Biochimica et Biophysica Acta, 1152 (1993) 243–252

BBAMEM 76108

Effect of cerulenin on the synthesis of very-long-chain fatty acids in microsomes from leek seedlings

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(Received 16 February 1993)

Key words: Acyl-CoA elongase; Cerulenin; Membrane; 3-Ketoacyl-CoA synthase; (*A. porrum*)

Cerulenin inhibits the elongation of stearoyl-CoA and eicosanoyl-CoA by microsomes from leek seedlings. The inhibition depends on the cerulenin concentration and affects the biosynthesis of docosanoic and tetracosanoic acids only slightly more than that of eicosanoic acid. A 30-min preincubation of the microsomes with cerulenin allows a quantitative inhibition of the elongation at 50 μM cerulenin (50% inhibition at 15 μM cerulenin). A kinetic study of the elongating activity in the presence or in the absence of the inhibitor suggests that the inhibition is non-competitive. Analysis of the products of the reaction suggests that 3-ketoacyl-CoA synthase is the target of cerulenin. A study of the partial reactions demonstrates that the inhibition affects almost exclusively the condensation step.

Introduction

Cerulenin ((2*S*,3*R*)2,3-epoxy-4-oxo-7,10-dodecadienoyl amide) was named after the strain which was shown for the first time to produce it, *Cephalosporium caerulens* [1]. Numerous studies carried out with purified fatty acid synthases from various sources have shown that cerulenin inhibits both multienzyme complexes and non-associated fatty acid synthases [2]. The enzyme catalyzing the condensation of fatty acyl-ACP with malonyl-ACP was rapidly suspected to be the target of cerulenin: the specific inactivation of this enzyme in *Escherichia coli* was demonstrated [3]. Cerulenin inhibited model reactions representing both the fatty acyl transacylase and the malonyl-ACP decarboxylase components of the 3-ketoacyl synthetase reaction. The inhibition was irreversible and it was associated with the binding of 1 mol of inhibitor per mol of enzyme when inhibition approached 100% [3]. Very similar results were observed with the fatty acid synthase from *Saccharomyces cerevisiae* [4]. 3 mol of cerulenin were bound to 1 mol of enzyme for quantitative loss of activity [4].

Shimakata and Stumpf [5] isolated two soluble 3-ketoacyl-ACP synthases from crude spinach leaf extracts. Synthase I, involved in de novo synthesis, was strongly inhibited by cerulenin (50% at 2 μM cerulenin), whereas synthase II, which had a specificity restricted to C_{14}-ACP and C_{16}-ACP (i.e., participating chiefly in C_{16}-ACP elongation), was relatively resistant to cerulenin (50% inhibition at 50 μM cerulenin [5]. Jaworski et al. [6] isolated a third 3-keto acyl-ACP synthase from *Spinacia oleracea*, which could use acetyl-CoA as a substrate, was specific for the synthesis of short-chain fatty acids and was cerulenin insensitive. This enzyme appeared to be highly similar to the acetoacetyl-ACP synthase found in *E. coli* [7]. Using radioactive [^{3}H]cerulenin, Siggaard-Andersen et al. [8] were able to label three dimeric barley chloroplast proteins and obtained the primary structure of a cerulenin-binding 3-ketoacyl-ACP synthase.

Compared to the studies devoted to soluble fatty acid synthases, little data concerning the effect of cerulenin on membrane-bound acyl-CoA elongases has been reported. A first study by Agrawal et al. [9] investigated the effect of cerulenin on the ATP-dependent elongation of (unknown) endogenous substrates by microsomes from leek epidermis. The activity was inhibited by 50% with 40 μM cerulenin, and the formation of C_{22} and C_{24} fatty acids was more affected than that of shorter homologues. Recently, a similar phenomenon was reported in developing seeds [10]. A 15000 $\times g$ particulate fraction from honesty (*Lunaria annua*) preincubated with various amounts of cerulenin re-

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vealed a weak sensitivity of the oleoyl-CoA elongation to cerulenin. As the assays were carried out without exogenous reductants, it was hypothesized that the 3-ketoacyl-CoA synthase could be the target of the inhibitor [10].

In leaves, and particularly in leek leaves, acyl-CoA elongases are exclusively associated with the microsomes. C_{18}-CoA elongase is chiefly located within the endoplasmic reticulum and C_{20}-CoA elongase in the Golgi apparatus [11]. The acyl-CoA elongase complex is presumably composed of four different enzymes (3-ketoacyl-CoA synthase, 3-ketoacyl-CoA reductase, 3-hydroxyacyl-CoA dehydrase and *trans*-2,3-enoyl-CoA reductase) [12]. The involvement of a 3-ketoacyl-CoA intermediate that is non-covalently bound to the enzyme has been demonstrated [13].

This investigation reports on the inhibition of the stearoyl-CoA and eicosanoyl-CoA elongation by cerulenin in microsomes from leek seedlings. The kinetics of the inhibition, the partition of cerulenin between the microsomes and the aqueous phase in the presence, or absence, of long-chain acyl-CoAs, the effects of a preincubation of the microsomes with cerulenin, or acyl-CoAs, on the elongation rate, the nature of the reaction products in the presence of the inhibitor and the effect of cerulenin on the partial activities of the membrane-bound acyl-CoA elongases, are presented and discussed.

Materials and Methods

Plant material, substrates and reagents

Leek seeds stored overnight at 4°C, were surface sterilized with sodium hypochlorite in the presence of Triton X-100 for 2 min and then washed with distilled water. They were grown for 7 days in the dark as described [11]. Chemicals were from Sigma (St. Louis, MO, USA).

[2-^{14}C]Malonyl-CoA (53 Ci/mol), [1-^{14}C]stearoyl-CoA (50 Ci/mol) were from Amersham International. [^{3}H]Cerulenin (2.9 Ci/mol) was a gift from Prof. P. Von Wettstein-Knowles and Dr. Siggaard-Andersen (Copenhagen, Denmark). *trans*-2,3-Octadecenoyl-CoA was a gift from Prof. D. Cinti (Farmington, CT, USA).

