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4-Bromo-2-octenoic Acid Specifically Inactivates 3-Ketoacyl-CoA Thiolase and Thereby Fatty Acid Oxidation in Rat Liver Mitochondria†

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ABSTRACT: In an attempt to develop a compound which would specifically inhibit 3-ketoacyl-CoA thiolase (EC 2.3.1.16) in whole mitochondria, 4-bromo-2-octenoic acid was synthesized and studied. After rat liver mitochondria were preincubated with 4-bromo-2-octenoic acid for 3 min, respiration supported by either palmitoylcarnitine or pyruvate was completely abolished, whereas no inhibition was observed with rat heart mitochondria. Addition of carnitine stimulated respiration supported by pyruvate without relieving inhibition of palmitoylcarnitine-dependent respiration. Hence, this compound seems to be a specific inhibitor of β-oxidation. When the enzymes of β-oxidation were assayed in a soluble extract prepared from mitochondria preincubated with 4-bromo-2-octenoic acid, only 3-ketoacyl-CoA thiolase was found to be inactivated. 4-Bromo-2-octenoic acid is metabolized by mitochondrial β-oxidation enzymes to 3-keto-4-bromooctanoyl-CoA which effectively and irreversibly inhibits 3-ketoacyl-CoA thiolase but not acetoacetyl-CoA thiolase (EC 2.3.1.9). Even though 3-keto-4-bromooctanoyl-CoA inhibits the latter enzyme reversibly, 4-bromo-2-octenoic acid does not inhibit ketogenesis in rat liver mitochondria with acetylcarnitine as a substrate. It is concluded that 4-bromo-2-octenoic acid specifically inhibits mitochondrial fatty acid oxidation by inactivating 3-ketoacyl-CoA thiolase in rat liver mitochondria.

Compounds which specifically inhibit key regulatory enzymes are important tools in studying the control of metabolic pathways. Fatty acid oxidation is one of the pathways whose regulation has been probed by use of several inhibitors (Olowe & Schulz, 1982; Declereq et al., 1987). The known inhibitors of this pathway affect one of three reactions [for a recent review, see Schulz (1987)]: those catalyzed by carnitine palmitoyltransferase I, a key regulatory enzyme in liver (McGarry & Foster, 1980); 3-ketoacyl-CoA thiolase, a suggested regulatory enzyme in heart (Olowe & Schulz, 1980); and acyl-CoA dehydrogenase, which catalyzes the first step of β-oxidation.

Although several inhibitors of 3-ketoacyl-CoA thiolase are known, none inactivates only this enzyme. For example, 4-bromocrotonic acid is metabolized intramitochondrially to 3-keto-4-bromobutyryl-CoA which effectively and irreversibly

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inactivates 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA thiolase, thereby inhibiting both fatty acid oxidation and ketone body degradation (Olowe & Schulz, 1982). 2-Bromooctanoic acid (Raaka & Lowenstein, 1979) and 4-pentenoic acid (Fong & Schulz, 1978) also inactivate both mitochondrial thiolases.

It was the aim of this study to develop a compound that could enter mitochondria and would be metabolized to a reactive derivative which in turn would irreversibly inhibit 3-ketoacyl-CoA thiolase without affecting acetoacetyl-CoA thiolase. In 4-bromo-2-octenoic acid we found such a compound.

Experimental Procedures

Materials. NAD^+,[1] NADH, CoASH, *n*-butyryl-CoA, *n*-decanoyl-CoA, and palmitoyl-CoA were purchased from Pharmacia Biotechnology, Inc. 2-Decenoic acid and 2-octynoic acid were obtained from Aldrich. Sigma was the source of acetoacetyl-CoA, crotonyl-CoA, pig heart 3-hydroxyacyl-CoA dehydrogenase, hexanal, and all standard biochemicals. Palmitoyl-L-carnitine, acetyl-L-carnitine, and L-carnitine were generously provided by Dr. K. Brendel, University of Arizona, College of Medicine. Pig heart 3-ketoacyl-CoA thiolase (Staack et al., 1978), rat heart acetoacetyl-CoA thiolase (Yang et al., 1987), and bovine liver enoyl-CoA hydratase (Steinman & Hill, 1975) were prepared as described. Coenzyme A thioesters of 2-decenoic acid, 2-octynoic acid, and 4-bromo-2-octenoic acid were synthesized by the mixed-anhydride method as described in principle by Goldman and Vagelos (1961). 3-Ketooctanoyl-CoA was prepared from 2-octynoyl-CoA as described by Thorpe (1986). The concentrations of all CoA derivatives were determined by the method of Ellman (1959) after cleavage of the thioester bond with hydroxylamine at pH 7.

