_{50}, concentration giving 50% inhibition.

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tetradecylthiobutyric acid, 3-octylthiopropionic acid and 2-nonylthioacetic acid were synthesized as described by Spydevold & Bremer (1989). The structures of these thia-substituted fatty acids are shown in Scheme 1.

Synthesis of acyl-CoA esters and acylcarnitine esters

Acyl-CoA esters were synthesized and their concentrations determined as described by Osmundsen *et al.* (1979).

Acylcarnitines were synthesized as described by Bremer (1968). Synthesis of carnitine esters of 2-tetradecylthioacetic acid and 3-tetradecylthiopropionic acid proved difficult. Instead, the carnitine esters of the related compounds 2-nonylthioacetic acid and 3-octylthiopropionic acid were prepared. A 25% excess of oxalylchloride was added to the thia-acid (600 mg), initially kept at 0 °C. During overnight standing at room temperature, the solid compounds were gradually converted into a fluid. Remaining oxalylchloride and volatile products were removed by brief exposure to a vacuum. The acid chlorides were added directly to (−)carnitine hydrochloride (200 mg) dissolved in 0.5 ml of trifluoroacetic acid. On overnight stirring, a single-phase mixture was formed. The acylcarnitines were precipitated on addition of 2–3 ml of acetone and excess diethyl ether. The precipitate was dissolved in 1 ml of methanol and re-precipitated as an oil with 10 ml of diethyl ether. The oil solidified on standing at 3 °C. The carnitine esters were analysed by t.l.c. on silica plates. The mobile phases used were chloroform/methanol/formic acid/water (65:35:4:4, by vol.) and methanol/acetone/formic acid (70:30:1, by vol.). Exposure to iodine vapour showed one spot, with R_F values of 0.55 and of 0.25 respectively in the two systems. Upon prolonged exposure to iodine vapour an additional weak spot corresponding to free carnitine was observed with R_F values of 0.07 and 0.15 respectively.

Dec-4-*cis*-enoic acid, synthesized from dec-4-*cis*-en-1-ol, was used to prepare dec-4-*cis*-enoyl-CoA as described previously (Osmundsen & Bjørnstad, 1985).

Animals

Male Wistar albino rats (250 g) were obtained from Veterinær Møllegaards Avlsstasjon, Havdrup, Denmark.

Rats were treated (by mouth) with 2-tetradecylthioacetic acid or 3-tetradecylthiopropionic acid (200 or 400 mg/kg body wt. per day for up to 7 days). They were kept under 12 h light/12 h dark cycles, with free access to food and water. The food contained 20% protein, 4% fat and 50% carbohydrates.

Isolation of mitochondrial and peroxisomal fractions

Rat liver mitochondria were isolated as described by Osmundsen & Bremer (1977). Peroxisomal fractions were isolated from livers of rats fed on a diet supplemented with 0.5% (w/w) clofibrate using Percoll density gradients as described previously (Neat *et al.*, 1981).

Assay of mitochondrial and peroxisomal β-oxidation

Mitochondrial β-oxidation was measured spectrophotometrically as rate of reduction of ferricyanide using acylcarnitines as substrate, as described by Osmundsen & Bremer (1977). Mitochondrial β-oxidation was also assayed as acid-soluble radioactivity released from [1-^{14}C]palmitoyl-CoA. The assay mixture contained 11 mM-$MgCl_2$, 11 mM-dithiothreitol, 5.6 mM-ADP, 0.20 mM-NAD^+, 0.6 mM-EDTA, 3.5 mM-KCN, 80 μM-[1-^{14}C]palmitoyl-L-carnitine, 100 μM-[1-^{14}C]palmitoyl-CoA, 2 mM-L-carnitine, 1–1.2 mg of protein and 80 mM-Hepes, pH 7.3. The incubation was carried out in a volume of 0.8 ml at 30 °C for 90 s and was stopped by addition of 500 μl of 4 M-$HClO_4$. After centrifugation at 10000 *g* for 12 min the radioactivity in the supernatants was measured.

Peroxisomal β-oxidation was measured spectrophotometrically as rate of acyl-CoA-dependent NAD^+ reduction, as described by Hovik & Osmundsen (1987). Peroxisomal β-oxidation was also assayed as acid-soluble radioactivity released from [1-^{14}C]palmitoyl-CoA, using solubilizing conditions of incubation as described by Osmundsen *et al.* (1987).

Peroxisomal acyl-CoA oxidase activity was measured spectrophotometrically as generation of dec-4-*cis*-2-*trans*-dienoyl-CoA from dec-4-*cis*-enoyl-CoA, as described by Hovik & Osmundsen (1989).

Spectrophotometric measurements were carried out using a Uvikon 810 spectrophotometer or an Aminco DW-2 spectrophotometer.