Microsome preparation

All operations were carried out at 4°C. 5–8 g of 7-day-old etiolated leek seedlings were ground in a mortar in 50 ml 0.08 M Hepes buffer (pH 7.2), containing 10 mM 2-mercaptoethanol and 0.32 M sucrose. The homogenate was filtered through two layers of miracloth (Calbiochem) and centrifuged at $3000 \times g$ for 5 min; the supernatant was centrifuged at $12000 \times g$ for 20 min. The resulting pellet was discarded and the supernatant was spun down at $189000 \times g$ for 15 min (Hitachi CS 100 ultracentrifuge). The microsomal pellet was resuspended in 2 ml 0.08 M Hepes buffer (pH 6.8) and 10 mM 2-mercaptoethanol and spun again at $189000 \times g$ for 15 min. The washed microsomes, resuspended in 2 ml 0.08 M Hepes (pH 6.8) and 10 mM 2-mercaptoethanol were used as the enzyme source.

Proteins were assayed according to Bradford [14].

Enzyme assays

The required amounts of cerulenin in methanolic solution were added to the test tubes and the methanol was evaporated. When a preincubation in the presence of cerulenin was desired, 50 μg of microsomal proteins were added and the volume was brought to 50 μl with 0.08 M Hepes buffer (pH 6.8); after the preincubation period, the incubation was started by the addition of the reaction mixture required for the assay of the activity under study (see below).

Assay of stearoyl-CoA elongase

50 μg of proteins in 0.08 M Hepes buffer (pH 7) containing 0.5 mM NADPH, 0.5 mM NADH, 2 mM DTT, 1 mM $MgCl_2$, 9 μM acyl-CoA and 17 μM [2-^{14}C]malonyl-CoA, were added to the tubes with or without cerulenin. The reaction mixture (0.1 ml) was incubated for various times at 30°C (routinely 60 min).

The reaction was stopped by the addition of 100 μl of 20% KOH in H_2O/CH_3OH (9:1 (v/v)) and the lipids were saponified for 1 h at 70°C. After acidification by 0.1 ml of H_2SO_4 (5 M), the fatty acids and lipidic material were extracted and the radioactivity of an aliquot was measured in a Tricarb 2000 CA Packard scintillation spectrometer. The label distribution was analyzed by thin-layer chromatography on Merck silica gel $60F_{254}$ plates eluted by hexane/diethyl ether/acetic acid (75:25:1 (v/v/v)).

The reaction products were identified by comparison with the R_f values of standards. The autoradiographs were made using Kodak DEF 5 films and scanned at 546 nm using a Camag TLC scanner.

Assay of 3-ketoacyl-CoA synthase

The activity was assayed as described previously [13]. Briefly, the reaction mixture is identical to that used for measuring the acyl-CoA elongation, except that NADH and NADPH were omitted. The time of incubation was 15 min (see text).

The reaction was stopped and the lipidic material was extracted. The radioactivity was measured and the reaction products (and particularly the methylketones) were resolved and identified as above. Alternatively, the reaction was stopped by addition of 0.8 ml of a solution containing 5 mg/ml $NaBH_4$, 0.1 M K_2HPO_4, 0.4 M KCl and 30% tetrahydrofuran. $NaBH_4$ was added extemporaneously. After adding the reducing mixture, the tubes were vigorously shaken for 45 min at 37°C. 0.8 ml benzene was then added and the solution

was vortexed. The benzene phase was recovered and used for radioactivity determination or for TLC analysis.

Assay of hydroxyacyl-CoA dehydratase activity

The activity of 3-hydroxyacyl-CoA dehydratase was assayed in the reverse reaction according to Osei et al. [15]. The assay medium contained: 80 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol, cerulenin (0–400 μM) and 50 μg of microsomal protein, in a final volume of 1 ml. After 6 min preincubation at room temperature, the reaction was started by the addition of *trans*-2,3-octadecenoyl-CoA. The change in absorbance at 280 nm was recorded in a Kontron Uvikon 810 UV-Vis spectrometer. The enzyme activity was calculated using an extinction coefficient of 3.6 $mM^{-1} cm^{-1}$.

trans-2,3-Enoyl-CoA reductase activity measurement

The assay medium contained 80 mM Tris-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 0–400 μM cerulenin and 72 μg microsomal proteins. The final volume was 1 ml. After 1 h of incubation at 30°C, the reaction was started by the addition of the *trans*-2,3-octadecenoyl-CoA (30 μM) and NADPH (150 μM). The change in absorbance at 340 nm was recorded in a Kontron Uvikon 810 UV-Vis spectrometer. The enzyme activity was calculated using an extinction coefficient of 6.2 $mM^{-1} cm^{-1}$.

Lipid analysis

60 μl of $CHCl_3$/CH_3OH (1 : 1 (v/v)) were added to 10-μl aliquots of the reaction mixture(s). The resulting homogeneous phase was submitted to TLC analysis on HPTLC 60 F_{254} Merck plates eluted by butanol/acetic acid/water (5:2:3 (v/v/v)), as described previously [16]. The reaction products were identified by comparison with the R_f values of standards and the autoradiographs were made with Kodak DEF 5 films and scanned at 546 nm using a Camag TLC scanner.

Radio-GLC of the fatty acids

Methyl esterification of the free fatty acids was performed by reaction with BF_3 reagent (14%) in methanol for 30 min at 70°C. The radio-gas liquid chromatography (radio-GLC) was performed using a Packard 429 chromatograph fitted with a 10% CPSil 58 Column. The effluent gases were continuously monitored for radioactivity in a Packard 894 gas proportional counter.

