Inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase and cholesterol biosynthesis by β -lactone inhibitors and binding of these inhibitors to the enzyme

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The β -lactones L-659,699 [(*E,E*)-11-[3-(hydroxymethyl)-4-oxo-2-oxetanyl]-3,5,7-trimethyl-2,4-undecadienoic acid] and its radioactive derivative ^3H -L-668,411 (the 2,3-ditritiated methyl ester of L-659,699) inhibited a partially purified preparation of rat liver cytosolic 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase with an IC_{50} of 0.1 μM . These compounds were also found to inhibit the incorporation of [^{14}C]acetate into sterols in cultured Hep G2 cells with an IC_{50} of 3 μM . New kinetic evidence indicated that inhibition of the isolated enzyme was irreversible. In contrast, sterol biosynthesis in cultured Hep G2 cells was rapidly restored upon removal of the compound from the medium of inhibited cultures, suggesting reversibility of inhibition in the cells. Radioactivity was found to be associated with a single cytoplasmic protein by SDS/PAGE of the cytoplasm of Hep G2 cells after incubation of the cells with the inhibitor ^3H -L-668,411. This protein was identified as cyto-

plasmic HMG-CoA synthase. Binding of the radioactive compound to the enzyme was decreased with time if the radioactive inhibitor was removed from the medium. Exposure of a gel containing the radioactive enzyme-inhibitor complex to neutral hydroxylamine also resulted in a loss of radioactivity from the gel. The purified rat liver enzyme reacted with the ^3H -ligand to form a stable enzyme-inhibitor complex which could be isolated by h.p.l.c. Radioactivity was also subsequently lost from this complex when it was incubated with neutral hydroxylamine. Incorporation of [^{14}C]acetate into cholesterol in mouse liver was inhibited in a reversible manner after oral administration of the β -lactone inhibitor. These studies, as well as the kinetic evidence presented, suggest that the β -lactone inhibitors acylate HMG-CoA synthase in a reaction which appears to be irreversible *in vitro*, but is easily reversed in cultured cells and in animals.

INTRODUCTION

Lovastatin, a natural product inhibitor of HMG-CoA reductase from *Aspergillus terreus* [1], was shown to lower plasma cholesterol in humans [2]. This discovery and subsequent studies on lovastatin and related compounds such as simvastatin [3] and pravastatin [4] were responsible for generating a great deal of interest in identifying other natural-product inhibitors of the enzymes of cholesterol biosynthesis. Natural-product inhibitors of the steps immediately before HMG-CoA reductase have been reported, e.g. a tryne carbonate (L-660,631) from an *Actinomyces* culture inhibited liver cytoplasmic acetoacetyl-CoA thiolase [5], and a β -lactone (L-659,699) from both *Fusarium* sp. [6] and *Scopulariopsis* sp. [7] inhibited HMG-CoA synthase. The studies demonstrated that the inhibition of HMG-CoA synthase *in vitro* was also translated into inhibition of sterol biosynthesis in cultured cells. The β -lactone L-659,699 inhibited cytoplasmic HMG-CoA synthase with an IC_{50} of 0.1 μM [6]. Inhibition of the synthase by this compound was also shown to be specific, inasmuch as other enzymes catalysing mechanistically similar reactions, such as HMG-CoA reductase, β -oxoacyl-CoA thiolase, acetoacetyl-CoA synthase and fatty acid synthase, were not affected by the compound [6].

Sterol biosynthesis from [^{14}C]acetate in cultured Hep G2 cells was also shown to be inhibited by the lactone, and the specificity of the inhibition was further indicated by the fact that acetate incorporation into fatty acids was not affected [6]. However, these studies left many questions unanswered, and we have

attempted to address some of these in this paper. (1) Is inhibition of sterol biosynthesis in cells directly related to specific inhibition of HMG-CoA synthase, as indicated by the reaction with the isolated synthase? (2) What is the mechanism of inhibition of the enzyme by the β -lactone inhibitor in the cells? (3) Does the compound inhibit cholesterol biosynthesis in animals as well as in cultured cells?

MATERIALS AND METHODS

Materials

L-659,699 was isolated from *Fusarium* sp. in the Merck Research Laboratories (Rahway, NJ, U.S.A.) by Dr. Robert Schwartz. The ditritiated methyl ester derivative of the β -lactone [2,3- ^3H]-L-668,411 (217.4 Ci/mmol), was prepared in the Merck Research Laboratories by Dr. Avery Rosegay and Mr. Roger Chen. The structures of these compounds are shown in Figure 1.

