Slow Binding Inhibition of 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase

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ABSTRACT: The mechanism of slow binding inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by lovastatin, fluindostatin, and related compounds was studied. Several of these compounds, including lovastatin, were found to be slow binding, while other less potent inhibitors were not. From a comparison of kinetic parameters obtained by steady-state measurements and progress curve analysis, it was concluded that the slow binding inhibitors bind by a mechanism which is more accurately described by biphasic binding than by single-step binding. The overall association rates of the slow binding inhibitors range from 1×10^6 to 4×10^7 M^{-1} s^{-1}, and the dissociation rates are in the range of 10^{-3} s^{-1}. The structures of slow binding and reversible inhibitors were compared by using molecular modeling methods. From these comparisons, it was proposed that the slow binding and very potent inhibition of, for instance, lovastatin, is not simply a result of binding of a transition state or reaction intermediate analogue. The various lipophilic groups of the inhibitors that do not seem to be related to structural features of the substrate may also play a crucial role in determining the mechanism of binding of HMGR inhibitors.

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR)[1] catalyzes the NADPH-dependent reduction of HMG-CoA to mevalonate. This reaction is the committed step in cholesterol biosynthesis and is a target for intervention in the treatment of hypercholesterolemia (Illingworth, 1987; McKenney, 1988). Several compounds such as lovastatin (Alberts et al., 1980; Alberts, 1988), pravastatin (Tsujita et al., 1986), and fluindostatin (XU 62-320) (Tobert, 1987) have been identified as extremely potent inhibitors of HMGR (Lee, 1987), but the mechanism of enzyme inhibition of these compounds has not been characterized in detail. An inhibitor closely related to lovastatin, compactin (mevastatin), has been identified as a slow binding inhibitor of yeast HMGR (Nakamura & Abeles, 1985). Compactin is not as potent an inhibitor of the mammalian enzymes and has not been investigated as a slow binding inhibitor of these enzymes. All of these inhibitors are believed to bind at the HMG-CoA binding site, although the only apparent resemblance of the inhibitor structure to that of the substrate is with the HMG portion of HMG-CoA. It has been proposed (Nakamura & Abeles, 1985) that the lipophilic portion of these inhibitors occupies a lipophilic binding site adjacent to, but not necessarily integral to, the active site and that occupancy of this site contributes to the extremely potent inhibition.

We compare here the binding poperties of a number of HMGR inhibitors with different lipophilic moieties. The compounds selected provide a structural comparison of inhibitors that exhibit slow binding with inhibitors which do not. Since elucidation of the kinetic details of slow binding can provide an improved understanding of the mode of interaction of inhibitor and enzyme, several of the compounds that exhibited slow binding were studied to determine a mechanism for binding. For instance, it has been proposed that the tight complexes formed by some slow binding inhibitors are analogous to transition-state or reaction intermediate complexes (Morrison, 1982; Morrison & Walsh, 1987; Schloss, 1988a). Many of these inhibitors bind by formation of an initial complex, followed by conversion to a complex in which the inhibitor is more tightly bound. In these cases, the appearance of slow binding in progress curves of the enzyme reaction can result from a slow rate of complex formation or slow dissociation from the final complex. From studies of the mechanism of slow binding inhibition of HMGR, we can propose a more detailed pathway for inhibition of the enzyme. By use of molecular modeling methods, structures which exhibit slow binding were compared with those that do not, providing a better understanding of the structural features which are important in slow binding and for potent inhibition.

Materials and Methods

Compounds. Lovastatin was obtained from Merck and Co., and compounds **2**–**8** were synthesized according to the following references: compound **2**, Kathawala (1984); compound **4**, Stokker et al. (1986a); compounds **7** and **8**, Hoffman et al. (1986b). The synthesis of compounds **3**, **5**, and **6** will be described.[2] Lovastatin was used as the active dihydroxy acid form, compound **1**, obtained by hydrolysis of the lactone in 0.1 N NaOH.

Enzyme Assays. A catalytic fragment of human HMGR, specifically HMGR-58, was prepared as previously described (Mayer et al., 1988). The enzyme used in these experiments was purified through the Blue–Sepharose step described in the reference. Because this enzyme exhibits some cold lability, the enzyme was allowed to stand at room temperature for at least 0.5 h before assays were begun to ensure full activity. HMGR was assayed continuously by monitoring the decrease in absorbance at 340 nm due to NADPH oxidation or at a

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[1] Abbreviations: HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; HMG-CoA, (*R*,*S*)-3-hydroxy-3-methylglutaryl-coenzyme A; HMG, 3-hydroxy-3-methylglutaryl; DTT, dithiothreitol.