Assay of carnitine acyltransferases

Activities of carnitine palmitoyltransferases (CPTs) I and II were measured essentially as described by Bremer (1981). The assay for CPT I contained 20 mM-Hepes, pH 7.4, 0.20 mM-[*methyl*-^{3}H]-L-carnitine (5000 c.p.m./nmol), 60 mM-KCl, 5 mM-KCN, 20 or 70 μM-palmitoyl-CoA, 10 mg of BSA/ml and 1 mg of mitochondrial protein/ml. When included, malonoyl-CoA was added to a concentration of 10 μM. Assay conditions for CPT II were identical, except that BSA was omitted and 1 mM-palmitoyl-D-carnitine was included to lyse mitochondria. The concentration of mitochondrial protein was 0.24 mg/ml.

Carnitine acetyltransferase was assayed by spectrophotometric measurement of dithiodipyridine reduction at 334 nm before and after addition of 1 mM-L-carnitine. The assay mixture contained 80 mM-Tris/HCl, pH 7.8, 60 μM-acetyl-CoA, 1 mM-palmitoyl-D-carnitine, 0.10 mg of dithiodipyridine/ml, 70 μg of mitochondrial protein/ml and 1 mM-L-carnitine. All assays were carried out at 30 °C.

Assay of protein

Protein was measured using the Pierce BCA reagent, with freeze-dried γ-globulins as standard.

RESULTS

β-Oxidation of 3-octylthiopropionoylcarnitine and effect of 3-octylthiopropionoylcarnitine and 2-nonylthioacetylcarnitine on mitochondrial palmitoylcarnitine oxidation

Addition of 3-octylthiopropionoylcarnitine to mitochondrial suspensions led to a transient stimulation of β-oxidation, as judged by rates of ferricyanide reduction (Fig. 1). β-Oxidation of 3-octylthiopropionoylcarnitine had a $V_{max.}$ of 80 nmol/s per g of protein, and a K_m of 3 μM. At concentrations greater than 25 μM, substrate inhibition was observed. 2-Nonylthioacetylcarnitine, as expected, failed to stimulate reduction of ferricyanide.

When 3-octylthiopropionoylcarnitine was added during palmitoylcarnitine oxidation, powerful progressive inhibition ensued, even following a transient oxalacetate-dependent stimulation of β-oxidation (Fig. 1). The order of addition of 3-octylthiopropionoylcarnitine and oxalacetate was of no consequence as regards the inhibitory effect on palmitoylcarnitine-dependent β-oxidation. These results suggests that generation of an inhibitor of β-oxidation occurs during β-oxidation of 3-octylthiopropionoylcarnitine.

Inhibition of β-oxidation at the level of CPT activity is one possible inhibitory mechanism. The CoA esters of the thia-fatty acids, however, were all excellent substrates for CPT (results not shown), rendering this an unlikely possibility.

Changes in mitochondrial β-oxidation *in vivo*

Mitochondria from rats treated with 3-tetradecylthiopropionic

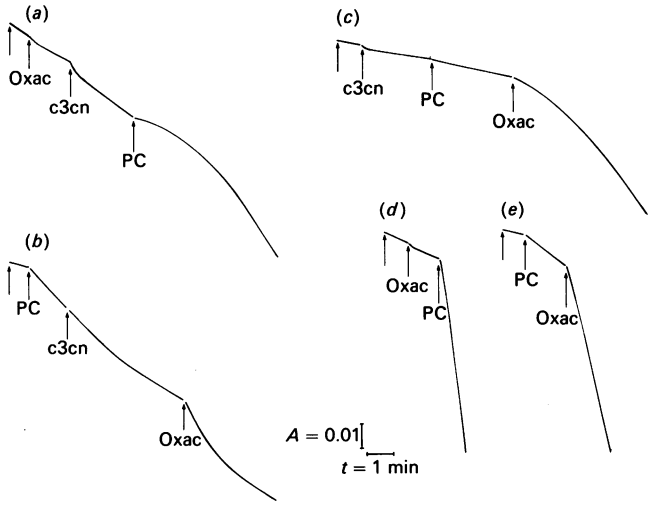

Fig. 1. Typical time course of mitochondrial β-oxidation: effect of 3-octylthiopropionoylcarnitine on β-oxidation of palmitoylcarnitine

Rates of mitochondrial β-oxidation were measured spectrophotometrically as rates of ferricyanide reduction as described in the Experimental section. (*a*) Typical time course of β-oxidation of 3-octylthiopropionoylcarnitine (11.3 μM) with palmitoylcarnitine (100 μM) added after the transient burst of β-oxidation obtained with 3-octylthiopropionoylcarnitine. Oxalacetate was added to the assay mixture before 3-octylthiopropionoylcarnitine. (*b*) Time course of mitochondrial β-oxidation of palmitoylcarnitine (25 μM), with 3-octylthiopropionoylcarnitine (450 μM) added after palmitoylcarnitine. Oxalacetate was added last. (*c*) Time course of β-oxidation observed when 35 μM-3-octylthiopropionoylcarnitine was added before 25 μM-palmitoylcarnitine and oxalacetate. (*d*) β-Oxidation of palmitoylcarnitine (100 μM) alone, using incubation conditions otherwise as in (*a*). (*e*) Time course of β-oxidation of 25 μM-palmitoylcarnitine alone. The points of addition of oxalacete (Oxac), palmitoylcarnitine (PC) and 3-octylthiopropionylcarnitine (c3cn) are indicated by arrows. The point of addition of mitochondria is indicated by an unlabelled arrow.

