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

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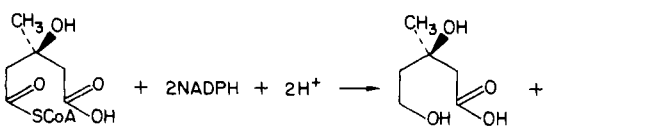
ABSTRACT: The sodium salts of compactin (**1**) and *trans*-6-[2-(2,4-dichloro-6-hydroxyphenyl)ethyl]-3,4,5,6-tetrahydro-4-hydroxy-2*H*-pyran-2-one (**3**) are inhibitors of yeast β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase. The dissociation constants are 0.24×10^{-9} and 0.28×10^{-9} M, 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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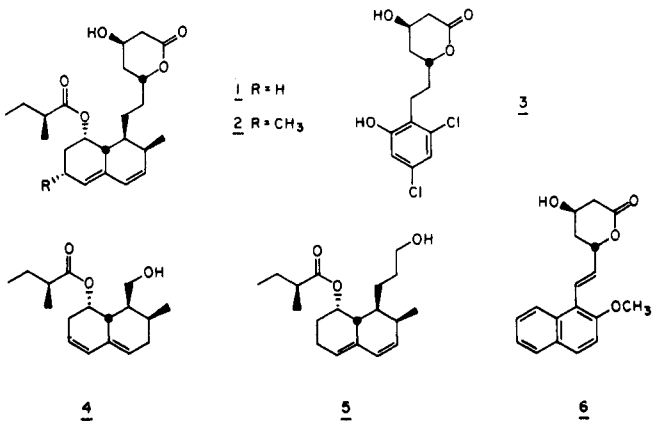
Chart I: Structures for Compounds 1–6[a]



[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.

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

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

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

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

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

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

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

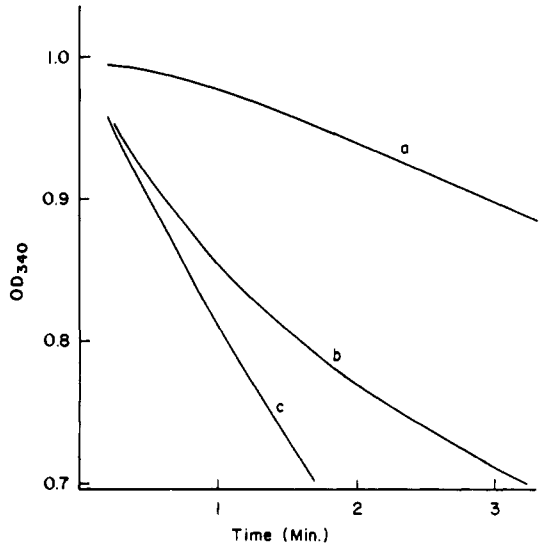


FIGURE 1: Inhibition of HMG-CoA reductase by compactin: effect of order of addition. Reactions contained 13 nM enzyme (tetramer), 160 μM NADPH, 300 μM HMG-CoA, 0.35 μM compactin, 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 5 mM DTT in a total volume of 1.0 mL at 25 °C. (a) Enzyme was added 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

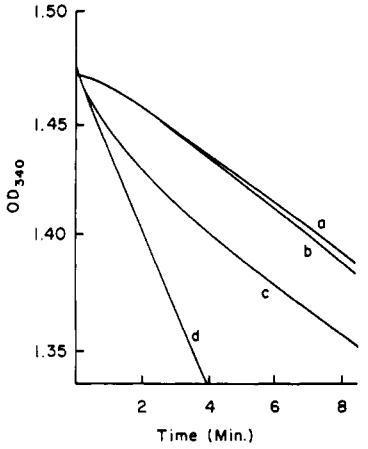


FIGURE 2: Inhibition of HMG-CoA reductase by compactin: effect of order of addition. Reactions conditions were as in Figure 1 except enzyme was 6 nM (tetramer) and NADPH was 230 μM. (a) Enzyme was added to a cuvette containing a premixed solution of NADPH, compactin, and buffer. After the solution was 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

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

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

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.

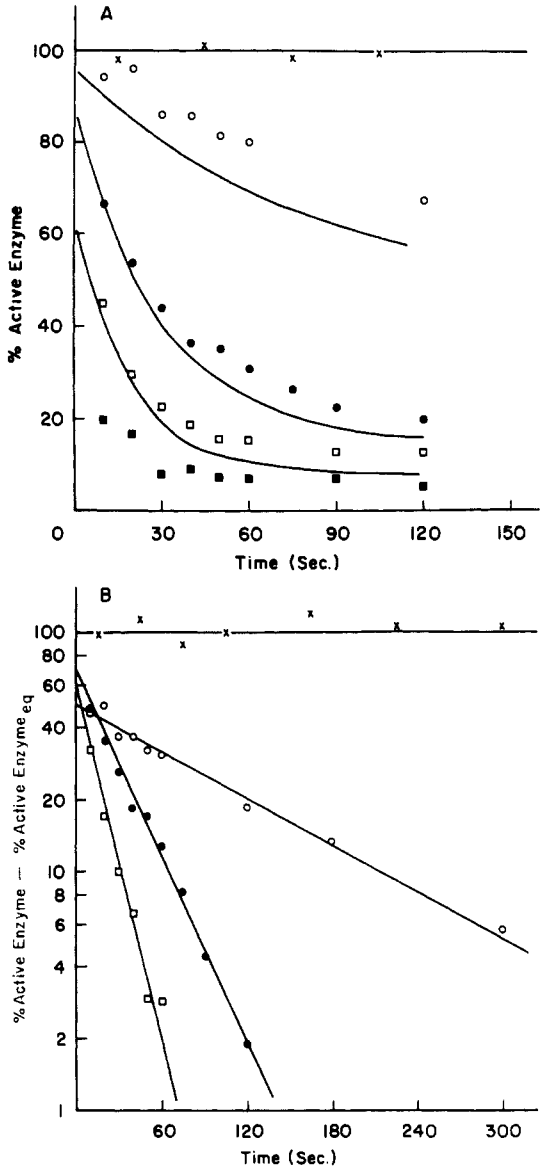


FIGURE 3: Rate of inhibition of HMG-CoA reductase by compactin in the presence of HMG-CoA. (A) Enzyme, 20 pmol (tetramer) in 5 μL, was added to a cuvette containing 150 μM HMG-CoA and various concentrations of compactin in a total volume of 970 μL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, 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

Table II: Rate and Equilibrium Constants for the Reaction of Enzyme with Compactin and HMG-CoA

reaction	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (M)
E + HMG-CoA $\underset{k_2}{\overset{k_1}{\rightleftharpoons}}$ ES	1.9×10^5 [b]	0.11 ± 0.09 [a]	$0.59 \pm 0.17 \times 10^{-6}$ [c]
E + compactin $\underset{k_4}{\overset{k_3}{\rightleftharpoons}}$ EC	$2.7 \pm 0.3 \times 10^7$ [a]	6.5×10^{-3} [b]	0.24×10^{-9} [d]

[a] Calculated from eq 7 as described in the text. [b] Calculated from the values of k_2 and k_3 obtained from eq 7 and the appropriate dissociation constant. [c] Determined from iodoacetic acid protection experiments as described in the text. [d] Determined from competition experiments with HMG-CoA as described in the text.