[2] J. D. Elliott, G. Dreyer, D. L. Bryan, and J. Leber, manuscript in preparation.

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fixed time by using the radiometric assay as described (Edwards et al., 1979; Mayer et al., 1988). The assay buffer contained 100 mM potassium phosphate, pH 7.2, 100 mM KCl, 1 mM EDTA, and 10 mM DTT. Except as noted, the concentrations of NADPH and HMG-CoA were 200 and 10 μM, respectively. HMG-CoA concentration was determined from the ratio of absorbance at 232 and 260 nm, and the K_m for HMG-CoA was taken to be 1.5 μM throughout. In the spectrophotometric assays, the enzyme concentration was 2 nM, calculated from the specific activity of the preparation, and the total reaction volume was 1 mL. The reaction was initiated by the addition of either enzyme or HMG-CoA after a 10-min preincubation at 37 °C of the remaining components of the assay. Initial velocities for each assay were determined by taking slopes as close as possible to the beginning of the reaction. In the radiochemical assay, the enzyme concentration was 30 pM in a final volume of 300 μL. The reaction was routinely initiated by the addition of NADPH after a 10-min preincubation to the otherwise complete reaction mixture, except as noted, and was routinely run for 20 min. Data analysis of the steady-state measurements was by either Dixon analysis (Dixon, 1953) or double-reciprocal plots (Cleland, 1979).

Progress Curves. Progress curves were measured under the same conditions as the spectrophotometric assay described above with enzyme added last to the remaining components and with substrate and inhibitor concentrations as indicated in the figure legends or in the text. Data were obtained on a Perkin-Elmer λ-4C spectrophotometer and digitally recorded and stored. Data points (200) for each curve were transferred to a VAX system for analysis. Because the curves were obtained as disappearance of cofactor rather than product appearance, the data were inverted to correspond to product formation so that the analysis for product formation previously described (Morrison & Walsh, 1987; Schloss, 1988b) could be used directly. Duplicate curves were averaged and fit to

$$P = v_f t + [(v_i - v_f)/k](1 - e^{-kt}) \quad (1)$$

and values for the parameters v_i (initial velocity), v_f (final velocity), and k (exponential rate constant) were obtained. Control curves (no inhibitor) at every substrate concentration were fit to a simple exponential with rate constant k'; no progress curves were used in the subsequent analysis of k for which k was not at least 8-fold larger than k'. For sets of progress curves that were analyzed as a function of substrate concentration, the value of k' was found to be a constant percentage (4%) of k over the substrate range used. Consequently, no attempt was made to correct any progress curves for lack of linearity in the controls.[3] The experimentally derived values for v_i, v_f, and k were analyzed further according to the equations for both a one-step (eqs 2 and 3) and a

$$v_i = VS/(K_m + S) \quad (2)$$

$$k = k_{off} + k_{on}[I/(1 + S/K_m)] \quad (3)$$

$$v_i = VS/[K_m(1 + I/K_i) + S] \quad (4)$$

$$k = k_4 + k_3[(I/K_i)/(1 + I/K_i + S/K_m)] \quad (5)$$

[3] The reason for the lack of linearity in the assay is unclear, but it is observed with the yeast and rat liver solubilized enzymes as well as the human enzyme. A number of experiments suggest that the curvature is dependent on time, but not number of turnovers, for a given substrate concentration and is not due simply to substrate depletion. Because the error introduced by not correcting for this curvature was small compared to other errors, data analysis was simplified by treating the controls as if they were linear.

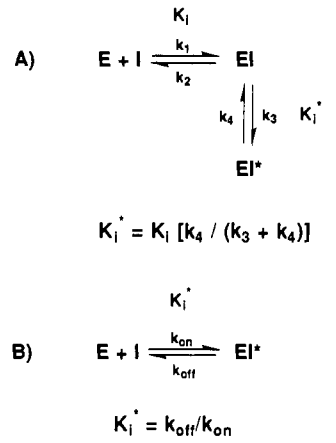

FIGURE 1: Possible mechanisms of slow binding inhibition. A two-step mechanism for binding is shown in (A), and a one-step mechanism is shown in (B).

