Studies on the catalytic site of rat liver HMG-CoA reductase: interaction with CoA-thioesters and inactivation by iodoacetamide

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Abstract The localization of reactive cysteines and characterization of the HMG-CoA binding domain of rat liver HMG-CoA reductase were studied using iodoacetamide (IAAD) and short-chain acyl-CoA thioesters. Freeze–thaw-solubilized HMG-CoA reductase is irreversibly inactivated by IAAD with a second order rate constant of 0.78 M^{-1} sec^{-1} at 37°C and pH 7.2. This IAAD inactivation is slowed down by pretreatment of the enzyme with disulfides, indicating that inactivation of HMG-CoA reductase occurs mainly through alkylation of specific cysteine residues in the protein. The substrate HMG-CoA, but not NADP(H), effectively protects the reductase from IAAD inactivation. When both HMG-CoA and NADP(H) are present, the reductase is inactivated by IAAD at a rate much faster than the inactivation in the presence of HMG-CoA alone. Of the two moieties of the HMG-CoA thioester, the CoA moiety confers protection from IAAD inactivation whereas HMG is totally ineffective. A series of CoA-thioesters of mono- and dicarboxylic acids of various size were tested for their effect on the activity of HMG-CoA reductase. The CoA analog, desulfo-CoA (des-CoA), and all CoA-thioesters of monocarboxylic acids of up to 6 carbons in length exhibit mixed-type inhibition of reductase activity. The competitive inhibition constants (K_i) for these compounds vary between 1 and 2 mM, whereas the noncompetitive component (K'_i) is relatively constant (540 ± 20 μM). As the acyl chain length increases beyond 6 carbons, the thioesters of monocarboxylic acids become more potent and acquire the characteristics of pure noncompetitive inhibitors. In contrast, the monothioesters of dicarboxylic acids are pure competitive inhibitors with K_i values which are similar to the K_i values of the corresponding thioesters of monocarboxylates. HMG does not affect reductase activity in concentrations of up to 2 mM, yet it greatly enhances the inhibition of the enzyme by des-CoA. Specifically, HMG affects only the K_i value of des-CoA by decreasing it from 1030 μM to 280 μM. The results indicate that reactive cysteine(s) are localized in the catalytic site of HMG-CoA reductase. Within the active site, these cysteines are closely associated with and probably participate in the binding of the CoA moiety of the substrate HMG-CoA. The results are also consistent with the existence of a noncatalytic hydrophobic site in HMG-CoA reductase.—**Roitelman, J., and I. Shechter.** Studies on the catalytic site of rat liver HMG-CoA reductase: interaction with CoA-thioesters and inactivation by iodoacetamide. *J. Lipid Res.* 1989. **30:** 97–107.

Supplementary key words active-site cysteines • enzyme hydrophobic pocket • short-chain acyl-CoA thioesters • desulfo-CoA • mixed-type inhibition • competitive inhibition

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34), catalyzes the two-step NADPH-dependent reduction of HMG-CoA to mevalonate. This reaction is considered to be the major rate-limiting step in the biosynthetic pathway of sterols and other isoprenoids (1–3).

HMG-CoA reductase is a resident glycoprotein of the endoplasmic reticulum (4) which appears to exist as a non-covalently linked dimer of 97 kDa subunits (5–7). Each subunit is composed of two major structural and functional domains (8): *i*) an elaborated membrane-spanning domain that plays a crucial role in the regulation of reductase degradation (9–11), *ii*) a cytoplasm-facing domain that contains the enzyme's catalytic site (8, 12). This cytosolic domain can be released from the endoplasmic reticulum by limited proteolysis as an enzymatically active species with subunit size of 52–63 kDa. The amino acid sequence of the two domains is highly conserved between rodents and humans (13, 14), and that of the cytoplasmic domain is also conserved in yeast (15).

Although considerable information is available regarding the overall structure of HMG-CoA reductase, very little is known about the nature and the spatial localization of specific residues that are involved in catalysis and in regulation of reductase activity. The pH-dependent properties of the yeast-derived reductase suggest that the formation of mevaldate thiohemiacetal, the putative enzyme-bound intermediate, is assisted by a protein carboxyl group, and that the catalytic group at the active site is a cationic acid, probably histidine (16). Indeed, HMG-CoA reductase is inactivated by reagents known to react with active site histidines (17). Phosphorylation of HMG-CoA reductase by

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IAAD, iodoacetamide; des-CoA, desulfo-CoA; DTT, dithiothreitol; MVA, mevalonate.

reductase kinase and ATP result in incorporation of phosphoryl groups into serine residues, concomitantly with loss of enzyme activity (18–20). Since reagents that react with active site serines do not affect enzyme activity (17), serines probably play an indirect role in catalysis.

Cysteines were also suggested to participate in the catalysis and regulation of HMG-CoA reductase (17, 21–28). These studies demonstrated that the in vitro activity of HMG-CoA reductase requires prior reduction of the enzyme by thiols. Moreover, disulfides, which most likely act by oxidizing specific reactive sulfhydryls, are potent inhibitors of reductase activity (24–26). Furthermore, reductase activity can be modulated in vitro depending on the type of the thiol used, its concentration, and the ratio of R-SH/R-SS-R (24, 25, 27). This modulation of reductase activity by sulfhydryl reducing agents is presumably regulated in vivo by the "Reductive Status" of the cell, through *mediation of cytosolic protein(s)* (26, 27, 29, 30).

In the present work we studied the involvement of cysteines in the catalytic site of HMG-CoA reductase using the thiol reagent iodoacetamide (IAAD). We provide evidence indicating that reactive cysteines are localized in the active site of HMG-CoA reductase. Within the active site, these cysteines are closely associated with the binding site of the CoA moiety of the substrate HMG-CoA. In addition, we found that HMG-CoA reductase is inhibited by short-chain acyl-CoA thioesters, suggesting the existence of a regulatory hydrophobic pocket in the enzyme.

MATERIALS AND METHODS

Materials

IAAD was obtained from Aldrich Chemical Co. (R,S)[^{14}C]HMG-CoA (55–57 mCi/mmol) was obtained from New England Nuclear; mevinolin was generously provided by Alfred Alberts from Merck, Sharp and Dohme; and all other reagents were purchased from Sigma. Solubilized HMG-CoA reductase was prepared by freeze–thawing of liver microsomes from rats fed with cholestyramine and mevinolin, as previously described (31). Under optimal assay conditions (31), HMG-CoA reductase specific activity was ~150 nmol MVA/min per mg protein.

