Regulation of Rat Liver 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase

EVIDENCE FOR THIOL-DEPENDENT ALLOSTERIC MODULATION OF ENZYME ACTIVITY*

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Rat liver microsomes devoid of free thiols were prepared in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer in the presence of 30 μM leupeptin. The activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase by GSH and dithiothreitol (DTT) in these microsomes was studied and compared to the activation by these thiols of an enzyme that was solubilized by freeze-thawing. An increase of V_max was observed, for the two enzyme preparations, with increasing concentrations of the two activating thiols. Reactions of GSH-activated microsomal enzyme with increasing concentrations of NADPH show sigmoidal kinetics with a Hill coefficient of 2.01 ± 0.07 at 2–4 mM GSH. Increase of the activating GSH concentrations resulted in a gradual change towards Michaelis-Menten kinetics, and a Hill coefficient of 1.08 ± 0.03 for NADPH was calculated at 25 mM GSH. Activation of the microsomal enzyme by DTT yielded similar results except that a Hill coefficient of 1.1 was observed already at 2.5 mM DTT.

Normal Michaelis-Menten kinetics were observed for HMG-CoA at all GSH concentrations.

Solubilization of HMG-CoA reductase by the widely used freeze-thaw procedure abolished the cooperative pattern, and normal Michaelis-Menten kinetics with a Hill coefficient of 1.0 was observed regardless of GSH concentration.

These results are compatible with a model in which HMG-CoA reductase activity is GSH-dependent, allosterically modulated under physiological hepatic conditions. In addition, the widely used assay conditions, using high DTT concentrations or employment of highly purified soluble enzyme precluded the observation of sigmoidal kinetics and the suggested model.

The enzyme hydroxymethylglutaryl-CoA reductase (NADPH) (EC 1.1.1.34) is the rate-limiting enzyme in the biosynthetic pathway leading to the formation of cholesterol (1, 2). Several reports showed an increase of the activity of this enzyme in the presence of thiols, especially GSH (3–6). Gilbert and Stewart (7) demonstrated the inhibition of the yeast-derived enzyme by various disulfides including GSSG, CoA disulfide, and Ellman’s reagent. This inhibition was completely reversed upon addition of thiols. Tormanen and Scallen (8) indicated that there is an inverse relationship between the activity of this enzyme and the dithioerythritol concentration required to obtain 50% of maximal activity. Our two previous reports (9, 10) as well as others (6), suggested a possible regulatory role of thiols and disulfides on this enzyme.

Previous reports from this laboratory demonstrated that rat hepatic thiol-deficient microsomal HMG-CoA reductase exists in a latent, inactive form that can be easily activated by the addition of thiols. In order to demonstrate the existence of the latent form, we studied the binding of soluble enzyme to the affinity resin blue dextran-Sepharose 4B and to agarose/hexane/HMG-CoA. These binding studies showed that the binding of the substrate HMG-CoA to the thiol-deficient, latent form of the enzyme is impaired whereas the thiol-activated form of this enzyme binds this ligand readily. These studies also indicated that NADPH, the cosubstrate for the reaction, binds to both forms of the enzyme (9). In a later report, we observed that various concentrations of thiols affect both the V_max and the apparent K_m for HMG-CoA and NADPH (10). These results could not be explained by a simple conversion of an inactive form of the enzyme to an active one. Furthermore, it was also demonstrated that various thiols caused different maximal degrees of activation of this enzyme (9). This, together with reports by others as well as by ourselves, that various disulfides inhibit this enzyme, suggested that HMG-CoA reductase activity may be modulated by the hepatic Thiol-Status as defined by Kosower and Kosower (11).

We undertook this study in order to further elucidate the effect of thiols on the modulation of hepatic HMG-CoA reductase activity.

EXPERIMENTAL PROCEDURES

Materials—NADPH, glucose 6-phosphate, glucose-6-P dehydrogenase, HMG-CoA, mevalonolactone, GSH, DTT, and leupeptin were from Sigma. R,S-3-[14C]HMG-CoA, 55.1 Ci/mol or 56.6 Ci/mol was from New England Nuclear and Amersham Radiochemical Center, respectively. Sepharose 4B was from Pharmacia, Amberlite XAD-2 was from Rohm and Haas Co. and all other chemicals were of reagent grade or better and were purchased from commercial sources.