Table I: Inhibition Constants for Compounds **1**–**8** Obtained by Dixon Analysis

compd	$K_{i,app}$[a] (nM)	K_i^*[b] (nM)
1	ND[c]	0.2
2	12	0.3
3	30	2.3
4	ND[c]	0.5
5	26	3.5
6	14	2.3
7	2.6	3.6
8	47	42

[a] Value obtained by Dixon analysis using a spectrophotometric assay where enzyme was added last; values are reproducible to ±25% in duplicate determinations. [b] Value obtained by Dixon analysis for K_i^* as defined in Figure 1 using a radiometric assay, a radiometric assay and a spectrophotometric assay where substrate was added last (compounds **6** and **7**), and a spectrophotometric assay only (compound **8**); values are reproducible to ±15% in duplicate determinations. [c] ND, not determined.

two-step (eqs 4 and 5) binding process as described in the references. Nonlinear least-squares fitting was done by using the program Superfit, based on the SAS software package from SAS Institute, Inc. (Marquardt, 1963). The rate constants and equilibrium constants in eqs 2–5 are defined in Figure 1, v_i and k derive from eq 1, S is the concentration of HMG-CoA, K_m is the Michaelis constant for HMG-CoA, V is the maximal velocity, and I is the concentration of inhibitor. As indicated in Figure 1, K_i^* is defined as the equilibrium constant for the formation of the final complex within either a one-step or a two-step mechanism, even though the mathematical representation is not the same in the two mechanisms.

Molecular Modeling. The model for the structure of HMG-CoA was taken from the coordinates of the CoA in the crystal structures of citrate synthase (Remington et al., 1982; Brookhaven Protein Data Bank, entry 2CTS and 3CTS; Bernstein et al., 1977), and the remaining atoms were added on the basis of the position of citrate in the same structures. A starting structure for compound **1** was established from the reported crystal structure of 6(*S*)-*epi*-mevinolin (Stokker et al., 1986b) and subsequently modified to the dihydroxy acid form by using the molecular modeling program MacroModel[4] and its implementation of the MM2 force field (Allinger, 1977). The remaining structures were fully generated by using

[4] W. C. Still, F. Mohamadi, N. G. J. Richards, W. C. Guida, R. Liskamp, M. Lipton, C. Caufield, G. Chang, and T. Hendrickson, MacroModel V1.5, Department of Chemistry, Columbia University, New York, NY 10027.

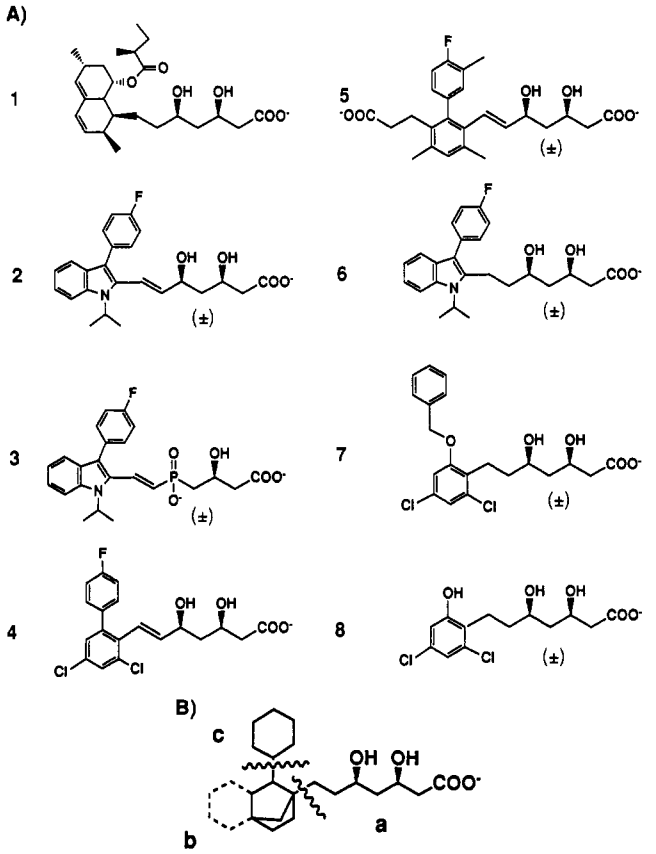

FIGURE 2: (A) Structures of the compounds referred to in the text. Compound **1** is the dihydroxy acid form of lovastatin, and compound **2** is also referred to as fluindostatin or XU 62-320. The absolute stereochemistry of compounds **1** and **4** is as shown, and the remaining compounds are racemic (±). The relative stereochemistry of diols **2** and **5–8** is syn as shown. (B) Schematic representation of compounds **1–8**, indicating different domains of the molecules. Part a is referred to in the text as the dihydroxy acid portion, part b as the proximal part of the lipophilic group, and part c as the distal part of the lipophilic group. In compound **1**, the ester attached to the decalin ring is considered to be in part c.