Inactivation of HMG-CoA reductase by IAAD

Soluble HMG-CoA reductase (11 μg of protein) was preincubated for 30 min at 37°C, in 180 μl of buffer A (50 mM NaCl/75 mM KPO_4^{2-}, pH 7.2) containing DTT and the tested compounds, as indicated in the figure legends. At time 0, 20 μl of IAAD stock solution (in H_20, adjusted to pH 7.2) was added to give the indicated final concentration. Aliquots were withdrawn immediately (time 0) and at the indicated time points were added to ice-cold buffer A containing 100 mM DTT and kept on ice. After the last sampling, the tubes were kept on ice for an additional 30 min, supplemented with NADPH, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase to final concentrations of 3 mM, 15 mM, and 5 units/ml, respectively, and incubated at 37°C for an additional 30-min period. Final concentration of DTT was 50 mM. Enzyme reactions were initiated by adding [^{14}C]HMG-CoA to a final concentration of 100 μM (50,000 dpm) and HMG-CoA reductase activity was measured as previously described (27, 31); "100% reductase activity" is the activity at time 0.

Inhibition of HMG-CoA reductase activity by CoA thioesters

Soluble HMG-CoA reductase was preincubated for 30 min at 37°C in buffer A containing 10 mM DTT, 3 mM NADPH, 15 mM glucose-6-phosphate, 5 units/ml of glucose-6-phosphate dehydrogenase, and the indicated concentrations of the various CoA thioesters. Reactions were initiated by adding [^{14}C]HMG-CoA at the concentrations indicated for each experiment. The reactions were terminated after 30 min, and reductase activity was determined as described earlier (27, 31).

Other assays

Protein was determined according to Peterson (32). Concentrations of des-CoA and CoA thioesters solutions were determined spectrophotometrically at 257 nm for des-CoA (E mM = 16.84) and at 260 nm for CoA thioesters (E mM = 16.4) (33).

Data analysis

Half-time ($t_1/_2$) values for reductase inactivation by IAAD were determined from best-fit linear regression of log remaining reductase activity as a function of time. Only the linear part of the curves was considered. Experimental data obtained from inhibition of HMG-CoA reductase by CoA thioesters were analyzed according to the methods of Dixon (1/v vs. [I]; 34, 35) and Cornish-Bowden ([S]/v vs. [I]; 36). Constants for competitive (K_i) and noncompetitive (K'_i) inhibition were determined graphically from the intersects of the Dixon and Cornish-Bowden plots, respectively (34, 36).

RESULTS

Inactivation of HMG-CoA reductase by IAAD

Rat liver HMG-CoA reductase is irreversibly inactivated by IAAD in a concentration- and time-dependent manner. Plotting log half-time values as a function of log IAAD con-

centration yields a straight line with a slope of − 0.94 (**Fig. 1**), indicating that the inactivation of HMG-CoA reductase by IAAD is a simple second order process (37). The observed pseudo-first order rate constant of inactivation was linearly dependent on IAAD concentration up to 10 mM. The calculated second order rate constant is 0.78 M^{-1} sec^{-1}.

Protection of HMG-CoA reductase from IAAD inactivation by disulfides

Although IAAD is considered to be a cysteine-specific reagent, it can also alkylate other residues. Since the active site of HMG-CoA reductase contains reactive histidine residues(s) (16, 17), it was necessary to verify that the IAAD-mediated inactivation of HMG-CoA reductase indeed occurs through modification of cysteines. Disulfides reversibly inactivate HMG-CoA reductase, presumably by oxidizing essential cysteines in the enzyme (24–26). Therefore, once oxidized, these protein disulfides should become resistant to modification by IAAD. **Fig. 2** shows that treating the solubilized reductase with glutathione disulfide (GSSG) effectively protects the enzyme from IAAD-mediated inactivation. Essentially the same results were obtained with CoA-SS-G mixed disulfide (data not shown). We conclude that IAAD-mediated inactivation of HMG-CoA reductase results mainly from modification of cysteine(s).

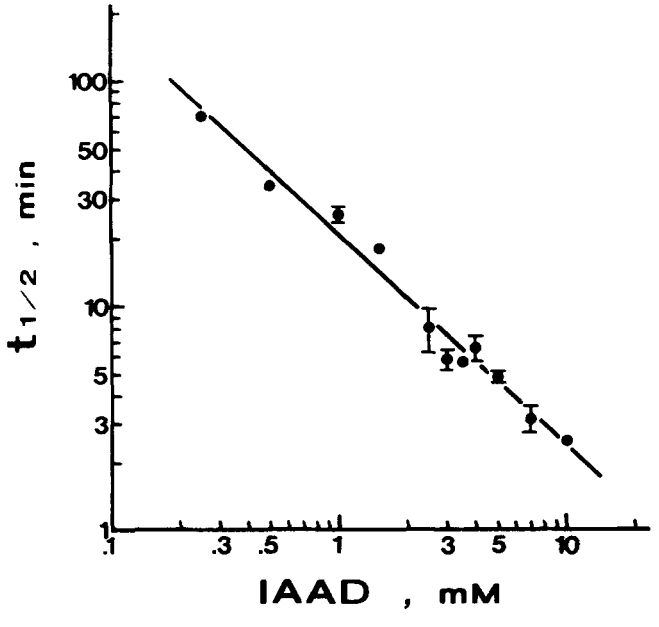


Fig. 1. Inactivation of rat liver HMG-CoA reductase by IAAD. Solubilized rat liver HMG-CoA reductase was inactivated by the indicated concentrations of IAAD, as described in the Materials and Methods section. In all cases, IAAD was present in excess molar ratio over added -SH equivalents. Half-time values were determined from best-fit linear-regressed plots of log residual activity versus time. The results presented are the mean values of two to eight different experiments. Standard deviation is given by the bars. The slope of the line is − 0.94; r = − 0.975.

