Mode of Interaction of β-Hydroxy-β-methylglutaryl Coenzyme A Reductase with Strong Binding Inhibitors: Compactin and Related Compounds†

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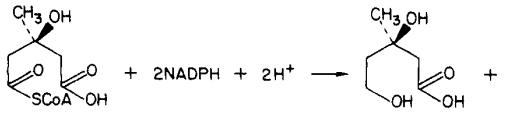
ABSTRACT: The sodium salts of compactin (**1**) and *trans*-6-[2-(2,4-dichloro-6-hydroxyphenyl)ethyl]-3,4,5,6-tetrahydro-4-hydroxy-2*H*-pyran-2-one (**3**) are inhibitors of yeast β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase. The dissociation constants are 0.24×10^{-9} and 0.28×10^{-9} M, respectively. Similar values have been reported for HMG-CoA reductase from mammalian sources [Endo, A., Kuroda, M., & Tanzawa, K. (1976) *FEBS Lett. 72*, 323; Alberts, A. W., et al. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3957]. The structures of these compounds marginally resemble that of any substrates of HMG-CoA reductase. We, therefore, investigated the basis for the strong interaction between HMG-CoA reductase and these inhibitors. HMG-CoA and coenzyme A (CoASH), but not reduced nicotinamide adenine dinucleotide phosphate (NADPH), prevent binding of compactin to the enzyme. HMG-CoA, but not CoASH or NADPH, prevents binding of **3** to the enzyme. We also investigated the inhibitory activity of molecules that resemble structural components of compactin. Compactin consists of a moiety resembling 3,5-dihydroxyvaleric acid that is attached to a decalin structure. The sodium salt of DL-3,5-dihydroxyvaleric acid inhibits HMG-CoA reductase competitively with respect to HMG-CoA and noncompetitively with respect to NADPH. The dissociation constant for DL-3,5-dihydroxyvaleric acid, derived from protection against inactivation of enzyme by iodoacetic acid, is $(2.1 \pm 0.9) \times 10^{-2}$ M. Two decalin derivatives (structurally identical with or closely related to the decalin moiety of compactin) showed no detectable inhibition. If the lack of inhibition is due to their limited solubility, the dissociation constant of these decalin derivatives may be conservatively estimated to be ≥0.5 mM. Simultaneous addition of decalin derivatives and DL-3,5-dihydroxyvaleric acid does not lead to enhanced inhibition. The sodium salt of (*E*)-6-[2-(2-methoxy-1-naphthalenyl)ethenyl]-3,4,5,6-tetrahydro-4-hydroxy-2*H*-pyran-2-one (**6**) inhibits HMG-CoA reductase competitively with respect to HMG-CoA and noncompetitively with respect to NADPH. The inhibition constant (vs. HMG-CoA) is 0.8 μM. CoASH does not prevent binding of **6** to enzyme. Compound **6**, therefore, behaves analogously to compound **3**. We propose that these inhibitors occupy two sites on the enzyme: one site is the hydroxymethylglutaryl binding domain of the enzyme active site and the other site is a hydrophobic pocket located adjacent to the active site. The high affinity of these inhibitors for HMG-CoA results from the simultaneous interaction of these inhibitors with two separate binding sites on the enzyme. The binding advantage gained from connecting the lactone and the decalin portions of compactin may be $\geq 5 \times 10^4$ M. When HMG-CoA (150 μM) and compactin (0.07–0.70 μM) are added to the enzyme, the enzyme is rapidly converted to enzyme–compactin and E·HMG-CoA. However, the amount of E·HMG-CoA formed exceeds that present at equilibrium. In a subsequent slow phase the excess E·HMG-CoA is converted to enzyme–compactin until equilibrium is reached. Under these conditions, formation of E–compactin is, thus, a biphasic process. The "overshoot" in the formation of E·HMG-CoA is a consequence of the concentration of HMG-CoA and compactin and the slow dissociation rate of E·HMG-CoA. From an analysis of this system we have determined the on- and off-rate constants of HMG-CoA to be 1.9×10^5 M^{-1} s^{-1} and 0.11 s^{-1}, respectively, and the on- and off-rate constants of compactin to be 2.7×10^7 M^{-1} s^{-1} and 6.5×10^{-3} s^{-1}, respectively. The on-rate constant for compactin is nearly diffusion limited and is 2 orders of magnitude faster than the on-rate constant for HMG-CoA.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the reduction of HMG-CoA to mevalonic acid; reduced nicotinamide adenine dinucleotide phosphate (NADPH) is required as a cofactor (eq 1). HMG-CoA