[1- ^{14}C]Acetyl-CoA (sp. radioactivity 46 mCi/mmol) and acetoacetyl-CoA were purchased from ICN and Pharmacia respectively. All other biochemical reagents were from Sigma or Boehringer Mannheim. Tissue-culture material was from Gibco, and SDS/PAGE was carried out with material and instruments from Bio-Rad. Other chemicals used were of analytical grade and were obtained from local sources.

Preparation and assay of cytoplasmic HMG-CoA synthase

Before purification of cytoplasmic HMG-CoA synthase, the

Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; PBS, phosphate-buffered saline; L-659,699, (*E,E*)-11-[3-(hydroxymethyl)-4-oxo-2-oxetanyl]-3,5,7-trimethyl-2,4-undecadienoic acid; L- ^3H -668,411, 2,3-ditritiated methyl ester of L-659,699.

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Figure 1 Structures of L-659,699 and ^3H -L-668,411

enzyme was maximally induced in the livers by feeding rats with 2% cholestyramine in milled Purina chow for 3 days, followed by 2% cholestyramine plus 0.5% lovastatin for 2 more days. The enzyme was purified by a procedure similar to that described by Mehrabian et al. [8], except that the chromatofocusing step was omitted, and the final step in the purification was by h.p.l.c. on a DEAE-5PW column (Bio-Rad). The enzyme was eluted with a gradient of 50–400 mM NaCl in 50 mM Tris (pH 7.0)/0.1 mM EDTA/0.5 mM dithiothreitol. This final step yielded a preparation showing a single protein band on SDS/PAGE. The enzyme obtained from the Blue Sepharose and the DEAE-5PW steps was very labile, and attempts to stabilize it with acetoacetyl-CoA, acetyl-CoA, glycerol at concentrations up to 20%, or the use of plastic tubing and tubes, were all unsuccessful. This loss of activity as the enzyme was purified made it impossible to calculate the purification efficiency. Except for the purification procedure in which the enzyme was assayed by the radioactive assay as described by Greenspan et al. [6], all the assays described in these studies were carried out by the continuous spectrophotometric assay of Clinkenbeard et al. [9]. This assay is based on the absorbance of the magnesium-complex of the enol-form of acetoacetyl-CoA ($\epsilon_{303} = 16\,100\text{ M}^{-1}\cdot\text{cm}^{-1}$). Standard assay conditions were defined as 200 μM acetyl-CoA and 50 μM acetoacetyl-CoA at 25 °C in 0.10 M Tris buffer containing 0.02 M MgCl_2 adjusted to pH 8.00 with HCl. Initial velocities remained constant for an absorbance change of at least 0.100 (12% reaction), and nearly constant over 98% of the total reaction, until acetoacetyl-CoA fell below 1 μM . At a typical control rate of 8.40×10^{-2} A unit/s, the assay had a 3% background caused by non-enzymic hydrolysis of acetoacetyl-CoA ($v = 0.254 \times 10^{-3}$ A unit/s), and 2% additional background of unknown source that was not inhibitable by excess L-659,699 (1 μM).

Preparation of HMG-CoA synthase- ^3H -L-668,411 complex

Rat liver cytoplasmic HMG-CoA synthase (0.5 mg) was incubated with 76 pmol of ^3H -L-668,411 (21.6 nCi/ μmol) in the presence of 0.06 M potassium phosphate buffer, pH 7.2, and 0.1 M KCl in a volume of 30 μl at room temperature. The unbound ligand was separated from the enzyme- ^3H -L-668,411 complex by a Fractogel 4W (Toso Industries) h.p.l.c. column (75 mm $\times$ 0.75 mm). The radioactive complex was eluted with aqueous buffer (0.05 M potassium phosphate, pH 7.2, and 0.05 M KCl) at 1.0 ml/min. After 4 min the flow rate was changed to 1.5 ml/min, and a 10 min gradient of 0–85% acetonitrile was started. The unbound ligand was eluted at about 65% acetonitrile. Fractions were collected every 1 min.

Gel electrophoresis of the cytosolic fraction from Hep G2 cells

Hep G2 cells (A.T.C.C. HB 8065) were grown as described by Greenspan et al. [6]. After a 48 h period in serum-free Higuchi medium [10], ^3H -L-668,411 was added as described in the Figure legends. At the end of the incubation period the medium was discarded, the cells were washed three times with PBS, and scraped with 0.1 ml of PBS containing 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride and 0.5 mM tosyl-lysylchloromethane hydrochloride. The cellular suspension was withdrawn from the flask and sonicated in a Branson Sonicator with a micro-probe at 50% power setting with three 20 s bursts, and centrifuged for 1 h at 100 000 g. A sample of the 100 000 g cytosolic extract was reduced and denatured by boiling in mercaptoethanol and SDS, and subjected to electrophoresis in 7.5%-polyacrylamide/SDS gels. The gels were generally stained with Coomassie Blue and cut into horizontal 0.5 cm segments. The gel sections were dissolved by heating at 60 °C overnight in scintillation vials with 0.8 ml of 30% H_2O_2 and 0.4 ml of 70% HClO_4 . Scintillation fluid was added and the radioactivity determined in each segment.