Preparation of Thiol-deficient Microsomes—Wistar rats were fed ad libitum with standard diet containing 10% (w/w) ground Amberlite XAD-2 resin for two weeks, as described (12). After this period, the rats were killed in the mid-dark cycle and their livers were removed into ice-cold 10 mM HEPES buffer solution, pH 7.5, containing 0.3 M sucrose and 30 μM leupeptin. The livers were minced, washed in the same buffer, and then homogenized in a glass-Teflon homogenizer (6.0 ml of buffer/g of liver). The homogenate was centrifuged for 15 min at 10,000 × g and the supernatant was centrifuged for 45 min at 150,000 × g. The microsomal pellet was suspended, centrifuged again,

1 The abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
and resuspended in minimal volume of the same buffer. The microsomes were applied onto a Sepharose 4B column (3 x 48 cm) which was pre-equilibrated with HEPES buffer (160 mM HEPES, 200 mM KCl, pH 7.5), and filtered through at room temperature. The fractions at the void volume, containing the microsomes, were pooled and 1.0-mL aliquots were frozen and stored in liquid N2. The gel filtration step was found necessary for the removal of endogenous thiols and pyridine nucleotides. Reductase activity in these microsomes was found to be completely latent and its expression was easily achieved by the addition of thiols and substrates.

Preparation of Latent Soluble Enzyme—The solubilization of HMG-CoA reductase was done similar to the procedure of Edwards et al. (15) up to and including the (NH4)2SO4 step except that the freeze-thawing step was repeated. Protein precipitated at 20–60% (NH4)2SO4 was dissolved in a small volume, applied onto and filtered through a HEPES buffer pre-equilibrated Bio-Gel P-4 column (1 x 25 cm). The fractions containing protein were combined and 200-μL aliquots were frozen and stored in liquid N2. This preparation was used for studies of soluble HMG-CoA reductase and did not display enzyme activity in the absence of thiols.

Assay of HMG-CoA Reductase Activity—Activity of HMG-CoA reductase was determined by a slight modification of a microradiosay procedure described by Shapiro et al. (14). Each assay was done in 0.2 mL 0.05 M HEPES buffer and contained 1.5 mM NADPH, 7.5 mM glucose 6-phosphate, 0.25 units of glucose-6-P dehydrogenase, and the thiols in concentrations as specified for each experiment. Total volume of the reaction was 100 μL.

Preincubation of the enzyme and cofactors was routinely made for 20–35 min at 37°C before reaction was initiated by the addition of the [14C]HMG-CoA to a final concentration of 30 μM. In NADPH-dependent kinetics experiments, NADPH was omitted from the preincubated mixtures and the reactions were initiated by the simultaneous addition of the two substrates, the radiolabeled HMG-CoA (30 μM) and NADPH to the specified concentrations. After the assay period, the reactions were terminated by the addition of 10 μL of 10 N HCl and 10 μL of 0.88 mM carrier mevalonolactone. Mevalonolactone was allowed to form during 1-h incubation at 37°C. Protein precipitated was removed by centrifugation and 60 μL of the supernatant were taken, and the radioactivity was determined by the method of Ellman (15) and protein was determined by the method of Lowry et al. (16).

Data Analysis—Vmax values of reactions were determined from double reciprocal plots of rate versus the first and second powers of substrate concentrations. Hill coefficient and S50 values were calculated from the Hill plots (17, 18).

RESULTS

The activation of latent rat hepatic HMG-CoA reductase in the presence of various concentrations of GSH and DTT was investigated. In these experiments, several measures of precaution must be taken to ensure that the enzyme is completely latent and devoid of relevant low molecular weight factors (e.g. pyridine nucleotides, CoA derivatives, and thiols) so that background activities in the absence of the tested factor(s) can be diminished to, or nearly to, those obtained with the enzyme. Thus, in addition to preparing the microsomes in the absence of thiols, they were filtered through a Sepharose 4B column at room temperature. A similar procedure was applied for the solubilized enzyme by filtering it through a Bio-Gel P-4 column. Table I shows that both microsomal and solubilized enzyme preparations obtained by these procedures exhibit HMG-CoA reductase activity which was totally dependent on addition of thiols and NADPH.