Synthesis of 4-Bromo-2-octenoic Acid. 2-Octenoic acid was synthesized from hexanal and malonic acid in the presence of pyridine as described in principle by Boxer and Linstead (1931). The procedure of Bellassoued et al. (1983) developed for the synthesis of 4-bromocrotonic acid was used to prepare 4-bromo-2-octenoic acid. To 8 g of 2-octenoic acid (56 mmol) and 9 mL of chlorotrimethylsilane (71 mmol) in 200 mL of anhydrous ether was added 6.2 mL of anhydrous pyridine dropwise under vigorous stirring. The resulting mixture was heated under reflux for 3 h after which time the formed precipitate was removed by filtration and the ether was distilled off under reduced pressure. The resultant 11.5 g of trimethylsilyl 2-octenoate (54 mmol) was used without further purification. Ten grams (47 mmol) of this compound dissolved in 70 mL of CCl_4 was combined with 8.9 g of *N*-bromosuccinimide (50 mmol). After the mixture was heated to the boiling point, 0.5 g of dibenzoyl peroxide was added to start the reaction, and the refluxing was continued for 3 h. The formed precipitate was removed by filtration, and CCl_4 was evaporated under reduced pressure. The remaining oil was distilled, and the fraction boiling between 130 and 135 °C at 2.5 mmHg was collected. This fraction was combined with an equal volume of water, shaken for 10 min, and extracted 3 times each with 5 mL of ether. The etheral extracts were combined and dried over Na_2SO_4. After evaporation of the ether, 6.1 g (27.6 mmol) of 4-bromo-2-octenoic acid was obtained (49% yield). The ^1H NMR spectrum of this compound gave the following resonances (TMS = 0 ppm): 4.17 ppm (–CHBr–), 5.54 ppm (=CH—COO—), and 6.6 ppm (R—CH=). Anal. Calcd for 4-bromo-2-octenoic acid ($C_8H_{13}O_2Br$): C, 43.44; H, 5.88; Br, 36.17. Found: C, 43.44; H, 5.86; Br, 35.46.

Isolation of Rat Liver Mitochondria and Preparation of a Soluble Extract from Rat Liver Mitochondria. Rat heart mitochondria were isolated according to the procedure of Chappel and Hansford (1969). Rat liver mitochondria were isolated in a similar manner except that 0.25 M sucrose was used instead of 0.21 M mannitol plus 0.07 M sucrose and the treatment with Nagarse was omitted. Mitochondria suspended in isolation buffer were sonicated at 0 °C 5 times for 5 s each with a Branson sonifier (Model W-185) equipped with a microtip. The resulting mixture was centrifuged at 105000*g* for 1 h. Protein concentrations of the mitochondrial suspension and the soluble mitochondrial extract were determined by the biuret method (Gornall et al., 1949).

Measurement of Oxygen Uptake by Rat Liver Mitochondria. Rat liver mitochondria (2 mg) were suspended in 1.9 mL of a basal isotonic incubation buffer containing 0.1 M KCl, 20 mM Tris-HCl (pH 7.4), 4 mM KP_i, 4 mM $MgCl_2$, and 0.1 mM EGTA. To this suspension were added in the indicated sequence bovine serum albumin (0.5 mg/mL) 0.5 mM L-malate, 1 mM ADP, and, 1 min later, varying amounts of 4-bromo-2-octenoic acid. The mixture was incubated for 3 min or the indicated periods of time. State 3 respiration was started by the addition of 30 μM of palmitoylcarnitine or 5 mM pyruvate. Oxygen uptake was measured polarographically with a Clark oxygen electrode attached to a Gilson oxygraph.

Assays of β-Oxidation Enzymes Present in Mitochondria Preincubated with 4-Bromo-2-octenoic Acid. Rat liver mitochondria were incubated with varying concentrations of 4-bromo-2-octenoic acid for 3 min as detailed in the preceding paragraph. No respiratory substrate had been added when samples were quickly frozen in a dry ice/methanol bath and stored at −76 °C until enzyme assays were performed. Acyl-CoA dehydrogenase (EC 1.3.99.3), butyryl-CoA dehydrogenase (EC 1.3.99.2), enoyl-CoA hydratase (EC 4.2.1.1), and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were assayed as described by Olowe and Schulz (1982). The activities of acetoacetyl-CoA thiolase (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) were determined as described by Binstock and Schulz (1981). Extinction coefficients of 5500 cm^{-1} M^{-1} and 13300 cm^{-1} M^{-1} were used when 3-ketooctanoyl-CoA and acetoacetyl-CoA were used as substrates, respectively. The activity of acetoacetyl-CoA thiolase was obtained by subtracting from the combined activities of both thiolases measured with acetoacetyl-CoA the part due to 3-ketoacyl-CoA thiolase. The activity of the latter enzyme with acetoacetyl-CoA as a substrate was calculated from its 3-ketooctanoyl-CoA thiolase activity, which is between 3.7 and 3.9 times greater than its activity with acetoacetyl-CoA (Middleton, 1975).