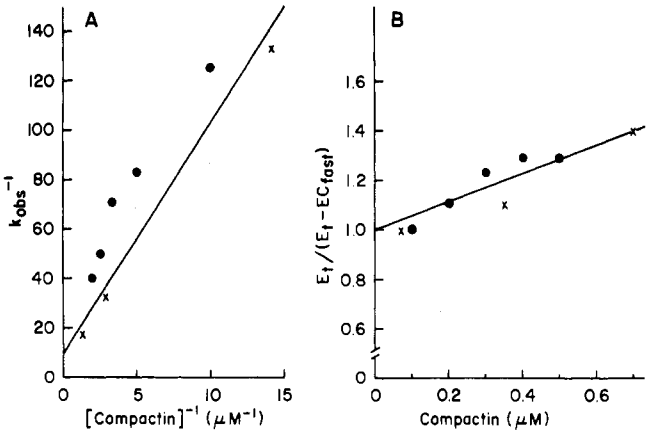


FIGURE 4: Inhibition of HMG-CoA reductase by compactin in the presence of HMG-CoA: determination of rate constants. Data obtained under conditions described in Figure 3A were treated as described in the text. (A) Observed pseudo-first-order rate constants were plotted according to eq 7. (B) Data was plotted according to eq 8. (×) Data from Table I. (●) Data from an experiment identical with that of Figure 3A except for compactin concentration. Lines were drawn by using mean values of slopes and intercepts from each experiment.

concentrations of compactin and substrate, it was determined that to a first approximation k_4 may be dropped from eq 6 to yield eq 7. The values of k_3 and k_2 can be calculated from the slope and intercept of a plot of $1/k_{obsd}$ vs. $1/[C]$. Analysis of two separate experiments according to eq 7 gave values of $(2.7 \pm 0.3) \times 10^7$ M^{-1} s^{-1} and 0.11 ± 0.09 s^{-1} for k_3 and k_2, respectively (Figure 4A). Values for k_1 and k_4 were calculated to be 1.9×10^5 M^{-1} s^{-1} and 6.5×10^{-3} s^{-1}, respectively, from the equilibrium expressions $K_{DS} = k_2/k_1$ and $K_{DC} = k_4/k_3$. Rate and equilibrium constants are summarized in Table II. 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. In spite of the difference between on-rate constants, free enzyme initially partitions favorably to ES. This is a reflection of the fact that HMG-CoA is present at much higher concentrations than compactin so that the on rates are more nearly equal. The initial formation of ES is kinetically favored but thermodynamically unstable. The slowness of the subsequent approach to equilibrium is due to the relatively slow dissociation of HMG-CoA from ES and the fact that free enzyme thus formed again partitions favorably to ES.

The values of EC_{fast} are of interest because the partitioning ratio of the burst is approximated by the expression $(E_t - EC_{fast})/EC_{fast} = k_1[S]/k_3[C]$, where $(E_t - EC_{fast})$ represents the amount of ES present immediately after the burst. If the formation of EC_{fast} and ES during the burst is sufficiently fast compared to the following slow rearrangement toward equilibrium, eq 8 is valid. A plot of $[E_t]/([E_t] - [EC_{fast}])$ vs. $[C]$

$$\frac{[E_t]}{[E_t] - [EC_{fast}]} = 1 + \frac{k_3[C]}{k_1[S]} \qquad (8)$$

is shown in Figure 4B. The ratio $k_3/k_1 = 110 \pm 10$ was obtained from the slope of Figure 4B according to eq 8. The value of the ratio k_3/k_1 obtained in this manner is independent of eq 7; thus, this result confirms the 100-fold larger on-rate constant for compactin compared to that for HMG-CoA.

In another approach, which assumes only the mechanism in eq 4, and no steady-state assumption, the exact solution to $\int d[EC]/dt$ was found to be eq 9 (see Appendix). The fraction

$$\frac{[EC]}{[E_t]} = \frac{\alpha}{\beta} + \frac{\alpha M e^{-0.5(\gamma-\delta)t}}{\delta} - \frac{\alpha N e^{-0.5(\gamma+\delta)t}}{\delta} \qquad (9)$$

of enzyme–compactin complex is described as a function of time by three terms: a constant and two exponential terms. The constant term represents the equilibrium fraction of EC while the two exponential terms represent the burst formation of EC_{fast} and the slow formation of EC_{slow}. The Greek letters represent functions of the rate constants shown in eq 4 and the concentrations of HMG-CoA and compactin (see Appendix). Each exponential term in eq 9 contains a variable (M and N; the values of M are negative) that is constant for a given set of initial conditions. The solutions for M and N under our conditions are reported in the Appendix. By use of the rate constants reported in Table II, the time-dependent formation of EC calculated by eq 9 compared favorably to the experimentally observed values (solid lines, Figure 3A). When the values of the rate constants are varied (less than a factor of 2), a significantly better fit can be obtained. The good fit between the experimentally observed and calculated values indicates that the steady-state assumption used above is valid. The deviation between calculated and experimentally observed values of EC at the lowest compactin concentration is due in part to the depletion of free compactin as the reaction reaches equilibrium (at equilibrium, the concentration of free compactin is 70% of the total compactin concentration).

Titration of HMG-CoA Reductase with Compactin. In order to determine the number of molecules of compactin that bind per tetramer of HMG-CoA reductase, the enzyme was titrated with compactin. Enzyme activity decreased linearly with increasing compactin concentration, and a stoichiometry of two compactin molecules per enzyme (tetramer) was required to obtain 100% inhibition (data not shown).

Competition Binding Experiments between Compactin and HMG-CoA, NADPH, or CoASH: Dilution Experiments. The active site of HMG-CoA reductase must contain a binding site for NADPH and HMG-CoA. The data cited so far show that the compactin binding domain overlaps, at least partially, the HMG-CoA binding site since HMG-CoA prevents binding of compactin to the enzyme. In order to more closely define the compactin binding domain, we investigated the effect of NADPH and CoASH upon compactin binding. An important feature of the compactin/HMG-CoA competition experiments, reported in the preceding sections, is that the distribution of EC and ES was determined without dilution of the enzyme species. The affinity of free enzyme for CoASH or NADPH is significantly lower than that for HMG-CoA. As a result,

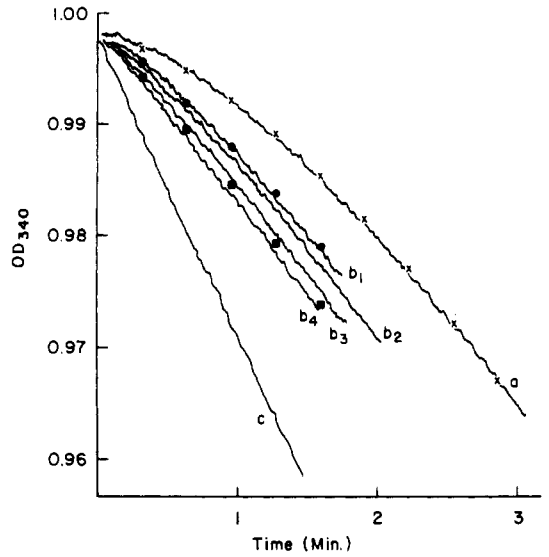


FIGURE 5: Inhibition of HMG-CoA reductase by compactin in the presence HMG-CoA: dilution assays. Four sets of experiments were performed in which enzyme, 140 pmol (tetramer), was incubated in 1.0 mL of 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 5 μM DTT containing 0.23 μM compactin and/or HMG-CoA at 25 °C. The sets differed only in [HMG-CoA]. At various times, 10-μL aliquots of enzyme were withdrawn and assayed spectrophotometrically at 340 nm until sequential traces were superimposable. At this point, the incubation was judged to be in equilibrium. The traces shown are traces when equilibrium was reached. The assays were performed at 25 °C, in 1.0 mL of the same buffer containing 300 μM HMG-CoA, 160 μM NADPH, and appropriate additions such that the assays within each set of experiments contained identical concentrations of reactants. (a) Enzyme was incubated with compactin (traces for all sets were not significantly different). (b) Enzyme was incubated with compactin and 38, 75, 150, or 300 μM HMG-CoA (b_1, b_2, b_3, and b_4, respectively). (c) Enzyme was incubated with HMG-CoA. Calculated points are shown for traces a (×), b_1 (●), and b_3 (■). Calculated points were generated as follows. EC_{eq}/E_t was calculated to be 0.82, 0.70, 0.63, 0.55, and 0.45 for trace a and traces b, respectively, from eq 5 with $K_{DS} = 0.59 \times 10^{-6}$ M and $K_{DC} = 0.24 \times 10^{-9}$ M. $k_{max} = 75 \times 10^{-9}$ M s^{-1} was obtained from trace c. The experimental data was then fit to eq 10, varying only the value of k_4. The value of k_4 that best fit the data was 4.8×10^{-3} s^{-1}.

experiments directly analogous to the compactin/HMG-CoA experiments described above could not be used to determine if CoASH or NADPH prevent binding of compactin to enzyme. The high concentration of CoASH that would be required to detect protection if CoASH and compactin are competitive would inhibit all enzyme activity. The high concentration of NADPH that would be required precludes the use of a spectrophotometric assay. In order to determine if CoASH or NADPH could protect enzyme from inhibition by compactin, it was necessary to preincubate the enzyme with compactin and CoASH or NADPH and determine activity by diluting an aliquot of the enzyme solution into an assay mixture.

