4-Bromotiglic Acid, a Novel Inhibitor of Thiolases and a Tool for Assessing the Cooperation between the Membrane-Bound and Soluble β-Oxidation Systems of Rat Liver Mitochondria[†]

Xiquan Liang and Horst Schulz*

Department of Chemistry, City College of the City University of New York, New York, New York 10031

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ABSTRACT: An inhibitor of long-chain 3-ketoacyl-CoA thiolase has been developed as a tool for probing the cooperation between the two fatty acid β-oxidation systems located in the inner mitochondrial membrane and in the mitochondrial matrix, respectively. 4-Bromotiglic acid was synthesized and found to inhibit palmitoylcarnitine-supported respiration of rat liver mitochondria in concentration-dependent and time-dependent fashions. Complete inhibition of respiration was achieved after incubating coupled mitochondria with 10 μM 4-bromotiglic acid for 2 min. Uncoupled mitochondria were resistant to the toxic effect of the inhibitor. Inhibition of octanoate-supported or octanoylcarnitine-supported respiration was partially reversed when the inhibitor was removed from the incubation medium. Such reversal was not observed with either palmitoylcarnitine or 2-methyldecanoic acid as the respiratory substrate. The severity of the irreversible inhibition declined with decreasing chain length of the acylcarnitine substrate. Of all β-oxidation enzymes, only thiolases were inactivated by the inhibitor. Under conditions at which acetoacetyl-CoA thiolase and long-chain thiolase were completely inactivated, 3-ketoacyl-CoA thiolase retained some activity. It is concluded that the degradation of palmitic acid and longer-chain fatty acids is initiated by the β-oxidation system of the inner membrane, whereas fatty acids shorter than palmitic acid can be oxidized to a certain degree by the matrix system alone. The effectiveness of the matrix system increases with decreasing chain length of the substrate.

Fatty acid degradation in mitochondria requires the participation of two enzyme systems, each of which catalyzes a full cycle of β-oxidation (*1*). One set of enzymes with a preference for long-chain fatty acids is bound to the inner mitochondrial membrane. The second set consists of soluble matrix enzymes that are most active toward short-chain and medium-chain fatty acids. Although investigations of inherited fatty acid oxidation disorders in humans have led to the conclusion that both enzyme systems are required for the β-oxidation of fatty acids (*2*), the cooperation of the two systems in the degradation of long-chain fatty acids remains largely unexplored.

It was the aim of this study to design, synthesize, and characterize an inhibitor that would irreversibly inactivate the long-chain specific β-oxidation system in intact mitochondria. Such an inhibitor would then be used to investigate the cooperation between the two mitochondrial β-oxidation systems.

EXPERIMENTAL PROCEDURES

Materials. Palmitoyl-CoA, decanoyl-CoA, butyryl-CoA, all acylcarnitines, CoASH, NADH, ADP, defatted bovine serum albumin, bovine liver enoyl-CoA hydratase (crotonase), and standard biochemicals were purchased from Sigma. The following compounds and their sources were the following: 2-methyldecanoic acid from Narchem Corp. (Chicago, IL); chloroacetaldehyde from Fisher; *tert*-butyl 2-bromopropionate from TCI America (Portland, OR); and triphenylphosphine, hexadecyltributylphosphonium bromide, 2-octenoic acid, and 2-octynoic acid from Aldrich. Acetoacetyl-CoA (*3*) and crotonyl-CoA (*4*) were prepared by established procedures. Most other acyl-CoA thioesters that were not commercially available were synthesized by the mixed anhydride method as detailed by Fong and Schulz (*5*). 3-Ketooctanoyl-CoA was prepared by the enzymatic hydration of 3-octynoyl-CoA catalyzed by crotonase as described in principle by Thorpe (*6*). The concentrations of all acyl-CoA thioesters were determined spectrophotometrically by measuring CoASH with Ellman's reagent (*7*) after cleaving the thioester bond with NH_2OH at pH 7.0 (*5*).

Synthesis of 4-Bromotiglic Acid. The synthesis of 4-bromotiglic acid (Scheme 1, compound 7) was based on a synthetic approach outlined by Stotter and Hill (*8*). One of the required building blocks was (carb-*tert*-butoxyethylidene)-triphenylphosphorane (Scheme 1, compound 3) which was prepared from triphenylphosphine (26 g) and *tert*-butyl 2-bromopropionate (Scheme 1, compound 1) (10.6 g) in a