Enzymatic Conversion of 4-Bromo-2-octenoyl-CoA to 3-Keto-4-bromooctanoyl-CoA and Its Effect on Purified Thiolases. To 1 mL of 20 μM 4-bromo-2-octenoyl-CoA in 0.1 M HEPES buffer (pH 8) was added 15 milliunits of enoyl-CoA hydratase. When the hydration reaction was completed as judged by no further decrease being observed in the absorbance at 263 nm, 1 mM NAD^+ and 20 mM $MgCl_2$ were added. The dehydrogenation reaction was started by the addition of 2 units of 3-hydroxyacyl-CoA dehydrogenase, and

[1] Abbreviations: EDTA, ethylenediaminetetraacetate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NAD^+, nicotinamide adenine dinucleotide, oxidized form; $NADP^+$, nicotinamide adenine dinucleotide phosphate, oxidized form; CoASH, coenzyme A.

the progress of the reaction was monitored spectrophotometrically at 340 nm. After the reaction had reached equilibrium, 10 milliunits of 3-ketoacyl-CoA thiolase or 4 milliunits of acetoacetyl-CoA thiolase was added and incubated for 3 min before the addition of 60 μM CoASH and either 12 μM 3-ketooctanoyl-CoA or 16 μM acetoacetyl-CoA. The absorbance of the solution was recorded at 303 nm.

Samples containing in 1 mL of 0.1 M HEPES buffer (pH 8), 20 μM 4-bromo-2-octenoyl-CoA, 15 milliunits of enoyl-CoA hydratase, 1 mM NAD^+, 20 mM $MgCl_2$, 2 units of 3-hydroxyacyl-CoA dehydrogenase, 10 milliunits of 3-ketoacyl-CoA thiolase, or 4 milliunits of acetoacetyl-CoA thiolase were incubated for 3 min and dialyzed for 36 h against 2 L of 0.75 M Tris-HCl (pH 8), containing 25% glycerol, 10 mM mercaptoethanol, and bovine serum albumin (1 mg/mL). The dialysis buffer was changed once after 20 h. After the dialysis was completed, 200 μL of the sample was transferred into 700 μL of H_2O containing 60 μM CoASH and 20 mM $MgCl_2$. Either 12 μM 3-ketooctanoyl-CoA or 16 μM acetoacetyl-CoA was added to start the reaction. Extinction coefficients of 14 400 cm^{-1} M^{-1} and 21 300 cm^{-1} M^{-1} were used to calculate rates for the thiolysis of 3-ketooctanoyl-CoA and acetoacetyl-CoA, respectively. The changes in volume that occurred during dialysis were corrected for by weighing of samples before and after dialysis.

Measurement of Acetoacetate Formation in Rat Liver Mitochondria. Liver mitochondria from rats fasted for 24 h were suspended in the incubation system used for respiration measurements. In addition 3.3 mM malonate was present. Reactions were started by addition of 30 μM palmitoylcarnitine or 3 mM acetylcarnitine and allowed to proceed for 5 min at which time the reaction was stopped by bringing the pH to 1.8 with $HClO_4$ followed immediately by adjusting the pH to 6 with 1 M KOH. The concentration of acetoacetate formed was determined as described by Krebs et al. (1969).

Results

Synthesis of 4-Bromo-2-octenoic Acid. Although 2-enoic acids can be directly, albeit slowly, brominated with *N*-bromosuccinimide at carbon atom 4, the resulting 4-bromo 2-enoic acids are obtained in poor yield, thus complicating their purification. In contrast bromination of the esters of 2-enoic acids with *N*-bromosuccinimide proceeds rapidly and yields mostly the desired product. The procedure developed by Beellassoued et al. (1983) for the synthesis of 4-bromocrotonic acid and some other short-chain 4-bromo 2-enoic acids was used to prepare 4-bromo-2-octenoic acid. According to their approach 2-octenoic acid was converted to its trimethylsilyl ester by reacting the acid with chlorotrimethylsilane in the presence of pyridine. The resulting trimethylsilyl 2-octenoate was brominated with *N*-bromosuccinimide to yield trimethylsilyl 4-bromo-2-octenoate which was hydrolyzed upon addition of water in the absence of base thereby preventing hydrolysis of the allylic bromide.

Effect of 4-Bromo-2-octenoic Acid on Fatty Acid Oxidation in Mitochondria. Since 4-bromocrotonic acid causes the inactivation of 3-ketoacyl-CoA thiolase as well as acetoacetyl-CoA thiolase, it inhibits both fatty acid oxidation and ketone body degradation (Olowe & Schulz, 1982). It was reasoned that a longer chain homologue of 4-bromocrotonic acid, like 4-bromo-2-octenoic acid, may inactivate only 3-ketoacyl-CoA thiolase and thereby inhibit fatty acid oxidation without affecting ketone body metabolism.