Results

Cerulenin effect on acyl-CoA elongation

The effect of cerulenin on stearoyl-CoA elongation was investigated using microsomes (preincubated for 10 min with the inhibitor) from 7-day-old leek seedlings, in the presence of labeled malonyl-CoA and NADH + NADPH as the reductants (Fig. 1). There was a sharp inhibition of the malonyl-CoA incorporation into fatty acids for cerulenin concentrations up to 150 μM and a plateau was reached at 400 μM, when about 70% of the total activity was inhibited. 45% of the maximal inhibition was observed at a cerulenin concentration of 50 μM. Almost identical results were observed when stearoyl-CoA was replaced in the reaction mixture by eicosanoyl-CoA, i.e, when the C_{20}-CoA elongase, was checked instead of the C_{18}-CoA elongase (Fig. 1, inset). At 50 μM cerulenin, the inhibition reached about 40% of the maximal effect (which was observed at 400 μM, when 70% of the C_{20}-CoA elongation was abolished).

Effect of 200 μM cerulenin on the elongation of various amounts of stearoyl-CoA as a function of time

The reaction mixtures containing the microsomes, [2-^{14}C]malonyl CoA, NADPH and NADH and variable amounts of unlabeled stearoyl-CoA were incubated for 0, 5, 10, 30 and 60 min at 30°C in the presence or absence of 200 μM cerulenin. The results are given in Fig. 2.

In the absence of cerulenin two different situations are observed:

(a) At low (i.e., limiting, subsaturating) stearoyl-CoA concentrations (9 μM), the maximal elongation is reached very rapidly (5 to 10 min), and only a small

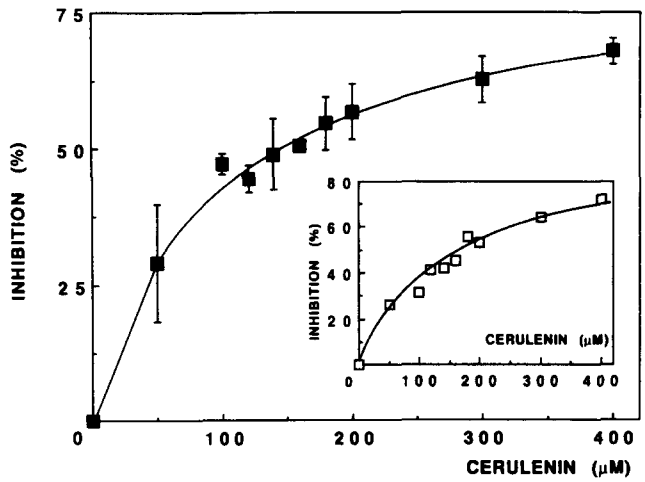

Fig. 1. Effect of cerulenin on microsomal acyl-CoA elongation activity. The incubations of the microsomes were carried out in the presence of 9 μM stearoyl-CoA, 17 μM [2-^{14}C]malonyl-CoA and various amounts of cerulenin as indicated. Other conditions were as described in Materials and Methods. The microsomes were preincubated for 10 min with cerulenin. Results are given as the % inhibition as compared to the control. Mean values ± S.D. of four experiments are given. Inset: Effect of cerulenin on the elongation of C_{20}-CoA. Same experimental conditions as in Fig. 1, except that the experiments were carried out in the presence of eicosanoyl-CoA (9 μM).

increase of the elongation is observed afterwards, due to the lack of substrate.
(b) At higher (22.5, 45 and 90 μM), non-limiting stearoyl-CoA concentrations, the synthesis increases for 60 min, but as the acyl-CoA concentration increases, there is a progressive decrease of the overall elongation rate.

In the presence of cerulenin, there is a marked decrease of the elongation under all experimental conditions investigated. Here again, two different situations may be distinguished:
(a) At limiting initial stearoyl-CoA concentrations (9 μM), the maximal inhibition is reached after 10 min, with only insignificant effects on the activities thereafter.
(b) At higher concentrations (22.5, 45 and 90 μM) cerulenin inhibits the elongation, which increases slowly, but linearly, for 60 min.

For the three concentrations used, the ratio of the slopes (specific activity in the presence of inhibitor/specific activity in its absence = V_i/V) were close to 0.4, irrespective of the substrate concentration used.

Partition of cerulenin between the microsomes and the aqueous phase

[^{3}H]Cerulenin was used for this study. It was shown (Fig. 3) that cerulenin partitions in the microsomes. Under the experimental conditions used in Fig. 3, the amount of [^{3}H]cerulenin associated with the membranes increases almost linearly as a function of the overall concentration, so that the amount in the membranes is always about 5% of the total amount added. This is fairly low when compared to that of stearoyl-CoA, which reaches 95% of added and illustrates a major difference between the two types of molecules (Fig. 4). However, the concentration of cerulenin within the membranes reaches high values: approx. 9 mM from 100 μM overall concentration, 16 mM at 200 μM and 25 mM at 400 μM. Under the experimental conditions used in this study, the cerulenin is concentrated by almost 60–100-times in the membrane. The amount of cerulenin bound to the membrane is identical when it is measured after 2, 5 or 60 min of incubation, so that no variations of the binding are likely to occur during the experiments reported here. On the other hand, when the partition of a constant amount of [^{3}H]cerulenin between a constant amount of microsomes (50 μg proteins) and a variable volume of aqueous phase was studied, we observed a decrease of [^{3}H]cerulenin concentration within the membrane, which correlated well with the decrease of the overall aqueous [^{3}H]cerulenin concentration (Fig. 3, inset). At the same time, the partition of the acyl-CoA between