Table 1. Inhibition of mitochondrial β-oxidation by thioether fatty acids

Measurements of rates of β-oxidation of palmitoylcarnitine or [1-^{14}C]palmitoyl-CoA in mitochondria isolated from livers of rats treated with 2-tetradecylthioacetic acid or 3-tetradecylthiopropionic acid were carried out using the dosages and durations of treatment shown. Rates of β-oxidation were measured spectrophotometrically or radiometrically as described in the Experimental section. Rates are expressed as percentages of the rates obtained with control mitochondria (means ± S.D.). These control values were 1.4 ± 0.2 μmol/s per g of protein ($n = 5$) for ferricyanide reduction and 72 ± 5 nmol/s per g of protein ($n = 4$) for release of acid-soluble radioactivity. Student's t test was used to test the significance of differences between population means. Significant differences from the control are indicated by: * $P < 0.01$; ** $P < 0.001$.

Treatment	Dose (mg/kg body wt.)	Duration (days)	Activity (% of control)	n
Rate of ferricyanide reduction:				
Control	0	—	100	
2-Tetradecylthio-acetic acid	200	2	121 ± 24	4
	200	4	93 ± 21	4
3-Tetradecylthio-propionic acid	200	2	50 ± 3*	2
	200	4	54 ± 6*	2
	400	4	30 ± 5**	2
	400	7	22 ± 3**	2
Acid-soluble radioactivity released:				
Control	0	—	100	
2-Tetradecylthio-acetic acid	150	1	140 ± 12*	4
	150	2	112 ± 16	4
	150	3	198 ± 12**	4
	150	5	140 ± 9**	4
	150	7	98 ± 16	4

acid showed inhibited β-oxidation (Table 1). The extent of inhibition was dose-dependent, and increased with duration of treatment. At most, about 80% inhibition was observed after 1 week (Table 1). The mechanism of inhibition may be different from that of the virtually instantaneous inhibition observed *in vitro* (see preceding section).

Identical treatment with 2-tetradecylthioacetic acid, on the other hand, led to a transient increase in rates of mitochondrial β-oxidation (Table 1), which returned to normal levels on continued treatment. The apparent increase in activity observed after 2 days of treatment with 2-tetradecylthioacetic acid was not statistically significant. This absence of significance was most likely caused by inter-individual variations.

Effects of treatment with 2-tetradecylthioacetic acid on carnitine acyltransferases

Treatment with clofibrate increases the activity of many mitochondrial enzymes, e.g. carnitine acetyltransferase, CPT II (Solberg *et al.*, 1972), acyl-CoA dehydrogenase (Furuta *et al.*, 1981) and enzymes of mitochondrial β-oxidation (Osmundsen *et al.*, 1982).

As treatment with 2-tetradecylthioacetic acid transiently increased mitochondrial β-oxidation activity (Table 1), we have investigated the effects of treatment with 2-tetradecylthioacetic acid on the activities of CPT I and CPT II. Fig. 2 shows a several-fold stimulation of carnitine acetyltransferase and CPT II, whereas the malonoyl-CoA-sensitive CPT I activity was virtually unchanged. Only a minor increase in the malonoyl-CoA-insensitive CPT I (which may be caused by exposure of CPT II) was observed after several days of treatment. These phenomena do not correlate directly with the observed transient increase in β-oxidative capacity (Table 1).

Inhibition of mitochondrial β-oxidation *in vitro*: effects of pre-treatment with 2-tetradecylthioacetic or 3-tetradecylthiopropionic acid

Increasing concentrations of 3-octylthiopropionoylcarnitine or 2-nonylthioacetylcarnitine led to progressive inhibition of mitochondrial β-oxidation *in vitro*, as judged by rates of ferricyanide reduction (Fig. 3). 3-Octylthiopropionoylcarnitine is the more potent of the two, with an IC_{50} (concn. giving 50% inhibition) of about 0.7 μM (Fig. 3*a*); 2-nonylthioacetylcarnitine has an IC_{50} of about 180 μM (Fig. 3*b*).

Mitochondria isolated from rats treated with 2-tetradecylthioacetic acid (200 mg/kg body wt.) for 4 days showed an increased IC_{50} value for 3-octylthiopropionoylcarnitine of 2.6 μM, whereas no significant effect was observed on the IC_{50} for 2-nonylthioacetylcarnitine (Fig. 3). With mitochondria from rats treated with 3-tetradecylthiopropionic acid, no significant effect was observed on inhibition of β-oxidation *in vitro* by 3-nonylthiopropionoylcarnitine or 2-octylthioacetylcarnitine (Fig. 3).

