INHIBITION OF LIVER ACETYL-COENZYME-A CARBOXYLASE BY 2-TETRADECANYLGLUTARATE

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In a previous study (A. ENDO, *et al.*, J. Antibiotics 38: 599~604, 1985), 2-alkyl glutarate and its derivatives isolated from cultures of *Gongronella butleri* were shown to inhibit animal acetyl-CoA carboxylase. In the present communication, the inhibition of liver acetyl-CoA carboxylase was investigated with several 2-alkyl glutarate and 2-alkyl succinate analogs. Their inhibitory potency increased with the chain length of the alkyl moiety, and 2-tetradecanylglutarate was most potent among the inhibitors tested. Kinetic analysis indicated that inhibition by 2-tetradecanylglutarate was non-competitive with respect to the substrates, ATP, HCO_3^- and acetyl-CoA, and competitive with respect to the allosteric regulator citrate, giving a *Ki* value of 40 μM. Sucrose density gradient centrifugation analysis showed that the citrate-induced polymerization of the enzyme was inhibited by 2-tetradecanylglutarate.

Acetyl-CoA carboxylase mediates the initial step in fatty acid biosynthesis and plays a critical role in its regulation. This enzyme is activated by hydroxytricarboxylic acids such as citrate and isocitrate[1,2], while it is reversibly inactivated by long-chain fatty acyl-CoA thioesters[3,4].

In the course of searching for acetyl-CoA carboxylase inhibitors, it has been found that a strain of *Gongronella butleri* mating (+) produces active substances, which include 2-octylglutaric acid, 9-decenyl-1-pentenedioic acid, and decanyl-2-pentenedioic acid[5]. In the present paper, the inhibition of liver acetyl-CoA carboxylase by these products and some related compounds was studied.

Materials and Methods

Materials

2-Octylglutaric acid, 9-decenyl-1-pentenedioic acid, decanyl-1-pentenedioic acid and decanyl-2-pentenedioic acid were isolated from culture broth of *G. butleri* M3180 as described previously[5]. Other 2-alkyl glutarate and succinate compounds were chemically synthesized by the method of malonic ester synthesis[6]. Other chemicals were the highest grade commercial products.

Purification and Assay of Acetyl-CoA Carboxylase

Purification of highly phosphorylated acetyl-CoA carboxylase from chicken liver was carried out with avidin affinity chromatography and Sepharose CL-2B gel filtration as described by WADA and TANABE[7]. The final enzyme preparations with a specific activity of 3.9 units/mg protein was dialyzed against 50 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 5 mM 2-mercaptoethanol and 1 mM EDTA and was stored at −80°C. Under these conditions, the enzyme was stable for at least 4 months.

Acetyl-CoA carboxylase activity was determined at 37°C by the spectrophotometric method in

combination with pyruvate kinase and lactate dehydrogenase reactions[8], except that BSA (bovine serum albumin) was removed for the kinetic analysis with 2-tetradecanylglutarate (TDG) in the presence of 10 mM of citrate, and 0.25 M sucrose was added to the reaction mixture for stabilization of the enzyme in experiments to determine *Ki* value of TDG. The reaction mixture containing all components and an inhibitor except $KHCO_3$ was subjected to prior incubation at 37°C for 10 minutes and the reaction was initiated by addition of $KHCO_3$. The coupling assay system was not affected by the concentration of inhibitors used.

Measurement of Critical Micelle Concentration

The CMC (critical micelle concentration) of the 2-alkyl glutarate and succinate (potassium salts) was determined by the method of ZAHLER *et al.*[9]. Increasing concentrations of tested compounds were added to 0.5 μM pinacyanol chloride in 50 mM Tris-HCl (pH 7.5) in glass cuvettes (1 cm path). The intersection of the two linear portions of the curves in relation plots between these concentrations and their absorbance was taken as the value of CMC.

Sucrose Density Gradient Centrifugation

Sedimentation studies were carried out by the procedure of OGIWARA *et al.*[3] as modified as follows. A mixture (150 μl) containing 2.6 μM acetyl-CoA carboxylase, 50 mM Tris-HCl (pH 7.5) 5 mM 2-mercaptoethanol, 1 mM EDTA in the presence and absence of 0.5 mM potassium citrate, and 50 or 100 μM TDG was preincubated for 10 minutes. An aliquot of 100 μl of the mixture was then applied onto 4.9 ml of a 5~20% (w/v) sucrose gradient in a tube. The tube was centrifuged using an Hitachi RPS-50 rotor at 10°C and 43,000 rpm for 190 minutes. After centrifugation, fractions of 250 μl were collected for protein determination by the method of SCHAFFNER and WEISSMAN[10]. Aliquots of preincubation mixtures were pooled and assayed for acetyl-CoA carboxylase activity.