the MacroModel program. The structures of compound **1** and the HMG-CoA model were compared visually by using the hydroxyl and carboxylic acid functionalities as the initial overlapping points. The overlays of compounds **1**, **2**, **4** and **7** were obtained similarly.

Results

Inhibition Constants. The inhibitors shown in Figure 2 represent a variety of structural groups known to inhibit HMGR, as well as some variations on these known structures, such as compounds **3** and **5**. The inhibition of HMGR by compounds **1–8** was studied by a combination of methods. The inhibition constant for the tight complex (K_i*, Figure 1) was determined for compounds **1–8** as indicated in Table I. By Dixon analysis, there was no difference in the inhibition constant obtained from the radiometric assay when initiated with enzyme or with substrate following a 10-min preincubation of the remaining components of the assay. Several of the inhibitors (**1**, **2**, **3**, **7**) were also shown to be competitive with respect to HMG-CoA under the conditions of the radiometric assay (data not shown). Estimates of the inhibition constants for the initial complex ($K_{i,app}$, by analogy to K_i, Figure 1) were determined by Dixon analysis of spectrophotometric assays in which enzyme was added last to initiate the assay. For those compounds where both values were determined and were significantly different, the ratio of $K_{i,app}$ to K_i* is approximately an order of magnitude. Compounds **7** and **8** showed no difference in the value of $K_{i,app}$ and K_i* and were assumed to bind in rapid equilibrium, while the remaining compounds were assumed to be slow binding. There is no clear relationship between the absolute value of K_i* and observation of slow binding, as, for example, comparing the results for compounds **3** and **7**. However, all of the inhibitors with K_i* values of less than 1 nM do appear to be slow binding (i.e., K_i* < $K_{i,app}$ in Table I).

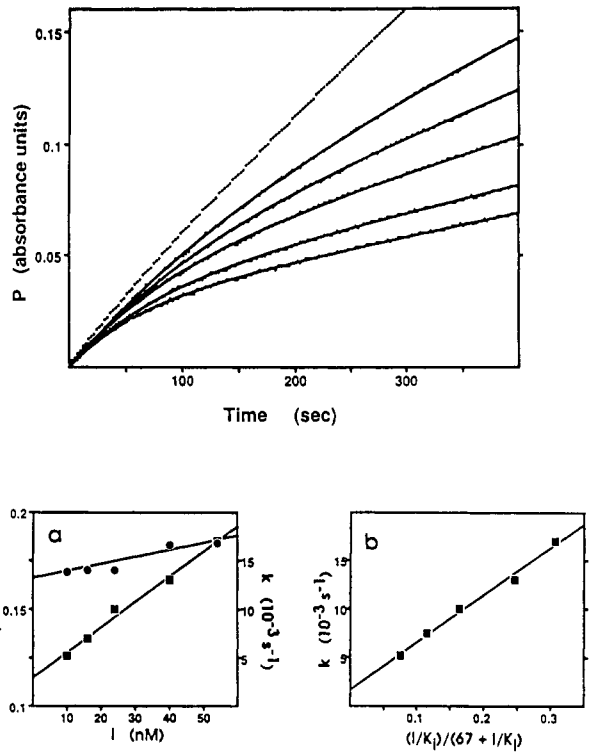

FIGURE 3: (Upper panel) Progress curves for the binding of compound **1** to HMGR at 100 μM HMG-CoA. The top curve, represented only by data points, is a control curve with no inhibitor. The remaining curves, from top to bottom, are for inhibitor concentrations of 10, 16, 24, 40, and 54 nM, respectively. The solid lines through the points represent the fitted curve according to eq 1 as described under Materials and Methods. (Lower panels) Plots of parameters from eq 1 according to the two pathways in Figure 1. (Panel a) Plot for k for analysis according to pathway B (■); plot of $1/v_i$ as a function of inhibitor concentration (●) for compound **1**. (Panel b) Plot of k for analysis according to pathway A for compound **1**.