When coupled rat heart mitochondria were preincubated with 4-bromo-2-octenoic acid, respiration supported by palmitoylcarnitine remained unaffected (data not shown). This finding was not completely surprising because 2-bromooctanoic acid, an effective inhibitor of fatty acid oxidation in rat liver mitochondria (Raaka & Lowenstein, 1981), also had no significant effect on the same pathway in rat heart mitochondria (Olowe & Schulz, 1982). However, when coupled rat liver mitochondria were preincubated for 3 min with 20 μM 4-bromo-2-octenoic acid, respiration supported by either palmitoylcarnitine (see Figure 1A,B) or pyruvate (see Figure 1C,D) was completely inhibited. Addition of carnitine to these inhibited mitochondrial suspensions restored pyruvate-supported respiration to 60% of the control level without relieving the inhibition of respiration sustained by palmitoylcarnitine (see Figure 1B,D). Thus, 4-bromo-2-octenoic acid seems to specifically inhibit fatty acid oxidation. When mitochondria were uncoupled with 2,4-dinitrophenol before being incubated with 4-bromo-2-octenoic acid, palmitoylcarnitine-supported respiration was not inhibited (see Figure 1E,F). Thus, it appears that the inhibitor is only effective when ATP or some other form of energy is available, possibly in order to meet the energy requirement for conversion of the inhibitor to its CoA thioester.

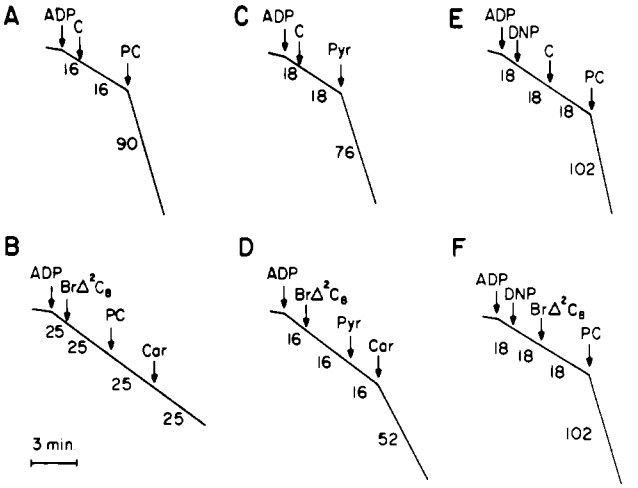

FIGURE 1: Effect of 4-bromo-2-octenoic acid on respiration in rat liver mitochondria. For experimental details, see Experimental Procedures. PC, 30 μM palmitoylcarnitine; C, solvent used to dissolve the inhibitor; $Br\Delta^2C_8$, 20 μM 4-bromo-2-octenoic acid dissolved in ethanol/incubation buffer (1:1); Car, 2.2 mM L-carnitine; Pyr, 6.25 mM pyruvate; DNP, 0.1 mM 2,4-dinitrophenol. The numbers give the rates of respiration in nanoatoms of oxygen min^{-1} (mg of protein)$^{-1}$.

In order to determine which reaction of the β-oxidation cycle may be inhibited by 4-bromo-2-octenoic acid, rat liver mitochondria were incubated in the presence 0.1 mM inhibitor for 3 min and rapidly frozen. After the mitochondrial suspension was quickly thawed, the enzymes of β-oxidation were assayed immediately. As is apparent from the data presented in Table I, 3-ketoacyl-CoA thiolase is the only enzyme that is significantly inhibited. The activity of acetoacetyl-CoA thiolase was obtained by assaying both types of mitochondrial thiolases with acetoacetyl-CoA and deducting from the measured value that part of the activity which was due to 3-ketoacyl-CoA thiolase (for details, see Experimental Procedures). The β-oxidation enzymes were released from the matrix space by solubilizing the mitochondrial membrane with Triton X-100. Since this treatment results in the extensive dilution of the matrix content, a reversible inhibition of 3-ketoacyl-CoA thiolase should not have persisted. Thus, it appars that the inhibition of 3-ketoacyl-CoA thiolase is at least in part due to an irreversible inactivation of the enzyme.

The effect of 4-bromo-2-octenoic acid on the activities of the two thiolases and on palmitoylcarnitine-supported respi-

Table I: Effect of 4-Bromo-2-octenoic Acid on the Activities of β-Oxidation Enzymes[a]

enzyme	substrate	sp act. [μmol min^{-1} (mg of protein)$^{-1}$] no inhibitor	+inhibitor[b]	remaining activity (%)
acyl-CoA dehydrogenase	*n*-butyryl-CoA	0.024	0.0235	98
	n-decanoyl-CoA	0.011	0.095	86
	palmitoyl-CoA	0.0095	0.0087	92
enoyl-CoA hydratase	crotonyl-CoA	5.6	6	107
	2-decenoyl-CoA	2.35	2.35	100
3-hydroxyacyl-CoA dehydrogenase	acetoacetyl-CoA	0.92	0.93	101
3-ketoacyl-CoA thiolase	3-ketooctanoyl-CoA	0.872	0.154	18
acetoacetyl-CoA thiolase	acetoacetyl-CoA	0.19	0.2	105

[a] For experimental details, see Experimental Procedures. [b] Inhibitor: 0.1 mM 4-bromo-2-octenoic acid.