Results

Inhibition by 2-Alkyl Glutarate and Succinate Derivatives

The four inhibitors of microbial origin inhibited chicken liver acetyl-CoA carboxylase 50% at a concentration of 200~330 μM (Table 1). Decanyl-1-pentenedioic and decanyl-2-pentenedioic acids were inhibitory to the same extent, indicating that the location of double bond in these compounds does not affect inhibitory activity. 2-Decanylglutarate derivatives were slightly more potent than 2-octylglutarate. Similar results were obtained with partially purified rat liver acetyl-CoA carboxylase (data not shown).

In the experiments shown in Table 2, 2-alkyl glutarates and their analogs, 2-alkyl succinates, with various hydrocarbon chain lengths were assayed for inhibitory activity. Since 2-alkyl glutarate and succinate derivatives with a long alkyl chain, were expected to have detergent effects, their CMC values were also determined. Of the derivatives tested, TDG was the most potent, giving 50% inhibition of acetyl-CoA carboxylase at a concentration of 65 μM, while in the succinate series the tridecanyl derivative was the most potent inhibitor. The concentrations required for significant inhibition of the enzyme were 7-fold or more lower than for CMC, suggesting that inhibition by dicarboxylic acids are not due to non-specific detergent effects.

Titration of Acetyl-CoA Carboxylase Activity with TDG

In order to study more detailed information about the inhibition by 2-alkyl glutarate, acetyl-CoA carboxylase was titrated with TDG in the presence and absence of BSA as shown in Fig. 1. BSA showed a protective effect on inhibition of the enzyme by TDG. These effects are possibly ascribed to competitive binding of the inhibitor by BSA in the assay mixture. Inhibition curves of TDG were a saturated type and were different from that of sodium dodecylsulfate, a non-specific inhibitor[11].

Table 1. Inhibition activity of inhibitors produced by a fungal strain of *Gongronella butleri* M3180.

	ID_{50} (μM)
2-Octylglutaric acid $CH_3(CH_2)_7CH(COOH)CH_2CH_2COOH$	330
9-Decenyl-1-pentenedioic acid $CH_2{=}CH(CH_2)_8C(COOH){=}CHCH_2COOH$	220
Decanyl-1-pentenedioic acid $CH_3(CH_2)_9C(COOH){=}CHCH_2COOH$	200
Decanyl-2-pentenedioic acid $CH_3(CH_2)_9CH(COOH)CH{=}CHCOOH$	200

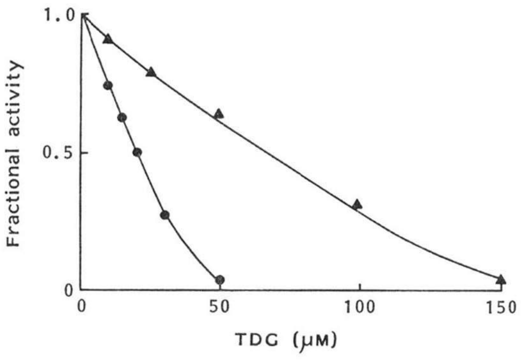
ID_{50} represents concentration required for 50% inhibition.

Table 2. Inhibition activity and critical micelle concentration of 2-alkyl glutarates and succinates.

		ID_{50} (μM)	CMC (mM)
$RCH(COOH)CH_2CH_2COOH$	R = $CH_3(CH_2)_7$	330	>4.0
	$CH_3(CH_2)_9$	200	4.0
	$CH_3(CH_2)_{12}$	85	3.0
	$CH_3(CH_2)_{13}$	65	0.5
$R'CH(COOH)CH_2COOH$	R′ = $CH_3(CH_2)_9$	200	>4.0
	$CH_3(CH_2)_{11}$	150	>4.0
	$CH_3(CH_2)_{12}$	65	3.5
	$CH_3(CH_2)_{13}$	120	0.8

Fig. 1. Inhibition of chicken liver acetyl-CoA carboxylase by TDG in the presence or absence of BSA.

Acetyl-CoA carboxylase was assayed in the presence (0.75 mg/ml) (▲) or absence (●) of BSA. Assay mixture contained 5.0 nM acetyl-CoA carboxylase.