(1)

reductase is rate limiting in cholesterol biosynthesis (Goldstein & Brown, 1977); thus, inhibitors of this enzyme are of considerable interest for their potential pharmacological value. The fungal metabolites compactin (**1**) and mevinolin (**2**) are extremely potent inhibitors of HMG-CoA reductase (Endo et al., 1976; Alberts et al., 1980) (Chart I). In clinical trials, compactin has been shown to reduce serum cholesterol levels in patients with hypercholesterolemia (Yamamoto et al., 1980; Mabuchi et al., 1981).

Inhibition of HMG-CoA reductase by compactin and mevinolin is competitive with respect to HMG-CoA. The active inhibitory species of these compounds are the hydroxy acids corresponding to the parent compound in which the lactone ring has been hydrolyzed.[1] Inhibition constants for

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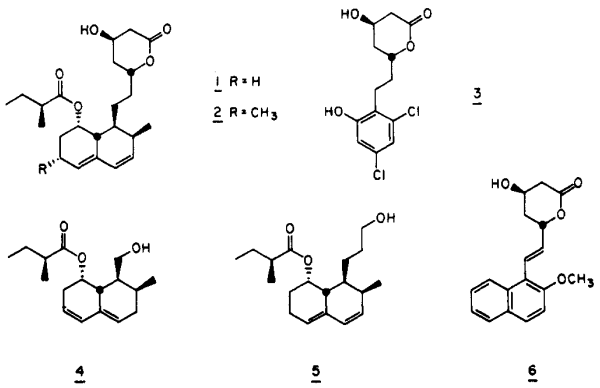
Chart I: Structures for Compounds **1**–**6**[a]



[a] Compounds **3** and **6** are racemic mixtures of the indicated compounds. Compounds **4** and **5** are diastereomeric mixtures of the indicated compounds. Each diastereomer has the *S* configuration in the appendage but the enantiomeric configuration at the four chiral centers of the decalin unit.

the inhibition of rat liver enzyme by compactin and mevinolin have been reported to be 1.4–10 nM and 0.64 nM, respectively (Endo et al., 1976; Alberts et al., 1980). It is not obvious why these inhibitors should bind to HMG-CoA reductase with such high affinity. The hydroxy acid side chain of compactin and mevinolin resemble mevalonic acid, and it is likely that the hydroxy acid moiety of these inhibitors occupies the hydroxymethylglutaryl binding site on the enzyme; however, mevalonic acid is not a potent inhibitor of HMG-CoA reductase. It is, however, not apparent how the "lower portion" (decalin moiety) facilitates binding of the inhibitor to the enzyme. The experiments reported here were designed to elucidate the basis for the strong interaction of compactin and related compounds with HMG-CoA reductase. In order to accomplish this, we carried out experiments to determine with what portion of the active site, if any, compactin and other inhibitors interact. For the purpose of this analysis it is convenient to subdivide the active site into three domains: (1) a domain which binds the hydroxymethylglutarate portion of HMG-CoA; (2) a domain which binds CoA; (3) a domain which binds NADP or NADPH. We then determined whether substances that specifically interact with each of these domains can prevent the binding of compactin or related inhibitors. We also determined how strongly fragments of compactin interact with the enzyme. From the binding constants of components of compactin, we can then evaluate the "binding advantage" gained from covalently linking the two components. Finally, in the course of this work, we evaluated k_{on} and k_{off} for compactin as well as the substrate HMG-CoA. It was shown that these constants could be determined for an inhibitor which dissociates relatively slowly, from a kinetic analysis of the reaction, i.e., without the necessity of directly detecting, as for instance, by spectroscopic methods, enzyme–substrate or enzyme–inhibitor complexes, or the use of rapid reaction techniques.

Materials and Methods

Chemicals. NADPH, coenzyme A (CoASH) lithium salt, DL–mevalonic acid δ-lactone, and iodoacetic acid were purchased from Sigma Chemical Co., β-hydroxy-β-methylglutaryl anhydride was from Calbiochem-Behring Corp., and agarose–hexane–CoA affinity gel, type V, was from P-L Biochemicals, Inc.