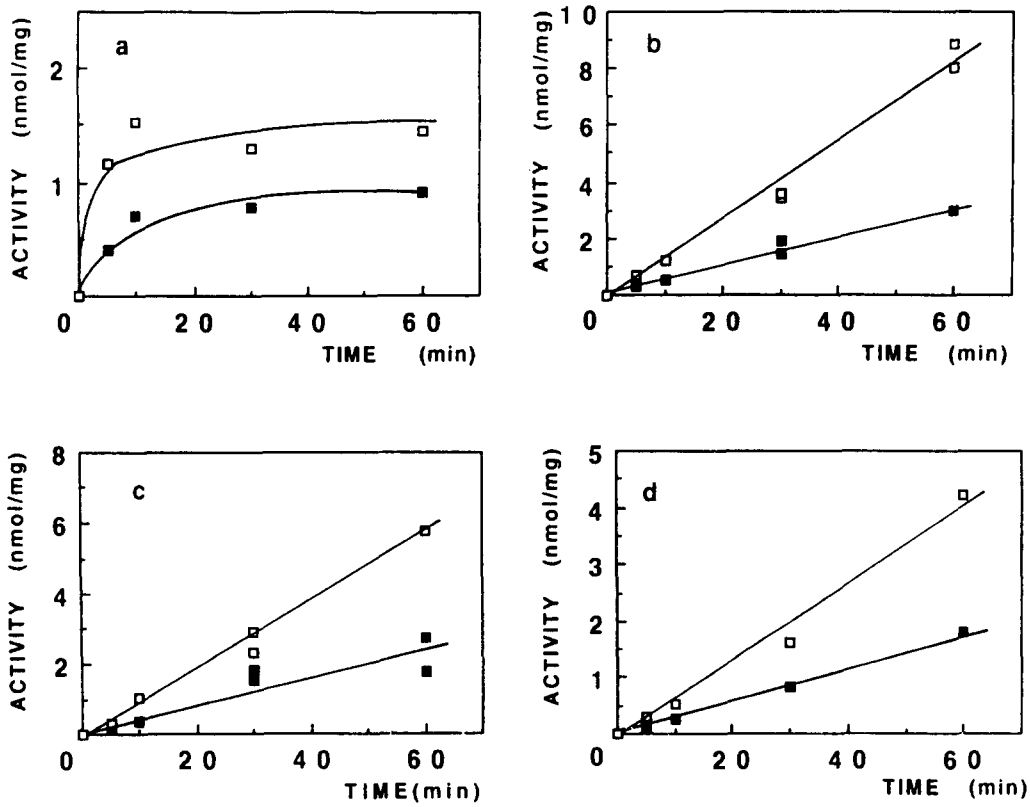

Fig. 2. Kinetics of cerulenin inhibition of the stearoyl-CoA elongation. Various amounts of stearoyl CoA (a, 9 μM; b, 22.5 μM; c, 45 μM; d, 90 μM) were incubated for 5, 10, 30 and 60 min with or without 200 μM cerulenin. Other conditions were the same as described in Materials and Methods. The results are expressed as nmol/mg protein. (□) C_{18}-CoA elongation in the absence of 200 μM cerulenin. (■) C_{18}-CoA elongation in the presence of 200 μM cerulenin.

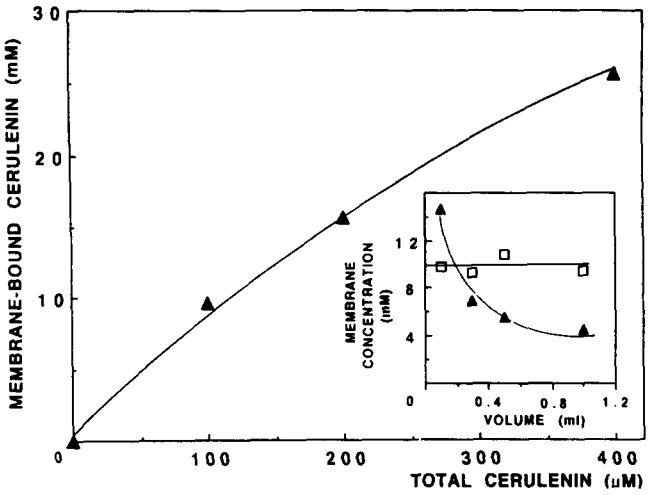

Fig. 3. Cerulenin binding to the microsomes. Various amounts of [^{3}H]cerulenin (13.4 Ci/mol) were incubated with 50 μg microsomes at 25°C. Results are given as the concentration (mM) of cerulenin within the microsomes. Inset: Microsomes (50 μg proteins) were incubated with 9 μM [1-^{14}C]stearoyl-CoA (50 Ci/mol) or 200 μM [^{3}H]cerulenin in 0.1 ml, 0.3 ml, 0.5 ml or 1 ml. Results are given as membrane-bound stearoyl-CoA (□) or cerulenin (▲) (mM).

the membranes and the aqueous phase did not vary as a function of the dilution. This confirms our previous observation that the amount, but not the concentration, of acyl-CoAs plays a role in their binding to a constant amount of microsomes from mouse sciatic nerves [17] and the conclusion that, as far as acyl-CoAs are concerned, these detergents form micelles (so that the bulk concentration may be far-off the expected concentration), the true parameter to be considered is the ratio of the amount of acyl-CoA to membrane [18,19].

As seen in Fig. 3, the membrane concentrations of cerulenin and stearoyl-CoA are of the same order of

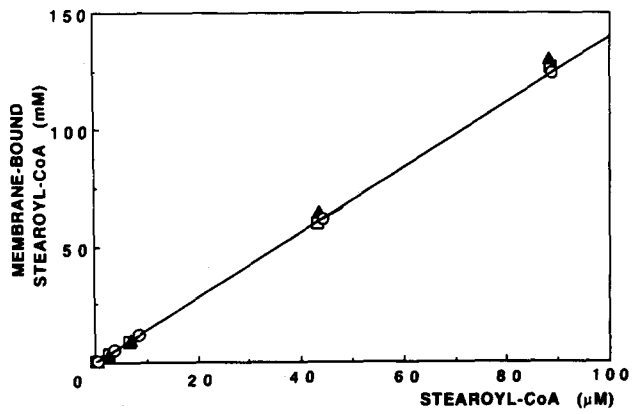

Fig. 4. Stearoyl-CoA binding to microsomes. Microsomes (50 μg proteins) were incubated with various amounts of [1-^{14}C]stearoyl-CoA in the presence of 100 μM (▲), 200 μM (○), or in the absence (□) of cerulenin. Results are given as membrane-bound stearoyl-CoA (mM).