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[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetate; EGTA, [ethylene bis(oxyethylenenitrilo)] tetraacetate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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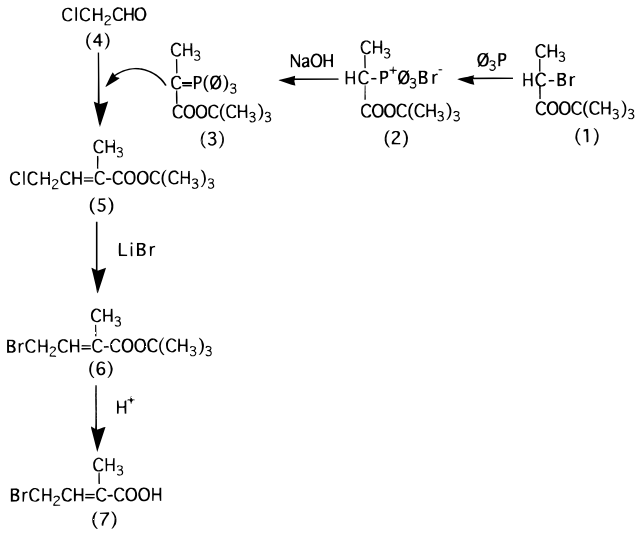
Scheme 1: Synthesis of 4-Bromotiglic Acid[a]



[a] Ø represents phenyl.

7:3 mixture of benzene and toluene (*9*, *10*). After the reaction mixture was stirred for 3 days at room temperature, the solvent was removed under reduced pressure and the residue was ground up in anhydrous ether. The crude phosphonium salt was dissolved in cold water and converted to the corresponding phosphorane by titrating the solution with 1 N NaOH to beginning alkalinity as indicated by phenolphthalein while simultaneously extracting the formed phosphorane into ether. After removal of the ether, 8 g (40%) of (carb-*tert*-butoxyethylidene)triphenylphosphorane was obtained. The product was further purified by recrystallization from hot ethyl acetate by the addition of petroleum ether. The final product was reacted with chloroacetaldehyde in a Wittig reaction to yield *tert*-butyl 4-chloro-2-methylbut-2-enoate (Scheme 1, compound 5) (*8*). The commercial preparation of aqueous chloroacetaldehyde (15 g) was dehydrated by mixing it with 60 mL of CH_2Cl_2 and keeping it over anhydrous $MgSO_4$ for 1 h. After removal of the drying agent by filtration, the filtrate was combined with (carb-*tert*-butoxyethylidene)triphenyl-phosphorane (6.5 g) and heated under reflux for 4 h. After evaporating the solvent, the residue was extracted three times with a 1:1 mixture of petroleum ether and ether. The pooled extracts were applied to a silica gel column which was equilibrated and developed with a 1:1 mixture of petroleum ether and ether. *tert*-Butyl-4-chloro-2-methyl-but-2-enoate, which was eluted first, was detected by thin-layer chromatography. Fractions containing this compound were combined and freed of solvent to yield 2.5 g (79% yield) of product. The mass spectrum of the product revealed the presence of two protonated molecular ions with *m*/*z* of 191 and 193 in a ratio of 3:1 corresponding to the isotopic mixture of the chlorinated ester. *tert*-Butyl 4-chloro-2-methylbut-2-enoate was converted to the bromo-substituted ester by reacting the former compound (2 g) with lithium bromide (18 g) dissolved in acetone for 12 h. After evaporating the acetone under reduced pressure, the residue was extracted three times with petroleum ether. The resultant *tert*-butyl 4-bromo-2-methylbut-2-enoate (Scheme 1, compound 6) was hydrolyzed under acidic, not alkaline, conditions to avoid the likely hydrolysis of the bromine residue. For this purpose, the ester (1.7 g) was added to a mixture of hexadecyltributylphosphonium bromide (0.367 g) and 10 M aqueous sulfuric acid (3.6 mL) and stirred for 4 h at 25° C. The resultant white−gray precipitate was isolated by filtration and washed with a small amount of petroleum ether. After recrystallization from petroleum ether/ether, 4-bromotiglic acid (Scheme 1, compound 7) (0.8 g; 62% yield) was obtained with a mp of 92.5−93.5 °C in good agreement with a mp of 93.4−94.8 °C reported by Löffler et al. (*11*). The proton NMR spectrum in $CDCl_3$ showed three signals: δ 1.93 (s, 3, H−Cβ'), δ 4.04 (d 2, H−Cγ), and δ 7.05 (m, 1, H−Cβ). NOE measurements did not show an enhancement of the signal corresponding to the H−Cb proton upon irradiation of the sample at the frequency of the H−Cb′ protons. This observation agrees with an E configuration of the double bond which was predicted by the synthetic approach (*8*). The mass spectrum revealed the presence of two protonated molecular ions with *m*/*z* values of 179 and 181, in a 1:1 ratio, reflecting the isotopic mixture of 4-bromotiglic acid.