The experimental approach to be used in these experiments was first tested with HMG-CoA. The validity of this approach can then be established by comparing results obtained in this experiment to the results obtained above. The experiment was carried out as follows: enzyme was incubated with compactin plus various concentrations of HMG-CoA, with compactin alone, and with HMG-CoA alone. After equilibrium had been established, aliquots were removed and diluted 100-fold into an assay mixture containing NADPH, and the disappearance of NADPH was recorded. The time courses of NADPH disappearance are shown in Figure 5. Traces b (Figure 5) show the time courses of NADPH disappearance when enzyme was incubated with compactin plus various concentrations of HMG-CoA and then diluted into the assay mixture. The initial rate of NADPH oxidation is dependent upon the amount of HMG-CoA present in the reaction mixture prior to dilution into the assay mixture. At high HMG-CoA concentrations the initial rate of NADPH oxidation was nearly equal to that observed in the absence of compactin (trace c). At low HMG-CoA concentrations the initial rate of NADPH disappearance was slow and approached that observed when enzyme was reacted with compactin alone (trace a). These results lead to the same qualitative conclusion as the experiment described above, i.e., that binding of compactin and HMG-CoA to enzyme is mutually exclusive. Furthermore, these results confirm that this approach can be used to determine whether substrates prevent compactin from binding to HMG-CoA reductase.

The results of Figure 5 can be analyzed quantitatively. We expect that the enzyme in the preincubation will partition between ES and EC, eventually forming an equilibrium mixture. Upon dilution into the assay mixture, the formation of product with respect to time will be quantitatively described by the sum of two terms, a linear and nonlinear term. Immediately upon dilution into the assay mixture, the fraction of enzyme that is originally present in the preincubation as ES will form product at a constant rate proportional to the maximal rate given by the uninhibited reaction (linear term). The remaining fraction of the enzyme, originally present in the preincubation as EC, will become active and form product only after compactin has dissociated from the EC complex (nonlinear term). Under the conditions of the assay, the dissociation of compactin from EC is irreversible. The rate at which active enzyme is formed from EC will be determined by the off rate of compactin from EC, and this rate should correspond to an observed exponentially increasing rate of product formation.

In the absence of product inhibition, the formation of product is described with respect to time by eq 10 (Cha, 1975)

$$P = k_{max}t - \frac{k_{max}EC_{eq}}{k_4E_t}(1 - e^{-k_4t}) \qquad (10)$$

where P is [NADP], k_{max} is the zero-order rate constant for the formation of NADP that is obtained from the uninhibited reaction, EC_{eq}/E_t is the fraction of EC in the preincubation when equilibrium is reached, and k_4 is the rate constant for the dissociation of compactin from EC. The experimental data of Figure 5 was fit to eq 10 by using $k_{max} = 75 \times 10^{-9}$ M s^{-1} (from trace c), calculated values of EC_{eq}/E_t (derived from the previously determined dissociation constants and the known concentrations of the reactants in the preincubation[3]), and varying only the value for the rate constant, k_4. The value for k_4 that best fit the data was found to be 4.8×10^{-3} s^{-1}, in good agreement with the value of the rate constant for the dissociation of compactin from EC determined elsewhere in this paper, 6.5×10^{-3} s^{-1}. Calculated points are shown in Figure 5.

The effect of NADPH upon compactin binding was investigated through the same experimental approach as that described for HMG-CoA. The results are shown in Figure 6. NADPH ([NADPH] = $2200K_{D\text{-}NADPH}$) has no effect on the binding of compactin to the enzyme or upon the rate of dissociation of compactin. This result suggests that both compactin and NADPH can bind to the enzyme simultane-

[3] For mutually exclusive binding of compactin and X to enzyme, $K_{D\text{-}X}[C_{free}][EX]/[E_t] = K_{D\text{-}C}[X][EC]/[E_t]$. $[EX]/[E_t] + [EC]/[E_t] = 1.0$, and $[C_{free}] = [C_{total}] - 2[EC]$ where [EC] is taken as a tetramer.

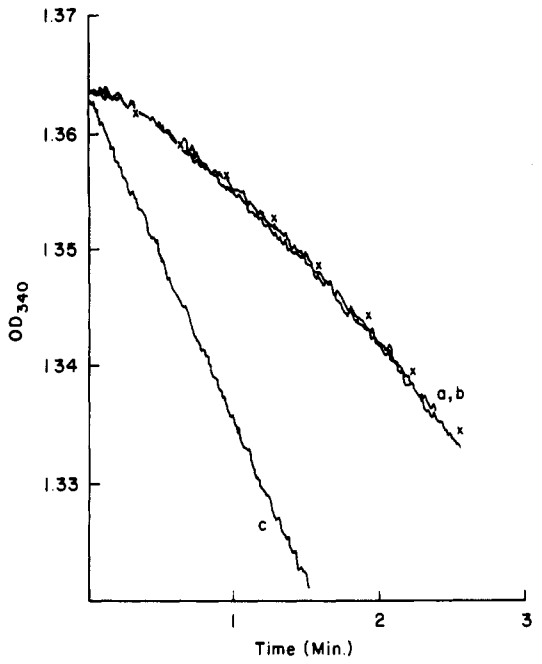


FIGURE 6: Inhibition of HMG-CoA reductase by compactin in the presence of NADPH: dilution assays. Enzyme was incubated with compactin and/or 22 mM NADPH. Experiments were performed as described in the legend to Figure 5 except that the concentration of NADPH in the assays was 220 μM. (a) Enzyme was incubated with compactin. (b) Enzyme was incubated with compactin and NADPH. (c) Enzyme was incubated with NADPH. (×) Calculated points. Calculated points were obtained as described in the legend to Figure 5. $k_{max} = 77 \times 10^{-9}$ M s^{-1} was obtained from trace c. The value of k_4 that best fit the data was 4.8×10^{-3} s^{-1}.

ously, which rules out the possibility that the binding sites for these compounds overlap.

In another set of experiments the effect of CoASH on the binding of compactin to enzyme was investigated. The results are shown in Figure 7. The initial rate of NADPH oxidation when enzyme is preincubated with CoASH plus compactin is faster than when enzyme is preincubated with compactin alone. This increase is proportionally greater at higher CoASH concentration. Values for EC_{eq}/E_t when enzyme was preincubated with compactin and CoASH were estimated by the best fit of eq 10 to the data (Figure 7, traces b_1 and b_2). By use of these values of EC_{eq}/E_t, the dissociation constant for the E·CoASH complex was calculated to be $(0.68 \pm 0.44) \times 10^{-3}$ M.[3] This value is in good agreement with $K_i = 0.23 \times 10^{-3}$ M for CoASH determined elsewhere in this paper from initial velocity experiments with CoASH vs. HMG-CoA. These results show that binding of compactin to HMG-CoA reductase is mutually exclusive with respect to CoASH.