All experiments involving the binding of the radioactive inhibitor to the enzyme were repeated at least twice to confirm the results.

Sterol synthesis in Hep G2 cells

Hep G2 cells were grown to about 80% confluency in minimum essential medium + 10% fetal-calf serum in 25 cm² tissue-culture flasks, then switched to Higuchi medium without serum [10] for 48 h with daily medium changes. At 3 h before the start of the experiment, the medium was removed, the cells were washed with 2 $\times$ 3 ml of sterile PBS, and 3 ml of fresh medium was added and the cells were preincubated with or without L-659,699 at 1.0 $\mu\text{g}/\text{ml}$ from a stock solution of L-659,699, at 200 $\mu\text{g}/\text{ml}$ in dimethyl sulphoxide. This preincubation was continued for 3 h, after which the medium was again removed, and the cells were washed 3 times with PBS. Sterol biosynthesis in the cells was determined by addition of 6.0 μCi of [$1\text{-}^{14}\text{C}$]acetate (sp. radioactivity 1.9 mCi/mmol) at zero time with 3 ml of fresh medium in the presence or absence of L-659,699 as indicated. At times of 0–3 h as specified, the medium was removed, the cells were washed again 3 times with PBS and the amount of radioactivity incorporated into sterols was determined by saponification, acidification, extraction into hexane and t.l.c. of extracts of the cells as described [6].

Inhibition of cholesterol biosynthesis in mice

Liver cholesterol biosynthesis was measured in Charles River CRL:CD-1 female mice (56 days old, average wt. 30.1 g). The mice were conditioned on Purina milled chow containing 2% cholestyramine for 5 days before the experiment. A suspension of L-659,699 prepared in aq. 2% Emulphor was administered orally to the mice. The crystalline compound was weighed out and mixed thoroughly in a glass homogenizer with the undiluted Emulphor. After complete dispersal, water was added slowly while mixing to give a final solution of 2% Emulphor. The solution was administered orally in the doses described. At 15 min before killing, 0.2 ml of [$1\text{-}^{14}\text{C}$]acetate (5 μCi ; sp. radioactivity 4.47 mCi/mmol) was also administered orally to the animals. After death, the incorporation of radioactivity into liver cholesterol was determined by addition of 5 ml of 2.9 M KOH in 80% ethanol (2.4 M KOH final concn.) per g of liver. This mixture was heated at 90 °C for 1 h, then acidified with concentrated HCl, and the total saponifiable and non-

saponifiable fraction was extracted with 2×5 ml of light petroleum (b.p. 35–60 °C). The combined light-petroleum fractions were dried under N_2 , and the residue was redissolved in 100 μ l of chloroform. Then 50 μ l of the chloroform solution was spotted on to 250 μ m-thick silica-gel GF thin-layer plates and developed in light petroleum/diethyl ether/acetic acid (75:25:1, by vol.). Lipids on the plate were detected in an iodine chamber, and those bands corresponding to standard cholesterol and oleic acid were scraped into scintillation vials, followed by 1.0 ml of 0.9% NaCl and 10 ml of Aquasol-2. Incorporation of radioactivity from acetate into sterols was linear for at least 15 min under these conditions.

RESULTS

Kinetics of inhibition by L-659,699

In our earlier paper [6] we reported that the β -lactone L-659,699 appeared to be a reversible inhibitor of the enzyme, principally on the basis of results of an experiment involving dilution of the inhibited reaction. Closer scrutiny indicated either that it was an irreversible inhibitor, or that it had a dissociation constant much

lower than the assayable enzyme concentrations. This was evident in the velocity data of Figure 2, in which the mole fraction of active enzyme at steady state (as v_i/v_0 , below) was plotted against the inhibitor concentration at two different enzyme concentrations. If the inhibition was freely reversible and of modest affinity, then the data for the two enzyme concentrations should be superimposable and fit the expression $v_i/v_0 = 1/(1 + [I]/K_i)$. Instead, the correlations were linear and discrete, consistent with titration of the enzyme by the inhibitor. The abscissa intercepts, representing the concentrations of inhibitor required for 100% inhibition, have values of 84 and 419 nM at the two enzyme concentrations. The ratio of these intercepts, 4.99, agrees closely with the ratio of enzyme concentrations, 5.00, indicative of systems where inhibition is irreversible or of such high affinity that the dissociation constant of the enzyme–inhibitor complex was much less than the enzyme concentrations in the assay. These results also were consistent with estimated enzyme concentrations in the assays, and predict a k_{cat} of 1.39 s^{-1} . These considerations precluded determination of the dissociation constant from steady-state velocities.