From reported data (13, 19, 20) and experiments that will be described elsewhere, we noted an effect of phosphate on the activity of the enzyme. Therefore, in this study, soluble and microsomal enzymes were obtained and assayed in HEPES buffer.

Microsomal enzyme kinetics as a function of increasing concentrations of NADPH, performed on enzyme activated with various concentrations of GSH, showed sigmoidal behavior, especially at low concentrations of the thiol. The maximal velocity of the reaction increased with the increase of the concentration of GSH (Fig. 1A). No linear Lineweaver-Burk plots were obtained for most kinetics especially at the low GSH concentrations (Fig. 1B).

The possibility that the sigmoidal kinetics at low GSH concentrations and the deviation from linear double reciprocal plots might be due to depletion of NADPH by other competing microsomal enzymatic reactions and that the NADPH-generating system (glucose-6-P/glucose-6-P dehydrogenase) is ineffective in replenishment of NADPH, was investigated. This was done by monitoring the absorbance at 340 nm. The reactions were carried out under assay conditions in which NADPH regeneration is most critical (i.e. 10 μM NADPH) with or without the glucose-6-P/glucose-6-P dehydrogenase system. While the rate of NADPH oxidation by the microsomal preparation in the absence of the NADPH-generating system was 550 nm/min, the addition of 7.5 mM glucose-6-P and 0.25 units of glucose-6-P dehydrogenase to the assay mixture slowed the NADPH oxidation rate to 15 nm/min. Addition of 30 μM HMG-CoA (the concentration of this substrate in the radioassay) increased this rate to 38 nm/min. From the above, it is apparent that even after 30 min (the usual time course of the reductase assay) about 90% of the NADPH is still available, ensuring linear measurements of initial rates.

Plotting the microsomal enzyme kinetic data into the Hill equation resulted in a series of straight lines (r = 0.989 ± 0.009) with slopes (Hill coefficients) greater than 1 (plots not shown). Further analysis of the kinetics revealed a relationship between the Hill coefficients and Vmax obtained for a given thiol concentration (Fig. 2). This figure contains data from experiments carried out with two different preparations of microsomes prepared under the same conditions (see "Experimental Procedures") yet differed from one another with respect to HMG-CoA reductase specific activity. However, in both preparations, maximal reductase activity was achieved at 25 mM GSH and did not alter significantly at higher GSH concentrations. Thus, Vmax values, of assays performed at the various GSH concentrations, are expressed as per cent of the maximal velocity obtained with 25 mM GSH. In this figure, an inverse relationship between Vmax and the Hill coefficient is clearly seen, with the latter ranging between 1 at high GSH
FIG. 1. Enzyme kinetics of microsomal HMG-CoA reductase as a function of NADPH concentration in the presence of various concentrations of GSH. The assays were carried out as described under "Experimental Procedures" and contained 4 mM (■), 6 mM (○), 8 mM (▲), and 10 mM (■) GSH. Microsomes were preincubated with the cofactors for 30 min at 37 °C in a total volume of 80 µl. The reactions were initiated by the addition of both substrates in 20 µl. Reactions ran for 30 min and [%]mevalonolactone was measured as described.

Fig. 2. Relationship between V_max and Hill coefficients for NADPH of microsomal HMG-CoA reductase and varying concentrations of GSH. V_max values were determined from double reciprocal plots of rate versus the first and second powers of NADPH concentrations and are expressed as percent of those obtained with 25 mM GSH (100%). Hill coefficients were determined from the slopes of Hill plots (log υ/V_max - υ versus log [GSH]).

Variations of the Hill coefficient at various concentrations of GSH, are between 1 and 2.