Progress Curves. The slow binding aspect of some of these inhibitors was also manifest as a decrease in velocity with time in the progress curves. This slow binding phenomenon was investigated in more detail by analysis of the progress curves. An example of the progress curves obtained over appropriate ranges of inhibitor and substrate is given in Figure 3 for the binding of compound **1**. The concentrations of inhibitor and substrate were chosen to give large variations in the values of k which were also much different from the values of k' determined from the control traces. Inhibitor concentrations well above that of the enzyme were employed; high HMG-CoA concentrations were used to maintain the substrate concentration significantly above K_m throughout the time course of the progress curve, as well as to allow measurement of activity at the high inhibitor concentrations. A series of progress curves of similar appearance to those in Figure 3 was obtained for compounds **2–4** by using appropriate concentration ranges of inhibitor: 75–300 nM compound **2** at 100 μM HMG-CoA; 60–360 nM compound **3** at 50 μM HMG-CoA; and 10–75 nM compound **4** at 50 μM HMG-CoA. From these curves, values of v_i, v_f, and k were obtained (eq 1); these data were further analyzed by the equations describing a one- or two-step

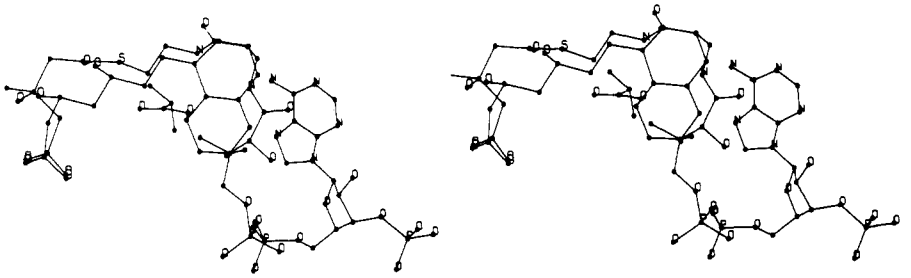

FIGURE 4: Comparison of the modeled structures of compound **1** and HMG-CoA. Structures were obtained as described under Materials and Methods.

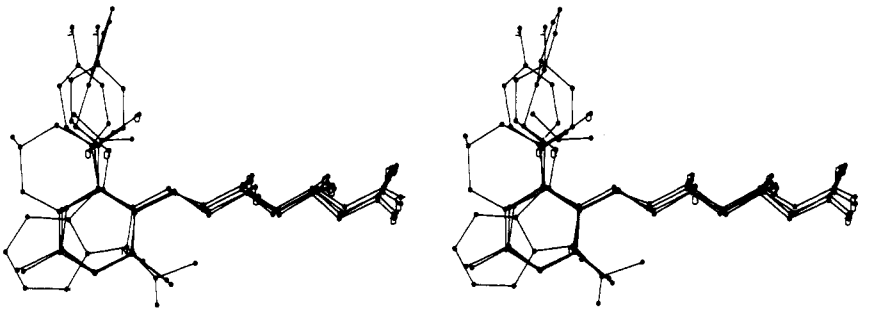

FIGURE 5: Comparison of modeled structures of compounds **1**, **2**, **4**, and **7**. The dihydroxy acid moiety and proximal part of the lipophilic group are overlaid as closely as possible, with the distal part of the lipophilic group taking an orientation allowed by the structure of the compound.

process. An example of this analysis is shown in Figure 3 for compound **1**. All of the plots of k vs [I] fit equally well to a linear model or to a hyperbolic model and appear to be linear. All of the values for rate constants obtained from this analysis with compounds **1**–**4** are summarized in Table II.

It can be seen that the rates of association with the enzyme (k_{on}) for all the compounds are relatively fast with either binding model, varying over an order of magnitude. The off-rates from the tight complex (k_{off}, k_4) are all similar, although the standard errors are relatively large. Under conditions where the concentration of inhibitor dissociating from the EI* complex was much less than K_i*, it was not possible to accurately measure the enzyme activity. Therefore, it was not possible to measure the off-rates directly by observing the regain of activity from the EI* complex. The results obtained from plots using the v_f values were discarded as the standard errors for v_f, and consequently for all results derived from v_f, were much larger than those for any other parameter.