Isolation of Mitochondria and Respiration Measurements. Rat liver mitochondria were isolated as described by Nedergaard and Cannnon (*12*). Protein concentrations were determined as described by Bradford (*13*) with bovine serum albumin as the protein standard. For respiration measurements, 1.5 mg of rat liver mitochondria was incubated in 1.9 mL of a basal medium containing 20 mM Tris-HCl[1] (pH 7.4), 4 mM KP_i, 0.1 M KCl, 4 mM $MgCl_2$, and 0.1 mM EGTA.[1] To this incubation mixture were added in the following indicated order: bovine serum albumin (0.5 mg/mL), 0.5 mM L-malate, 1 mM ADP, and varying amounts of 4-bromotiglic acid. After preincubation for 2 min, respiration was stimulated by the addition of the indicated substrates of fatty acid oxidation. Rates of respiration were measured polarographically with a Clark oxygen electrode attached to an oxygraph. When the reversibility of the inhibition caused by 4-bromotiglic acid was evaluated, mitochondria were incubated for 2 min as detailed above for respiration measurements except that the respiration substrate was omitted. The mitochondrial suspension was then centrifuged at 13000*g* for 30 s, and the pellet was resuspended in the same incubation medium except that the inhibitor was omitted. Respiration was initiated by the addition of substrate.

Determination of Enzyme Activities in Mitochondria Preincubated with or without 4-Bromotiglic Acid. Rat liver mitochondria were incubated with various concentrations of 4-bromotiglic acid for 2 min under conditions used to measure respiration except that the substrate of fatty acid oxidation was omitted. Samples were quickly frozen in a dry ice/methanol bath and stored at −80 °C until assayed. Acyl-CoA dehydrogenases (EC 1.3.99.2 and EC 1.3.99.3), enoyl-CoA hydratases (EC 4.2.1.17 and EC 4.2.1.74), and L-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) were assayed as described by Olowe and Schulz (*14*). The activities of acetoacetyl-CoA thiolase (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) were determined as detailed by Binstock and Schulz (*15*).

Separation of Matrix and Membrane-Bound Thiolases. Rat liver mitochondria were incubated under conditions used for measuring respiration except that the respiratory substrate was omitted. Two samples were incubated for 3 min in the presence and absence of 30 μM 4-bromotiglic acid, respectively. The samples were immediately centrifuged at 7800*g*

Table 1: Effect of 4-Bromotiglic Acid on the Activities of β-Oxidation Enzymes in Coupled Rat Liver Mitochondria

enzyme	substrate	specific activities[a] (nmol min^{-1} mg^{-1}) no inhibitor	specific activities[a] (nmol min^{-1} mg^{-1}) plus inhibitor	remaining activity[b] (%)
acyl-CoA dehydrogenase	butyryl-CoA	56	53	95
	decanoyl-CoA	31	30	97
	palmitoyl-CoA	29	29	100
enoyl-CoA hydratase	crotonyl-CoA	7000	7300	104
	octenoyl-CoA	6600	6300	95
3-hydroxyacyl-CoA dehydrogenase	acetoacetyl-CoA	1700	1600	94
thiolase	acetoacetyl-CoA	380	33	9
	3-ketooctanoyl-CoA	500	120	24

[a] Mitochondria (1 mg/mL) were incubated with 30 μM 4-bromotiglic acid for 3 min and assayed for β-oxidation enzymes as described under Experimental Procedures. [b] The values are means based on 3−5 measurements with standard deviations of less than 10% of the mean values.

for 10 min, and the pellets were suspended in 20 mM potassium phosphate (pH 7.0) containing 5 mM mercaptoethanol and 1 mM EDTA[1] (buffer A), sonicated 8 times for 10 s each at 4 °C. The resultant suspensions were applied separately to DEAE−cellulose columns (1 × 10 cm) which had been equilibrated with buffer A. The columns were washed with buffer A containing 0.1 M KCl, and fractions of 2 mL were collected until protein ceased to be eluted. The columns were then developed with a 0.1−1 M KCl gradient in buffer A. Fractions of 1 mL were collected. Flow-through and gradient fractions were assayed for 3-hydroxyacyl-CoA dehydrogenase with 3-ketooctanoyl-CoA as substrate and for thiolase activities with acetoacetyl-CoA and 3-ketooctanoyl-CoA as substrates.

RESULTS

Synthesis and Evaluation of 4-Bromotiglic acid: A Potential Inhibitor of Fatty Acid Oxidation. The most direct synthetic route to 4-bromotiglic acid is the bromination of tiglic acid or tiglic ester with *N*-bromosuccinimide. Since this approach yields an inseparable mixture of methyl 4-bromotiglate and methyl 2-bromomethylcrotonate (*11*), the desired product was synthesized from chloroacetaldehyde and (carb-*tert*-butoxyethylidene)triphenylphosphorane by a Wittig reaction (*8*). After the chlorine was replaced with a bromine residue followed by acid hydrolysis of the ester, 4-bromotiglic acid was obtained in good yield.