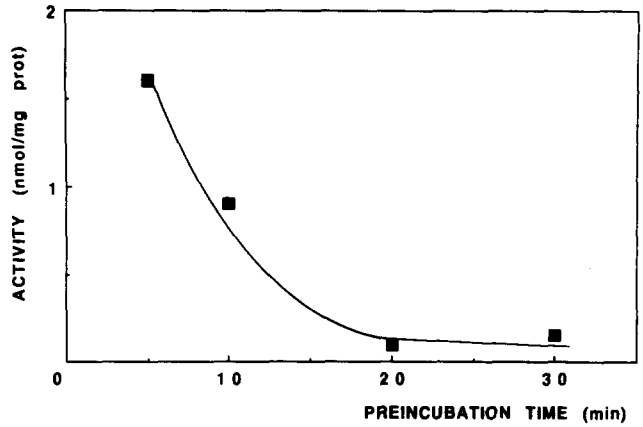

Fig. 5. Effect of the preincubation time of microsomes with cerulenin on stearoyl-CoA elongation. Microsomes were preincubated with 200 μM cerulenin for the times indicated and then incubated with the elongation mixture (45 μM stearoyl-CoA, [2-^{14}C]malonyl-CoA, NADPH and NADH as described in Materials and Methods). Results are given as nmol/mg protein.

magnitude, although the initial overall concentrations differ greatly (200 μM cerulenin, 8 μM stearoyl-CoA).

The reciprocal effects of the presence of cerulenin (0, 100 μM, 200 μM) on the binding of various amounts of stearoyl-CoA (0, 4.5 μM, 9 μM, 45 μM and 90 μM) were investigated. The binding of any initial amount of stearoyl-CoA to the microsomes was unaffected up to 200 μM cerulenin (Fig. 4). Conversely, the addition of increasing amounts of stearoyl-CoA to the reaction mixture did not modify the binding of cerulenin to the membranes (5% of added). This experiment shows that the inhibition by cerulenin cannot be due to a decrease of the acyl-CoA concentration within the membrane provoked by the modification of its partition.

Effect of preincubation of the microsomes with cerulenin on the stearoyl-CoA elongation

The effect of preincubating 200 μM cerulenin with microsomes for various times on the elongation reaction time (30 min) of 45 μM stearoyl-CoA was investigated. A 5-min preincubation with cerulenin did not change the inhibition level observed without preincubation period. The inhibition was greatly increased by a 10-min preincubation and was almost total at 20 and/or 30-min preincubation with cerulenin (Fig. 5). This observation prompted us to analyze the effect of a 30-min preincubation of microsomes with increasing amounts of cerulenin on the elongation of 45 μM and 9.2 μM stearoyl-CoA. As shown in Fig. 6, an inhibition of almost 100% is observed at 50 μM cerulenin. Basically similar results were obtained when the initial stearoyl-CoA concentration was 9.2 μM instead of 45 μM.

When $1/V_i$ was plotted vs. the cerulenin concentration (V_i being the activity in the presence of inhibitor), two straight lines were obtained for 9.2 and 45 μM of

stearoyl-CoA (Fig. 6, inset). The ordinates at [cerulenin] = 0 are 0.62 and 0.92.

Such values of $1/V$ (activity in the absence of cerulenin) are close to those observed in Fig. 2 (0.66 and 0.95, respectively).

Moreover, the ratios of these ordinates and of the slopes are very similar (0.65 and 0.61, respectively) and, for both straight lines, the ratio of the ordinate at [cerulenin] = 0/slope is constant. These results greatly suggest a non-competitive inhibition ($1/V_i = 1/V \cdot (1 + I/K_I)$), from which a K_I value of around 15 μM can be determined.

Analysis of the products of acyl-CoA elongation upon cerulenin addition

(a) Under the experimental conditions described in Fig. 1 (effect of cerulenin on stearoyl-CoA elongation), we studied the label distribution among the various lipids after incubation of the microsomes in the presence and absence of cerulenin. An homogeneous phase of the whole reaction mixture was prepared as previously described [16] and subjected to thin-layer chromatographic analysis using butanol/acetic acid/water (5:2:3 (v/v/v)) as the eluent. The radioactivity was located chiefly in the long-chain acyl-CoA band and, to a lesser extent, in phosphatidylcholine and free fatty acids. Besides this metabolism, some degradation of [2-^{14}C]malonyl-CoA had also occurred, yielding labeled malonic acid and labeled acetyl-CoA. The increase in cerulenin concentration was accompanied by a decrease of the label of the long-chain acyl-CoA band, but this observation gave no clue as to the step(s) affected by the inhibitor, since the method does not allow to separate 3-ketoacyl-CoAs from unsubstituted saturated, or monounsaturated acyl-CoAs. After saponification of the reaction mixture, acidification and extraction of lipidic material, TLC analysis revealed the presence of label exclusively in free fatty acids. No label was associated with methyl-ketones, which could arise from the degradation of 3-ketoacyl-CoAs during saponification [13], and which have already been observed in leek microsomes only when NADPH and NADH were omitted from the reaction mixture, i.e., when the overall elongation was likely to be blocked at the 3-ketoacyl-CoA reduction step.

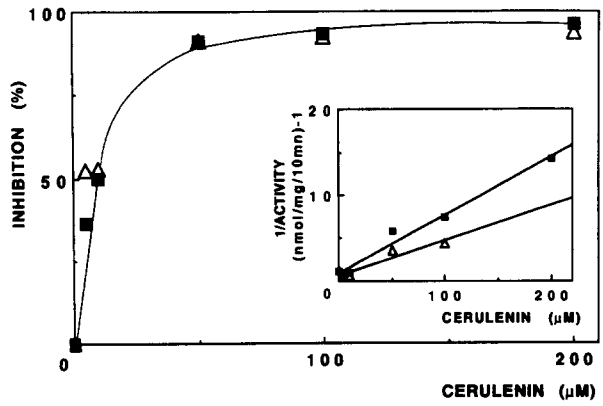

Fig. 6. Effect of preincubation of microsomes with various amounts of cerulenin on stearoyl-CoA elongation. Microsomes (50 μg protein) were preincubated with various amounts of cerulenin for 30 min. Incubations with the elongation mixture were performed as described in Materials and Methods, with 9 μM (△) or 45 μM (■) stearoyl-CoA. Results are expressed as % inhibition. Inset: Same legend as Fig. 6. Results are expressed as (nmol/mg per 10 min)$^{-1}$.