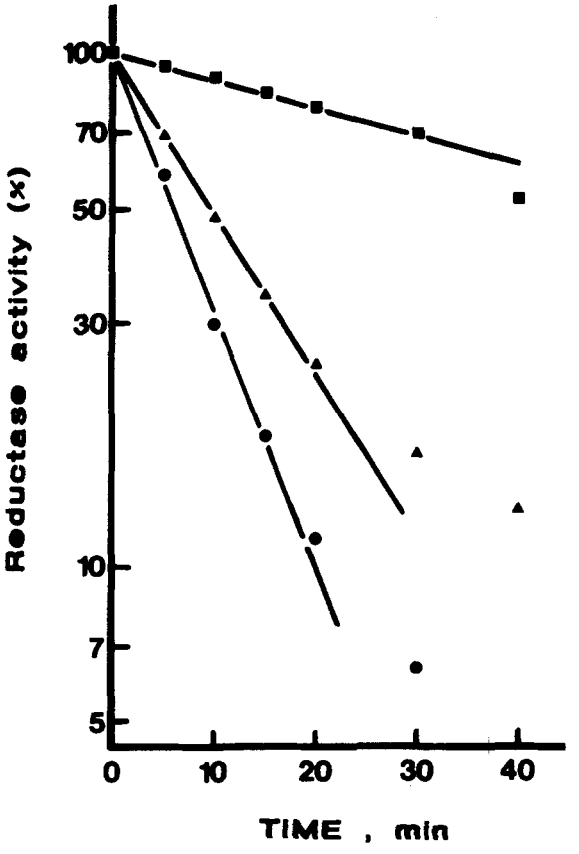


Fig. 2. GSSG-dependent protection of HMG-CoA reductase from inactivation by IAAD. Soluble HMG-CoA reductase was incubated at 37°C in the presence of 1 mM DTT. After 20 min, buffer (●) or GSSG was added to give the final concentrations of 0.25 mM (▲), or 5 mM (■). Ten min later (time zero), IAAD was added to a final concentration of 3 mM and samples were withdrawn at the indicated time points. Samples were processed, and residual HMG-CoA reductase activity was determined as described under Materials and Methods section; $t_1/_2$ values are: ●, 6 min; ▲, 9.5 min; ■, 58.5 min.

Protection of HMG-CoA reductase from IAAD inactivation by HMG-CoA but not by NADP(H)

In order to localize the essential cysteines of the reductase, we turned to protection experiments using substrates and substrate analogs. **Fig. 3** shows that binding of HMG-CoA effectively protects the reductase from IAAD inactivation. This protection of HMG-CoA reductase is not due to neutralization of IAAD by HMG-CoA, since mixing HMG-CoA with IAAD does not result in any change in the chromatographic properties of either compounds. Moreover, HMG-CoA provides the same extent of protection whether the enzyme is exposed to a mixture of HMG-CoA and IAAD or when HMG-CoA is added prior to IAAD (data not shown). NADPH, the co-substrate for the reaction, is totally ineffective against IAAD inactivation of HMG-CoA reductase when present by itself (Fig. 3). However, the presence of NADPH decreases the protec-

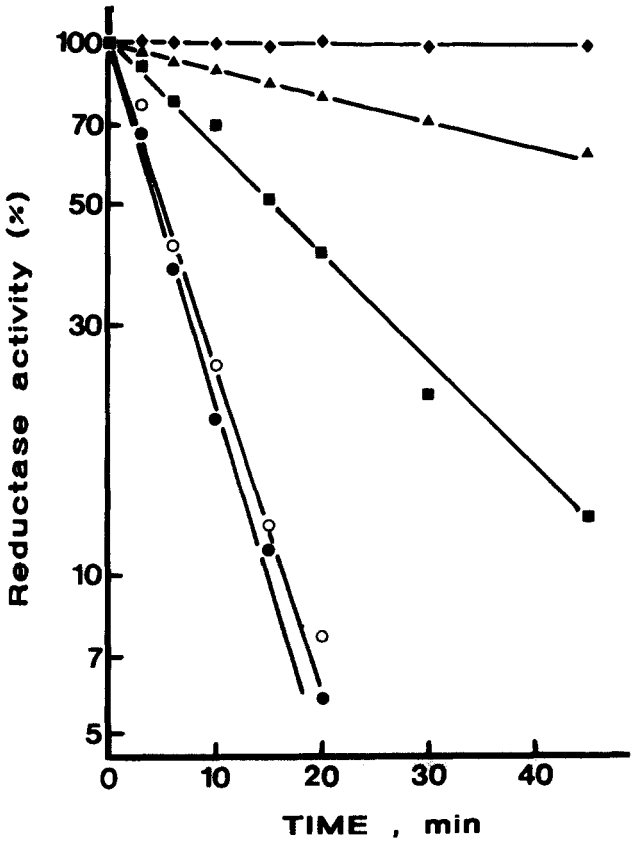


Fig. 3. HMG-CoA, but not NADPH, protects HMG-CoA reductase from inactivation by IAAD. Time kinetics experiments for the inactivation of solubilized reductase by IAAD were performed essentially as described in the Materials and Methods section. HMG-CoA reductase was activated with 1 mM DTT and alkylated by 5 mM IAAD. Alkylation mixtures contained: no addition (●, $t_1/_2$ = 4.5 min); 5 mM NADPH (○, $t_1/_2$ = 5.0 min); 2 mM HMG-CoA (▲, $t_1/_2$ = 63.5 min); 2 mM HMG-CoA plus 5 mM NADPH (■, $t_1/_2$ = 15.5 min); or 2 mM HMG-CoA plus 5 mM NADPH without IAAD (♦, $t_1/_2$ > 4500 min).

tive effect provided by HMG-CoA. When similar experiments were performed with $NADP^+$ rather than with NADPH, essentially the same results were obtained (data not shown). Taken together, these experiments demonstrate that the reactive cysteine(s) are specifically protected by HMG-CoA but not by NADP(H). Moreover, the results suggest that, when NADP(H) is present, conformational changes occur at the catalytic site so that HMG-CoA can no longer provide maximal protection to the reactive cysteine(s).

Protection of HMG-CoA reductase from IAAD inactivation by CoA but not by HMG

To further define the moiety of HMG-CoA that confers protection on the reductase from IAAD inactivation, we tested the effect of HMG, CoA, and the combination of both. We used desulfo-CoA (des-CoA), a derivative of CoA lacking the sulfhydryl group, in order to avoid possible protective effects of contaminating CoA disulfide. **Fig. 4** shows that HMG-CoA reductase is inactivated by 3 mM IAAD with a $t_1/_2$ of 5.7 min. HMG provides no significant protection to the enzyme ($t_1/_2$ = 6.2 min). Des-CoA is effective in protecting the reductase ($t_1/_2$ = 34.7 min) albeit only half as effective as HMG-CoA ($t_1/_2$ = 62.3 min). The mixture of HMG and des-CoA is slightly more effective ($t_1/_2$ = 43.2 min) than des-CoA by itself, yet less effective than the HMG-CoA thioester. Clearly, the CoA moiety of HMG-CoA is responsible for protecting the reductase from IAAD inactivation.