When coupled rat liver mitochondria were preincubated with 10 μM 4-bromotiglic acid for 2 min, respiration supported by palmitoylcarnitine was completely inhibited (compare panels 1 and 4 of Figure 1). However, when mitochondria were first incubated with 2,4-dinitrophenol to uncouple respiration from oxidative phosphorylation, 4-bromotiglic acid was ineffective (see panels 2 and 5 of Figure 1). This observation supports the assumption that ATP[1] is required for the activation of 4-bromotiglic acid which thereafter may be further metabolized intramitochondrially to yield the activated inhibitor. When 2,4-dinitrophenol was added after 4-bromotiglic acid, palmitoylcarnitine-supported respiration remained completely inhibited (data not shown). An experiment with *n*-octanoate instead of palmitoylcarnitine as the respiratory substrate produced a different result. As is apparent from Figure 1, panels 3 and 6, octanoate-stimulated respiration initially was almost completely inhibited when mitochondria were pretreated with 4-bromotiglic acid. However, within 1 min, 90% of the original activity was recovered. This result demonstrated that the inhibition of octanoate-supported respiration is for the most part

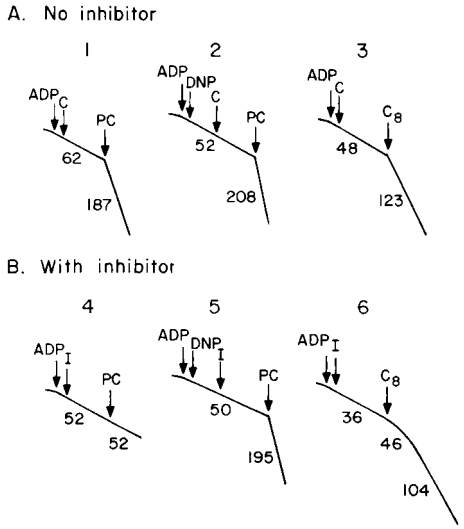


FIGURE 1: Effect of 4-bromotiglic acid on mitochondrial respiration supported by either palmitoylcarnitine or octanoate. For experimental details see Experimental Procedures: PC, 15 μM palmitoylcarnitine; C_8, 0.1 mM octanoate; I, 10 μM 4-bromotiglic acid in 1 M Tris-HCl (pH 7.2) containing 1 M KCl; C, carrier of inhibitor; and DNP, 0.1 mM 2,4-dinitrophenol. The numbers represent the rates of respiration in nanoatoms of O_2/min/mg of protein.

reversible. Moreover, octanoate, possibly by competing with 4-bromtiglic acid for mitochondrial activation or possibly uptake, seems to prevent the further formation of the reversible inhibitor.

Since 4-bromotiglic acid caused the complete inhibition of palmitoylcarnitine-supported respiration, the target of the inhibitor might be one or several of the β-oxidation enzymes. To identify the site of the inhibition, coupled mitochondria were incubated with 4-bromotiglic acid and assayed for the enzymes of β-oxidation. As is apparent from the results shown in Table 1, only the thiolase activities were significantly reduced. The activity detected with acetoacetyl-CoA was inhibited to a greater extent than was the activity measured with the medium-chain substrate 3-ketooctanoyl-CoA. Since the inhibition of thiolases was detected after their extensive dilution by the assay medium, the inhibition seems to be irreversible. The loss of thiolase activity and the decrease of palmitoylcarnitine-supported respiration were functions of the inhibitor concentration (see Figure 2A). The complete inhibition of respiration was achieved with 10 μM 4-bromotiglic acid after 2 min of incubation. At a fixed inhibitor concentration of 10 μM, the rate of respiration declined in a time-dependent manner (see Figure 2B). At

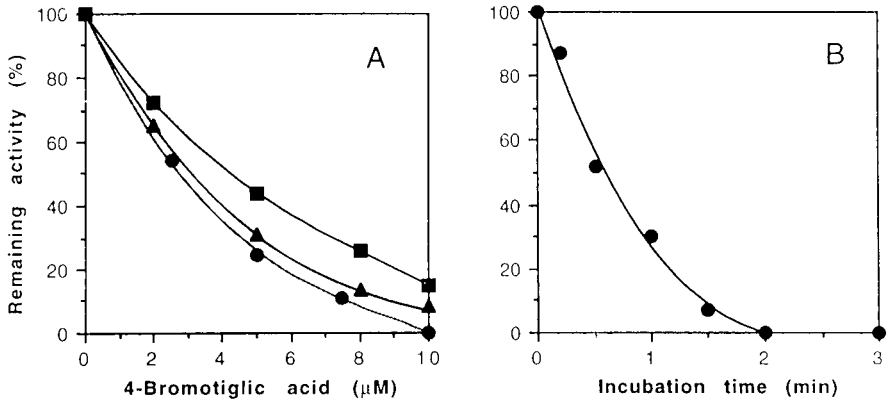


FIGURE 2: Inhibitions of respiration and thiolase activities in coupled rat liver mitochondria as functions of the 4-bromotiglic acid concentration (A) and the incubation time (B): A, The incubation was 2 min; B, the concentration of 4-bromotiglic acid was 10 μM; (●) respiration supported by palmitoylcarnitine; (▲) thiolase assayed with acetoacetyl-CoA; (■) thiolase assayed with 3-ketooctanoyl-CoA. Thiolase activities are based on three measurements with standard deviations of 6% or less of the mean values.