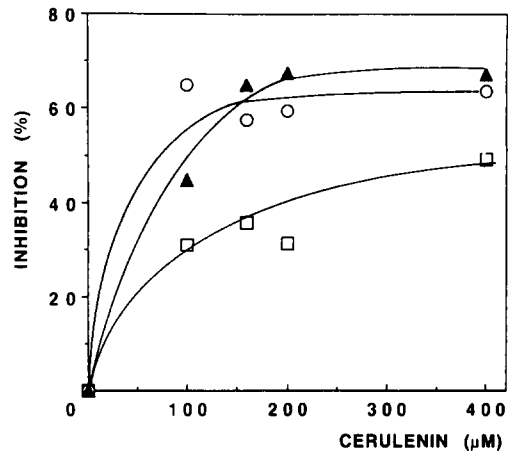

Fig. 7. Cerulenin effect on the products of stearoyl-CoA elongation by microsomes. The conditions were as described in Materials and Methods. Fatty acid methyl esters, obtained by the methylation of fatty acids derived from the saponification of the reaction mixture used for the assay of microsomal stearoyl-CoA elongation, were submitted to radio-gas-liquid chromatography. Results are given as % inhibition compared to control. (□), C_{20}; (○), C_{22}; (△), C_{24}.

No label was observed in 3-hydroxy fatty acids, suggesting that the cerulenin did not preferentially affect the 3-hydroxyacyl-CoA dehydrase. As the method does not separate saturated and unsaturated free fatty acids, it is not possible at this stage to decide whether some inhibition of the *trans*-2,3-enoyl-CoA reductase (or reductase II) could have occurred. However, these results strongly suggest that the main block is located at the level of the condensing enzyme. This is also true when C_{20}-CoA replaces C_{18}-CoA in the reaction mixture, since only one radioactive band comigrating with the free fatty acids was observed at all cerulenin concentrations.

(b) The effect of cerulenin on the label distribution among the fatty acids elongated from stearoyl-CoA was studied by radio GLC (Fig. 7). Cerulenin affected the labeling of all the very long-chain fatty acids, but some differences could be observed: the eicosanoic acid formation was less affected by cerulenin than that of docosanoic acid and lignoceric acid. At 100 μM cerulenin, the inhibition of C_{22} synthesis (approx. 60%) is higher than that of eicosanoic acid (30%). At 400 μM cerulenin, C_{20} formation is inhibited by about 50%, as compared to approx. 65% for C_{22} and C_{24}. These

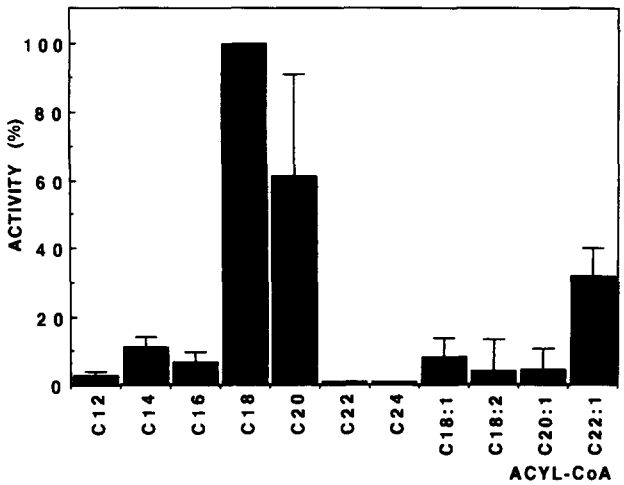

Fig. 8. Substrate specificity of 3-ketoacyl-CoA synthase. Experimental conditions: 75 μg microsomal proteins and 17 μM [2-^{14}C]malonyl-CoA; 9 μM acyl-CoAs. Other conditions were as described in Materials and Methods. Results are given as relative activity. The value of the assay with stearoyl-CoA was taken as 100%. Mean values ± S.D. of three experiments are given.

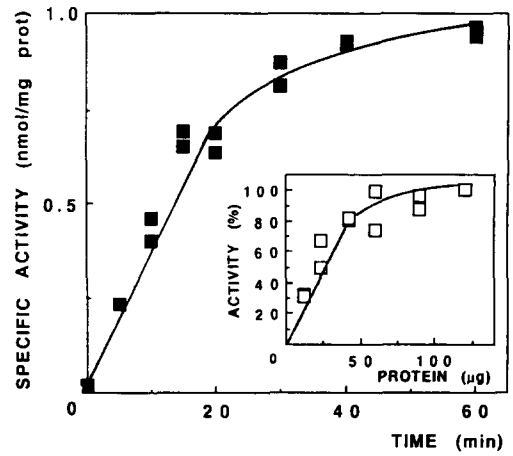

Fig. 9. Kinetics of 3-ketoacyl-CoA synthase activity. 75 μg microsomal proteins were incubated for various times with [2-^{14}C]malonyl-CoA (17 μM) and 9.2 μM stearoyl-CoA, 2 mM DTT and 1 mM $MgCl_2$ in the absence of NADH and NADPH. Final volume 100 μl. Results are given as nmol/mg protein. Inset: Activity of 3-ketoacyl-CoA synthase as a function of the amount of microsomal protein. Other experimental conditions were as above. Incubation time was 20 min. Results are given as % of maximal activity.

results indicate that the formation of C_{20} is always slightly less inhibited by cerulenin than that of the higher homologues. In addition, these results confirm the absence of labeled methylketones (originating from 3-ketoacyl-CoAs) and 3-hydroxyacids and show that the *trans*-2,3-unsaturated fatty acids, if present, are not abundant when compared to saturated ones.

This is true whatever the cerulenin level, and again supports the hypothesis that cerulenin chiefly affects (if not uniquely) the condensation step.