Compactin was a generous gift from Dr. A. Endo; compounds **3** and **6** were from Dr. F. Kathawala, Sandoz, Ltd.; compound **4** was from Professor C. H. Heathcock; compound **5** was from Professor B. B. Snider; DL-3,5-dihydroxyvaleric acid δ-lactone was from Professor P. Dowd.

Preparation of Substrates and Inhibitors. DL-HMG-CoA was prepared as described by Louw et al. (1969) except a 10-fold excess of β-hydroxy-β-methylglutaryl anhydride was used. The crude HMG-CoA was purified by gel filtration over Sephadex G-10 equilibrated in 1 mM HCl. The resulting HMG-CoA was >98% pure as determined by high-performance liquid chromatography (HPLC) on a 0.39 × 30 cm Waters Associates (Milford, Ma) μBondapak C_{18} with UV detection at 254 nm. The solvent system was 12% methanol in 10 mM potassium phosphate, pH 5.8. The concentration of HMG-CoA was determined by UV absorbance at pH 3.0 by using $\epsilon_{260} = 16.4 \times 10^3$ M^{-1} cm^{-1} (Stadtman, 1957), which was consistent with values obtained by measuring the total decrease in absorbance at 340 nm in an incubation of HMG-CoA, HMG-CoA reductase, and an excess of NADPH. The concentration of NADPH was determined by UV absorbance at pH 7.0 by using $\epsilon_{340} = 6.2 \times 10^3$ M^{-1} cm^{-1} (Horecker & Kornberg, 1948). The concentration of CoASH was determined by UV absorbance at pH 3.0 by using $\epsilon_{257} = 16.8 \times 10^3$ M^{-1} cm^{-1} (Morris & Redfearn, 1978), which was consistent with values obtained by the method of Ellman (1959). Solutions containing HMG-CoA or CoASH were treated with dithiothreitol (DTT) as described (Gilbert & Stewart, 1981) to prevent artifacts due to enzyme inactivation by CoASH disulfide. In experiments where enzyme was preincubated with CoASH, no inactivation was observed. The activity of enzyme preincubated with CoASH alone for a time period equal to that of the experiment was not significantly different from an identical assay in which there was no preincubation.

The hydrolysis of compactin and compounds **3** and **6** to their respective sodium salts was performed as described (Endo et al., 1976). Solutions of the sodium salts of DL-mevalonic acid and DL-3,5-dihydroxyvaleric acid were obtained by stirring their respective lactones in a 1 N NaOH solution, at room temperature, such that 5% excess base was present. After hydrolysis was complete, the solution was adjusted to pH 7.0 with the addition of 1 N HCl. The absence of lactone in the resulting solutions was indicated by thin-layer chromatography on silica (Eastman Kodak Co.) in benzene/acetone (1:1) with visualization by iodine (Shapiro et al., 1969). Stock solutions of compounds **4** and **5** were made in acetonitrile. Compounds **4** and **5** showed UV absorbance in acetonitrile with λ_{max} 227, 234, and 242 nm (log ϵ = 4.3 , 4.4, and 4.2) and λ_{max} 228, 236, and 244 nm (log ϵ = 4.2, 4.3, and 4.1), respectively. The concentration of compactin was determined prior to hydrolysis by UV absorbance in ethanol using $\epsilon_{237} = 2.00 \times 10^4$ M^{-1} cm^{-1} (Brown et al., 1976). All other inhibitor concentrations were determined by weight.

Enzyme Isolation. HMG-CoA reductase was prepared from Fleischmann's bakers' dry yeast by the procedure of Qureshi et al. (1976a) through the concentration step following DEAE-cellulose chromatography. The specific activity of the partially purified enzyme was 0.3–0.6 μmol of NADPH oxidized min^{-1} (mg of protein)$^{-1}$ under the conditions specified in the above reference. In a single experiment, enzyme was further purified to a specific activity of 3.2 by using a CoASH

[1] In this paper, inhibition of HMG-CoA reductase by the sodium salts of compactin and compounds **3** and **6** is referred to by the parent lactone; it is to be understood that all experiments were carried out with the sodium salts. The lactone moiety of compactin and related compounds will be referred to as the hydroxy acid or the "upper" portion. The decalin moiety of compactin and related compounds will be referred to as the decalin or "bottom" portion.