The induction of CPT II activity (and also presumably of other β-oxidation enzyme activities; see Furuta *et al.*, 1981) by 2-tetradecylthioacetic acid may explain the observed desensitization of β-oxidation to inhibition by 3-octylthiopropionoylcarnitine in mitochondria isolated from rats treated with 2-tetradecylthioacetic acid (Fig. 3). Treatment with clofibrate has similarly

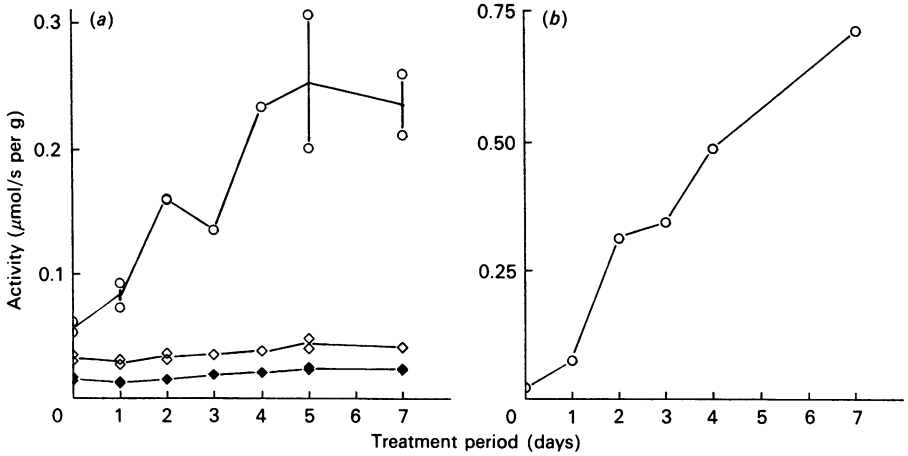

Fig. 2. Effect of treatment with 2-tetradecylthioacetic acid on the activities of CPTs I and II and of carnitine acetyltransferase in rats

The activities of CPT I and CPT II (*a*) and of carnitine acetyltransferase (*b*) in mitochondria isolated from rats treated with 2-tetradecylthioacetic acid (200 mg/kg body wt. per day) were measured as described in Experimental section. The rats were treated for the number of days indicated before isolation of liver mitochondria. (*a*) CPT I activity: ◇, no added malonoyl-CoA; ◆, plus 10 μM-malonoyl-CoA; ○, CPT II activity.

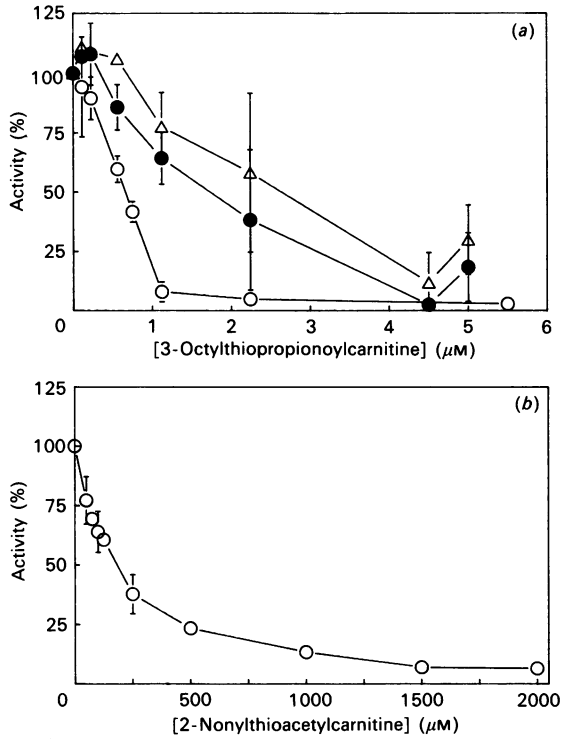

Fig. 3. Inhibition of mitochondrial β-oxidation *in vitro* by 3-octylthiopropionoylcarnitine or 2-nonylthioacetylcarnitine: effects of pretreatment of rats with 2-tetradecylthioacetic acid or 3-tetradecylthiopropionic acid