all inhibitor concentrations, the decline of the thiolase activities was less severe than was the inhibition of respiration (see Figure 2A). The medium-chain thiolase activity was inhibited to a lesser extent than was the acetoacetyl-CoA thiolase activity. This observation is indicative of a differential inactivation of the various thiolases present in mitochondria.

Rat liver mitochondria contain at least three types of thiolases. They are acetoacetyl-CoA thiolase, which only acts on acetoacetyl-CoA, 3-ketoacyl-CoA thiolase with a broad chain length specificity, and long-chain 3-ketoacyl-CoA thiolase, which acts on all substrates except for acetoacetyl-CoA (*1*). For determining the effects of 4-bromotiglic acid on the individual thiolases, mitochondria were treated with 30 μM 4-bromotiglic acid for 3 min, sonicated, and subjected to fractionation on DEAE−cellulose. Acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase, both of which are soluble matrix enzymes, did not bind to the column and emerged in the forerun (see Figure 3, panels A and B). Long-chain 3-ketoacyl-CoA thiolase, which is a protein of the inner mitochondrial membrane, was retained by the column and was eluted with a KCl gradient (see Figure 3, panels C and D). A comparison of the control (Figure 3, panels A and C) with the inhibitor-treated sample (Figure 3, panels B and D) demonstrated the complete inactivation of long-chain 3-ketoacyl-CoA thiolase (Figure 3, panel D) while a fraction of the 3-ketoacyl-CoA thiolase (approximately 15%) was still active. Separation of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase by chromatography on phosphocellulose revealed the complete inactivation of the former enzyme, whereas a fraction of the latter thiolase remained active (data not shown). Since acetoacetyl-CoA thiolase is not involved in fatty acid oxidation (*1*), 3-ketoacyl-CoA thiolase is assumed to be the only thiolase responsible for the degradation of medium-chain and short-chain fatty acids.

Probing Mitochondrial β-Oxidation with 4-Bromotiglic Acid. The cooperation between the two mitochondrial β-oxidation systems was evaluated by using 4-bromotiglic acid to completely inactivate long-chain 3-ketoacyl-CoA thiolase while retaining part of the 3-ketoacyl-CoA thiolase activity. For this purpose, mitochondria were preincubated for 2 min with various concentrations of 4-bromotiglic acid. The remaining capacity to oxidize fatty acids was then analyzed by measuring respiration supported by several

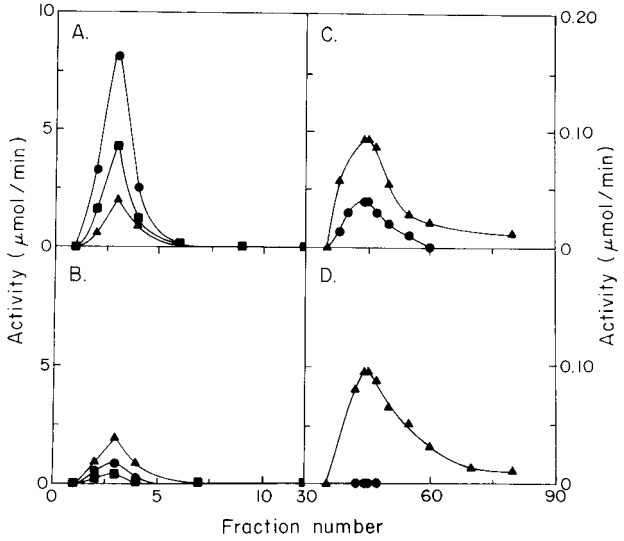


FIGURE 3: Separation of thiolases present in a homogenate of rat liver mitochondria by chromatography on DEAE−cellulose. (A, B) Flow-through fractions containing soluble 3-ketoacyl-CoA thiolase, acetoacetyl-CoA thiolase, and 3-hydroxyacyl-CoA dehydrogenase. (C, D) Fractions eluted with a KCl gradient contained long-chain 3-ketoacyl-CoA thiolase and long-chain 3-hydroxyacyl-CoA dehydrogenase. (A, C) Homogenate from control mitochondria. (B, D) Homogenate from mitochondria preincubated with 4-bromotiglic acid. For experimental details see under Experimental Procedures: thiolase activities detected with acetoacetyl-CoA (■) and with 3-ketooctanoyl-CoA (●); 3-hydroxyacyl-CoA dehydrogenase measured with 3-ketooctanoyl-CoA as substrate (▲).