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affinity column as described (Qureshi et al., 1976a). Protein concentration was determined by the method of Lowry et al. (1951). Enzyme concentration was calculated by assuming a M_r 2.6×10^5 (tetramer) and a specific activity of 10 μmol of NADPH oxidized min^{-1} (mg of pure enzyme)$^{-1}$ (Qureshi et al., 1976a; Veloso et al., 1981). The enzyme was stored at −20 °C in the presence of 5 mM DTT and 10% (v/v) glycerol.

Enzyme Assay and Inhibition. Immediately before use, enzyme was incubated at 30 °C for 30 min with an additional 5 mM DTT. HMG-CoA reductase was assayed by following the decrease in absorbance at 340 nm due to the disappearance of NADPH. All measurements were carried out on a Perkin-Elmer spectrophotometer, Model 559, equipped with a thermostated sample compartment set at 25 °C. All stock solutions were temperature equilibrated to 25 °C prior to use. For standard initial velocity measurements, 2–5 milliunits of enzyme was assayed in a total volume of 1.0 mL containing appropriate concentrations of substrates and inhibitors in 0.1 M potassium phosphate buffer, pH 7.0, 1 mM ethylenediaminetetraacetic acid (EDTA), and 5 mM DTT. The enzyme assay was generally initiated by the addition of enzyme. Initial velocity was corrected for background NADPH "oxidase" activity obtained from control experiments where HMG-CoA was omitted. In some cases, 5–10% acetonitrile was included in the assay to increase the solubility of inhibitor; in these cases, acetonitrile was also included in the assays without inhibitor. Prolonged incubation of enzyme in buffer containing acetonitrile was avoided; loss of enzyme activity was observed at longer incubation times (approximately 8% at 15 min).

Inhibition of HMG-CoA reduction was determined at six to eight concentrations of HMG-CoA varied between 2 and 20 μM with the NADPH concentration fixed at 160 μM or six to eight concentrations of NADPH varied between 8 and 160 μM with the HMG-CoA concentration fixed at 10 μM. Values for slopes and ordinate intercepts of linear (initial velocity)$^{-1}$ vs. [substrate]$^{-1}$ plots were calculated by linear regression analysis, weighing each point equally, and did not vary significantly from values obtained graphically. Inhibition constants were obtained from replots of the slopes or intercepts of $1/v$ vs. $1/S$ plots vs. at least two inhibitor concentrations. The indicated error limits were calculated as standard deviation of two or more independent experiments. Inhibition constants reported without error limits were obtained from a single experiment and are thought to be accurate within 50%.

Titration of HMG-CoA Reductase with Compactin. Enzyme, 120 pmol (tetramer) was incubated with 0–210 pmol of compactin in a total volume of 100 μL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 5 mM DTT at 25 °C; 10-μL aliquots of enzyme were assayed spectrophotometrically at 340 nm in a total volume of 1.0 mL of the same buffer at 25 °C containing 300 μM HMG-CoA and 160 μM NADPH. Identical initial velocities were obtained when enzyme was incubated with compactin for 2 or 20 min.

Inactivation of HMG-CoA Reductase by Iodoacetic Acid. Immediately before use, enzyme was incubated at 30 °C for 30 min with 5 mM fresh DTT. The enzyme solution, 0.5 mL, was subsequently passed through a 2 × 6 cm Sephadex G-10 column equilibrated at room temperature in 0.1 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA to remove the DTT. The enzyme was then placed on ice. All other solutions were made in the same buffer and temperature equilibrated to 25 °C. The pH of all solutions were checked and adjusted to pH 7.0 with HCl or KOH as necessary.

Iodoacetic acid (final concentration 0.125–0.5 mM in a final volume of 0.5 mL) was added to 60–90 pmol of enzyme (tetramer) which had been previously temperature equilibrated at 25 °C. At timed intervals after the addition of iodoacetic acid, 25- or 50-μL aliquots were withdrawn and assayed immediately by adding the aliquot to a cuvette, usually containing 300 μM HMG-CoA and 160 μM NADPH in 0.1 M potassium phosphate buffer, pH 7.0, and 1 mM EDTA. When the effect of a ligand on the inactivation of enzyme by iodoacetic acid was being tested, the ligand was incubated with enzyme for a minimum of 30 s prior to addition of iodoacetic acid. Enzyme incubated with ligand in the absence of iodoacetic acid showed no change in activity.