Inhibition *in vitro* of mitochondrial β-oxidation of palmitoylcarnitine (20 μM) by 3-octylthiopropionoylcarnitine (*a*) and 2-nonylthioacetylcarnitine (*b*) after pretreatment with 2-tetradecylthioacetic acid or 3-tetradecylthiopropionic acid is shown. Mitochondrial β-oxidation was measured in the presence of various concentrations of 3-octylthiopropionoylcarnitine (*a*) or 2-nonylthioacetoylcarnitine (*b*). β-Oxidation was measured as rate of ferricyanide reduction, as described in the Experimental section. (*a*) Mitochondrial from untreated control rats ($n = 3$) ○, and from rats pretreated with 2-tetradecylthioacetic acid [200 mg/kg body wt. per day for 2 ($n = 4$) (●) or 4 ($n = 4$) (△) days]. Mitochondria isolated from rats pretreated with 3-tetradecylthiopropionic acid showed no significant difference from the controls, and are therefore not shown separately for the sake of clarity. (*b*) Mitochondria isolated from untreated control rats (○) ($n = 2$). Mitochondria isolated from rats pretreated with 3-tetradecylthiopropionic acid or 2-tetradecylthioacetic acid showed no significant differences from the controls, and are therefore not shown separately for the sake of clarity.

been reported to protect against inhibition of β-oxidation by hypoglycin (van Hoof *et al.*, 1985), a metabolite of which is known to cause irreversible inhibition of butyroyl-CoA dehydrogenase (EC 1.3.99.2) (Osmundsen & Sherratt, 1975).

Peroxisomal β-oxidation of 2-tetradecylthioacetyl-CoA, 3-tetradecylthiopropionoyl-CoA and 4-tetradecylthiobutyroyl-CoA

Both 3-tetradecylthiopropionoyl-CoA and 4-tetradecylthiobutyroyl-CoA produced self-limiting pulses of NAD^+-reduction, and by 3–4 min after addition only a low rate of NAD^+ reduction was measured (Fig. 4). The fraction of substrate which was β-oxidized during this time represented about 10% of the amount added, as judged by the stoichiometry of NAD^+ re-

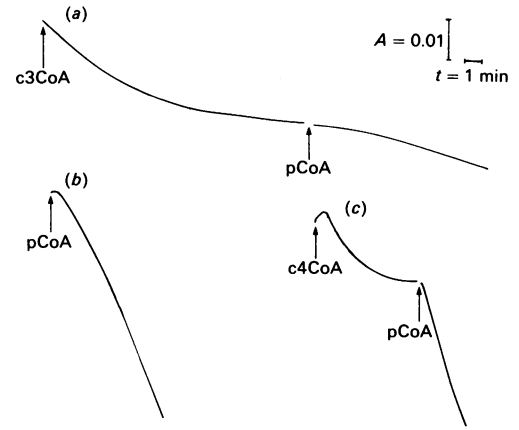

Fig. 4. Effects of 3-tetradecylthiopropionoyl-CoA and 4-tetradecylthiobutyroyl-CoA on time courses of peroxisomal β-oxidation of palmitoyl-CoA

Rates of peroxisomal β-oxidation were measured spectrophotometrically as rates of acyl-CoA-dependent NADH generation, as described in the Experimental section. (*a*) Typical time course of peroxisomal β-oxidation of 50 μM-3-tetradecylthiopropionoyl-CoA, with 50 μM-palmitoyl-CoA added after the transient burst of NAD^+ reduction caused by addition of 3-tetradecylthiopropionoyl-CoA. (*b*) Typical recorder trace obtained for β-oxidation of 50 μM-palmitoyl-CoA alone. (*c*) Typical time course obtained for β-oxidation of 43 μM-4-tetradecylthiobutyroyl-CoA, with 10 μM-palmitoyl-CoA added following the self-limiting pulse of NAD^+ reduction caused by 43 μM-4-tetradecylthiobutyroyl-CoA. The times of addition of palmitoyl-CoA (pCoA), 3-tetradecylthiopropionyl-CoA (c3CoA) and 4-tetradecylthiobutyroyl-CoA (c4CoA) are indicated with arrows.

duction (assuming only one cycle of β-oxidation per substrate molecule). Based on the initial rates of NAD^+ reduction, oxidation of 3-tetradecylthiopropionoyl-CoA had a $V_{max.}$ of 180 nmol/s per g of protein, and 4-tetradecylthiobutyroyl-CoA had a $V_{max.}$ of 330 nmol/s per g of protein. These values correspond to about 80% and 140% respectively of the $V_{max.}$ for β-oxidation of palmitoyl-CoA using the same peroxisomal preparation. K_m values of 20 μM with 3-tetradecylthiopropionoyl-CoA and 70 μM with 4-tetradecylthiobutyroyl-CoA were measured (results not shown). Both compounds exhibited marked substrate inhibition at concentrations higher than about 20 μM, which is similar to the situation observed previously with long-chain fatty acyl-CoA esters (Hovik & Osmundsen, 1987). β-Oxidation of palmitoyl-CoA added to incubations during the self-limiting pulse of β-oxidation of 3-tetradecylthiopropionoyl-CoA was powerfully inhibited (Fig. 4). The extent of inhibition decreased in a time-dependent fashion, β-oxidation of palmitoyl-CoA added later in the course of the reaction being inhibited less than when the palmitoyl-CoA was added at an earlier time point. Also, increasing degrees of inhibition were observed with increasing concentrations of 3-tetradecylthiopropionoyl-CoA (results not shown).