Inhibition of HMG-CoA Reductase by trans-6-[2-(2,4-Dichloro-6-hydroxyphenyl)ethyl]-3,4,5,6-tetrahydro-4-hydroxy-2H-pyran-2-one (3). Compound 3 is an inhibitor of mammalian HMG-CoA reductase (Willard et al., 1983). Compound 3 resembles compactin in that its "upper" portion contains the same lactone ring. The structure of its "lower" portion, however, is entirely different from that found in compactin. Compound 3 is an effective inhibitor of yeast HMG-CoA reductase. Its inhibitory properties were investigated by the same techniques described for compactin above. Compound 3 was found to be competitive with HMG-CoA with $K_D = 0.28 \times 10^{-9}$ M, a value identical with that found for compactin. NADPH did not affect the binding of compound 3. In these experiments concentrations of NADPH up to $3000K_D$-NADPH were used. In this respect compound 3 also behaves in a manner analogous to compactin. Compound

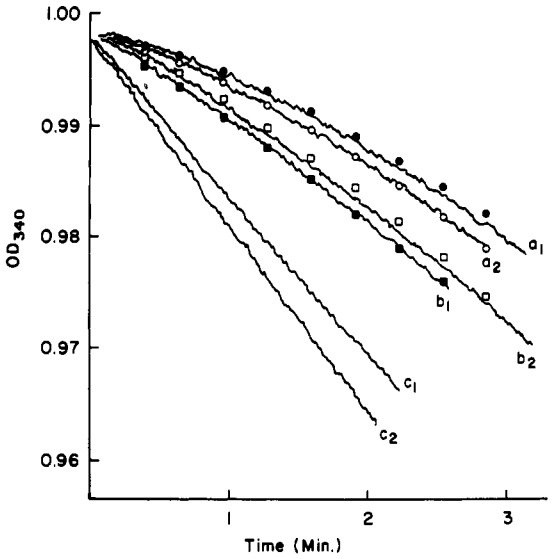


FIGURE 7: Inhibition of HMG-CoA reductase by compactin in the presence of CoASH: dilution assays. Two sets of experiments were performed in which enzyme was incubated with compactin and/or CoASH. The sets differed only in that [CoASH] in the incubations was 60 and 120 mM. Experiments were performed as described in the legend to Figure 5. (a_2 and a_1) Enzyme was incubated with compactin and then assayed in the assay mixture plus 0.6 or 1.2 mM CoA, respectively. (b_2 and b_1) Enzyme was incubated with compactin and 60 or 120 mM CoA, respectively. (c_2 and c_1) Enzyme was incubated with 60 or 120 mM CoA, respectively. Calculated points are shown for a_1 (●), a_2 (○), b_1 (■), and b_2 (□). Calculated points were generated from as s follows. k_{max} values of 47×10^{-9} and 40×10^{-9} M s^{-1} were obtained from traces c_2 and c_1, respectively. $k_4 = 4.0 \times 10^{-3}$ s^{-1} was obtained from best fit of eq 10 to traces a_2 and a_1 by using $EC_{eq}/E_t = 0.82$ (see legend to Figure 5) and the appropriate value for k_{max}. Traces b_2 and b_1 were then fit to eq 10, varying only the value for EC_{eq}/E_t. The values for EC_{eq}/E_t which best fit eq 10 to traces b_2 and b_1 were found to be 0.70 and 0.52, respectively.

3 differed in an important respect from compactin: CoASH did not affect the binding of compound 3. These results indicate that compound 3 only interacts with the hydroxymethylglutarate binding domain of the active site.

Inhibition of HMG-CoA Reduction by Compounds Structurally Related to Compactin and Compound 3. In order to define how the structure of compactin and compound 3 contributes to the extremely strong interaction with the enzyme, the inhibitory effect of a number of compounds structurally related to compactin and compound 3 were examined. Of particular interest were compounds that resembled either the upper portion or the lower portion of compactin.

The sodium salts of DL-mevalonate and DL-3,5-dihydroxyvalerate (which resemble the upper portion of compactin) inhibited HMG-CoA reduction competitively with respect to HMG-CoA. The slope inhibition constants, K_{is} (Cleland, 1963), for these inhibitors are shown in Table III. If $K_D = K_{is}$, the dissociation constants for these compounds are approximately 8 orders of magnitude higher than that for compactin.

Analogues of the hydrophobic lower portion of compactin, compounds 4 and 5, were tested for inhibition of HMG-CoA reduction with HMG-CoA and NADPH concentrations of 10 and 160 μM, respectively. No inhibition was observed for 4 or 5 at concentrations approaching their solubility limits, 700 and 330 μM, respectively. Inhibition of HMG-CoA reduction by 4 or 5 was also determined in the presence of DL-3,5-dihydroxyvalerate (3,5-dihydroxyvalerate was present at concentrations which by itself inhibited the reaction 25–50%); no increase in inhibition was observed.

Inhibition of HMG-CoA reduction by compound 6 was

Table III: Inhibition Constants for the Reduction of HMG-CoA to Mevalonic Acid[a]

inhibitor	variable substrate	pattern[b]	inhibition constant (mM)[c] K_{is}	K_{ii}
DL-mevalonate	HMG-CoA	C	11 ± 1	
DL-3,5-dihydroxy-valerate	HMG-CoA	C	33 ± 6	
compound **4**[d]	*f*			
compound **5**[d]	*f*			
CoA	HMG-CoA	NC	0.23	<0.15[e]
compound **6**	HMG-CoA	C	0.8×10^{-3}	
	NADPH	NC	3.1×10^{-3}	4.5×10^{-3}

[a] Experiments were performed as described under Materials and Methods. [b] C, competitive; NC, noncompetitive. [c] The K_M for HMG-CoA and NADPH were 5.4 ± 0.5 μM and 18 ± 4 μM, respectively. Inhibition constants were obtained as described under Materials and Methods. [d] Assays were performed in 5–10% acetonitrile. [e] The inhibitory effect on V_{max} saturates below a CoA concentration of 0.15 mM; V_{max} is reduced approximately 40%. [f] No inhibition observed.

found to be competitive with respect to HMG-CoA and noncompetitive with respect to NADPH. In this respect, inhibition of HMG-CoA reductase by compound **6** is similar to the inhibition observed for compactin and the more potent compactin analogues.

Inhibition of HMG-CoA Reduction in the Presence of Compound 6 plus CoASH. Inhibition of HMG-CoA reductase by compound **6** was further investigated in order to determine if the binding site for compound **6** overlaps the CoASH binding site. Inhibition of HMG-CoA reduction was examined in the presence of compound **6** plus CoASH by the method of Chou & Talalay (1981). The inhibition of HMG-CoA reduction was determined at a fixed concentration of HMG-CoA and NADPH with varied concentrations of inhibitor(s) which were present alone or together. Fractional velocities [f_v = (velocity in the presence of inhibitor)/(velocity in the absence of inhibitor)] were determined for the inhibited reactions, and I_{50}'s (the concentration of inhibitor that gave 50% inhibition) were determined for each inhibitor. If linear Michaelis–Menten kinetics are observed, a plot of log (f_v^{-1} – 1) vs. log (I/I_{50}) for reactions containing only one inhibitor will fall on a single straight line with a slope of 1. Values of log (f_v^{-1} – 1) for reactions containing both inhibitors will fall on the same line when these values are plotted vs. either log ($I_1/I_{50,1} + I_2/I_{50,2}$) or log [$I_1/I_{50,1} + I_2/I_{50,2} + (I_1I_2)/(I_{50,1}I_{50,2})$] depending upon whether the inhibitors are mutually exclusive (cannot bind simultaneously) or mutually nonexclusive (can bind simultaneously), respectively. The subscripts 1 and 2 in the above expressions refer to the two different inhibitors, and in the latter case, it is assumed that the dissociation constant for the dissociation of I_1 from the binary $E{\cdot}I_1$ complex is identical with the dissociation constant for the dissociation of I_1 from the ternary $E{\cdot}I_1{\cdot}I_2$ complex.