Slow recovery of enzymic activity

The enzyme–inhibitor complex slowly dissociated to regenerate active enzyme, with a half-life of 55 h ($k = 0.0125 \pm 0.0005 \text{ h}^{-1}$). In comparison, the free lactone hydrolysed non-enzymically, with a half-life 2-fold faster under the same conditions ($k = 0.0267 \pm 0.0025 \text{ h}^{-1}$; $t_{1/2} = 26 \text{ h}$), as determined from its ability to inhibit the enzyme. These data are shown in Figure 3. Mercaptoethanol (0.1 M) accelerates the non-enzymic hydrolysis of free lactone to a half-life of less than $\frac{1}{2} \text{ h}$, but has little effect on the rate of recovery of active enzyme. Since the rate of non-enzymic hydrolysis was faster than regeneration of active enzyme from enzyme–inhibitor complex, it was not possible to ascertain which of several mechanisms prevailed: (1) reversal of a tightly bound non-covalent enzyme–inhibitor complex, (2) reversal of a covalent acyl-enzyme complex by recycization to the lactone, or (3) hydrolysis of a covalent acyl-enzyme complex to products.

As described above, steady-state velocities cannot be used to estimate a dissociation constant for the enzyme–inhibitor complex, owing to the potency of the inhibitor. In the sense that the inhibition was in practice reversible, regardless of the covalent or non-covalent nature of the enzyme–inhibitor complex, the rate constants for inhibition ($k_1 = 3 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$) and re-activation ($k_2 = 3 \times 10^{-6} \text{ s}^{-1}$) predicted a steady-state dissociation constant $K_1 = k_2/k_1 = 1 \times 10^{-11} \text{ M}$ in the absence of non-enzymic hydrolysis in the inhibitor.

Inhibition of sterol biosynthesis in cultured cells

Since we have previously shown that L-659,699 inhibited [^{14}C]acetate incorporation into sterol in cultured Hep G2 cells [6], we considered it important to determine whether the inhibition of sterol biosynthesis in these cells, like that of the isolated enzyme, was also irreversible. For this experiment, cells were grown to near confluency, then switched to serum-free Higuchi medium for 48 h to induce sterol synthesis. The cells were then preincubated for 3 h with the β -lactone inhibitor at 1 $\mu\text{g}/\text{ml}$ (3 μM) as described in the Materials and methods section. At the end of this period the medium was removed, the cells were washed extensively with PBS, fresh medium was added (with or without inhibitor), and the incorporation of [^{14}C]acetate into sterol was measured at various times. Incorporation of [^{14}C]acetate, which was virtually 100% inhibited at the beginning of the experiment, returned to approx. 48% of the non-inhibited

Figure 2 Tight-binding (or irreversible) inhibition

The mole fraction of free enzyme (v_i/v_0), taken from initial velocities when reaction is initiated by addition of the two substrates to equilibrated solutions of enzyme and inhibitor (v_i), expressed relative to that in the absence of inhibitor (v_0), is shown as a function of total inhibitor concentration for two enzyme concentrations differing by a factor of 5.

Figure 3 Non-enzymic hydrolysis of lactone and slow re-activation of enzyme

The plot shows the mole fraction of active enzyme (E/E_0) per unit of time. The upper curve (●) shows the rate of non-enzymic hydrolysis of the lactone under the standard assay conditions, as determined by the ability of a solution of lactone to inhibit an equimolar solution of enzyme as a function of time. The lower curve (■) shows the recovery of enzyme activity when the same equimolar enzyme and inhibitor solutions were combined at zero time and then assayed periodically. The lines are theoretical for the first-order processes.