Pseudo-first-order rate constants (k_{obsd} for the inactivation of enzyme were calculated from $k_{obsd} = 0.693/t_{1/2}$. Half-times were determined graphically from a plot of log (activity − activity$_\infty$) vs. time by using a minimum of six time points. Binding constants were determined from a minimum of three concentrations of ligand as described below. The second-order rate constant for the inactivation of enzyme by iodoacetic acid was determined with iodoacetic acid recrystalized (petroleum ether, bp 60–100 °C) before use; however, values obtained from commercial iodoacetic acid were not significantly different, and this step was generally omitted.

Assuming enzyme inactivation by iodoacetic acid (IAA) is a simple second-order process, two mechanisms by which a ligand may affect enzyme inactivation are shown in eq 2a and 3a. Free enzyme is inactivated by iodoacetic acid with a

$$\begin{array}{ccc} E + IAA & \xrightarrow{k_1} & E\text{-}IAA_{inactive} \\ \pm X \Big\downarrow\uparrow K_D & & \\ E\cdot X & & \end{array} \qquad (2a)$$

$$\left(\frac{k_{obsd}}{[IAA]}\right)^{-1} = \frac{1}{k_1} + \frac{1}{k_1 K_D}[X] \qquad (2b)$$

$$\begin{array}{ccc} E + IAA & \xrightarrow{k_1} & E\text{-}IAA_{inactive} \\ \pm X \Big\downarrow\uparrow K_D & & \\ E\cdot X + IAA & \xrightarrow{k_2} & X\cdot E\text{-}IAA_{inactive} \end{array} \qquad (3a)$$

$$\left(\frac{k_{obsd}}{[IAA]} - k_1\right)^{-1} = \frac{1}{a} + \frac{K_D}{a}\frac{1}{[X]} \qquad (3b)$$

$$a = k_2 - k_1 \qquad (3c)$$

second-order rate constant, k_1. The enzyme–ligand complex, E·X, is either inert to inactivation by iodoacetic acid (eq 2a) or inactivated by iodoacetic acid with a second-order rate constant, k_2 (eq 3a). The inactivation process in the presence of a large excess of iodoacetic acid is quantitatively described by a pseudo-first-order rate constant. Assuming free enzyme and ligand are in rapid equilibrium, the observed pseudo-first-order rate constant of inactivation, k_{obsd}, is described by eq 2b or 3b and 3c. Dissociation constants, K_D, were determined from a plot of $(k_{obsd}/[IAA])^{-1}$ vs. [X] according to eq 2b or a plot of $[(k_{obsd}/[IAA]) - k_1]^{-1}$ vs. $[X]^{-1}$ according to eq 3b. In all cases, estimation of the dissociation constant of the E·X complex by the concentration at which the half-maximal effect was observed was consistent with the calculated dissociation constants.

Results

Inhibition of HMG-CoA Reductase by Compactin: Effect of Order of Addition of Reactants. The effect of HMG-CoA and NADPH on the interaction of enzyme with compactin was investigated. Enzyme was first incubated with compactin, then the other components of the reaction (HMG-CoA and

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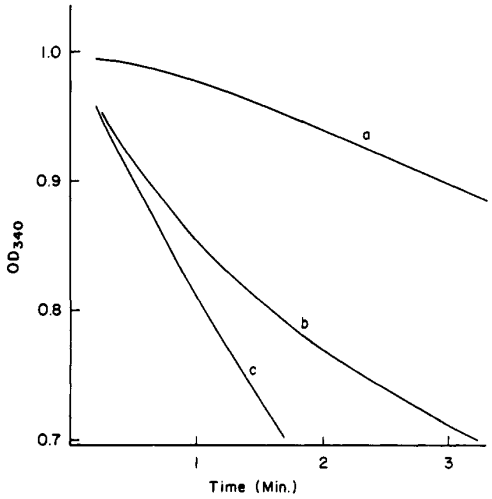

FIGURE 1: Inhibition of HMG-CoA reductase by compactin: effect of order of addition. Reactions contained 13 nM enzyme (tetramer), 160 μM NADPH, 300 μM HMG-CoA, 0.35 μM compactin, 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 5 mM DTT in a total volume of 1.0 mL at 25 °C. (a) Enzyme was added to a cuvette containing a premixed solution of compactin and buffer. After the solution was rapidly mixed, the reaction was initiated immediately with the simultaneous addition of HMG-CoA and NADPH contained in a total volume of 75 μL. (b) The reaction was initiated with the addition of 10 μL of enzyme. (c) Control assay without compactin.