Inhibition of HMG-CoA reductase activity by short-chain acyl-CoA thioesters

In order to assess whether the acyl moiety assists the binding of the thioester to the reductase active site, we tested a variety of short-chain acyl CoA thioesters for their protection against IAAD inactivation. In all cases, the CoA thioesters of short-chain acids inhibited the activity of HMG-CoA reductase (see below). Preliminary experiments

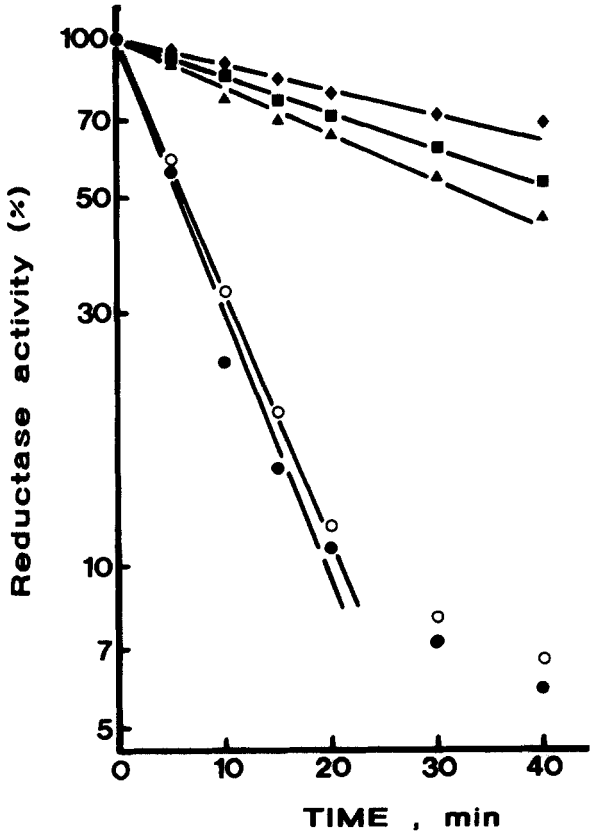


Fig. 4. CoA, but not HMG, protects HMG-CoA reductase from inactivation by IAAD. Inactivation of HMG-CoA reductase was performed as described under Materials and Methods. Solubilized reductase was inactivated by 3 mM IAAD in the presence of 2 mM of each of the following: no additions (●, $t_1/_2$ = 5.7 min); HMG (○, $t_1/_2$ = 6.2 min); des-CoA (▲, $t_1/_2$ = 34.7 min); HMG plus des-CoA (■, $t_1/_2$ = 43.2 min); HMG-CoA (♦, $t_1/_2$ = 62.3 min).

showed that these thioesters also protect the enzyme against inactivation by IAAD (data not shown). However, we noted that the various acyl-CoA thioesters exhibited different patterns of inhibition of activity of solubilized HMG-CoA reductase. Therefore, we turned to a more systematic study of the effect of these compounds on reductase activity.

Inhibition of HMG-CoA reductase activity by CoA thioesters of monocarboxylic acids

Fig. 5 depicts the inhibition of reductase activity by des-CoA and by CoA thioesters of monocarboxylic acids successively increasing in length. The data were analyzed according to Dixon (34) and Cornish-Bowden (36). The use of these plots allows the determination of the type of inhibition as well as the values for inhibitor constants (35, 36). Linear plots were obtained for all compounds tested, suggesting that there is no cooperativity in the inhibition. The pattern in both the Dixon and the Cornish-Bowden plots is characteristic of mixed-type inhibition (note that the intersections of the lines in both plots are not on the [I] axis). The competitive (K_i) and the noncompetitive K'_i) inhibition constants were determined from the Dixon and the Cornish-Bowden plots, respectively, and are summarized in **Fig. 6**. As seen in Fig. 6, des-CoA and all short-chain acyl-CoA thioesters of up to 6 carbons in length are rather weak inhibitors of HMG-CoA reductase; the apparent K_i values for these compounds are in the mM range. However, while the apparent K_i values vary between 1 and 2 mM, the apparent K'_ivalues are relatively constant (540 ± 20 μM).

Inhibition of HMG-CoA reductase activity by CoA thioesters of dicarboxylic acids

In contrast to the mixed-type inhibition pattern observed for the CoA thioesters of monocarboxylic short-chain acids, the inhibition of reductase activity by CoA monothioesters of dicarboxylic acids (**Fig. 7**) is characteristically competitive. Note that the lines in the Dixon plots intersect above the [I] axis and Cornish-Bowden plots yield parallel lines at low HMG-CoA concentrations. Thus, the noncompeti-