Inhibition of HMG-CoA reductase by CoASH was also examined as a function of HMG-CoA concentration by more classical methods. Inhibition of HMG-CoA reduction by CoASH exhibited a slope and an intercept effect (Figure 8). A replot of slope vs. [CoASH] was linear and yielded K_{is} = 0.23 mM (Table III). In contrast, the intercept in the presence of CoASH concentrations above 150 μM did not vary and corresponded to 60% of the V_{max} observed in the absence of CoASH. We interpret this finding to mean that in the reduction of HMG-CoA to mevalonate, the release of products is ordered in that CoASH is preferentially released before mevalonate and/or NADP; however, when sufficient free CoASH is present, this pathway is blocked, and product release occurs through an alternative, slightly slower, pathway.

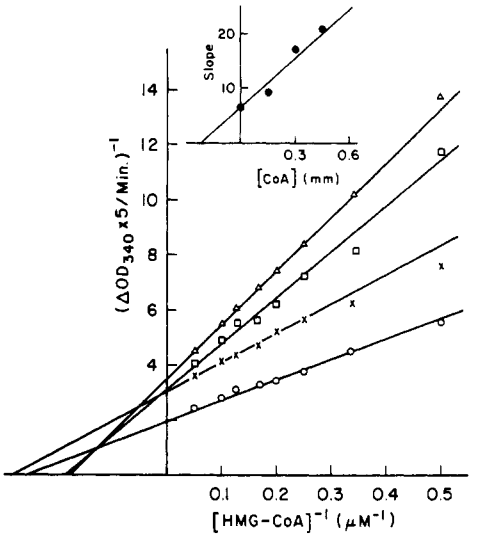


FIGURE 8: Inhibition of HMG-CoA reductase by CoASH. Inhibition of HMG-CoA reductase by CoASH was determined as described under Materials and Methods. (○) CoASH = 0; (×) CoASH = 0.15 mM; (□) CoASH = 0.30 mM; (△) CoASH = 0.45 mM. [NADPH] was fixed at 160 μM. Insert: Slope replotted as a function of CoASH concentration as described under Materials and Methods.

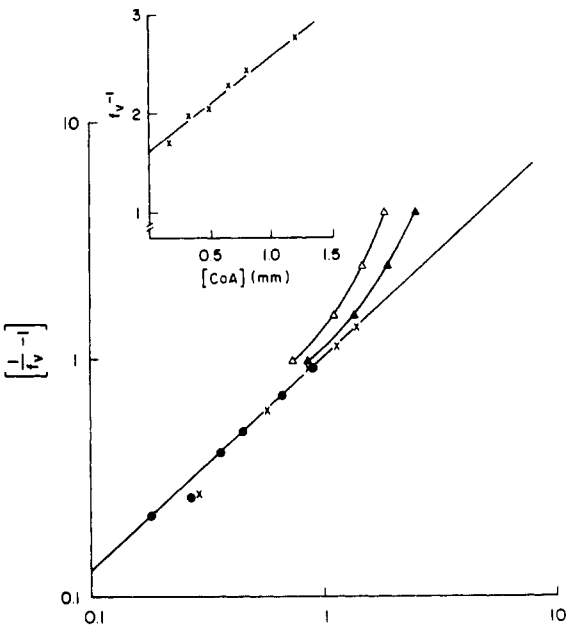


FIGURE 9: Inhibition of HMG-CoA reductase by CoASH and compound **6**. Initial velocity of NADPH oxidation was determined in the presence and absence of inhibitors with HMG-CoA fixed at 20 μM and NADPH fixed at 160 μM as described under Materials and Methods. Inhibition by CoASH alone was determined at 0.32–1.6 mM, inhibition of compound **6** was determined at 1.67–8.33 μM, and inhibition by CoASH plus compound **6** was determined at CoASH concentrations of 0.32–0.80 mM with a [CoASH]/[compound **6**] ratio of 96/1. The data were treated as described by Chou & Talalay (1981). I_{50} values of 1.8 mM and 5.8 μM for CoASH (normalized; see text) and compound **6**, respectively, were obtained from plots of log [$(f_v^{-1}) - 1$] vs. log (I). log [$(f_v^{-1}) - 1$] for CoASH (normalized) or compound **6** inhibitions is plotted vs. log (I/I_{50}): (●) CoASH alone; (×) compound **6** alone. log [$(f_v^{-1}) - 1$] for CoASH plus compound **6** inhibitions (normalized) is plotted vs. (△) $(I/I_{50})_{CoASH} + (I/I_{50})_{cmpd6}$ or (▲) $(I/I_{50})_{CoASH} + (I/I_{50})_{cmpd6} + [(I/I_{50})_{CoASH}(I/I_{50})_{cmpd6}]$. Insert: f_v^{-1} for inhibition by CoA alone are plotted vs. [CoASH]. Extrapolated intercept at [CoASH] = 0 corresponds to V_{max} in the presence of CoASH and is equal to $0.62V_{max}$ in the absence of CoASH. Fractional velocities for inhibitions containing CoASH are normalized by division by 0.62.

The effect of the presence of compound **6**, CoASH, or compound **6** plus CoASH on HMG-CoA reduction by the

Table IV: Dissociation Constants Obtained from Protection against Inactivation by Iodoacetic Acid[a]

compound	dissociation constant (M)
HMG-CoA	$0.59 \pm 0.17 \times 10^{-6}$
DL-mevalonate	$4 \pm 2 \times 10^{-3}$
DL-3,5-dihydroxyvalerate	$21 \pm 9 \times 10^{-3}$
NADPH	$10 \pm 1 \times 10^{-6}$

[a] Experiments were performed as described under Materials and Methods.

method of Talalay is shown in Figure 9. In agreement with the results reported above, inhibition of HMG-CoA reduction by CoASH exhibits an intercept (V_{max}) component which is saturated at CoASH concentrations above 150 μM (Figure 9, inset). Since the intercept component of CoA inhibition is independent of the CoA concentrations used in this experiment, the data for the reactions inhibited by CoASH have been normalized to reflect only the slope effect. The normalized data for reactions containing CoASH alone and the data for reactions containing compound **6** alone define a straight line as required. The normalized data for the reactions containing compound **6** plus CoASH fall above the line regardless of which abscissa parameter is used; therefore, more inhibition is observed than is predicted by either the mutually exclusive or mutually nonexclusive inhibitions discussed above. The simplest explanation that is consistent with these results is that compound **6** and CoASH inhibit HMG-CoA reductase in a mutually nonexclusive fashion in which each inhibitor binds more strongly in a ternary $E \cdot I_1 \cdot I_2$ complex than in a binary $E \cdot I_1$ complex. Compound **6** and compound **3** are similar in that the binding site for the "bottom" pieces of both compounds do not overlap the CoASH binding site on the enzyme.

Inactivation of HMG-CoA Reductase by Iodoacetic Acid: Protection Studies. In order to draw meaningful conclusions from the results of the experiments reported in the preceding sections in which single substrates were used to prevent the binding of compactin (or compound **3**) to HMG-CoA reductase, the binding constants of the substrates must be known. We, therefore, have obtained dissociation constants from experiments in which substrates were used to protect HMG-CoA reductase against inactivation by iodoacetic acid. HMG-CoA reductase is subject to inactivation by a number of sulfhydryl reagents (Durr & Rudney, 1960; Kirtley & Rudney, 1967). Iodoacetic acid was found to efficiently inactivate HMG-CoA reductase. The decrease in activity of enzyme incubated with excess iodoacetic acid was pseudo first order and linear for at least three half-times. Enzyme not treated with iodoacetic acid showed no loss of activity during the time required to perform a single experiment. The observed pseudo-first-order rate constant of inactivation was linearly dependent upon iodoacetic acid concentration, and no saturation was observed up to 0.5 mM iodoacetic acid. The second-order rate constant of inactivation was found to be 28 M^{-1} s^{-1}.