A similar analysis was performed with a set of progress curves obtained at a constant concentration of compound **3** (500 nM) while the HMG-CoA concentration was varied (30–400 μM). The results were comparable to those in Figure 3 and yielded rate constants that were similar to those from the previous experiment as shown in Table II. The agreement of results from the two data sets supports the validity of the assumptions made in the calculations in each experiment.

Molecular Modeling. A model for the conformation of enzyme-bound HMG-CoA was derived from crystal structures of citrate synthase containing CoA and citrate. In this analogy, it is assumed that the HMG moiety of the HMG-CoA would occupy roughly the same relative position as the citrate. The transformation of citrate into HMG was accomplished with minimal distortion of the coordinates of several atoms conserved between the structure and the model. Figure 4 shows the three-dimensional model of HMG-CoA together with that of compound **1** plotted as a stereo pair. The overlay represents simply a visual orientation of the compound on the model, using the hydroxyl and carboxylic acid groups as the coincident points. This orientation places the decalin ring system over

Table II: Rate Constants and Inhibition Constants Derived from Progress Curve Analysis

(A) Derivation According to Pathway A in Figure 1

compd	K_i (nM)	k_3 (s^{-1})	k_4 (10^{-3} s^{-1})[a]	k_{on} (10^6 M^{-1} s^{-1})	K_i* (nM)
1	1.8 (0.4)[b]	0.048 (0.003)	2 (0.7)	26 (6)	0.07 (0.03)
2	11 (1.8)	0.036 (0.006)	1 (0.4)	3.4 (0.6)	0.3 (0.1)
3	31 (9)	0.044 (0.01)	4 (2)	1.4 (0.5)	2.3 (1.4)
4	2.5 (0.3)	0.086 (0.007)	1 (0.8)	35 (6)	0.03 (0.02)
3[c]	14 (2)	0.027 (0.003)	4 (1)	1.9 (0.3)	2.1 (0.5)

(B) Derivation According to Pathway B in Figure 1

compd	k_{on} (10^6 M^{-1} s^{-1})	k_{off} (10^{-3} s^{-1})	K_i* (nM)
1	2.1 (0.4)	4 (1)	0.2 (0.06)
2	2.1 (0.4)	2 (0.4)	1 (0.2)
3	1.0 (0.3)	5 (2)	5 (1)
4	17 (0.5)	5 (0.3)	0.3 (0.03)
3[c]	0.62 (0.09)	7 (0.5)	11 (2)

[a] Units with exponents are as written. [b] Numbers in parentheses are the standard errors. [c] Results for compound **3** derived from the progress curves where the concentration of substrate, rather than inhibitor, is varied, as described in the text.

the pantotheinate section of CoA, which is of similar dimension. The unexpected fit of the decalin ring within the CoA structure with minimal modification of the original structure is the feature that makes this model interesting. The energy-minimized structures of the remaining inhibitors (represented by compounds **2**, **4**, and **7**) were overlaid on the structure of compound **1**, holding the structurally conserved carboxylic acid portion fixed while finding the best overlap of the lipophilic structures visually (Figure 5). While the decalin portion of compound **1** and the proximal part (see Figure 2) of the lipophilic group could be easily superimposed on the HMG-CoA model, the distal part of the lipophilic group did not necessarily fit within the bounds of the HMG-CoA structure. No attempt was made, therefore, to force a common orientation for these lipophilic portions of the inhibitors.

Discussion

Two principal mechanisms have been proposed to describe slow binding inhibition: binding of inhibitor in a single step with a slow rate of dissociation, as in Figure 1B, or binding

via an initial complex followed by conversion to a more tightly bound complex, either formed slowly or from which dissociation is slow, as in Figure 1A. Various physical interpretations of these mechanisms have been proposed (Morrison, 1982; Morrison & Walsh, 1987; Erion & Walsh, 1987).