Effect of cerulenin on the condensation step

The condensing enzyme (3-ketoacyl-CoA synthase) can be studied simply by omitting NADH and NADPH, thereby yielding 3-ketoacyl-CoAs which are easily degraded into methylketones [13] and can be analyzed by thin-layer chromatography.

The effect of the acyl chain length and degree of unsaturation of the acyl-CoAs on the activity of the condensing enzyme was investigated (Fig. 8). The results clearly establish that the condensing enzyme uses exclusively C_{18}-CoA and C_{20}-CoA, in excellent agreement with the presence of two elongases in the microsomes from leek seedlings. Thus, the specificity of the two elongases seems to be entirely explained by the specificity of the condensing enzyme. Interestingly, oleoyl-CoA, linoleoyl-CoA and eicosenoyl-CoA are not accepted by the condensing enzyme, whereas docosenoyl-CoA, which is not likely to be present in this plant is almost as efficiently condensed as eicosanoyl-CoA. As a consequence of the results given in Fig. 8, stearoyl-CoA was used for all subsequent assays on the condensing enzyme. The reaction is linear for 20 min and then tends towards a plateau which is reached within 60 min (Fig. 9). The activity increases almost linearly with the protein amount up to 50 μg, and then tends towards a plateau; no activity variations are observed between 60 and 120 μg proteins (Fig. 9, inset).

The effect of various amounts of cerulenin on the condensation step and on two supposed partial reactions of the overall elongation pathway, were further investigated. Apart from the 3-ketoacyl-CoA synthase, the dehydrase reaction (3-hydroxyacyl-CoA dehydrase)

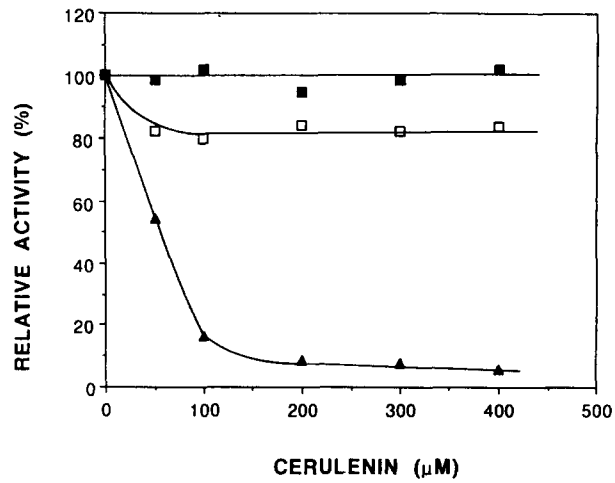

Fig. 10. Effect of cerulenin on partial elongation activities. (▲), 3-ketoacyl-CoA synthase; (□), 3-hydroxyacyl-CoA dehydrase and (■), *trans*-2,3-enoyl-CoA reductase were assayed as described in Materials and Methods. Partial activities were measured following a 10-min preincubation of microsomes with various amounts of cerulenin. Results are given as relative % of the control activity (100%). Condensing enzyme: mean value of three experiments.

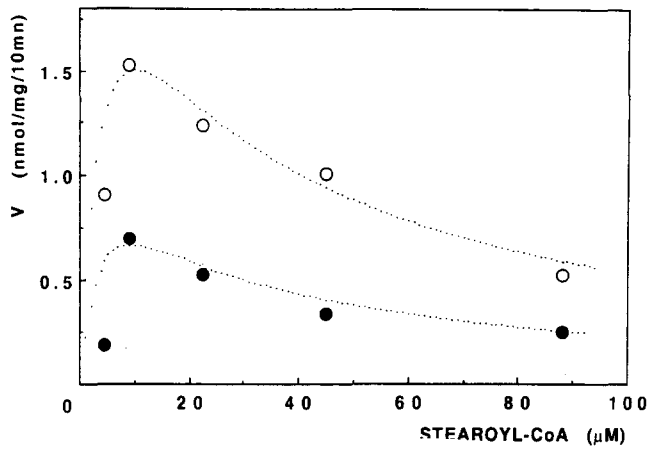

Fig. 11. Elongating activity as a function of stearoyl-CoA concentration. The activities were calculated after a 10-min preincubation in the absence (○) or presence (●) of cerulenin (200 μM). Results replotted from Fig. 2.

and the reductase II (*trans*-2,3-enoyl-CoA reductase) were studied. The results are given in Fig. 10. It is noteworthy that cerulenin up to 400 μM had no effect on the reductase II activity and only a reduced effect on the dehydration step: a small decrease (15%) of this activity was observed after incubation of the microsomes with 50 μM cerulenin and increasing the cerulenin concentration did not produce any additional inactivation. On the other hand, cerulenin had a drastic effect on the condensation reaction: a 46% inhibition was observed at 50 μM, reaching 85% at 100 μM cerulenin and 91% at 200 μM before reaching a plateau, with almost no residual activity. Combined with the facts that: (i) in the presence of cerulenin the residual activity yields exclusively unsubstituted acyl moieties and not 3-ketoacyl, or 3-hydroxyacyl-moieties and (ii) almost no unsaturated acyl moieties have been detected, these data demonstrate that, in membrane-bound acyl-CoA elongases, cerulenin acts exclusively on the β-ketoacyl-CoA synthase, and this inhibition is entirely responsible for the decrease of the overall elongation.

Discussion

The results reported here demonstrate that cerulenin affects exclusively the 3-ketoacyl-CoA synthase of the elongation complex, i.e. it inhibits the same step as in soluble fatty acid synthases. This is interesting, because fatty acid synthases and acyl-CoA elongases differ in many respects:

(1) Whereas acyl-CoA-elongases are strictly membrane bound, the fatty acid synthases are soluble multienzymes; in higher plants they are plastidial, easily resolved into their various constituents, with the consequence that the spatial organization of the plant fatty acid synthases is virtually unknown.