TDG also inhibited the enzyme activated with citrate in a time-dependent manner. But the activated enzyme was slightly more resistant to TDG than the untreated enzyme (data not shown).

Kinetic Analysis of Inhibition by TDG

Steady state kinetics were obtained and the

Fig. 2. Lineweaver-Burk plots for the inhibition of acetyl-CoA carboxylase by TDG with ATP (A), HCO_3^- (B) and acetyl-CoA (C) as the varied substrate concentration.

All assays were carried out in the absence of BSA using 7.5 nM acetyl-CoA carboxylase. ● 0 μM (control); ▲ 12.5 μM; ■ 25 μM of TDG.

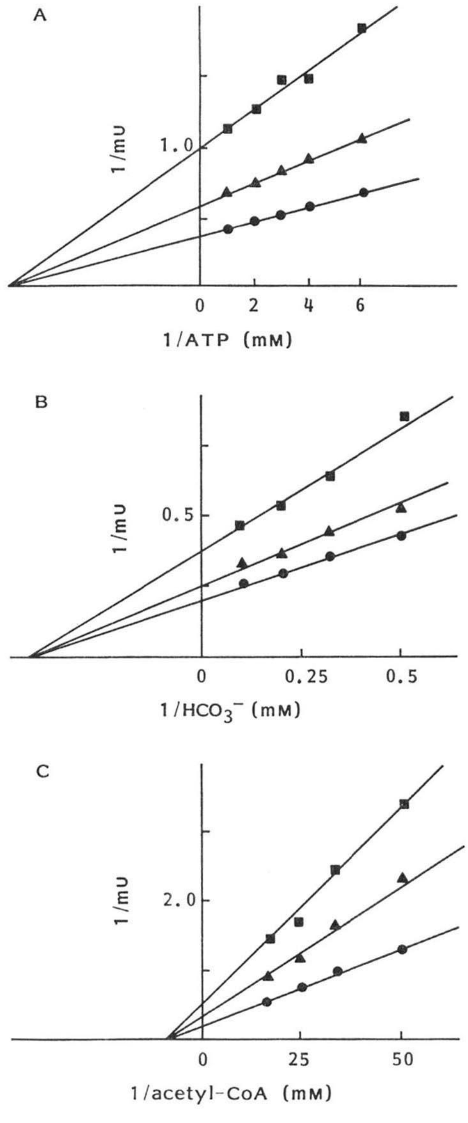
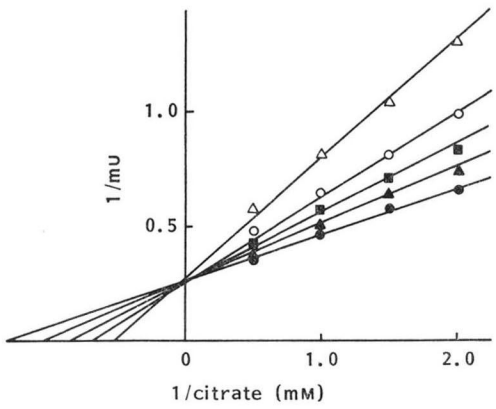


Fig. 3. Lineweaver-Burk analysis of inhibition by TDG with citrate as the varied component.

Assays were carried out in the presence of 0.25 M sucrose and absence of BSA, using 12.5 nM acetyl-CoA carboxylase. ● 0 μM (control); ▲ 10 μM; ■ 0 μM; ○ 25 μM; △ 30 μM of TDG.



data are summarized in Fig. 2. TDG inhibited acetyl-CoA carboxylase non-competitively with respect to the three substrates. *Km* for ATP, HCO_3^- and acetyl-CoA was 125 μM, 2.2 mM and 100 μM, respectively. TDG inhibited the enzyme competitively with respect to citrate, but a *Ki* value could not be determined in the standard assay condition because of instability of the enzyme at low concentrations of citrate. Thus, sucrose was added to the assay mixtures as a stabilizer and kinetic analysis for Dixon plots were performed. The results shown in Fig. 3 indicated that inhibition was competitive with respect to the activator citrate, giving a *Ki* value of 40 μM.