The values of $K_{i,app}$ and K_i^* presented in Table I were obtained by analogy with the two steps as shown in Figure 1A. The comparison of the values of $K_{i,app}$ and K_i^* in Table I is merely an indication of the observation that some of the inhibitors are slow binding ($K_i^* < K_{i,app}$) while others are not ($K_i^* = K_{i,app}$). The $K_{i,app}$ values are calculated by assuming that the velocity determined by taking the slope at the beginning of the reaction after enzyme is added to the assay is in fact the initial velocity or the velocity at the first part of the binding curve, not at some later point along the binding curve. They do not necessarily represent K_i (Figure 1), and these steady-state experiments do not prove that an initial reversible complex exists. The values for K_i^* represent the equilibrium constants in the final complex, regardless of the mechanism by which that complex is formed, because the enzyme and inhibitor are allowed to equilibrate for a length of time sufficient to form the final complex and substrate and inhibitor equilibrate over the time of the assay.

Further information about the slow binding can be obtained from analysis of the progress curves. As with the steady-state results, for an appropriate interpretation of the progress curve results we must consider whether the parameter defined as v_i is an approximation of the real initial velocity in the binding reaction, or whether inhibitor binding is so fast that the velocity which can be measured by manual mixing is not a reasonable approximation of this initial velocity. Considering the rates of association presented in Table II, it is clear that compounds **1** and **4** exhibit considerably faster association rates than compounds **2** and **3**. Using these k_{on} values to estimate the half-life for the association reaction, it is apparent that the "initial velocities" (the values determined for the parameter v_i) for compounds **1** and **4** represent velocities several half-lives into the reaction because of the lag due to mixing. In contrast, the initial velocities determined with compounds **2** and **3** would be measured within two half-lives of the binding reaction, so these values are a reasonable estimate of the rates of initial formation of the EI complex.

The criterion usually used to distinguish between the two mechanisms shown in Figure 1 is the dependence of k on [I]. If this dependence is hyperbolic, then the mechanism can be unambiguously identified as pathway A. However, in these experiments, it was not possible to obtain accurate data at sufficiently high concentrations of inhibitor to conclusively determine whether or not a plot of k vs [I] is hyperbolic. A set of theoretical curves of k vs [I] can be constructed for various values of K_i by using appropriate values for I, S, and K_m, showing that the dependence of k on [I] would not appear to be hyperbolic over the concentration ranges of I used unless K_i were in fact smaller than the values of $K_{i,app}$ in Table I or K_i in Table IIA. Since the true values of K_i are almost certainly somewhat larger than these, we cannot clearly distinguish between binding mechanisms by this criterion.

Because of this limitation in the data, we have tried to make some distinction between the mechanisms by comparing the values of K_i^* from the progress curves (Table II) with those from steady-state analysis (Table I). For compounds **2** and **3**, the analysis according to a two-step process is in excellent agreement with the steady-state results, while for compounds **1** and **4**, the results for a one-step process are in better agreement with the steady-state results. The results are consistent with the conclusion that the progress curves for compounds **1** and **4** represent a later phase of binding and the values determined for v_i and consequently for K_i from these curves are not accurate due to the lag in measurement time. Conversely, the curves for compounds **2** and **3** represent a more complete binding curve, as the association rates for these compounds at the concentrations of inhibitor used would be slower.

This comparison of the K_i^* values for compounds **2** and **3** suggests that the progress curves are not adequately described by mechanism B. The existence of an initial reversible complex cannot be clearly demonstrated, but cannot be ruled out. Mechanism A, as the other extreme case, may also not be a completely accurate description, but a mechanism similar to mechanism A (Allen & Abeles, 1989) can be proposed for the slow binding of compounds **2** and **3**, and, by analogy, for compounds **1** and **4**. This conclusion would be in agreement with that of Nakamura and Abeles (1985) where compactin binds to yeast HMGR with a fast burst followed by a second, slower phase. By a somewhat different analysis, they obtained rate constants of 2.7×10^7 M^{-1} s^{-1} (k_{on}) and 0.0065 s^{-1} (k_{off}) for the binding of compactin, similar to those obtained here for the closely related compound **1**. The overall rates of association are extremely fast by comparison with slow binding inhibitors of a variety of other enzymes (Schloss, 1988a), while the rates of dissociation are relatively fast for a slow binding inhibitor.

Other inhibitors of HMGR with slightly different structures do not exhibit slow binding, such as compounds **7** and **8**, and are not as potent inhibitors of the enzyme (Hoffman et al., 1986b; Stokker et al., 1986a). The most significant structural differences between compounds that do or do not exhibit slow binding or are much less potent inhibitors where slow binding has not been investigated (Lee, 1987) are in the distal portion of the lipophilic group.