(2) The substrates of the fatty acid synthases are exclusively soluble, short-chain molecules (acetyl-CoA or acetyl-ACP, and malonyl-CoA), which are covalently linked to the enzyme(s). The membrane-bound acyl-CoA elongases use membrane-bound long-chain acyl-CoAs and soluble malonyl-CoA and do not require covalent binding of the substrates to the enzyme(s).

A priori, the partition of cerulenin makes it possible that it acts either in the aqueous phase, or in the membrane, or both. However, the preincubation of the enzyme with malonyl-CoA had no effect on the inhibition by cerulenin and, thus, did not prevent cerulenin from binding to the enzyme. On the other hand, preincubation with 45 μM C_{18}-CoA, had a marked protecting effect, since the inhibition of the elongation by 200 μM cerulenin dropped to 25%. The latter result may be compared with the amplification of the inhibition observed upon preincubation with cerulenin and suggests that membrane-bound cerulenin could be the active fraction of the inhibitor.

In addition, the potential protection of the activity upon stearoyl-CoA addition explains that when cerulenin and acyl-CoA are used without preincubation of the microsomes with either of them, the inhibition of the elongating activity cannot be complete, as is the case when cerulenin is incubated in the absence of stearoyl-CoA.

However, one of the most interesting points to be discussed concerns the mechanism of the inhibition by cerulenin. The data gathered in this paper allow to address this question.

The variations of the activity (10 min incubation) as a function of the stearoyl-CoA concentration are reported in Fig. 11 (data replotted from Fig. 2). The results obtained in the absence of inhibitor (open circles) are typical of an inhibition due to an excess of substrate. It seems, therefore, that there are two sites for stearoyl-CoA, the catalytic site (site 1) and a second one (site 2) that binds the substrate with a constant K'_m.

Hence:

$$E + S \leftrightharpoons ES_1 \rightarrow E + P \text{ with } K_m = E \cdot S/[ES_1] \quad (1)$$

$$E + S \leftrightharpoons ES_2 \text{ with } K'_m = E \cdot S/[ES_2] \quad (2)$$

$$ES_1 + S \leftrightharpoons ES_1S_2 \text{ with } K'_m = [ES_1] \cdot S/[ES_1S_2] \quad (3)$$

$$(ES_2 + S_1 \leftrightharpoons ES_1S_2 \text{ with } K_m = [ES_2] \cdot S/[ES_1S_2]) \quad (4)$$

Such a situation leads to the following expression of the activity:

$$V = \frac{V_{max}\left(1 + 2\frac{K_m}{K'_m} + \frac{K_m}{K'_m}\right)}{1 + \frac{K_m}{S} + \frac{S}{K'_m} + \frac{K_m}{K'_m}} = \frac{V_{max} \cdot g_{min}}{g(S)} \quad (5)$$

where

$$g(S) = 1 + \frac{K_m}{S} + \frac{S}{K'_m} + \frac{K_m}{K'_m} \quad (6)$$

and g_{min} is the minimum value of $g(S)$, obtained for $S = K_m \cdot K'_m$.

From Fig. 11, it appears that V_{max} is close to 1.5 nmol/mg per 10 min and that $K_m \cdot K'_m$ is approx. 10. The curve obtained using $V_{max} = 1.5$, $K_m = 6$ μM and $K'_m = 18$ μM (upper dashed line) mimicks correctly the experimental values. It can be noticed that the value of K_m (6 μM) determined here is in perfect agreement with the values described previously [19].

The results giving $1/V_i$ vs. the cerulenin concentration (Fig. 6, inset) greatly suggested a non-competitive inhibition with a $K_I = 15$ μM.

On the other hand, the ratios of the slope (V_i/V) obtained (in the absence of preincubation) for the highest concentrations of stearoyl-CoA (Fig. 2) were close to 0.4. Taken together, these results show that, without preincubation, around 40% of the enzymes remain inaccessible to the inhibitor (this value can be compared to the values of 30% and 0% found after 10 and 30 min of preincubation, respectively).

Therefore, in the absence of preincubation, the theoretical value of activity in the presence of cerulenin is described by:

$$V_i = 0.4V + \frac{0.6V}{1 + \frac{I}{K_I}}$$

or

$$V_i = \frac{V_{max} \cdot g_{min}}{g(S)} \left(0.4 + \frac{0.6}{1 + \frac{I}{K_I}} \right) \quad (8)$$

The corresponding curve is reported in Fig. 11 (lower dashed line) and (using $I = 200$ μM and $K_I = 15$ μM) fits correctly with the experimental values (closed circles).

However, the value of K_I (15 μM) seems difficult to reconcile with the data shown in Fig. 1. This is due to the fact that, as far as the inhibition by cerulenin is concerned, 60% of the activity is inhibited by cerulenin, even in the absence of preincubation; 10 to 15% is inhibited only after 10 min of preincubation, and 25 to 30% is not inhibited at all, even at high cerulenin concentrations. Here, all enzyme molecules do not have the same affinity (exposition?) for cerulenin, and this allows to interpret the results of Fig. 1.

In conclusion, this study has brought new information concerning the inhibition of acyl-CoA elongation by cerulenin, and has elucidated the step which is specifically affected by the presence of the inhibitor. Further analysis of the kinetic data has allowed to propose an interpretation of the effect of cerulenin, and the results are highly compatible with a non-competitive inhibition in which the two inhibition constants are most likely identical. The model briefly presented in the discussion accounts for all the data and is able to interpret not only the kinetic results, but also the various experiments of preincubation and, in particular, the activity observed after preincubation with the inhibitor, or the substrate.

Acknowledgements

The generous gift of a tritium-labeled sample of cerulenin by Prof. P. von Wettstein-Knowles and Dr. Siggaard-Andersen is gratefully acknowledged. We are indebted to Prof. D. Cinti for the gift of acyl-CoA intermediates. This study has been conducted under the BIOAVENIR programme/Groupe de recherches Barrières Cuticulaires financed by Rhone-Poulenc with the contribution ot the Ministère de la Recherche et de l'Espace and the Ministère de l'Industrie et du Commerce Extérieur. F.S. is the recipient of a grant from RPA.

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