With 4-tetradecylthiobutyroyl-CoA, in contrast, no inhibition of β-oxidation of palmitoyl-CoA (added to the incubation-mixture after completion of the self-limiting pulse of β-oxidation of 4-tetradecylthiobutyroyl-CoA) was observed.

Kinetic studies of inhibition of peroxisomal β-oxidation by 3-tetradecylthiopropionoyl-CoA and 2-tetradecylthioacetyl-CoA

Inhibition of acyl-CoA oxidase by 3-tetradecylthiopropionoyl-CoA or by 2-tetradecylthioacetyl-CoA was investigated directly using dec-4-enoyl-CoA to selectively monitor acyl-CoA oxidase activity (Fig. 5). Both 3-tetradecylthiopropionoyl-CoA and 2-tetradecylthioacetyl-CoA were competitive inhibitors (Fig. 5), with K_i values of 0.24 μM and 2.6 μM respectively.

Fig. 6 shows Lineweaver–Burke plots obtained using 3-tetradecylthiopropionoyl-CoA and 2-tetradecylthioacetyl-CoA as inhibitors of palmitoyl-CoA-dependent NAD^+-reduction. From these plots it appears that both compounds are mixed-type inhibitors. Replots of slopes (or y intercepts) against inhibitor concentrations gave non-linear plots (results not shown), suggesting that more than one site of inhibition is involved. This mixed pattern of inhibition may alternatively result from the presence of CoA in the assay mixture. CoA inhibits acyl-CoA oxidase in a complex fashion (Hovik & Osmundsen, 1989), and the combined effects of the two inhibitors can give the observed pattern of inhibition.

Inhibition of release of acid-soluble radioactivity from [1-^{14}C]palmitoyl-CoA

The effect of 3-tetradecylthiopropionoyl-CoA on the release of acid-soluble radioactivity from [1-^{14}C]palmitoyl-CoA is shown in

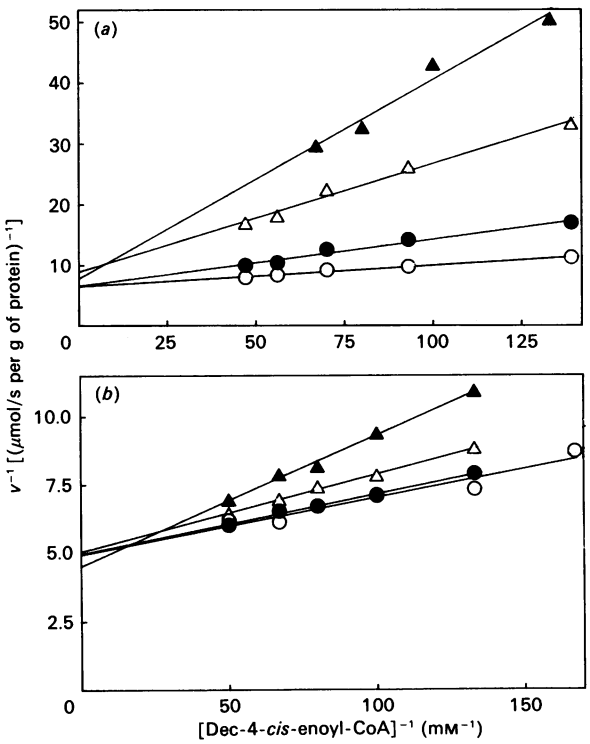

Fig. 5. Inhibition of peroxisomal acyl-CoA oxidase by 3-tetradecylthiopropionoyl-CoA and by 2-tetradecylthioacetyl-CoA

The inhibitory effects of 3-tetradecylthiopropionoyl-CoA (*a*) and of 2-tetradecylthioacetyl-CoA (*b*) on acyl-CoA oxidase were examined by classical enzyme kinetics using isolated peroxisomal fractions. The results are presented as double-reciprocal plots of rate of acyl-CoA oxidase activity versus concentration of dec-4-*cis*-enoyl-CoA. (*a*) Effect of different concentrations of 3-tetradecylthiopropionoyl-CoA: ○, 0 μM; ●, 0.37 μM; △, 0.66 μM; ▲, 1.30 μM. (*b*) Effect of different concentrations of 2-tetradecylthioacetyl-CoA: ○, 0 μM; ●, 0.30 μM; △, 1.2 μM; ▲, 3.0 μM. Acyl-CoA oxidase activity was measured as generation of dec-2-*trans*-4-*cis*-dienoyl-CoA, as described in the Experimental section.