Figure 4 Reversibility of inhibition by L-659,699 of sterol biosynthesis in Hep G2 cells

Hep G2 cells were incubated with L-659,699 for 3 h as described in the Materials and methods section. The medium was removed, and [¹⁴C]acetate incorporation into sterols was determined in the presence or absence of L-659,699 at the indicated times as described. Each time point represents the average of two plates of cells.

control level after 1 h, and to almost 72% of the non-inhibited control level after the inhibitor had been removed for 3 h (Figure 4). Sterol biosynthesis in the control cells with L-659,699 in both the pre- and post-incubations was virtually completely inhibited during the time course of the experiment. Although the rapid release of the inhibition suggested that the inhibition was actually reversible in the cells, the incompatibility with the findings *in vitro* raised the question of whether new enzyme protein was synthesized during the time period. Table 1 shows that addition of cycloheximide at a concentration which inhibited [¹⁴C]leucine incorporation into total cellular protein by over 90% had no effect on the reconstitution of cholesterol biosynthesis after inhibition by L-659,699. Differences in the percentage reversibility were noted after 1 h in the experiments described in Figure 4 and Table 1 (48 and 71% of the non-inhibited control respectively). It was thought that these differences were due to the fact that each experiment was carried out on different days with different sets of cells. Small differences in inoculum size, confluency of the cells at the time of the experiment and age of the medium were noted to affect the rate of sterol biosynthesis in the cells. For this reason both non-inhibited and completely inhibited controls were included in both experiments. What is important in these experiments is that qualitatively sterol biosynthesis in the cells was reversed by removal of the inhibitor from the medium, and this reversal of

inhibition could not be ascribed to synthesis of new enzyme by the cells during this time.

Binding of ³H-L-688,411 to HMG-CoA synthase in Hep G2 cells

The dinitrated partially reduced methyl ester of L-659,699, referred to as ³H-L-688,411 (see Figure 1), inhibited sterol biosynthesis at a level comparable with that of L-659,699 in the cells (results not shown). Thus it was feasible to use this compound to examine the interaction of the enzyme with the inhibitor in the cells.

In the experiment illustrated by Figure 5 the radioactive inhibitor was incubated with the cells for 1 h, after which the cells were sonicated, a 100000 g cytosolic extract was prepared, and a portion of this extract was subjected to SDS/PAGE. The gel was stained, cut into 0.5 cm sections, and the sections were dissolved and counted for radioactivity as described above. One protein band contained most of the radioactivity. This protein band co-migrating with the radioactivity reacted with a cytosolic HMG-CoA synthase antibody in a Western-blot analysis. Binding of the radioactive inhibitor to a specific protein in the cytoplasm of the cell is also the basis of the experiments described in Figures 6 and 7.

Inhibition of HMG-CoA synthase *in vitro* and the fact that the compound inhibited sterol biosynthesis in the cells, along with the appearance in the gel of a single radioactive protein migrating with HMG-CoA synthase, suggested that this band to which ³H-L-688,411 was bound was in fact cytosolic HMG-CoA synthase. If this were true, then reversal of inhibition of sterol biosynthesis should be reflected in the dissociation of the radioactive inhibitor from the protein with time when it was omitted from the medium. The results of this study are shown in Figures 6(a) and 6(b). The radioactive β -lactone was preincubated with Hep G2 cells for 1 h, the medium was removed, the cells were washed and fresh medium was added back with (Figure 6a) or without (Figure 6b) the radioactive inhibitor, and the incubation was continued for 90 min more. The cells were then sonicated and centrifuged at 100000 g for 1 h, and a portion of each supernatant was subjected to SDS/PAGE. This was followed by cutting the gels and counting them for radioactivity as described in the Materials and methods section. Almost a complete loss of radioactivity was seen in that protein band which corresponded to HMG-CoA synthase in the sample which had been incubated for 90 min without the radioactive inhibitor (Figure 6b). This experiment was repeated three times with similar results.

The gels in this particular experiment were not stained and destained in order to determine whether there was any radioactivity associated with, but not covalently bound to, a protein.

Table 1 Effect of cycloheximide on the incorporation of [¹⁴C]acetate into cholesterol in Hep G2 cells

The results are averages of two sets of cells. Where present, cycloheximide was 10 μ g/ml, and L-659,699 was 1 μ g/ml.

Conditions		[¹⁴ C]Acetate incorporation into cholesterol			
		— Cycloheximide		+ Cycloheximide	
Preincubation	Incubation	(c.p.m./mg)	(% inhibition)	(c.p.m./mg)	(% inhibition)
3 h medium only	1 h medium	10452	0	8741	0
3 h + L-659,699	1 h medium	7391	29	7594	13
3 h + L-659,699	1 h L-659,699	1089	90	1419	84

Figure 5 Binding of ${}^3\text{H}$ -L-668,411 to a protein in the cytosol of Hep G2 cells

The radioactive inhibitor ${}^3\text{H}$ -L-668,411 was incubated with cells for 1 h, after which a sample of the cell extract was submitted to SDS/PAGE. The gel was stained (left panel) and then cut into segments, and the radioactivity determined (right panel), as described in the Materials and methods section.