HMG-CoA and the sodium salts of mevalonic acid and 3,5-dihydroxyvaleric acid protected HMG-CoA reductase from inhibition by iodoacetic acid in a manner consistent with complete protection at infinite concentration of ligand. Plots of $[IAA]/k_{obsd}$ vs. [HMG-CoA] were linear as required by eq 2b. The dissociation constants determined for HMG-CoA, mevalonic acid, and 3,5-dihydroxyvaleric acid by this method are summarized in Table IV and were found to be consistent with their respective inhibition constants determined from initial velocity experiments (Table III).

NADPH decreased the rate of inactivation of enzyme by iodoacetic acid. The rate of inactivation decreased to a finite value with increasing NADPH concentration. This result is consistent with formation of an E·NADPH complex which is inactivated at a reduced rate as compared to free enzyme.[4] A plot of $[(k_{obsd}/[IAA]) - k_1]^{-1}$ vs. $[NADPH]^{-1}$ according to eq 3b was linear and yielded K_D-NADPH = 10 ± 1 μM (data not shown).

Discussion

Compactin (**1**) and compound **3** are powerful reversible inhibitors of yeast HMG-CoA reductase. We have determined the dissociation constants for these inhibitors to be 0.24×10^{-9} M and 0.28×10^{-9} M, respectively. The dissociation constant for compactin is close to the values of inhibition constants previously determined for the rat liver enzyme, $(1.4–10) \times 10^{-9}$ M (Endo et al., 1976; Alberts et al., 1980). This inhibition is surprising since the structures of these inhibitors only marginally resemble any of the substrates which participate in the normal catalytic reaction. In order to understand the basis of the strong binding of compactin and compound **3** to HMG-CoA reductase, we have performed experiments designed to define the domains of the substrate binding site(s) on HMG-CoA reductase with which compactin and compactin analogues interact. These experiments are largely based on the ability of single substrates (HMG-CoA, NADPH, or CoASH) to prevent the binding of compactin to HMG-CoA reductase. In separate experiments, it was established that these substrates bind to HMG-CoA reductase, and their binding constants were determined.

It was found that HMG-CoA or CoASH prevented the binding of compactin to HMG-CoA reductase, but NADPH had no effect (Figures 5–7). The binding of compound **3** was prevented by HMG-CoA but not by CoASH or NADPH. These results show that compactin interacts with the hydroxymethylglutarate domain and the CoASH domain of the active site, whereas compound **3** only interacts with the hydroxymethylglutarate domain of the active site.

Binding of compound **6**, which is 3 orders of magnitude lower than that of compactin, was investigated by measuring competition in initial velocity experiments. It was found that **6** inhibits HMG-CoA reduction competitively with HMG-CoA noncompetitively with NADPH. It was also established that **6** and CoASH can bind to the enzyme simultaneously. It was concluded that compound **6** only interacts with the hydroxymethylglutarate domain of the active site.

We also measured the interaction of the upper portion of **3** and compactin and the lower portion of compactin with HMG-CoA reductase. The inhibition constants of DL-mevalonate and DL-3,5-dihydroxyvalerate (determined from inhibition of HMG-CoA reductase vs. HMG-CoA in initial velocity experiments) were found to be 11 ± 1 mM and 33 ± 6 mM, respectively. These values are consistent with dissociation constants obtained by protection of HMG-CoA reductase from inactivation by iodoacetic acid, 4 ± 2 mM and 21 ± 9 mM, respectively. These compounds resemble the upper portion of compactin and compound **3**. No inhibition was detected with compounds **4** and **5** which resemble the lower portion of compactin.[5] Simultaneous addition of these

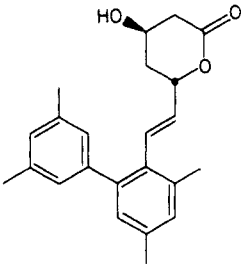
[4] NADPH did not consistently protect enzyme from inactivation by iodoacetic acid. When protection was observed, the second-order rate constant for the inactivation of the E·NADPH complex was 12 ± 2 M^{-1} s^{-1}, and the K_D for NADPH was 10 ± 1 μM. The ability of NADPH to prevent inactivation of enzyme by iodoacetic acid appeared to decrease with increasing time after enzyme purification. No other effect of age of enzyme was observed; e.g., the K_M for NADPH did not change. Enzyme that did not exhibit NADPH protection from iodoacetic acid inactivation was protected from inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) with a $K_D \leq 160$ μM (data not shown).

compounds with DL-3,5-dihydroxyvalerate did not enhance inhibition over that observed in the presence of DL-3,5-dihydroxyvalerate alone.

The dissociation constant for compactin is 8 orders of magnitude lower than that for DL-3,5-dihydroxyvalerate. Clearly, the lower portion of compactin (and that of compound **3**) must make a large contribution to the binding interaction of these compounds to HMG-CoA reductase. Furthermore, the fact that the upper and lower portions are covalently connected must be essential for the strong interaction of compactin and compound **3** with the enzyme. We propose that the upper portion of compactin and compound **3** bind at the hydroxymethylglutarate domain and the lower portion binds at a hydrophobic region located near the active site. Since the lower portion of compactin is larger than that of **3**, it overlaps with the CoASH domain, although interaction with that domain may not contribute appreciably to binding. The interaction of compound **3** with HMG-CoA reductase is as strong as the interaction of compactin with the enzyme although compound **3** does not interact with the CoASH binding site. The proposed mode of binding of these inhibitors is consistent with the results which we have obtained: HMG-CoA competes with compactin and **3**: CoASH competes only with compactin, while NADPH has no effect on the binding of these compounds.

Inhibition of HMG-CoA reductase by **6** is consistent with the proposed binding mode. The lower affinity of this compound for HMG-CoA reductase may be due to the structure of the lower portion and/or to the fact that the presence of the double bond may freeze the lower portion into a less favorable interaction with the hydrophobic region.

The hydrophobic region can accommodate a range of structures as evidenced by the fact that compactin and **3** bind equally well to HMG-CoA reductase, although the lower portions are quite different. A number of substituted biphenyls connected to the "top" piece of compactin such as **7** exhibit

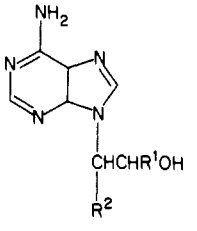


7

inhibitory activities approaching but not significantly surpassing that of compactin (Willard et al., 1983). The interaction between the hydrophobic "pocket" and compactin shows less demanding structural requirements than the interaction between the enzyme and substrate. The interaction between the decalin portion of compactin and the hydrophobic pocket may be equivalent to a number of interactions of low affinity rather than a single interaction of higher affinity (Jencks, 1980). It is apparent, however, that the hydrophobic region shows some specificity since substitution of naphthyl or phenyl for the lower portion produces inhibitors with dissociation constants at least 3 orders of magnitude higher than that of compactin.

We believe that the high affinity of compactin and **3** for HMG-CoA reductase is due to simultaneous interaction at two separate binding areas: the hydroxymethylglutarate domain and the hydrophobic region. It has been pointed out that a compound which interacts simultaneously with two binding sites of an enzyme can show a very high binding constant (Jencks, 1981). If one represents the compound as A–B and the separate components as A and B, then the binding "advantage" in connecting A and B is equivalent to the ratio of dissociation constants, $(K_{D\text{-}A}K_{D\text{-}B})/K_{D\text{-}A\text{-}B}$. This advantage can be as high as 10^8 M. It is possible that compounds **4** and **5** bind to HMG-CoA reductase without affecting catalytic efficiency; however, it is more likely that the absence of observed inhibition by these compounds is due to their limited solubility. A lower limit for their dissociation constants may be conservatively estimated to be 0.5 mM. The advantage of connecting the lactone and decalin portions of compactin together is then $\geq 5 \times 10^4$ M $[(0.5 \times 10^{-3})(21 \times 10^{-3})/(0.24 \times 10^{-9})]$. The number 10^8 M represents a maximum value that would be observed if binding portion A (or B) of the compound so restricted portion B (or A) that portion B "fit" on the enzyme without further loss of entropy. This is an improbable situation that would not be expected to be observed.