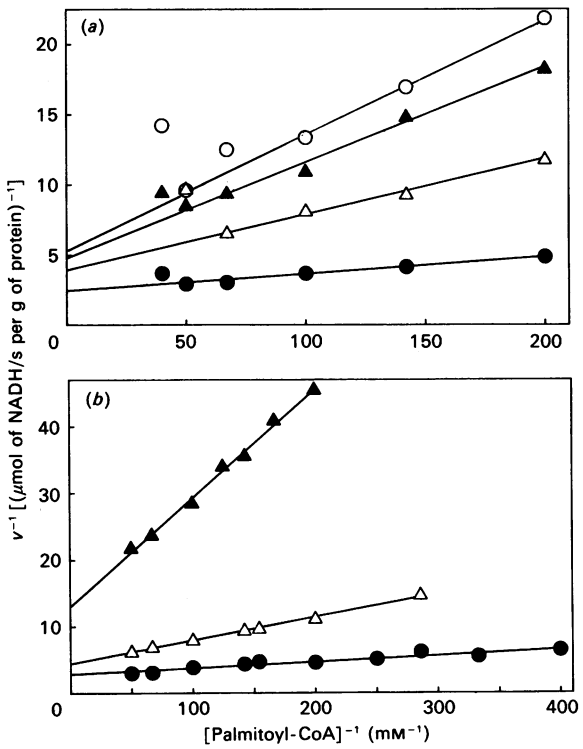

Fig. 6. Inhibition of peroxisomal β-oxidation by 3-tetradecylthiopropionoyl-CoA and by 2-tetradecylthioacetyl-CoA

The inhibitory effects of 3-tetradecylthiopropionoyl-CoA (*a*) and 2-tetradecylthioacetyl-CoA (*b*) on peroxisomal β-oxidation were examined by classical enzyme kinetics using isolated peroxisomal fractions. The results are presented as double-reciprocal plots of rate of palmitoyl-CoA-dependent NAD^+ reduction versus concentration of palmitoyl-CoA. (*a*) Inhibition by various concentrations of 3-tetradecylthiopropionoyl-CoA: ●, 0 μM; △, 1.25 μM; ▲, 5.0 μM; ○, 10.0 μM. (*b*) Inhibition by various concentrations of 2-tetradecylthioacetyl-CoA: ●, 0 μM; △, 48 μM; ▲, 96 μM. Rates of palmitoyl-CoA-dependent NAD^+ reduction were measured as described in the Experimental section.

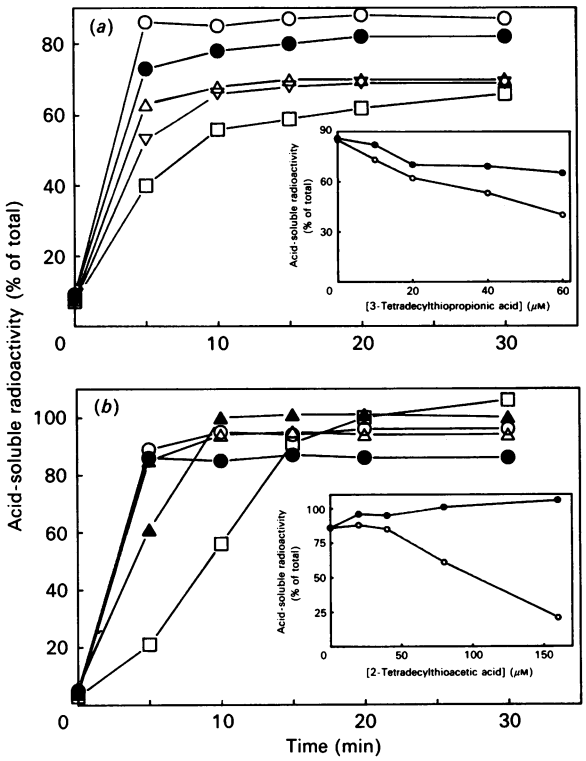

Fig. 7. Inhibition of peroxisomal β-oxidation of [1-^{14}C]palmitoyl-CoA by 3-tetradecylthiopropionoyl-CoA and by 2-tetradecylthioacetyl-CoA

The inhibitory effects of 3-tetradecylthiopropionoyl-CoA (*a*) and 2-(tetradecylthio)acetyl-CoA (*b*) were investigated by measuring the release of acid-soluble radioactivity from [1-^{14}C]palmitoyl-CoA by isolated peroxisomal fractions. (*a*) Inhibition of β-oxidation of [1-^{14}C]palmitoyl-CoA by various concentrations of 3-tetradecylthiopropionoyl-CoA: ○, 0 μM; ●, 10 μM; △, 20 μM; ▽, 40 μM; □, 60 μM. Inset: amount of acid-soluble radioactivity released after 5 min (○) and 30 min (●). (*b*) Inhibition of β-oxidation of [1-^{14}C]palmitoyl-CoA by various concentrations of 2-tetradecylthioacetyl-CoA: ●, 0 μM; ○, 20 μM; △, 40 μM; ▲, 80 μM; □, 160 μM. Inset: amount of acid-soluble radioactivity released after 5 min (○) and 30 min (●). The assay was carried out using solubilizing conditions of incubation, as described in the Experimental section.