Similar observations were made when the activating thiol was DTT (Fig. 3). Sigmoidal kinetics are observed only at very low DTT concentrations (0.5 and 1.0 mM) while at higher DTT concentrations (2.5 and 5.0 mM) apparent Michaelis-Menten kinetics are observed (Fig. 3A). This shift, from sigmoidal to Michaelis-type kinetics with the increase of thiol concentration, is more pronounced in the double reciprocal plots (Fig. 3B). As shown, the curvature of the reciprocal plots observed at low DTT concentrations diminishes and the plots approach linearity at higher concentrations. As was reported by us earlier (10) and as shown above for GSH, V_max increases with the increase of DTT concentrations. Again, qualitatively similar inverse relationship between V_max and the Hill coefficient is obtained for various concentrations of activating DTT (Fig. 4). Despite a tendency of the Hill coefficient to increase at the lower DTT concentrations, in this and other experiments, we never calculated a value of 2 (or near 2) even at very low DTT concentrations. Thus, the maximal value of the Hill coefficient obtained from reactions activated by DTT is lower than that obtained for GSH.

Fig. 5 illustrates another difference between GSH and DTT in the activation of microsomal reductase. It is clear that the concentrations of NADPH necessary to achieve half-maximal velocity (S_0.5) in assays carried out in the presence of DTT decrease as DTT concentrations increase, whereas S_0.5 values of reactions carried out in the presence of up to 25 mM GSH remain relatively constant (82 ± 11 µM NADPH).

In contrast to the sigmoidal kinetics of the microsomal...
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**FIG. 3.** Enzyme kinetics of microsomal HMG-CoA reductase as a function of NADPH concentration in the presence of various concentrations of DTT. Microsomes were incubated for 30 min at 37 °C with 20 mM DTT and then passed through a Sepharose 4B column (1 × 15 cm). The assays were carried out as described under "Experimental Procedures" and contained no DTT (○—○), 0.5 mM (●—●), 1.0 mM (▲—▲), 2.5 mM (■—■), and 5 mM (■—■) of DTT. Microsomes were preincubated with the cofactors for 30 min at 37 °C in a total volume of 80 μl. The reactions were initiated by the addition of both substrates in 20 μl. Reactions ran for 30 min and [14C]mevalonolactone was measured as described.

**FIG. 4.** Relationship between $V_{max}$ and Hill coefficients for NADPH of microsomal HMG-CoA reductase and varying concentrations of DTT. $V_{max}$ values were determined from double reciprocal plots of rate versus the first and second powers of NADPH concentrations. Hill coefficients were determined from the slopes of Hill plots (log $v/V_{max} = v$ versus log [S]).

The enzyme, normal Michaelis-Menten kinetics and linear double reciprocal plots of rates versus NADPH concentrations were observed with the solubilized enzyme (Fig. 6). Similar to the microsomal enzyme, the solubilized enzyme shows an increase of $V_{max}$ for NADPH concomitant to the increase in the activating GSH concentrations. However, unlike the microsomal enzyme, the Hill coefficient for NADPH remains constantly 1, regardless of GSH concentration (Fig. 7). Moreover, a 44% decrease in the apparent $K_m$ is observed as GSH concentration increases from 1 to 10 mM (Fig. 6B).

In an earlier report from this laboratory (10), we showed...
that enzyme kinetics for HMG-CoA at different activating DTT concentrations displayed Michaelis-Menten behavior and linear Lineweaver-Burk plots. In light of the above results, we examined the kinetic pattern for HMG-CoA with an enzyme activated by various concentrations of GSH. As shown in Fig. 8, normal Michaelis-Menten curves (inset) and linear Lineweaver-Burk plots are obtained at all tested concentrations of GSH. Increase of the activating GSH concentration resulted in an increase of both $V_{\text{max}}$ and the apparent $K_m$ for HMG-CoA. In contrast to the NADPH-dependent kinetics, it is clear that for HMG-CoA the Hill coefficient remains constantly 1 regardless of GSH concentration or $V_{\text{max}}$ (Fig. 9).