Figure 6 Reversibility of ${}^3\text{H}$ -L-668,411 binding to HMG-CoA synthase in Hep G2 cells

These studies were carried out as described in Figure 5. Two sets of Hep G2 cells were incubated with ${}^3\text{H}$ -L-668,411 (${}^3\text{H}$ -411) as described for 1 h, after which the cells were washed with 2×3 ml of PBS, and then further incubated for 90 min in the absence (a) or the presence (b) of the tritiated inhibitor.

No excess radioactivity was seen except at the gel front, which was the free radioactive lactone.

Since the foregoing experiment suggested a covalent attachment of the inhibitor to the enzyme, studies were carried out to investigate the nature of this attachment. After SDS/PAGE of a sample of the cytosolic extract from an inhibited sample of the cells, the gel was cut in half vertically, and one half of this gel was

Figure 7 Sensitivity of the HMG-CoA synthase- ${}^3\text{H}$ -L-668,411 complex of Hep G2 cells to hydroxylamine

The incubation and gel electrophoresis were carried out as described in Figure 5. The radioactive cytosol was spread over the entire length of the gel and electrophoresed. After electrophoresis, the gel was cut in half vertically and each half was incubated overnight with either 1 M Tris/HCl or 1 M hydroxylamine, pH 7.2. The gels were then sliced horizontally into 0.5 cm segments, and radioactivity in the segments was determined as described in the Materials and methods section.

incubated overnight in 1 M Tris, pH 7.2, and the other half in 1 M hydroxylamine/HCl, pH 7.2. The analysis of the gels for radioactivity is shown in Figure 7; the black bars represent that portion of the gel incubated with hydroxylamine, and the white bars the gel incubated with Tris. Practically all of the radioactivity had been lost from the protein after the hydroxylamine treatment of the gel. In contrast, most of the radioactivity was still present in the gel incubated in Tris/HCl (20 642 d.p.m. in the peak before hydroxylamine treatment, and 18 155 d.p.m. in the peak after overnight incubation with Tris/HCl).

Binding of ${}^3\text{H}$ -L-668,411 to isolated cytoplasmic HMG-CoA synthase

The hydroxylamine-sensitivity of the purified rat liver enzyme-inhibitor complex was also examined in the experiment of Figure 8. The radioactive enzyme-ligand complex was pre-

Figure 8 Sensitivity of isolated rat liver HMG-CoA synthase- ^3H -668,411 complex to hydroxylamine

An equivalent of 25 μg of ^3H -L-668,411-HMG-CoA synthase complex containing 1.64×10^6 c.p.m. (isolated as described in the Materials and methods section) in a volume of 50 μl was added to 50 μl of solutions of either 2 M Tris/HCl or 2 M hydroxylamine, pH 7.2. At the indicated times, 10 μl of each mixture was chromatographed on a Fractogel h.p.l.c. column, and samples of the eluate were collected and counted for radioactivity as described in the Materials and methods section.

Figure 9 Inhibition of $[^{14}\text{C}]$ acetate incorporation into mouse liver cholesterol by L-659,699

$[^{14}\text{C}]$ Acetate and L-659,699 were administered as indicated in the Materials and methods section. Each point in the experiment represents the mean $\pm$ S.E.M. from seven animals.

pared and separated from the tritiated inhibitor that had not reacted by h.p.l.c. as described in the Materials and methods section. The isolated radioactive complex was incubated with either 1 M Tris/HCl or 1 M hydroxylamine/HCl, pH 7.2, and at the times indicated in Figure 8 samples from each incubation mixture were rechromatographed on the same h.p.l.c. column. ^3H in the aqueous buffer fraction from the column indicated that the inhibitor was still bound to the enzyme. Similar results were seen in this experiment to those in the previous experiment with the extract from the Hep G2 cells: the enzyme-bound radioactive inhibitor was lost on incubation of the complex with hydroxylamine, whereas little release of radioactivity was seen with enzyme incubated with Tris. Attempts to isolate a radioactive hydroxamate derivative of ^3H -L-668,411 in the hydroxylamine incubation were unsuccessful. The single time point at 24 h was repeated with similar results.