NADPH) were added, and the rate of disappearance of NADPH was measured. The results are shown in Figure 1. The rate of NADPH disappearance is initially near zero and gradually increases until a rate is reached, which is less than that observed in the absence of compactin (25% of the uninhibited rate). In other experiments, the reaction was initiated by the addition of enzyme, or enzyme was first added to HMG-CoA and NADPH and compactin was then added (the results of these experiments were identical). The initial velocity is now identical with the uninhibited rate and gradually decreases to that observed in the above experiment. The effect of the order of addition of substrates and inhibitor on the kinetic course of the reaction has been previously observed with rat liver HMG-CoA reductase and was attributed to slow establishment of equilibrium (Alberts et al., 1980). The results obtained so far establish that compactin rapidly inhibits free enzyme; this rules out the possibility that compactin is a "slow binding" inhibitor. The data also indicate that when enzyme is added last, free enzyme initially partitions favorably to some enzyme–substrate specie(s) which does not interact with compactin. HMG-CoA and NADPH can prevent the binding of compactin. The slow decrease in rate of NADPH oxidation observed when compactin is added last is due to a low concentration of free enzyme.

The effect of the separate substrates was next examined. NADPH and compactin were added to the enzyme, and the reaction was started by the addition of HMG-CoA. The results are shown in Figure 2. The course of the reaction is identical with that seen when enzyme and compactin are first mixed and the reaction is started with NADPH and HMG-CoA. When enzyme was added to HMG-CoA and compactin and the reaction was initiated with NADPH, the rate of NADPH disappearance was initially nearly identical with that of the uninhibited reaction and gradually approached that of the inhibited reactions. These results show that HMG-CoA competes with compactin for the enzyme and, thus, compactin binds at least partially in the HMG-CoA binding domain. NADPH, on the other hand, at the concentration used, does

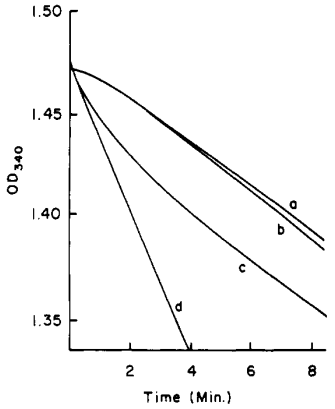

FIGURE 2: Inhibition of HMG-CoA reductase by compactin: effect of order of addition. Reactions conditions were as in Figure 1 except enzyme was 6 nM (tetramer) and NADPH was 230 μM. (a) Enzyme was added to a cuvette containing a premixed solution of NADPH, compactin, and buffer. After the solution was rapidly mixed, the reaction was initiated immediately by the addition of 20 μL of HMG-CoA solution. (b) Enzyme was added to a solution of compactin and buffer. The reaction was initiated immediately by the simultaneous addition of HMG-CoA and NADPH contained in a total volume of 40 μL. (c) Enzyme was added to a solution of HMG-CoA, compactin, and buffer. The reaction was initiated immediately with the addition of 20 μL of NADPH solution. (d) Control assay without compactin.

not prevent binding of compactin. The effect of NADPH upon compactin binding will be examined further.

Inhibition of HMG-CoA Reductase by Compactin in the Presence of HMG-CoA: Determination of K_D for the Enzyme–Compactin Complex and Rate Constants for the Reaction of Enzyme with Compactin and HMG-CoA. The equilibrium that exists in a solution containing enzyme, compactin, and HMG-CoA is shown in eq 4. Equation 5 relates

$$EC \underset{k_4}{\overset{k_3[C]}{\rightleftharpoons}} E \underset{k_2}{\overset{k_1[S]}{\rightleftharpoons}} ES \qquad (4)$$

$$\frac{[E_t]}{[E_t] - [E_{act\ eq}]} = 1 + \frac{K_{DC}[S]}{K_{DS}} \frac{1}{[C]} \qquad (5)$$