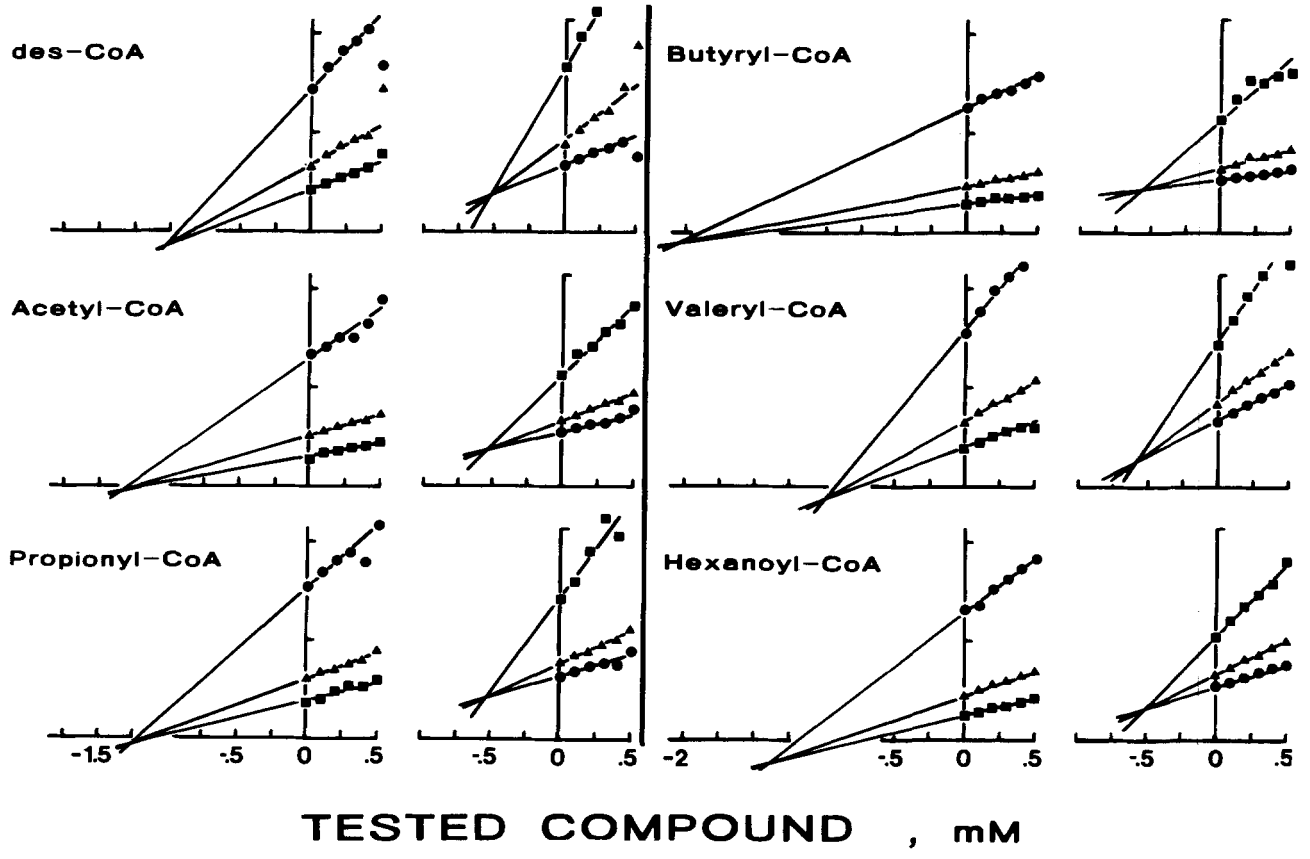


Fig. 5. Inhibition of HMG-CoA reductase by des-CoA and CoA thioesters of short chain monocarboxylic acids. Soluble reductase was preincubated for 30 min at 37°C with 10 mM DTT, NADPH (3 mM), an NADPH-regenerating system (15 mM glucose-6-phosphate; 5 units/ml glucose-6-phosphate dehydrogenase), and increasing concentrations of the indicated compounds. Reactions were initiated by adding 16.8 (± 0.8)μM (●), 53.8 (± 3.0)μM (▲), or 160.7 (± 6.2)μM (■) of [^{14}C]HMG-CoA. Reactions were terminated after 30 min. The results were analyzed according to Dixon (1/v vs. [I], left plot for each compound) and Cornish-Bowden ([S]/v vs. [I], right plot for each compound). For clarity, values along 1/v and [S]/v axes were omitted. Full scale for the 1/v axis in the Dixon plots is 70 min/nmol. Full scale for the [S]/v axis in the Cornish-Bowden plots is 3 $\times 10^3$ min/ml. Each compound was tested at least twice and the results presented are the mean values of these experiments.

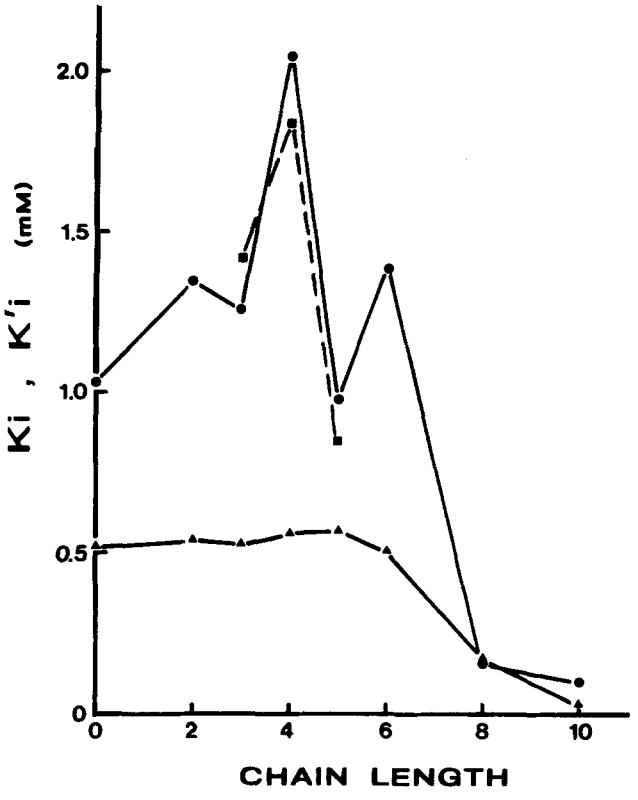


Fig. 6. Inhibition constants (K_i, K'_i) of CoA thioesters as a function of the acyl chain length. K_i values were determined graphically from the intersects of the lines of the Dixon plots shown in Figs. 5, 7, and 10. (●) K_i for CoA thioesters of monocarboxylates; (■) K_i for CoA thioesters of dicarboxylates. K'_i values (▲) were determined from the intersects of the lines of the Cornish-Bowden plots shown in Figs. 5 and 10.

tive component (K'_i) cannot be determined. Again, these thioesters are weak competitive inhibitors of the reductase, and the K_i values of these compounds are similar to the K_i values of the corresponding monocarboxylic acyl-CoA thioesters (Fig. 6).

It appears that the free carboxyl group of the thioester of dicarboxylates is responsible for the qualitative difference between the inhibition by mono- and dicarboxylic acyl-CoA thioesters. Therefore, we tested how, and to what extent, a free dicarboxylic acid affects the inhibition of reductase activity by des-CoA. We examined HMG since it is the acyl moiety of the natural substrate. **Fig. 8** shows that HMG by itself does not affect reductase activity in concentrations up to 2 mM. Yet, it greatly stimulates the inhibition of HMG-CoA reductase by des-CoA. **Fig. 9** shows the inhibition of the reductase by increasing equimolar concentrations of HMG and des-CoA. Analysis of the data demonstrates that HMG profoundly affects the competitive component of the inhibition by des-CoA (lowering the K_i value from 1030 μM to 280 μM) without affecting the noncompetitive component (K'_i = 520 μM in the absence of HMG and 540 μM in its presence).

Inhibition of HMG-CoA reductase activity by CoA thioesters of monocarboxylic long-chain acids

As chain length increases (Fig. 6 and **Fig. 10**), the thioesters of monocarboxyacyl-CoA become progressively more potent inhibitors of reductase activity. Moreover, the differences between K_i and K'_i values diminish and the inhibition patterns become more characteristic to noncompetitive inhibitors (e.g., octanoyl-CoA). This increase in enzyme inhibition with the increase in acyl chain length persists beyond decanoyl-CoA (**Table 1**).