Inhibition of cholesterol biosynthesis in animals

In order to examine the effectiveness of the compound as an inhibitor of sterol biosynthesis in animals, it was administered orally to mice at a concentration of 25 mg/kg in a suspension,

followed by the oral administration of a single dose of 5.0 μCi of $[^{14}\text{C}]$ acetate. Incorporation of radioactive acetate into liver cholesterol after oral dosing was linear for 15 min (results not shown), therefore the dose of $[^{14}\text{C}]$ acetate was given in each case 15 min before killing, regardless of the time of administration of the β -lactone inhibitor. Figure 9 shows that liver cholesterol biosynthesis was inhibited by 80 % after only 30 min of an oral dose of L-659,699. By 180 min cholesterol biosynthesis in the liver had returned to control levels, indicating reversibility of the inhibitor in the liver, as observed in the cultured cells.

DISCUSSION

The β -lactone L-659,699 inhibited the activity of isolated rat liver cytoplasmic HMG-CoA synthase. A previous paper from this laboratory reported that the inhibition of this enzyme was reversible [6]. The more careful kinetic studies described in the present paper indicated that the reaction of the β -lactone with the isolated rat liver synthase was in fact not reversible. The irreversibility of the inhibition is also in agreement with the studies reported by Mayer et al. [12], who showed both by kinetic methods and by attempts to dilute and dialyse away the inhibitor that the reaction of the lactone, as well as a number of derivatives, with the enzyme was irreversible [12]. It was puzzling therefore to find that inhibition of acetate incorporation into sterols in Hep G2 cells were reversible simply by removing the compound from the medium. This reversibility of inhibition of sterol biosynthesis in cells was also noted by Mayer et al. [12]. The fact that the recovery of activity was not sensitive to cycloheximide was further confirmation that the recovery of sterol-biosynthesis activity was probably due to dissociation of the inhibitor from the enzyme rather than to the resynthesis of new HMG-CoA synthase protein during the intervening period.

Studies with ^3H -L-668,411, a ditritiated derivative of the β -lactone L-659,699, were designed to answer two of the questions posed in the Introduction of this paper: (1) is inhibition of sterol synthesis in the cells directly related to specific inhibition of HMG-CoA synthase, and (2) what is the mechanism of inhibition of the enzyme by the β -lactone inhibitor?

Although we cannot positively rule out the possibility that the inhibitor might act as a non-covalent inhibitor at a step after HMG-CoA synthase, the fact that mevalonate incorporation into sterols is hardly affected in the cells by the β -lactone [6] makes this unlikely. The coincidence of an irreversible inhibition of the purified rat liver cytosolic HMG-CoA synthase, the inhibition of incorporation of acetate but not of mevalonate into sterols [6], and the covalent binding of the radioactive derivative to a protein which co-migrated with and reacted with an anti-(HMG-CoA synthase) antibody are strongly suggestive that inhibition of sterol synthesis in the Hep G2 cells is due to specific inhibition of HMG-CoA synthase.

The mechanism of inhibition of the enzyme is not as clear-cut. The irreversibility of the inhibition of the purified enzyme, the stability of this enzyme-inhibitor complex and the Hep G2 enzyme-inhibitor complex on SDS gels, and the sensitivity of both complexes to neutral hydroxylamine, verify that the interaction is covalent. This raises the interesting question of why the inhibition of the isolated enzyme is irreversible, whereas inhibition in the cells (and presumably in animals also) is reversible?

That the inhibitor in fact comes off the enzyme in the cells is fairly certain, since both sterol biosynthesis as well as loss of the radioactive inhibitor were seen after incubation of the cells for a relatively short period of time (1–3 h) in the absence of the inhibitor. This is in stark contrast with the spontaneous dis-

sociation of the isolated enzyme-inhibitor complex, which has a half-life of 55 h (Figure 3).

The sensitivity of the enzyme complexes to neutral hydroxylamine is suggestive that the covalent attachment of the inhibitor to the enzyme is by acylation of a cysteine thiol group. The choice of a thioester rather than an oxygen ester (both of which react with neutral hydroxylamine) is primarily based on the earlier studies by Miziorko et al. [13] with the mitochondrial HMG-CoA synthase, which showed that the mechanism of the synthesis of HMG-CoA involves the acylation of a cysteine at the active site of the enzyme by acetyl-CoA. The analogy between the mitochondrial and cytoplasmic enzyme can also be extended by other studies by Miziorko group [14], which demonstrated that an active-site peptide of 20 amino acids containing the active cysteine of the mitochondrial HMG-CoA synthase was the same as a sequence in the hamster cytosolic enzyme, suggesting that, although the structures of each enzyme were not the same, their mechanisms of reaction were probably similar.