According to our interpretation the interaction of compactin and **3** with the hydrophobic region (hydrophobic anchor) is important in the binding of these inhibitors to HMG-CoA reductase. The participation of "hydrophobic anchors" in the binding of several inhibitors has been demonstrated. For example, derivatives of pyrazole with *n*-alkyl substituents in the 4-positions are potent inhibitors of liver alcohol dehydrogenase [see Eklund et al. (1982) and references therein]. The affinity of the substituted pyrazole for liver alcohol dehydrogenase increases with increasing chain length. The ratio $K_{\text{i-pyrazole}}/K_{\text{i-4-(}n\text{-heptyl)pyrazole}}$ is >730. The structure of liver alcohol dehydrogenase, derived from X-ray crystallography, shows a hydrophobic channel near the active site which accommodates linear but not branched or bulky substituents on position 4 of pyrazole. Another example of the participation of a hydrophobic anchor in the efficacy of an inhibitor is provided by the inhibition of calf intestinal mucosal adenosine deaminase by 9-(1-hydroxy-2-alkyl)adenines (Schaeffer & Schwender, 1971). The affinity of these inhibitors (**8**) for this



8

enzyme also increases with increasing chain length (R^1 = H, R^2 = methyl to *n*-nonyl). The ratio of inhibition observed for ($R^1 = R^2$ = H)/(R^1 = H, R^2 = n-nonyl) is 230. Clearly, the hydrophobic moiety does not interact with the ribose binding site. We suggest that it interacts with a hydrophobic region near the active site. 9-(*erythro*-2-Hydroxy-3-nonyl)adenine (EHNA) (**8**; $R_1 = CH_3$; R_2 = *n*-hexyl) inhibits adenosine deaminase from mammalian and avian sources but does not inhibit the enzyme from *Plasmodium* (Schaeffer & Schwender, 1974; Schimandle & Sherman, 1983). This suggests that the environment in the vicinity of the active site differs in these enzymes. The enzymes that are not subject to inhibition by EHNA do not have a hydrophobic region near the active site which interacts with EHNA. In contrast, the transition-state analogue coformycin inhibits the enzyme from all sources (Sawa et al., 1967; Snyder & Henderson, 1973; Schimandle

[5] No significant inhibition was observed by compound **4** in a rat microsomal system (Heathcock et al., 1982).

& Sherman, 1983). Its efficacy is based on the structure of the active site which would be expected to be similar for all enzymes that deaminate adenosine by the same mechanism.

The utilization of hydrophobic anchors should be useful in the design of enzyme inhibitors. The deliberate design of inhibitors that bridge a portion of the active site and a hydrophobic region should be possible since the three-dimensional structure from X-ray crystallography is now available for many enzymes. The design of inhibitors that bridge domains of the active site and a hydrophobic region is less formidable than the design of inhibitors that bridge two domains of the active site. As demonstrated above, the specificity requirement for interactions with a hydrophobic region is less severe than for interactions with an active site domain.

In the course of this work we have also determined the rate constant for the dissociation of compactin from the enzyme–compactin complex to be 6.5×10^{-3} s^{-1} and the rate constant for the association of compactin and enzyme to be 2.7×10^7 M^{-1} s^{-1}. The rate constant for the association of HMG-CoA and enzyme is 1.9×10^5 M^{-1} s^{-1}. Remarkable is the fact that the on rate for compactin is nearly diffusion controlled and is over 100-fold faster than the on rate for HMG-CoA. This suggests that compactin is so "designed" that it fits readily into the active site of HMG-CoA reductase. Interaction of HMG-CoA and enzyme requires that either the enzyme, the substrate, or both must undergo a conformational change in order to form a productive complex, hence, the slower on rate.

We have also determined the rate constant for the dissociation of HMG-CoA from the E·HMG-CoA complex to be approximately 0.1 s^{-1}. The value of this rate constant, together with the data of Qureshi et al. (1976b), allows the determination of the relative rates for the dissociation of substrates from the E·HMG-CoA·NADPH complex. The reduction of HMG-CoA to mevalonic acid proceeds via an intermediate at the oxidation state of an aldehyde. The overall reduction of HMG-CoA to mevalonic acid is irreversible; however, HMG-CoA reductase catalyzes a reverse half-reaction, the oxidation of the aldehyde mevaldic acid ($CHOCH_2$–COH-CH_3–CH_2COOH) plus CoASH to HMG-CoA. If an E·HMG-CoA complex is an obligatory intermediate in the reverse half-reaction, the rate constant for the dissociation of HMG-CoA from the E·HMG-CoA complex must be equal or greater than k_{cat} for the reverse half-reaction. Qureshi et al. (1976b) have reported V_{max} for the oxidation of mevaldic acid plus CoASH to HMG-CoA. Using this V_{max} value, we calculate k_{cat} for the oxidation of mevaldic acid plus CoASH to be approximately 5 s^{-1}.[6] The k_{cat} for the reverse half-reaction is 50 times faster than the rate constant for the release of HMG-CoA from E·HMG-CoA. This indicates that in the reverse half-reaction essentially no E·HMG-CoA is formed; therefore, release of product is ordered. HMG-CoA must dissociate from E·HMG-CoA·NADPH significantly faster than NADPH can dissociate.

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APPENDIX

The system illustrated in eq A1 is described by eq A2–A5.

$$EC \underset{k_4}{\overset{k_3[C]}{\rightleftharpoons}} E \underset{k_2}{\overset{k_1[S]}{\rightleftharpoons}} ES \quad \text{(A1)}$$

$$d[E]/dt = k_2[ES] + k_4[EC] - (k_1[S] + k_3[C])[E] \quad \text{(A2a)}$$

$$d[ES]/dt = k_1[S][E] - k_2[ES] \quad \text{(A3)}$$

$$d[EC]/dt = k_3[C][E] - k_4[EC] \quad \text{(A4)}$$

$$[E_{total}] = [E] + [ES] + [EC] \quad \text{(A5a)}$$

Steady-State Assumption. If free enzyme reaches a low steady-state concentration after the burst

$$d[E]/dt = 0 \quad \text{(A2b)}$$

and

$$[E_{total}] = [ES] + [EC] \quad \text{(A5b)}$$

Combination of eq A2a,b and A5b yields an expression for [E] which substituted into eq A4 gives eq A6a,b. Rearrangement and integration of eq A6b between the limits t and t^* (where t^* represents a time immediately after the burst) gives eq A7. $[EC]_{fast}$ is the amount of EC that is formed during the burst. At infinite time, $[EC] = [EC]_{eq}$, where $[EC]_{eq}$ is the concentration of EC at equilibrium. From eq A7, at $t = \infty$

$$\frac{d[EC]}{dt} = \frac{k_2k_3[C][E_t]}{k_1[S] + k_3[C]} - \left[\frac{k_3(k_2 - k_4)[C]}{k_1[S] + k_3[C]} + k_4\right][EC] \quad \text{(A6a)}$$

$$d[EC]/dt = a - b[EC] \quad \text{(A6b)}$$

$$\ln \frac{a - b[EC]}{a - b[EC]_{fast}} = -bt \quad \text{(A7)}$$

$$[EC]_{eq} = a/b \quad \text{(A8)}$$

Combination of eq A5b, A7, and A8 gives

$$\ln ([ES] - [ES_{eq}]) = \ln ([EC_{eq}] - [EC]_{fast}) - bt \quad \text{(A9)}$$

Equation A9 is a description of the semilog plot given in Figure 3B of the text where $k_{obsd} = b$.

$$k_{obsd} = \frac{k_3(k_2 - k_4)[C]}{k_1[S] + k_3[C]} + k_4$$

$$k_{obsd} = \frac{k_3K_{DS}[C]/[S] + k_4}{1 + k_3[C]/k_1[S]} \quad \text{(A10)}$$