substrates of β-oxidation. Respiration measurements were carried out in the presence and absence of the inhibitor to distinguish between irreversible and reversible modes of inhibition. The assumption was that the reversible inhibition would only persist if the inhibitor was present in the incubation medium. With palmitoylcarnitine as the substrate, the rate of respiration declined almost linearly as the inhibitor concentration was raised from 0 to 10 μM (see Figure 4A). The same results were obtained whether the inhibitor was present during the respiration measurements. However, with octanoylcarnitine as the substrate, the degree of the inhibition was greatly reduced when 4-bromotiglic acid was removed from the incubation medium before the respiration was measured (see Figure 4C). A possible explanation of these different results is that palmitoylcarnitine was first acted upon by the long-chain β-oxidation system which was completely

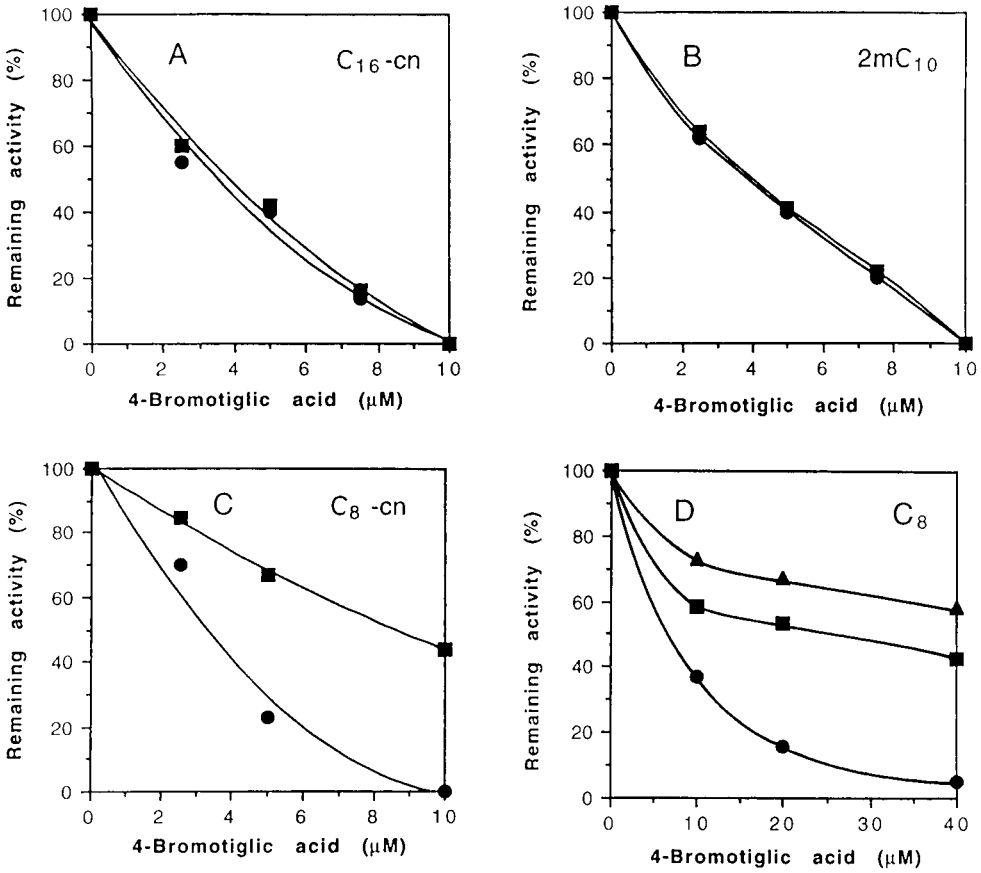


FIGURE 4: Inhibition of respiration supported by various substrates of β-oxidation as a function of the concentration of 4-bromotiglic acid. Coupled rat liver mitochondria (0.5 mg/mL) were preincubated for 2 min with the indicated concentrations of 4-bromotiglic acid. Substrates were added and rates of respiration were measured in the presence of the inhibitor (●) or after removing the inhibitor (■) by centrifuging the mitochondrial suspension for 30 s at 13000*g* and resuspending the pellet in the same incubation buffer except that 4-bromotiglic acid was omitted. Substrates were A, palmitoylcarnitine (C_{16}-cn); B, 2-methyldecanoic acid ($2mC_{10}$); C, octanoylcarnitine (C_8-cn); and D, octanoic acid (C_8). (D) Relative rates of respiration: (●) initial rates in the presence of the inhibitor; (▲) steady-state rates in the presence of the inhibitor; and (■) rates after removal of the inhibitor. The relative rates are based on the means of three measurements with standard deviations of 10% or less of the mean values.