the dissociation constants K_{DC} ($K_{DC} = k_4/k_3$) and K_{DS} ($K_{DS} = k_2/k_1$) to the concentration of reactants when $[S]/K_{DS} \gg 1$. E_t represents the total amount of enzyme, and $E_{act\ eq}$ represents the total amount of active enzyme at equilibrium. $E_{act\ eq}$ is the sum of the equilibrium values of ES and E. An experiment was carried out to evaluate K_{DC}. Enzyme was added to a solution of HMG-CoA and compactin.[2] At timed intervals after the addition of enzyme, the reaction was initiated by the addition of NADPH, and the initial rate of NADPH disappearance was recorded. The rate of NADPH disappearance is a measure of the concentration of active enzyme, i.e., enzyme not complexed with compactin. As shown in Figure 3A , a time-dependent decrease in the concentration of active enzyme was observed after enzyme was added to HMG-CoA and compactin [identical results were obtained with HMG-CoA reductase further purified to a specific ac-

[2] In these experiments, the total concentrations of HMG-CoA and compactin are sufficiently higher than the total concentration of enzyme so that the concentrations of free HMG-CoA and compactin may be taken as their respective total concentration. In addition, the concentration of HMG-CoA is significantly higher than K_{DS} (determined elsewhere in this paper), and the concentration of compactin is significantly higher than published values of its inhibition constant (Endo et al., 1976; Alberts et al., 1980). Under these conditions active enzyme is equal to ES.

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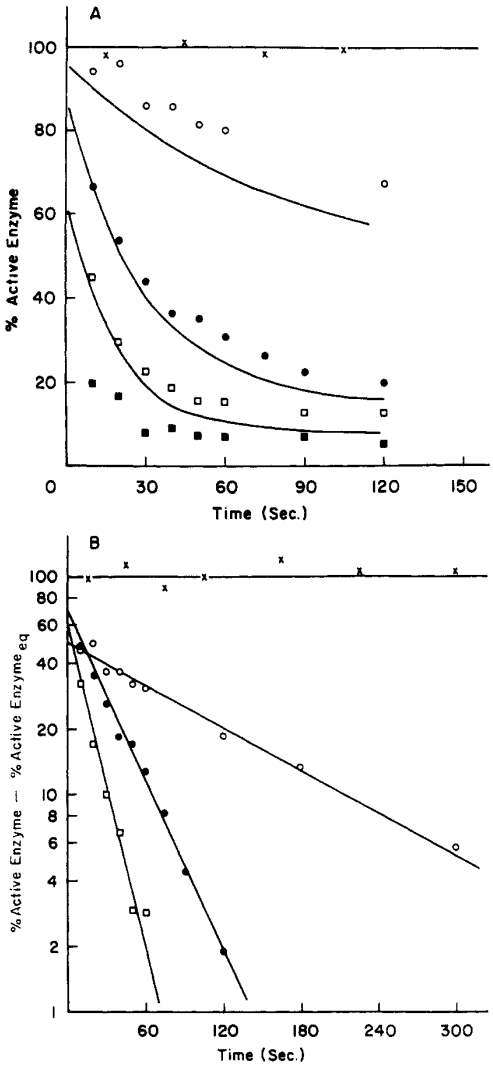

FIGURE 3: Rate of inhibition of HMG-CoA reductase by compactin in the presence of HMG-CoA. (A) Enzyme, 20 pmol (tetramer) in 5 μL, was added to a cuvette containing 150 μM HMG-CoA and various concentrations of compactin in a total volume of 970 μL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 5 mM DTT at 25 °C. At timed intervals after the addition of enzyme, 25 μL of an NADPH solution was added to a final concentration of 160 μM, and the initial rate of NADPH oxidation was immediately monitored spectrophotometrically at 340 nm. The initial velocity was determined from the first 0.5 min of the spectrophotometric trace. Compactin concentrations were 0 μM (×), 0.07 μM (○), 0.35 μM (●), 0.70 μM (□), and 1.4 μM (■). Solid lines were calculated from eq 9 as described in the text. (B) the data in (A) was replotted as log (percent active enzyme − percent active enzyme equilibrium) vs. time by using experimentally observed endpoints.