Fig. 11 shows that the inhibition of reductase activity by octanoyl-CoA can be reversed upon dilution of the inhibitor and does not require a cytosolic fraction, unlike that reported for oleyl-CoA (38). Moreover, the inhibition of HMG-CoA reductase by the long-chain CoA thioesters is not due to a possible inhibition of glucose-6-phosphate dehydrogenase (39, 40), since high concentrations of NADPH were used in our experiments.

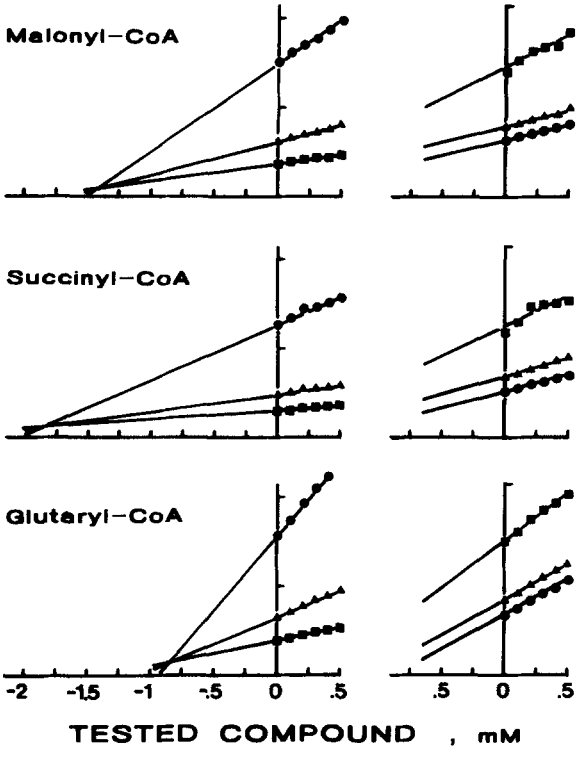


Fig. 7. Inhibition of HMG-CoA reductase by CoA thioesters of short chain dicarboxylic acids. The experiments were performed exactly as described in the legend of Fig. 5. Left plots for each compound: Dixon plots; right plots for each compound: Cornish-Bowden plots. For clarity, values along 1/v and [S]/v axes were omitted. Full scale for the 1/v axis in the Dixon plots is 70 min/nmol. Full scale for the [S]/v axis in the Cornish-Bowden plots is 3×10^3 min/ml. Each compound was tested at least twice and the results presented are the mean values of these experiments.

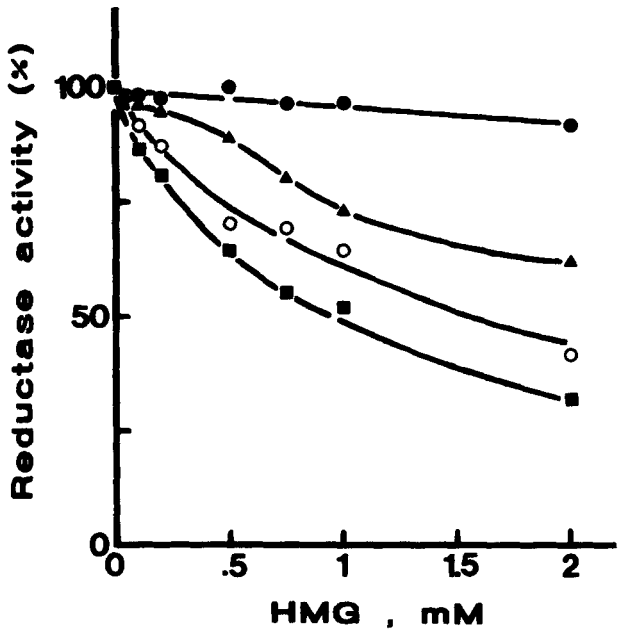


Fig. 8. HMG enhances the inhibition of HMG-CoA reductase activity by des-CoA. Solubilized reductase was preincubated for 30 min at 37°C with 10 mM DTT, 3 mM NADPH, an NADPH regenerating system, and the indicated concentrations of HMG in the presence of 100 μM (▲), 250 μM (○), 500 μM (■), or without (●) des-CoA. Reactions were started by adding 100 μM [^{14}C]HMG-CoA, and stopped 30 min later. "100%" Activities (without HMG) are: 60.0, 54.8, 48.1, and 42.7 pmol MVA/min for 0, 100, 250, and 500 μM des-CoA, respectively.

DISCUSSION

It was previously demonstrated that treatment of HMG-CoA reductase by thiols is necessary for the binding of HMG-CoA to the enzyme. This suggested that there are protein sulfhydryls that are essential for binding of this substrate and for catalysis (25, 26). However, these observations did not directly demonstrate that the essential sulfhydryls are confined to the active site(s). It is possible that conformational changes resulting from the reduction of protein disulfides that are remote from the active site may affect the binding of HMG-CoA at the catalytic site. Evidence for such thiol-induced conformational changes was provided by the marked alterations in the antigenicity (41, 42) and the kinetic properties of the reductase (27). The results presented in this report show that HMG-CoA, which binds exclusively to the catalytic site (see below), effectively protects the reductase from IAAD inactivation. This suggests that the reactive sulfhydryls are located within the enzyme's active site.

Kinetic and immunological evidence (43, 44) indicate that HMG-CoA reductase can bind each of its substrates (HMG-CoA, NADPH) independently. This suggests that the catalytic site is composed of two major domains that do not sterically overlap: an NADP(H) domain and a HMG-CoA binding domain. Inasmuch as protection from IAAD inactivation is conferred by HMG-CoA but not by NADP(H) (Fig. 3) and since the binding of only HMG-CoA requires that the enzyme be first reduced by thiols (25, 26), the reactive cysteine(s) should be confined to the HMG-CoA binding domain. The finding that NADP(H) decreases the protective effect exerted by HMG-CoA against IAAD inactivation suggests that the conformational changes induced in the reductase protein by the binding of NADP(H) to its domain (27) also affect the binding domain of HMG-CoA. Nakamura and Abeles (45) suggested that the HMG-CoA domain may be further divided to the HMG binding site and the CoA site. This is also supported by our results. Since only des-CoA, but not HMG, could protect the enzyme from IAAD inactivation (Fig. 4), it stands to reason that the reactive cysteines are specifically required for the binding of the CoA moiety to the enzyme's catalytic site.