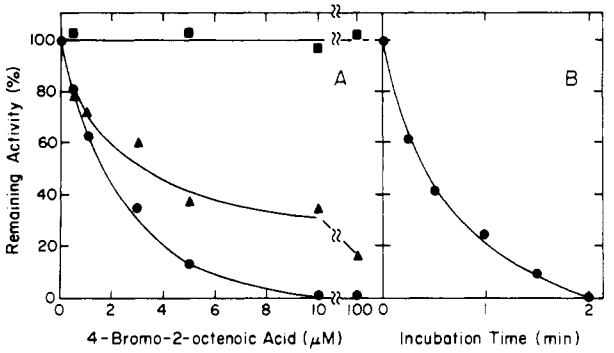

FIGURE 2: Effect of 4-bromo-2-octenoic acid on palmitoylcarnitine-supported respiration and thiolase activities in rat liver mitochondria. (A) Inhibition of respiration supported by palmitoylcarnitine and of the activity of 3-ketoacyl-CoA thiolase as a function of the 4-bromo-2-octenoic acid concentration. Coupled rat liver mitochondria were preincubated with the inhibitor for 3 min before respiration measurements, and thiolase assays were performed as described under Experimental Procedures. (B) Inhibition of palmitoylcarnitine-supported respiration by 4-bromo-2-octenoic acid as a function of the time. Coupled rat liver mitochondria were preincubated with the inhibitor. (●) Respiration supported by palmitoylcarnitine; (▲) activity of 3-ketoacyl-CoA thiolase measured with 3-ketooctanoyl-CoA; (■) activity of acetoacetyl-CoA thiolase determined as described under Experimental Procedures.

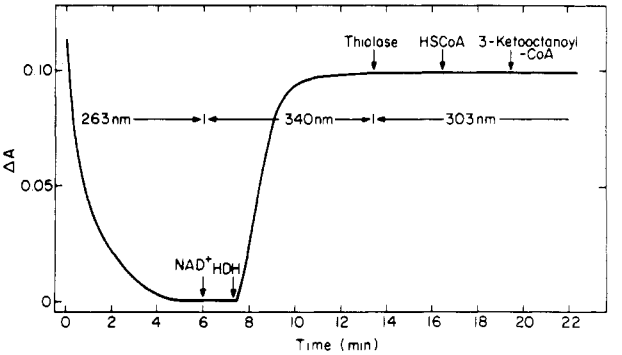

FIGURE 3: Spectrophotometric evidence for the metabolism of 4-bromo-2-octenoyl-CoA by β-oxidation enzymes. To 20 nmol of 4-bromo-2-octenoyl-CoA in 1 mL of 0.1 M HEPES (pH 8.1) was added 15 milliunits of enoyl-CoA hydratase. After no further change in absorbance at 263 nm could be observed, the wavelength was changed to 340 nm, and 20 mM $MgCl_2$ and 1 mM NAD^+ were added followed by 2 units of L-3-hydroxyacyl-CoA dehydrogenase. After completion of the reaction, the wavelength was changed to 303 nm, and 10 milliunits of 3-ketoacyl-CoA thiolase was added followed by 60 μM CoASH and finally 12 μM 3-ketooctanoyl-CoA.

ration as a function of the inhibitor concentration was studied in rat liver mitochondria. As shown in Figure 2A, 10 μM 4-bromo-2-octenoic acid completely inhibited respiration under the conditions used in this experiment. Inhibition by 50% was obtained with approximately 2 μM inhibitor. In contrast, 3-ketoacyl-CoA thiolase was only partially inhibited while the activity of acetoacetyl-CoA remained unaffected by the inhibitor up to a concentration of 0.1 mM. It should be noted that the thiolase activities were assayed after the mitochondrial membrane was dissolved and the contents of the matrix space were highly diluted so that a reversible inhibition would not have been detected. When carnitine was present during the preincubation of mitochondria with 4-bromo-2-octenoic acid, the inhibition was less severe than that observed in the absence of carnitine (data not shown). The inhibition of respiration caused by 20 μM 4-bromo-2-octenoic acid increased with time up to 2 min when respiration was completely inhibited (see Figure 2B).