tivity of 3.2 μmol of NADPH oxidized min^{-1} (mg of protein)$^{-1}$ at compactin concentrations of 0.35 and 0.7 μM]. At constant HMG-CoA concentration, the extent and rate of inhibition increased with increasing compactin concentration. Under these conditions the initial velocity of NADPH oxidation approached limiting low values, and 100% inhibition was not observed. The initial velocity of NADPH oxidation after long preincubation times is a measure of the active enzyme at equilibrium. $[E_t]/([E_t] - [E_{act\ eq}])$ was then plotted vs. $1/[C]$. A straight line was obtained as required by eq 5 (data not shown). From the slope of the line, $K_{DC} = 0.24 \times 10^{-9}$ M was obtained by using a value for $K_{DS} = 0.59 \times 10^{-6}$ M which was obtained from an experiment described elsewhere in this paper.

Table I: Inhibition of HMG-CoA Reductase by Compactin in the Presence of HMG-CoA

[compactin] (μM)	k_{obsd}[a] (s^{-1})	% of total enzyme		
		$E_{act\ eq}$[b]	EC_{slow}[c]	EC_{fast}[d]
0.07	0.0075	49	50	1
0.35	0.030	18	71	11
0.70	0.058	12	58	30
1.40	not done	5		

[a] k_{obsd} is the observed rate of formation of EC and was calculated from $0.693/t_{1/2}$ where the half-time was obtained graphically from Figure 3B. [b] $E_{act\ eq}$ is the amount of active enzyme in the preincubation at equilibrium. This quantity is directly measured. [c] EC_{slow} is the total amount of EC formed in the slow phase. EC_{slow} is equal to the extrapolated intercept of the lines in Figure 3B at $t = 0$. [d] EC_{fast} is the total amount of EC formed in a fast phase that could not be directly observed. The value for EC_{fast} was obtained by the relationship $EC_{fast} = E_{total} - E_{act\ eq} - EC_{slow}$.

The time-dependent decrease in initial velocity of NADPH oxidation (equivalent to the formation of EC complex) shown in Figure 3A was further examined. Figure 3B shows a semilogarithmic plot of the data in Figure 3A. The observed rate of inhibition was first order and increased as a function of compactin concentration. The extrapolated intercept at $t = 0$ represents the percent enzyme that forms enzyme–compactin complex in the observed first-order process. At higher compactin concentrations, the sum of the $t = 0$ intercept and the percent active enzyme at equilibrium is less than 100% (Table I, $EC_{slow} + E_{act\ eq}$). The difference increases as compactin concentration increases. These results show that formation of EC is a biphasic process; i.e., a fraction of EC is formed in a burst too rapid to be measured, and the remainder is formed during the observed first-order process shown in Figure 3B. We shall refer to the fraction of EC formed in the rapid process as EC_{fast} and to that formed in the slower first-order process as EC_{slow}. Upon mixing, free enzyme rapidly partitions between enzyme–compactin (EC_{fast}) and enzyme–HMG-CoA (ES). This rapid phase is characterized by an "overshoot" formation of ES; i.e., the amount of ES initially formed exceeds that present at equilibrium. The directly observed second phase is a slow conversion of overshoot ES to EC, presumably by way of free enzyme (eq 4). Finally, equilibrium is established between ES, EC, and E. In these experiments the total amount of EC that can be measured at any time is equal to EC_{fast} plus the amount of EC_{slow} that has been formed in the first-order process described above.

The rate of inhibition when enzyme is added to HMG-CoA and compactin is determined by the concentrations of reactants and the rate constants illustrated in eq 4. The first-order formation of EC_{slow}, together with the values of the dissociation constants K_{DS} and K_{DC}, is sufficient information to determine the absolute values of the individual rate constants. Assuming that free enzyme reaches a steady-state value after the burst (the validity of this assumption is addressed below), together with the fact that the concentration of E is much lower than the concentrations of either ES or EC, allows the determination of an expression for k_{obsd}. The derivation of equations are reported in detail in the Appendix. The observed rate constant for the inhibition of enzyme activity is a nonlinear function with respect to compactin concentration (eq 6). From the

$$k_{obsd} = \frac{k_3 K_{DS}[C]/[S] + k_4}{1 + k_3[C]/(k_1[S])} \quad (6)$$

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{[S]}{k_3 K_{DS}} \frac{1}{[C]} \quad (7)$$

previously determined dissociation constants and the known

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