Several reports have demonstrated the inhibition of HMG-CoA reductase by CoA, long chain fatty acids, and their CoA thioesters (22, 38, 45-52). These studies showed that inhibition by long-chain fatty acids and their CoA thioesters is exerted on both the microsomal and the solubilized reductase, indicating that the membrane-embedded domain of the enzyme is not required for such inhibition

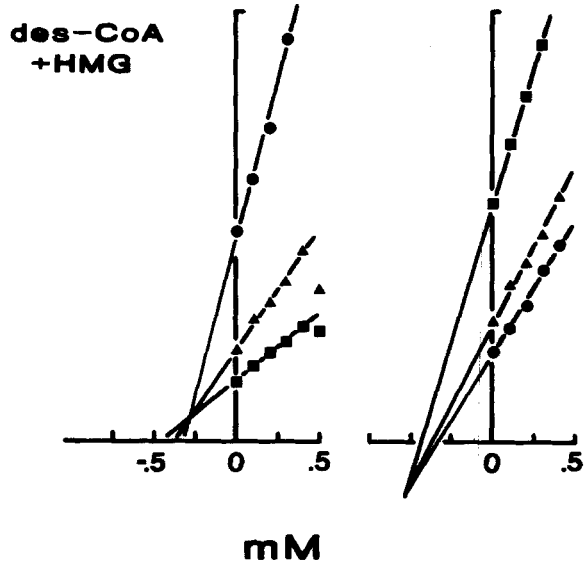


Fig. 9. Inhibition of HMG-CoA reductase by HMG plus des-CoA. Soluble HMG-CoA reductase was preincubated for 30 min at 37°C with 10 mM DTT, 3 mM NADPH, an NADPH-regenerating system, and indicated concentrations of an equimolar mixture of HMG and des-CoA. Reactions were initiated by adding 17.4 μM (●), 52.0 μM (▲), or 158.0 μM (■) of [^{14}C]HMG-CoA. Reactions were terminated after 30 min. The results were analyzed according to Dixon (1/v vs. [I], left plot) and Cornish-Bowden ([S]/v vs. [I], right plot). For clarity, values along 1/v and [S]/v axes were omitted. Full scale for the 1/v axis in the Dixon plot is 135 min/nmol. Full scale for the [S]/v axis in the Cornish-Bowden plot is 5.5 × 10^3 min/ml.

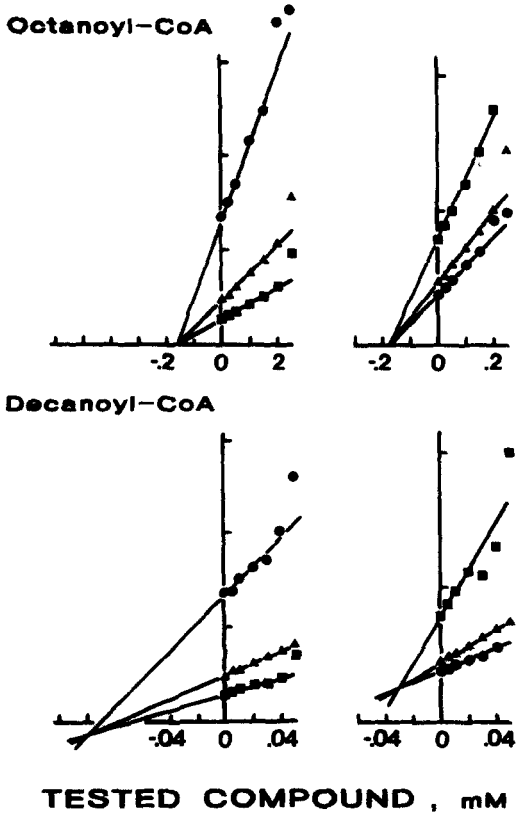
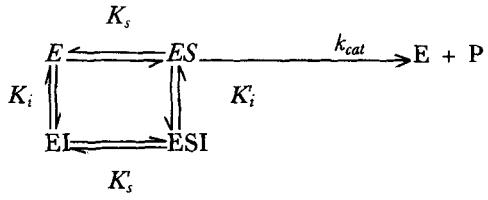


Fig. 10. Inhibition of HMG-CoA reductase by octanoyl- and decanoyl-CoA thioesters. The experiments were performed exactly as described in the legend of Fig. 5. Left plots: Dixon plots; right plots: Cornish-Bowden plots. For clarity, values along 1/v and [S]/v axes were omitted. Full scale for the 1/v axis in the Dixon plots is 105 min/nmol. Full scale for the [S]/v axis in the Cornish-Bowden plots is 4×10^3 min/mol. Each compound was tested at least twice and the results presented are the mean values of these experiments.

(38). Moreover, as chain length increases, the inhibition potency of the fatty acids and their corresponding thioesters increases as well (46). No correlation was found between the degree of enzyme inhibition and the detergent properties of these compounds (38, 46). Kinetic studies indicated that CoA and various CoA thioesters display either noncompetitive or mixed-type inhibition with respect to HMG-CoA. Unlike reports that showed that HMG-CoA reductase is noncompetitively inhibited by CoA with respect to HMG-CoA (45, 50), we observe mixed-type inhibition, in agreement with Bach and Lichtenthaler (52). One possible explanation for this discrepancy is that we used the combination of the Dixon and the Cornish-Bowden treatments for data analysis. This enables us to distinguish between mixed-type and pure noncompetitive patterns.

In this report we show that HMG-CoA reductase is inhibited by short-chain acyl CoA thioesters. We find that thioesters of dicarboxylates are competitive inhibitors whereas those of monocarboxylic acids are mixed-type inhibitors of the reductase. Fatty acyl CoA thioesters longer than 8 carbons acquire the characteristics of noncompetitive inhibitors (Figs. 6 and 10 and refs. 38 and 46), and are progressively stronger inhibitors as chain length increases (Table 1).

Mixed-type inhibition can be regarded as a case in which a fully noncompetitive effect (represented by K'_i) is associated with a competitive action (represented by K_i), according to the following scheme:



Our results fit into such a scheme if we invoke the existence of two separate sites: a catalytic site and a noncatalytic hydrophobic site, as proposed by Lehrer et al. (38). Both sites have affinity to and can bind the CoA moiety of acyl CoA thioesters. K_i and K'_i represent the dissociation constants of the thioesters from the catalytic site and the hydrophobic site, respectively. Short-chain monocarboxylic acyl-CoAs (up to 6 carbons in length) can be accommodated by the catalytic site and therefore compete with the binding of HMG-CoA. As evident by the high K_i values, this interference is rather weak. The acyl moiety appears to weaken this competitive binding since the K_i values for most of the short-chain monocarboxyacyl CoAs are greater than that of des-CoA. Thus, the binding to this site is probably dictated by the CoA moiety. A similar effect is observed for the dicarboxylic thioesters.