inactivated. In contrast, octanoylcarnitine is a substrate of the medium-chain/short-chain system which remained partially active. The results obtained with octanoic acid as the substrate (see Figure 4D) confirm this interpretation even though the inhibition is generally less severe than is the inhibition of octanoylcarnitine-supported respiration (compare the inhibitor concentrations in Figure 4, panels C and D). Surprisingly, the removal of the inhibitor did not relieve the inhibition observed with 2-methyldecanoic acid as the respiratory substrate. This result is attributed to the complete inhibition of long-chain 3-ketoacyl-CoA thiolase which, in contrast to the matrix 3-ketoacyl-CoA thiolase, is active with 2-methyl branched substrates (*16*). It seems that 3-ketoacyl-CoA thiolase and thereby the β-oxidation system present in the mitochondrial matrix system remained partially active while the long-chain system was completely inactivated. This situation provided the opportunity for determining the capacity of the matrix system to degrade fatty acids of various chain lengths. As is apparent from Figure 5, 10 μM 4-bromotiglic acid caused the complete inhibition of respiration supported by any of the tested acylcarnitines from octanoylcarnitine to palmitoylcarnitine (Figure 5). However, when the inhibitor was removed from the incubation medium before the respiration was measured, complete inhibition was only observed with palmitoylcarnitine as the substrate. Shorter-chain substrates did support residual rates of respiration. A clear trend was detectable, with the remaining relative respiration increasing from 0% to almost 70% as the acyl chain length decreased from 16 to 8 carbon atoms

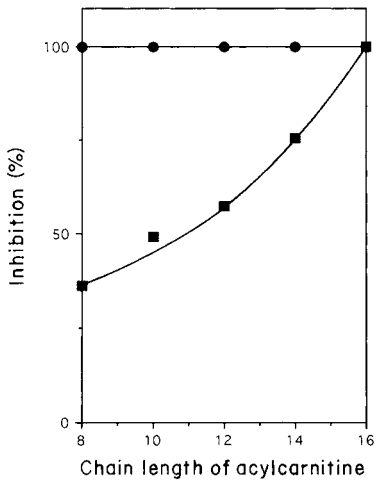


FIGURE 5: Inhibition of respiration as a function of the acylcarnitine chain length. Acylcarnitines with acyl chains having 8−16 carbon atoms served as substrates. Inhibition observed in the presence (●) and absence (■) of inhibitor after preincubating mitochondria (0.5 mg/mL) with 10 μM 4-bromotiglic acid for 2 min. Values of inhibition are based on the means of three measurements with standard deviations of 6% or less of the mean values.

(see Figure 5). Interestingly, even a presumed long-chain substrate like myristoylcarnitine sustained roughly 25% of the optimal respiration rate in mitochondria with a long-chain β-oxidation system that was completely inactive toward palmitoylcarnitine.

DISCUSSION

The first aim of this project was the design of an inhibitor that would irreversibly inactivate the long-chain specific system of fatty acid β-oxidation located in the inner mitochondrial membrane. Of the four enzymes that constitute the long-chain β-oxidation system, namely very long-chain acyl-CoA dehydrogenase, long-chain enoyl-CoA hydratase, long-chain 3-hydroxyacyl-CoA dehydrogenase, and long-chain 3-ketoacyl-CoA thiolase, thiolase is most amenable to chemical inactivation owing to its essential sulfhydryl group. A number of thiolase inhibitors have been designed and tested (*17*). A very effective one is 4-bromocrotonic acid which is metabolized in coupled mitochondria to 3-keto-4-bromobutyryl-CoA (*14*). This metabolite inactivates acetoacetyl-CoA thiolase (*14*), 3-ketoacyl-CoA thiolase (*14*), and long-chain 3-ketoacyl-CoA thiolase.[2] The plan was to design a derivative of 4-bromocrotonic acid that would inactivate the long-chain thiolase but not 3-ketoacyl-CoA thiolase. The effect of the inhibitor on acetoacetyl-CoA thiolase would be inconsequential because acetoacetyl-CoA thiolase is not involved in fatty acid β-oxidation (*1*). 4-Bromocrotonic acid with a 2-methyl substituent (4-bromotiglic acid) was a potential inhibitor of long-chain thiolase because it was expected to be metabolized intramitochondrially to 3-keto-4-bromo-2-methylbutyryl-CoA which is a likely substrate of long-chain 3-ketoacyl-CoA thiolase but not of 3-ketoacyl-CoA thiolase (*16*). Since the inhibition of thiolases by this group of inhibitors is assumed to be mechanism-based (*17*), the preferential inactivation of long-chain 3-ketoacyl-CoA thiolase was predicted.