Metabolism of 4-Bromo-2-octenoic Acid by β-Oxidation Enzymes and the Effect of Its Metabolites on the Activity of 3-Ketoacyl-CoA Thiolase. Since 4-bromo-2-octenoic acid does not cause the inhibition of fatty acid oxidation in rat heart mitochondria or uncoupled rat liver mitochondria, it is likely that this compound must first be activated to its CoA thioester before becoming inhibitory. However, it remained unclear whether 4-bromo-2-octenoyl-CoA itself or a metabolite derived from it causes the inactivation of 3-ketoacyl-CoA thiolase. With the aim of clarifying this point, 4-bromo-2-octenoyl-CoA was synthesized, and its metabolism via β-oxidation was studied. Purified enzymes of β-oxidation were used in a spectrophotometric evaluation of the β-oxidation of 4-bromo-2-octenoyl-CoA. In the presence of enoyl-CoA hydratase, 4-bromo-2-octenoyl-CoA was hydrated as evidenced by the decrease in absorbance at 263 nm shown in Figure 3. The reaction product must have been L-3-hydroxy-4-bromooctanoyl-CoA because the addition of L-3-hydroxyacyl-CoA dehydrogenase together with NAD^+ gave rise to an absorbance change at 340 nm due to the formation of NADH (see Figure 3). If so, 3-keto-4-bromooctanoyl-CoA should have been formed which in the presence of $MgCl_2$ gives rise to a Mg^{2+}–enolate complex with an absorbance maximum around 300 nm. Since the addition of 3-ketoacyl-CoA thiolase and CoASH did not cause an absorbance decrease at 303 nm (see Figure 3), 3-keto-4-bromooctanoyl-CoA is not a substrate of thiolase. In fact, this compound caused the inhibition of 3-ketoacyl-CoA thiolase since the final addition of the thiolase substrate 3-ketooctanoyl-CoA did not result in its thiolytic cleavage detectable at 303 nm. Spectroscopic evidence for the formation of 3-keto-4-bromooctanoyl-CoA was obtained by incubating 4-bromo-2-octenoyl-CoA with enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase in the presence of NAD^+ and $MgCl_2$. An absorbance, which increased with time, was detected around 320 nm with a shoulder at 340 nm (see Figure 4A). Addition of EDTA to the absorbing material corresponding to curve 3 in Figure 4A resulted in the disappearance of the absorbance close to 320 nm without affecting the absorbance centered at 340 nm. The absorbance at 340 nm was due to NADH since it disappeared upon addition of pyruvate and lactate dehydrogenase. Thus, the material giving

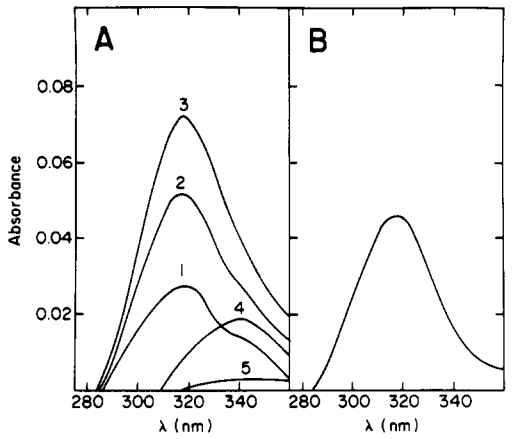

FIGURE 4: Spectrum of the Mg^{2+}–enolate complex of 3-keto-4-bromooctanoyl-CoA. The reaction mixture contained in 1 mL of 0.1 M HEPES (pH 8.1) 20 nmol of 4-bromo-2-octenoyl-CoA, 15 milliunits of enoyl-CoA hydratase, 20 μmol of $MgCl_2$, and 1 μmol of NAD^+. The reaction was started by the addition of 2 units of L-3-hydroxyacyl-CoA dehydrogenase. (A) Spectra (1) after 20 s, (2) after 1.5 min, (3) after 8 min, (4) after addition of 30 μmol of EDTA to the solution corresponding to spectrum 3, and (5) after addition of pyruvate and lactate dehydrogenase to the solution corresponding to spectrum 4. (B) Spectrum of the Mg^{2+}–enolate complex of 3-keto-4-bromooctanoyl-CoA obtained by subtracting spectrum 4 of panel A from spectrum 3 of panel A.