The short-chain monocarboxylic CoA thioesters may also bind to the noncatalytic, hydrophobic pocket. The relatively constant value of K'_i for thioesters of up to 6 carbons in length (Figs. 5 and 6) indicates that the acyl moiety does not contribute significantly to this binding, and the bind-

TABLE 1. Inhibition of HMG-CoA reductase by long-chain fatty acyl-CoA thioesters

Tested Compound	Chain Length	IC_{50}[a]
		μM
Decanoyl-CoA	10	65
Lauryl-CoA	12	22
Myristoyl-CoA	14	10
Palmitoyl-CoA	16	3

Soluble reductase was preincubated for 30 min at 37°C with 10 mM DTT, NADPH (3 mM), an NADPH-regenerating system, and increasing concentrations of the indicated compounds. Reactions were initiated by adding [^{14}C]HMG-CoA to a final concentration of 100 μM. Reactions were stopped after 30 min and reductase activity was measured.

[a]Concentrations of acyl-CoAs that produce 50% inhibition.

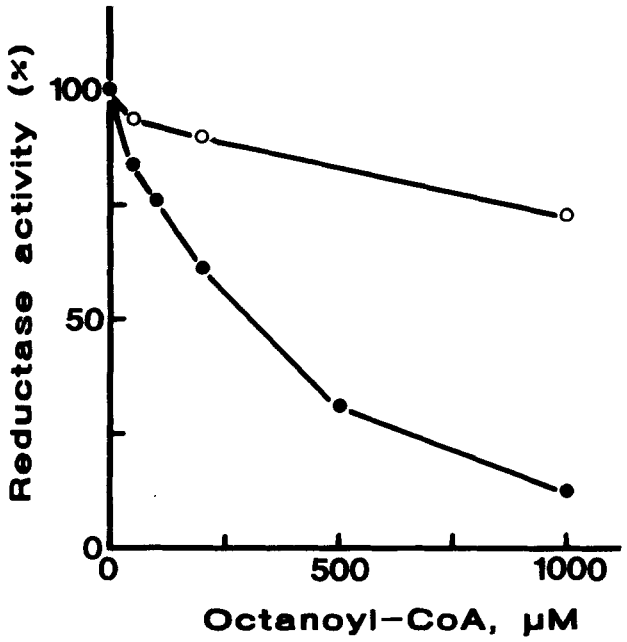


Fig. 11. Reversal upon dilution of the inhibition of HMG-CoA reductase by octanoyl-CoA. Solubilized HMG-CoA reductase was preincubated for 30 min at 37°C with 1.5 mM DTT and the indicated concentrations of octanoyl-CoA. Samples were diluted five-fold with phosphate-buffered saline, supplemented with DTT, NADPH (final concentrations of 50 and 3 mM, respectively) and an NADPH-regenerating system. Reactions were started by adding [^{14}C]HMG-CoA. Parallel, undiluted samples were assayed under the same conditions; ●, undiluted samples; ○, diluted samples.

ing is due mainly to the CoA moiety. This is also supported by the fact that des-CoA has the same K'_i value as the short-chain monocarboxylic thioesters. The postulated hydrophobic pocket of HMG-CoA reductase is not affected by, and most likely does not bind to, the short-chain thioesters of dicarboxylic acids probably because of their hydrophylic nature due to the extra carboxyl group. Accordingly, these compounds are pure competitive inhibitors without any measurable K'_i (Figs. 6 and 7).

The observation that valeryl- and glutaryl-CoA have K_i values similar to that of des-CoA indicates that an acyl chain of 5 carbons in length interferes the least (if at all) with the binding of the CoA moiety to the enzyme's active site. Perhaps this is because these acyl moieties have the same translatory length as does HMG and, thus, fit to the HMG-CoA binding site better than all other tested short-chain acyl CoAs. It is noteworthy that HMG, although not affecting reductase activity by itself, greatly enhances the inhibition of reductase activity by des-CoA (Fig. 8). Moreover, HMG decreases only the K_i value (competitive component) for des-CoA without affecting K'_i (the noncompetitive component) (Fig. 9). These results suggest that the natural substrate, HMG-CoA, binds exclusively to the active site of the reductase. This is also in agreement with earlier reports from this laboratory that, unlike NADPH, HMG-CoA does not display allosteric kinetics (27, 31). The efficient binding of HMG-CoA to the active site of the reductase may also be partially dictated by the 3-methyl group. This conclusion is based on previous findings that 3-hydroxy-3-methyl-4-carboxybutyryl-CoA is a strong competitive inhibitor of the reductase, as compared to 3,4-dicarboxy-3-hydroxybutyryl-CoA (53).

As the acyl chain of the thioesters increases in length, the steric interference in their binding to the catalytic site increases since this site can no longer accommodate the long acyl chains. The binding affinity to the hydrophobic pocket is, however, progressively increased, most likely due to the increase in the hydrophobicity of the hydrocarbon moiety. This is suggested by the increased inhibitory potency as chain length increase (Table 1). The resulting net effect is the preferential binding to the reductase's hydrophobic region and, again, the apparent disappearance of the competitive component yielding plots characteristic to noncompetitive inhibitors (e.g., octanoyl-CoA; and refs. 38 and 46).

It is possible that the postulated hydrophobic site plays some regulatory function, as was proposed by Lehrer et al. (38). Hydrophobicity plots of the amino acid sequence comprising the cytoplasmic domain of hamster HMG-CoA reductase revealed two regions, predicted to form β-structures, each of which has a central relatively hydrophobic core. These two β-domains also contain 10 out of the 27 cysteines of the reductase, and were postulated to form at least part of the active site of the enzyme (8). A hydrophobic pocket near the active site was also suggested from studies of the mode of interaction of HMG-CoA reductase with compactin-like derivatives (45). If the same hydrophobic site binds the decalin moiety of compactin-like compounds and long-chain fatty acyl chains, then clearly the binding specificities of this site are not strict. The enzyme ligand binding domains disclosed in this study, as well as the residues participating in catalysis, should be identified once the three-dimensional structure of HMG-CoA reductase is resolved.

This work was supported by Grant 1426AR1 from the Council for Tobacco Research-USA., Inc.

Manuscript received 28 April 1988 and in revised form 5 July 1988.

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