Sucrose Density Gradient Centrifugation Analysis

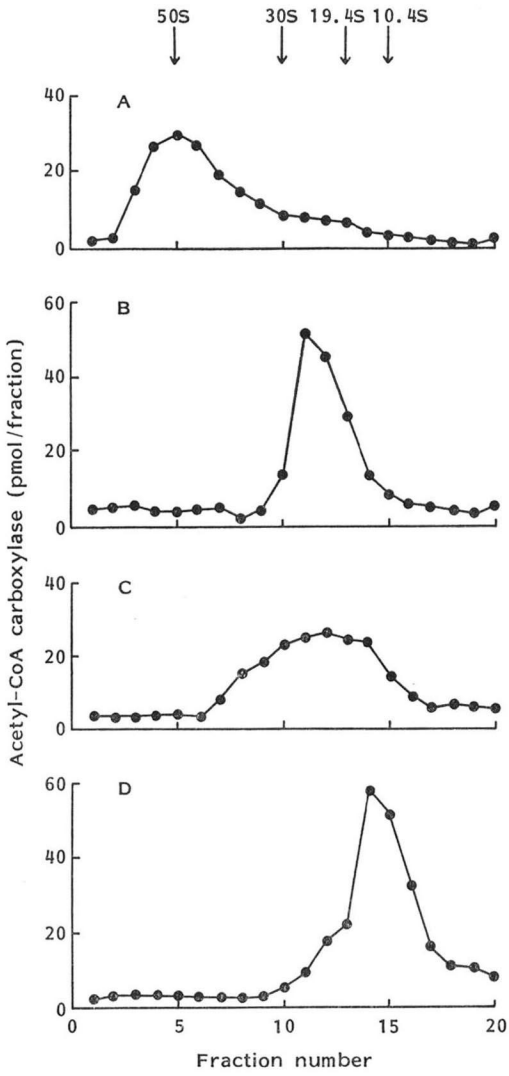
TDG inhibited the activation of acetyl-CoA carboxylase competitively with respect to citrate. These results suggested the possibility that polymerization of the enzyme was also inhibited by TDG. This possibility was confirmed by sucrose density gradient centrifugation analysis as shown in Fig. 4. The enzyme treated with 0.5 mM citrate showed a sedimentation coefficient (S_{20}, w)

Fig. 4. Effects of citrate and TDG on the sedimentation pattern of acetyl-CoA carboxylase.

Acetyl-CoA carboxylase was preincubated for 10 minutes in a mixture containing 0.5 mM citrate (A), no citrate (B), 0.5 mM citrate plus 50 μM TDG (C) and 0.5 mM citrate plus 100 μM TDG (D). After preincubation, an aliquot of 100 μl of the mixture was applied onto the top of sucrose gradient and analyzed as described in Materials and Methods.

External markers, *Bacillus subtilis* ribosome (50, 30S), and rabbit muscle pyruvate kinase (10.4S) were used.

Relative enzyme activity of the preincubation mixtures were 100 (A), 26 (B), 35 (C) and 8 (D), respectively.



of about 50S, while the untreated enzyme showed 25S, a value for the protomeric form of the enzyme. In the presence of 0.5 mM citrate and 50 μM TDG, polymerization of the enzyme was inhibited to give a broad peak of protein (10~40S). Complete dissociation of the enzyme into the monomeric form (10S) occurred at a higher concentration of 100 μM TDG in the presence of citrate.

Discussion

The present investigation has demonstrated that 2-alkyl glutarates and succinates inhibited animal acetyl-CoA carboxylase and that inhibitory activity varied with chain length of the alkyl group. Inhibition by the most potent compound, TDG, was competitive with respect to the activator, citrate. Similar mode of action has also been shown with long chain fatty acyl-CoA[3,4], 2-methyl-2-*p*-(1,2,3,4-tetrahydro-1-naphthyl) phenoxyl propionate (TPIA), 2-(*p*-chlorophenyl)-2-methyl-propionate (CPIB)[11,12], phosphoinositides[13], salicylate[14] and cyano-4-hydroxycinnamate[15]. However, it should be noted that the glutarate moiety of TDG is structurally related to citrate, the activator of acetyl-CoA carboxylase.

Kinetic constants of chicken liver acetyl-CoA carboxylase were also reported by BEATY and LANE[16]. Their *Km* values for ATP and HCO_3^- are in agreement with those obtained in the present study. The *Km* value for acetyl-CoA was, however, ten times smaller in their study than in ours. This discrepancy is possibly ascribed to the different phosphorylation state of the enzyme used in the experiments, since the highly phosphorylated rat liver enzyme has been shown to exhibit a smaller *Km* value for acetyl-CoA than the dephosphorylated form of the enzyme[17].

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