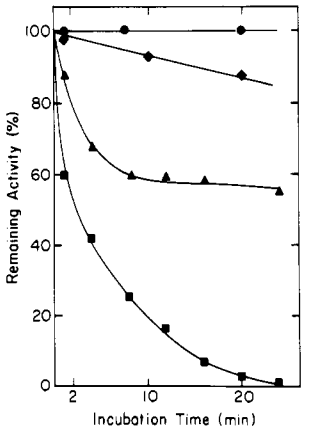

FIGURE 5: Effect of 4-bromo-2-octenoyl-CoA and its β-oxidation metabolites on the 3-ketoacyl-CoA thiolase activity present in a soluble extract of rat liver mitochondria. A soluble extract of rat liver mitochondria (0.278 mg of protein in 1 mL of buffer used in respiration measurements) was incubated with 4 mM NAD^+ (●), 20 μM 4-bromo-2-octenoyl-CoA (▲), 4 mM NAD^+ plus 20 μM 4-bromo-2-octenoyl-CoA (■), or 4 mM NAD^+ plus 20 μM 4-bromo-2-octenoyl-CoA plus 1 mM 3-ketooctanoyl-CoA (◆). Samples of 10 μL were taken at the indicated times and assayed for 3-ketoacyl-CoA thiolase with 3-ketooctanoyl-CoA as a substrate.

rise to the absorbance around 320 nm must have been the Mg^{2+}–enolate complex of 3-keto-4-bromooctanoyl-CoA. The spectrum of this compound without interference by the overlapping spectrum of NADH is shown in Figure 4B; its absorbance maximum is at 317 nm.

In an attempt to determine which of the metabolites of 4-bromo-2-octenoic acid causes the inactivation of 3-ketoacyl-CoA thiolase, a soluble extract of rat liver mitochondria was incubated with either 4-bromo-2-octenoyl-CoA or 4-bromo-2-octenoyl-CoA plus NAD^+. Since only in the latter experiment 3-ketoacyl-CoA thiolase was rapidly and completely inactivated, 3-keto-4-bromooctanoyl-CoA, which can only be formed in the presence of NAD^+, must be the metabolite causing the inactivation of this thiolase (see Figure 5). Protection against inactivation is provided by the thiolase substrate 3-ketooctanoyl-CoA (see Figure 5). This observation suggests that the inhibitor 3-keto-4-bromooctanoyl-CoA is directed against the active site of the enzyme. The limited inactivation of 3-ketoacyl-CoA thiolase seen in the presence of only 4-bromo-2-octenoyl-CoA (see Figure 5) may reflect the formation of a small amount of 3-keto-4-bromooctanoyl-CoA due to the presence of some NAD^+ in the soluble mitochondrial extract.

The question as to whether 3-keto-4-bromooctanoyl-CoA causes the reversible inhibition of 3-ketoacyl-CoA thiolase or its irreversible inactivation was attempted to be answered by treating the enzyme with 20 μM inhibitor which was subsequently removed by dialysis. The results of this experiment demonstrate an insignificant reactivation of 3-ketoacyl-CoA thiolase from 8.8% of the control activity before dialysis to 12.4% after dialysis. Thus, 3-ketoacyl-CoA thiolase seems to be inactivated by 3-keto-4-bromooctanoyl-CoA in an irreversible fashion. Surprisingly, acetoacetyl-CoA thiolase was inhibited by 50% in the presence of 20 μM 3-keto-4-bromooctanoyl-CoA, but this inhibition was completely reversed upon dialysis.

Effect of 4-Bromo-2-octenoic Acid on Ketogenesis in Rat Liver Mitochondria. Since 4-bromo-2-octenoic acid causes the irreverisbly inhibition of the β-oxidation enzyme 3-ketoacyl-CoA thiolase but does not inactivate acetoacetyl-CoA thiolase which is assumed to function in ketone body formation, the inhibitor was expected to inhibit ketogenesis from fatty acids but not from acetate. To test this hypothesis we determined the effect of 0.1 mM 4-bromo-2-octenoic acid on acetoacetate formation in rat liver mitochondria. As expected, acetoacetate formation from palmitoylcarnitine declined by 85% from 30.2 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in control mitochondria to 4.7 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in mitochondria preincubated with 0.1 mM 4-bromo-2-octenoic acid. In contrast, synthesis of acetoacetate from acetylcarnitine remained unchanged at 9.4 nmol $(5\ min)^{-1}$ $(mg\ of\ protein)^{-1}$ in the presence of the inhibitor. Hence, 4-bromo-2-octenoic acid seems to be a specific inhibitor of fatty acid oxidation.

Discussion

With 4-bromo-2-octenoic acid we have identified a compound which inhibits fatty acid oxidation without affecting ketone body synthesis from acetate and possibly amino acids in rat liver mitochondria. This selectivity can be attributed to the chain length of the inhibitor which after conversion to 3-keto-4-bromooctanoyl-CoA will inactivate the β-oxidation enzyme 3-ketoacyl-CoA thiolase but not acetoacetate-CoA thiolase which is assumed to function in ketogenesis only. The inhibitor also exhibits tissue specificity as reflected by its innertness in heart mitochondria in contrast to being a good inhibitor in liver mitochondria. The different effects of the inhibitor in heart and liver may be a consequence of 4-bromo-2-octenoic acid being converted to its CoA thioester in liver but not in heart mitochondria. This interpretation agrees with the reported specificities of medium-chain acyl-CoA synthetase in liver and heart mitochondria; the latter of the two has a much narrower specificity than the liver enzyme (Webster et al., 1965; Mahler et al., 1953). The foregoing interpretation is identical with the explanation presented for the observed inhibition of fatty acid oxidation in rat liver mitochondria by 2-bromooctanoic acid which is almost ineffective in rat heart mitochondria and myocytes (Schulz, 1987). Underlying the preceding discussion is the assumption that 4-bromo-2-octenoic acid must be converted to its CoA thioester in order to become inhibitory. Evidence in support of this assumption is provided by the finding that the inhibitor is ineffective in uncoupled rat liver mitochondria which are

deprived of ATP necessary for CoA thioester formation.