Dividing the two terms in the numerator of eq A10 by k_3 gives an expression containing dissociation constants, K_{DC} and K_{DS}, and concentrations, S and C, that allows the relative contribution of the two terms on k_{obsd} to be assessed. At the lowest concentration of compacin used in our experiments, the k_4 term contributes equally to the numerator of eq A10. At the highest

[6] Qureshi et al. (1976b) have reported V_{max} for the oxidation of mevaldic acid + CoA to be 236 ± 5 μmol of NADP reduced min^{-1} (mg of protein)$^{-1}$ and V_{max} for the reduction of HMG-CoA to mevalonic acid to be 2331 ± 40 μmol of NADPH oxidized min^{-1} (mg of protein)$^{-1}$. The experiments from which the V_{max} values were obtained were performed with a partially purified enzyme preparation. The specific activity of this enzyme preparation was not stated. We have assumed that the specific activity of the enzyme preparation was 1.86 μmol of NADPH oxidized min^{-1} (mg of protein)$^{-1}$; this value was obtained from Qureshi et al. (1976a) which was copublished with the above reference. We have calculated k_{cat} for these reactions using a molecular weight for HMG-CoA reductase of 2.6×10^5 (Qureshi et al., 1976a) and a specific activity for the reduction of HMG-CoA to mevalonic acid of 10 μmol of NADPH oxidized/min^{-1} (mg of pure enzyme)$^{-1}$ (Veloso et al., 1981). We calculate k_{cat} for the oxidation of mevaldic acid + CoA to be approximately 5 s^{-1} and k_{cat} for the reduction of HMG-CoA to mevalonic acid to be approximately 50 (NADPH oxidized) or 25 s^{-1} (mevalonate formed). The latter value is consistent with our observations (data not shown).

concentration of compactin, the k_4 term represents only 10% of the numerator. To a first approximation, the rate constant k_4 may be dropped from the numerator of eq A10, and rearrangement yields

$$\frac{1}{k_{obsd}} = \frac{1}{k_2} + \frac{[S]}{k_3 K_{DS}} \frac{1}{C]} \quad \text{(A11)}$$

The values of k_3 and k_2 can be estimated from the slope and intercept of a plot of $1/k_{obsd}$ vs. $1/[C]$ according to eq A11. This approximation is subject to error when (1) the rate constant k_4 contributes to the expression for k_{obsd} and (2) the concentration of free compactin changes significantly during the course of the experiment.

Integration of the Rate Equation d[EC]/dt. Equation A12 is obtained from eq A2–A5:

$$\frac{[EC]}{[E_t]} = \frac{\alpha}{\beta} + \frac{\alpha M e^{-0.5(\gamma-\delta)t}}{\delta} - \frac{\alpha N e^{-0.5(\gamma+\delta)t}}{\delta} \quad \text{(A12)}$$

where

$$\alpha = k_2 k_3 [C]$$

$$\beta = k_1 k_4 [S] + k_2 k_4 + k_2 k_3 [C]$$

$$\gamma = k_1 [S] + k_2 + k_3 [C] + k_4$$

$$\delta = \sqrt{\gamma^2 - 4\beta}$$

Equation A12 describes the fraction of enzyme present as EC as a function of time where α/β represents the equilibrium value, $\alpha M/\delta = EC_{slow}$, and $\alpha N/\delta = EC_{fast}$ (see text). The values of M and N are determined by the initial conditions of the reaction as follows. The initial conditions are given in eq A13 and A14.

at $t = 0$

$$[EC] = 0 \quad \text{(A13)}$$

at $t \rightarrow 0$

$$d[EC]/dt = k_3 [C][E_t] \quad \text{(A14)}$$

Equations A12 and A13 and eq A12 and A14 require that

$$\delta/\beta = N - M \quad \text{(A15)}$$

$$k_3 [C] = \frac{\alpha}{2\delta} N(\gamma + \delta) - M(\gamma - \delta) \quad \text{(A16)}$$

Solving eq A15 and A16 gives

$$M = \frac{k_3 [C]\delta}{\alpha\gamma} - \frac{\delta(\gamma + \delta)}{2\beta\gamma} \quad \text{(A17)}$$

$$N = \frac{k_3 [C]}{\alpha} - \frac{\delta - \gamma}{2\beta} \quad \text{(A18)}$$

An equation analogous to eq A12 has previously been reported (Lowry & John, 1910).

References

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-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., & Springer, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3957.

Brown, A. G., Smale, T. C., King, T. J., Hasenkamp, R., & Thompson, R. H. (1976) *J. Chem. Soc., Perkin Trans. 1*, 1165.

Cha, S. (1975) *Biochem. Pharmacol. 24*, 2177.

Chou, T. C., & Talalay, P. (1981) *Eur. J. Biochem. 115*, 207.

Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 173.

Durr, I. F., & Rudney, H. (1960) *J. Biol. Chem. 235*, 2572.

Eklund, H., Samama, J. P. & Wallen, L. (1982) *Biochemistry 21*, 4858.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

Endo, A., Kuroda, M., & Tanzawa, K. (1976) *FEBS Lett. 72*, 323.

Gilbert, H. F., & Stewart, M. D. (1981) *J. Biol. Chem. 256*, 1782.

Goldstein, J. L., & Brown, M. S. (1977) *Annu. Rev. Biochem. 46*, 897.

Heathcock, C. H., Taschner, M. J., Rosen, T., Thomas, J. A., Hadley, C. R., & Popjak, G. (1982) *Tetrahedron Lett. 23*, 4747.

Horecker, B. L., & Kornberg, A. K. (1948) *J. Biol. Chem. 175*, 385.

Jencks, W. P. (1980) *Mol. Biol. Biochem. Biophys. 32*, 3–25.

Jencks, W. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4046.

Kirtley, M. E., & Rudney, H. (1967) *Biochemistry 6*, 230.

Louw, A. I., Bekersky, I., & Mosbach, E. H. (1969) *J. Lipid Res. 10*, 683.

Lowry, M. T., & John, W. T. (1910) *J. Chem. Soc., Trans. 97*, 2634.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. T. (1951) *J. Biol. Chem. 193*, 265.

Mabuchi, H., Haba, R., Tatami, R., Miyamoto, S., Sakai, Y., Wakasugi, T., Watanabe, A., Koizumi, J., & Takeda, R. (1981) *N. Engl. J. Med. 305*, 478.

Morris, J. G., & Redfearn, E. R. (1978) in *Data for Biochemical Research* (Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M., Eds.) p 194, Oxford University Press, Oxford.

Qureshi, N., Dugan, R. E., Nimmannit, S. Wu, W. H., & Porter, J. W. (1976a) *Biochemistry 15*, 4185.

Qureshi, N., Dugan, R. E., Cleland, W. W., & Porter, J. W. (1976b) *Biochemistry 15*, 4191.

Sawa, T., Fukugawa, Y., Homma, I., Takeuchi, T., & Umezawa, H. (1967) *J. Antibiot., Ser. A 20*, 227.

Schaeffer, H. J., & Schwender, C. F. (1971) *J. Pharm. Sci. 60*, 1204.

Schaeffer, H. J., & Schwender, C. F. (1974) *J. Med. Chem. 17*, 6.

Schimandle, C. M., & Sherman, I. W. (1983) *Biochem. Pharmacol. 32*, 115.

Shapiro, D. J., Imblum, R. L., & Rodwell, V. W. (1969) *Anal. Biochem. 31*, 383.

Snyder, F. F., & Henderson, J. F. (1973) *J. Biol. Chem. 248*, 5899.

Stadtman, E. R. (1957) *Methods Enzymol. 3*, 931.

Veloso, D., Cleland, W. W., & Porter, J. W. (1981) *Biochemistry 20*, 887.

Willard, A. K., Cragoe, E. J., Jr., Novello, F. C., & Hoffman, W. F. (1983) U.S. Patent 4 375 475.

Yamamoto, A., Sudo, H., & Endo, A. (1980) *Atherosclerosis (Shannon, Irel.) 35*, 259.