The observed inhibition of palmitoylcarnitine-supported respiration by 4-bromotiglic acid in the absence of a severe effect on octanoate-driven respiration agrees with the expected inactivation of the long-chain β-oxidation system by this compound without having a serious effect on the medium-chain/short-chain β-oxidation system. In addition, this result proves that the inhibitor does not affect reactions downstream of β-oxidation. The anti-inhibitory effect of 2,4-dinitrophenol in mitochondria incubated with 4-bromtiglic acid was most likely due to the abolishment of ATP synthesis and the resultant failure of 4-bromotiglic acid to undergo activation to its CoA thioester. The same observation had been made with 4-bromocrotonic acid and 4-bromo-2-octenoic acid (*14*, *18*) which additionally underwent partial β-oxidation before becoming inhibitors of thiolases and thereby of β-oxidation. Similarly, 4-bromotigloyl-CoA is assumed to be hydrated and dehydrogenated, presumably by enoyl-CoA hydratase (crotonase) and short-chain 3-hydroxy-2-methylacyl-CoA dehydrogenase, respectively (*16*), to yield 3-keto-4-bromo-2-methylbutyryl-CoA which may be the actual inhibitor of thiolases. The inactivation of all three known thiolases by 4-bromotiglic acid, in the absence of significant effects on other enzymes of β-oxidation, was demonstrated. However, long-chain 3-ketoacyl-CoA and acetoacetyl-CoA thiolase were inactivated more severely than was 3-ketoacyl-CoA thiolase. In addition to being irreversibly inactivated, the thiolases also were reversibly inhibited. However, the reversible inhibition persisted only as long as 4-bromotiglic acid was present in the incubation mixture and was continuously metabolized intramitochondrially. Competition of octanoic acid and 4-bromotiglic acid for activation by medium-chain acyl-CoA synthetase or, less likely, competition for uptake relieved the reversible inhibition as did the removal of the inhibitor from the incubation medium. This observation supports the notion that the compound responsible for the reversible inhibition, possibly 4-bromo-3-keto-2-methylbutyryl-CoA, is short-lived due to its instability or further metabolism. Once the reversible inhibition was abolished, medium-chain substrates such as octanoylcarnitine and octanoate, in contrast to the long-chain substrate palmitoylcarnitine, were oxidized. The only exception was the medium-chain substrate 2-methyldecanoic acid which did not serve as a respiratory substrate, presumably because it is acted upon only by long-chain 3-ketoacyl-CoA thiolase but not by 3-ketoacyl-CoA thiolase (*16*). This interpretation agrees with the observation that long-chain 3-ketoacyl-CoA thiolase was totally inactivated while 3-ketoacyl-CoA thiolase retained part of its activity.

The differential inactivation of the two thiolases permitted an evaluation of their functions in the β-oxidation of fatty acids with different chain lengths. The use of acylcarnitines assured that all substrates entered mitochondria via the same uptake system. However, both carnitine palmitoyltransferase II and carnitine acetyltransferase may be required for the intramitochondrial acyl transfers from carnitine to CoASH. With palmitoylcarnitine as the substrate no respiration was detected, whereas shorter-chain substrates exhibited residual activities that increased with decreasing acyl chain length. Since the matrix 3-ketoacyl-CoA thiolase is highly active with all 3-ketoacyl-CoA intermediates formed during the β-oxidation of palmitate (*19*), the chain length specificity of the residual β-oxidation capacity is not explained by the remaining thiolase activity. The results fit a model based on the assumption that palmitoyl-CoA must be chain shortened by the membrane-bound β-oxidation system, whereas shorter-chain substrates can be degraded, at least in part, by the β-oxidation system in the matrix. The degradation of dodecanoyl-CoA and shorter-chain substrates seems to be affected only slightly by the inactivation of the long-chain β-oxidation system.

In conclusion, it seems that palmitoyl-CoA and most likely longer-chain acyl-CoA thioesters are obligatory substrates of the long-chain β-oxidation system. Moreover, it appears that the inactivation of one component enzyme of this β-oxidation system, for example, long-chain 3-ketoacyl-CoA thiolase, leaves the whole system inactive. This finding agrees with the reported intermediate channeling on the trifunctional β-oxidation enzyme (*20*), which catalyzes three of the four reactions of the long-chain β-oxidation system. Altogether, the observations fit a model of two mitochondrial β-oxidation systems cooperating in the degradation of long-chain fatty acids. The main transfer of intermediates between the two systems seems to occur after the completion of one or several full cycles of β-oxidation when the length of the acyl chain has been reduced to 14 or 12 carbon atoms.

[2] Cheng, H., and Schulz, H., unpublished observation.

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