The mechanism of hydrolysis of the enzyme-inhibitor complex in the cells is not understood. We presume that an unknown nucleophile must attack the thioester bond of the complex, resulting in a free active enzyme and a modified or open lactone. The identity of this nucleophile is not known, and preliminary studies to identify a derivative of ^3H -L-668,411 in the cell cytoplasm were unsuccessful. We were also unsuccessful in our attempts to demonstrate reversibility of the isolated enzyme-inhibitor complex by incubation of the h.p.l.c.-isolated radioactive complex with CoA, glutathione or ATP. It is quite possible that the actual nucleophile could be water, since a water molecule is involved in the hydrolysis of the bound product of the enzymic reaction, HMG-CoA. Thus the inherent thioesterase activity in the enzyme itself may be responsible for separating the inhibitor from the enzyme. If this is in fact the mechanism, it is not clear why reversibility was not seen with the purified enzyme *in vitro*, particularly since it appeared that a thioester was also the product of the reaction. The enzyme may perhaps have been modified in some subtle way during the purification procedure or the inhibitor may have shifted to another hydroxylamine-sensitive residue on the enzyme, for instance from a cysteine to a serine.

Another interesting question about the interaction of the β -lactone inhibitor with HMG-CoA synthase is the specificity of the inhibition. Fatty acid synthase, which like HMG-CoA

synthase catalyses the condensation of two acyl-CoA molecules and involves the formation of an acyl-cysteine intermediate on the enzyme before condensation with another thioester [15], was barely inhibited by L-659,699. This lack of activity against fatty acid synthase was reflected in the fact that [^{14}C]acetate incorporation into fatty acids in the cell was not affected by the inhibitor [6].

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REFERENCES

- 1 Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schönberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J. and Springer, J. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 3857–3961
- 2 Tobert, J. A., Bell, G. D., Birtwell, J., James, I., Kukovetz, W. R., Pryor, W. R., Buntinz, A., Holmes, J. B., Chao, Y. S. and Bolognese, J. A. (1982) *J. Clin. Invest.* **69**, 913–919
- 3 Alberts, A. W., Chen, J. S., Huff, J. W., Kuron, G. W. and Mucha, W. (1988) 8th Int. Symp. Atherosclerosis, Rome, abstr., p. 8
- 4 Endo, A. (1985) *J. Med. Chem.* **28**, 166–173
- 5 Greenspan, M. D., Yudkovitz, J. B., Chen, J. S., Hanf, D. P., Chang, M. N., Chaing, P. Y. C., Chabala, J. C. and Alberts, A. W. (1989) *Biochem. Biophys. Res. Commun.* **163**, 548–553
- 6 Greenspan, M. D., Yudkovitz, J. B., Lo, C.-Y. L., Chen, J., Alberts, A. W., Chang, M. N., Yang, S. S., Thompson, K. L., Chiang, Y.-C. P., Chabala, J. C., Monaghan, R. L. and Schwartz, R. L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7488–7492
- 7 Omura, S., Tomoda, H., Kumagai, H., Greenspan, M. D., Yudkovitz, J. B., Chen, J. S., Alberts, A. W., Martin, I., Mochales, S., Chabala, J. C., Schwartz, R. E. and Patchett, A. A. (1987) *J. Antibiot.* **40**, 1356–1357
- 8 Mehrabian, M., Callaway, K. A., Clarke, C. F., Tanaka, R. D., Greenspan, M. D., Lusi, A. J., Sparkes, R. S., Mohandas, T., Edmond, J., Fogelman, A. M. and Edwards, P. A. (1986) *J. Biol. Chem.* **261**, 16249–16255
- 9 Clinkenbeard, K. D., Reed, W. D., Mooney, R. A. and Lane, M. D. (1975) *J. Biol. Chem.* **250**, 3108–3116
- 10 Higuchi, K. (1970) *J. Cell. Physiol.* **75**, 65–72
- 11 Lowe, D. M. and Tubbs, P. K. (1985) *Biochem. J.* **227**, 591–599
- 12 Mayer, R. J., Louis-Flamberg, P., Elliott, J. D., Fisher, M. and Leber, J. (1990) *Biochem. Biophys. Res. Commun.* **169**, 610–616
- 13 Miziorko, H. M., Clinkenbeard, K. D., Reed, W. D. and Lane, M. D. (1975) *J. Biol. Chem.* **250**, 5768–5773
- 14 Miziorko, H. M. and Behnke, C. E. (1985) *J. Biol. Chem.* **260**, 13513–13516
- 15 Greenspan, M. D., Alberts, A. W. and Vagelos, P. R. (1969) *J. Biol. Chem.* **244**, 6477–6485