Fig. 7(*a*). The initial rate was inhibited and the final extent of β-oxidation was decreased. An inhibitory effect was apparent at a concentration as low as 10 μM, as shown in the replot of extent of inhibition versus inhibitor concentration (Fig. 7*a*, inset).

A similar experiment carried out using 2-tetradecylthioacetyl-CoA as inhibitor showed that inhibition was only significant at concentrations of 2-tetradecylthioacetyl-CoA higher than 40 μM (Fig. 7*b*). Only the initial rate of β-oxidation was inhibited, the final extent of β-oxidation being identical (about 100% of ^{14}C radioactivity being released), irrespective of the presence of the inhibitor. This is clearly expressed in the replot (Fig. 7*b*, inset), showing inhibition of about 80% at 5 min of incubation. At 30 min of incubation, however, no inhibition was apparent.

3-Tetradecylthiopropionoyl-CoA-dependent inhibition of peroxisomal β-oxidation was not critically dependent on the concentration of peroxisomal protein. At most, about 50% inhibition was usually observed with [1-^{14}C]palmitoyl-CoA as substrate, irrespective of the amount of peroxisomal protein present in the incubations (results not shown).

DISCUSSION

These studies have clearly demonstrated that a fatty acid with a sulphur atom in the 4-position can be a powerful inhibitor of both mitochondrial and peroxisomal β-oxidation. Results obtained with mitochondria and peroxisomes suggest that apparently irreversible inhibitors are generated during β-oxidation of 3-octylthiopropionoylcarnitine and 3-tetradecylthiopropionoyl-CoA respectively. In contrast, fatty acids with the sulphur atom in the 3- and 5-position (2-tetradecylthioacetic acid, 4-tetradecylbutyric acid) behaved as simple reversible inhibitors of β-oxidation.

The finding that rats treated with 3-tetradecylthiopropionic acid develop hepatic lipidosis (Berge *et al.*, 1989*b*) correlates well with the present observed inhibition of mitochondrial β-oxidation. A similar correlation has been observed with another inhibitor of β-oxidation, namely hypoglycin (Sherratt, 1986).

However, it is not apparent why 3-tetradecylthiopropionic acid causes minor induction of peroxisomal β-oxidation, whereas 2-tetradecylthioacetic acid is a relatively potent inducer (Berge *et al.* 1989*a*). In contrast, 3-tetradecylthiopropionic acid does induce peroxisomal β-oxidation in cultured hepatoma cells (Spydevold & Bremer, 1989).

The nature of the time course of β-oxidation of 3-octylthiopropionoylcarnitine suggests that an inhibitor of β-oxidation is generated during this reaction. An initial fast rate of β-oxidation, which then rapidly declines towards zero, was observed with both mitochondrial and peroxisomal β-oxidation (Figs. 1 and 4). A similar self-limiting pulse of β-oxidation has previously been observed with pent-4-enoate (Osmundsen, 1978), another potent inhibitor of β-oxidation (Billington *et al.*, 1978).

The finding of Lau *et al.* (1988) that 3-butylthiopropionoyl-CoA is a good substrate for medium-chain acyl-CoA dehydrogenase is in agreement with our findings. Similarly, 3-octylthiopropionoyl-CoA is also a substrate for long-chain acyl-CoA dehydrogenase (R. K. Berge, unpublished work). Recently, Lau *et al.* (1989) have shown that 3-butylthiopropionoyl-CoA is rapidly oxidized to 4-thia-2-*trans*-octenoyl-CoA. The latter binds very strongly to medium-chain acyl-CoA dehydrogenase (K_d = 0.47 μM), and may accordingly cause inhibition of β-oxidation. A similar mechanism of inhibition is also plausible for 4-thia-2-*trans*-enoyl-CoA esters of longer chain length. The mechanism of inhibition of peroxisomal β-oxidation may similarly involve the acyl-CoA oxidase.

The ferricyanide assay measures the flux of substrate through the acyl-CoA dehydrogenase directly. An inhibited substrate flux through these enzymes does not, however, necessarily imply that the enzymes are directly inhibited. Inhibition may also be caused by inhibition of an enzyme further along the β-oxidation pathway, causing secondary inhibition of flux through acyl-CoA dehydrogenases.

The inhibitory effect of 2-nonylthioacetylcarnitine, however, must be mechanistically rather different, as the corresponding acyl-CoA is not a substrate for long-chain acyl-CoA dehydrogenase. It is therefore likely to function as a competitive substrate with respect to CPT and as a competitive inhibitor with respect to acyl-CoA dehydrogenase (EC 1.3.99.3) (Lau *et al.*, 1988).

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Received 19 January 1990/15 March 1990; accepted 1 May 1990