Since compounds **1–8** have these structural distinctions as well as differences in mode of binding, a comparison was made of the different lipophilic groups using molecular modeling methods. The energy-minimized structures of selected inhibitors were compared with each other and with a model of HMG-CoA. The model is based on the conformation of CoA in the crystal structure of citrate synthase. This enzyme has no obvious mechanistic or evolutionary relationship to HMGR, and no significant homology was identified by attempting to align the sequences of HMGR and citrate synthase (Devereux et al., 1984); the model must therefore be considered speculative. A more recent crystal structure of a protein–CoA complex, chloramphenicol acetyltransferase with acetyl-CoA bound (Leslie et al., 1988), shows a conformation of CoA in which the pantotheinate is fully extended. While this structure was not available at the time, it does not have the features that make the citrate synthase structure a provocative model. The model is useful as a framework for comparing the different compounds, but is not intended as an adequate representation of the conformation of HMG-CoA. Comparing Figures 4 and 5, all the compounds can be oriented so that the dihydroxy acid portions are held constant with the proximal part of the lipophilic group fitting into the CoA turn region and the distal part extending away from the CoA structure. Compound **5** was synthesized to test the hypothesis that if compound **4** was binding in the orientation shown, the propionic acid group would fit into the diphosphate binding site of the CoA. The inhibition constant for compound **5** without the propionic acid moiety would be expected to be similar to that of a racemic mixture of compound **4** (Stokker et al., 1986a), or approxi-

mately 1 nM from the results given here. Since the K_i^* value for compound **5** is not smaller than 1 nM, the propionic acid apparently does not engage a new binding site but may be partially or fully solvated when the inhibitor is bound.

While no definitive conclusions can be drawn from these structural comparisons, it is clear that the entire lipophilic group of each of these molecules cannot be placed in exactly the same position, if the dihydroxy acid moiety is held fixed. Since the absolute stereochemistry of the two hydroxy groups is consistent with the stereochemistry of the substrate and the known stereochemistry of reduction of HMG-CoA (Blattman & Retey, 1970; Lee, 1987; Stokker et al., 1986b), it is assumed that the binding of this portion of the molecule is not flexible. The importance of the distal lipophilic group can be inferred from the observations that an analogue of compound **1** lacking the ester side chain is a 700-fold less potent inhibitor of HMGR than compound **1** (Hoffman et al., 1986a), and compound **8**, with no distal group at all, is the least potent of all the inhibitors discussed here. While it is not necessarily the case, the distal lipophilic groups of compounds **1**, **2**, and **4** can occupy approximately the same position. On the other hand, compounds **7** and **8**, which do not exhibit slow binding, clearly cannot occupy the same site as the distal part of the lipophilic group for the remaining compounds.

As has been proposed for other slow binding inhibitors, binding of compounds **1**–**6** could be interpreted as binding of a "transition-state analogue" (Morrison & Walsh, 1987). It is also possible that slow binding has another component which involves orientation of the lipophilic group. The binding of the lipophilic group could involve only hydrophobic interactions but could include another interaction such as a hydrogen bond to an oxygen or fluorine atom within the lipophilic structure. For example, an analogue of compound **4** lacking the fluorine is approximately 20-fold less potent than compound **4** (Stokker et al., 1986a; it has not been determined whether this compound is slow binding).

The binding of compound **3**, where the C-5 hydroxyl group has been replaced with a phosphinate moiety, also suggests that there is a component in binding which is not related to the binding of a transition state. There is a significant decrease in binding potency of compound **3** vs that of compound **2**, but the slow binding effect as measured by k_{off} is maintained. While not all phosphinate analogues exhibit slow binding and they are in general weaker inhibitors[5] than the corresponding alcohols, some phosphinate compounds can form the tighter complex. From pH studies (data not shown), phosphinate compounds such as compound **3** bind to a protonated form of the enzyme, while the dihydroxy compounds bind to a deprotonated form of the enzyme. Since binding of compounds **2** and **3** cannot result in the same enzyme interactions, it is unlikely that the slow binding arises from specific interactions at the active site, such as those which give rise to transition-state stabilization. The rather extensive number of lipophilic groups which lead to dissociation constants of nanomolar or less and the interesting comparison of these structures forces consideration that another binding site distinct from and adjacent to that of the substrate is not only available but critical to the binding of these HMGR inhibitors.

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[5] R. J. Mayer and P. L. Flamberg, unpublished results.

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