Although respiration supported by either pyruvate or palmitoylcarnitine is inhibited by 4-bromo-2-octenoic acid, inhibition of the former pathway is partially relieved by the addition of carnitine. It is likely that the inhibition of pyruvate oxidation is a consequence of the inhibitor tying up CoASH, thereby causing the inhibition of metabolic pathways dependent on free CoA. If, however, carnitine is present during the preincubation of mitochondria with the inhibitor, fatty acid oxidation is less severely inhibited than it is in the absence of carnitine. Although we have not further studied this effect of carnitine, it seems possible that in the presence of carnitine one or several metabolites of 4-bromo-2-octenoic acid may be transferred by carnitine acetyltransferase from CoASH to carnitine thereby lowering the intramitochondrial concentration of the inhibitory metabolite 3-keto-4-bromooctanoyl-CoA.

When the activities of the β-oxidation enzymes were assayed in solubilized mitochondria preincubated with the inhibitor, only 3-ketoacyl-CoA thiolase was found to be significantly inhibited. Since solubilization of mitochondria resulted in the dilution of the matrix content by several hundred fold, only an irreversible inhibition should have persisted. This suggestive conclusion was confirmed by the demonstration that the inhibition of 3-ketoacyl-CoA thiolase by 3-keto-4-bromooctanoyl-CoA persisted even after prolonged dialysis. Surprisingly, acetoacetyl-CoA thiolase was also inhibited, but its inhibition was reversed by dialysis and thus seems to be reversible. It is possible that 3-keto-4-bromooctanoyl-CoA binds reversibly to both acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase. The protection provided by 3-ketooctanoyl-CoA against inactivation of 3-ketoacyl-CoA thiolase by 3-keto-4-bromooctanoyl-CoA suggests that the inhibitor binds to the active site of the enzyme. If 3-keto-4-bromooctanoyl-CoA is not a substrate of the enzyme or is a poor one, the inhibition will be reversible until a reaction between inhibitor and enzyme leads to the covalent modification and thus inactivation of the enzyme. The most likely reaction between inhibitor and enzyme would be a displacement of the α-keto bromide by a nucleophilic group on the enzyme. This covalent modification of the enzyme could occur either before or after the initial catalytic event in which an α-bromohexanoyl-S-enzyme intermediate would be formed. If inactivation of the enzyme is the consequence of an intramolecular reaction of the α-bromohexanoyl-S-enzyme intermediate, it is obvious that acetoacetyl-CoA thiolase will not be irreversibly inactivated because this enzyme is inactive toward substrates with acyl chains having more than four carbon atoms. A reversible inhibition of 3-ketoacyl-CoA thiolase, which would not have been detected in experiments with dissolved mitochondria, may account for the apparently less severe inhibition of 3-ketoacyl-CoA thiolase as compared to the inhibition of palmitoylcarnitine-supported respiration. Thus it is possible that the combined reversible and irreversible inhibitions of the enzyme in whole mitochondria may be equal to the inhibition of respiration.

The observed reversible inhibition of acetoacetyl-CoA thiolase by 3-keto-4-bromooctanoyl-CoA prompted the question as to whether ketogenesis from substrates other than fatty acids may be affected by the inhibitor. The answer is apparently no since acetoacetate formation in rat liver mitochondria with acetylcarnitine as a substrate was not inhibited by 4-bromo-2-octenoic acid. The absence of an effect of 4-bromo-2-octenoic acid on ketogenesis from acetate may be the consequence of an insufficient accumulation of 3-keto-4-bromooctanoyl-CoA in mitochondria so that acetoacetyl-CoA thiolase is not inhibited significantly. In addition, this enzyme may not catalyze the rate-limiting step in acetoacetate formation under the conditions of this study. Thus, 4-bromo-2-octenoic acid is the desired specific inhibitor of fatty acid oxidation in rat liver mitochondria.

Registry No. 3-Ketoacyl-CoA thiolase, 9029-97-4; 4-bromo-2-octenoic acid, 115185-86-9; 3-keto-4-bromooctanoyl-CoA, 115185-87-0; 2-octenoic acid, 1470-50-4; chlorotrimethylsilane, 75-77-4; trimethylsilyl 2-octenoate, 115185-88-1.

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