**DISCUSSION**

The kinetics of HMG-CoA reductase shows that the apparent order of the reaction for NADPH is variable as expressed by the Hill coefficient for this substrate. This variation can be inversely related to the thiol concentration in the reaction (Figs. 2 and 4). As shown, at high DTT and GSH concentrations, the Hill coefficient for NADPH approaches the value of 1, whereas at low thiol concentrations higher values are achieved. This higher order kinetics for NADPH was not reported before because most investigators assay this enzyme at high thiol concentrations (usually 10 mM DTT). Under these conditions, apparent Michaelis-Menten kinetics is observed with a Hill coefficient of 1 (21). Comparison between the effects of GSH and DTT on microsomal HMG-CoA reductase shows several differences. At low thiol concentrations, Hill coefficients for NADPH obtained with GSH are higher than those obtained with DTT (see Figs. 2 and 4). Also, while an increase in the concentration of both thiols causes an increase in the $V_{\text{max}}$ of the reaction, the $S_{0.5}$ value for NADPH does not change significantly at various concent-
enzyme kinetics observed. The first one is based on the molecularity of the reaction catalyzed by HMG-CoA reductase. This reaction is bimolecular for NADPH and being such, a Hill coefficient of 2 is expected (18). Indeed, this value is observed at low GSH. However, the Hill coefficient is changed to 1 at higher GSH concentrations. If the value of 2 observed at low GSH was merely due to the molecularity of the reaction, we have to assume that the higher thiol concentrations caused a change in one of the reductive steps, catalyzed by this enzyme, to become rate-limiting relative to the other. Under these conditions, apparent first order kinetics would have been observed. The second explanation for the observed effect of high thiol concentrations on the Hill coefficient for NADPH is that thiols, by reducing a protein disulfide bridge(s), change the NADPH binding properties of the enzyme catalytic subunits from a fully cooperative mode to an independent one, affecting the sigmoidal kinetics towards this substrate.

The first possibility (molecularity) is rejected on the basis of comparison between the solubilized and the microsomal enzyme kinetics. If GSH affects one of the reductive steps (see above), it should be equally expressed in both enzyme preparations. Yet, the changes of the Hill coefficient caused by GSH are observed only with the microsomal reductase. On the other hand, the shortening of the enzyme polypeptide chains by the solubilization procedure (see below) might have removed segment(s) of the protein which affect the cooperativity in substrate binding, resulting in a transition from an allosteric enzyme to a nonallosteric form. Therefore, we consider the second possibility to be the proper explanation for the observed sigmoidal kinetics.

In an earlier report, we showed that activation of latent hepatic HMG-CoA reductase by thiols is necessary in order to cause binding of the enzyme to agarose/hexane/HMG-CoA affinity column and by analogy to HMG-CoA (9). The results presented here suggest that thiols affect the microsomal enzyme in more than a single site. A fully latent enzyme, when exposed to low concentrations of GSH, is activated and displays sigmoidal kinetics as demonstrated by a Hill coefficient of 2. Exposure of the enzyme to higher concentrations of thiols further modifies it to create a different form of the enzyme with a Hill coefficient of 1 towards NADPH. The latter modification is accompanied by an increase of \( V_{max} \) which can not be explained merely by an activation of additional enzyme molecules, since such activation would not affect the Hill coefficient. A possible explanation for the difference in the effect of GSH and DTT on the Hill coefficient values for NADPH may lie in the tendency of GSH to form protein-S-SG mixed disulfides whereas DTT is known to reduce protein disulfides completely to the thiol form (22–25), an illustrated by the following scheme.

It is possible that some of the backward reactions may involve low molecular weight disulfides.

Rogers and Rudney (26) recently provided evidence, based
on immunoinhibition curves, that the binding of the substrates to HMG-CoA reductase induces a conformational change in the enzyme and a related alteration in antigenicity. A conformational change of the enzyme caused by the binding of NADPH may explain the cooperativity effect towards this substrate. If HMG-CoA binding also causes conformational change, it is not expressed in cooperative kinetics towards this substrate (Fig. 9).

The freeze-thaw procedure for solubilization of HMG-CoA reductase results in an enzyme of $M_r = 104,000$ (9, 21). This solubilization is inhibited by leupeptin (27). The subunits’ $M_r$ of the enzyme from rat liver, Chinese hamster liver, and UT-1 cells that was solubilized by freeze-thawing and purified in the absence of leupeptin are 50,000 and 55,000 (21, 28–30). Addition of leupeptin and solubilization of the UT-1 cells with the detergent Zwittergent 3–14 yielded a protein of $M_r = 62,000$ as determined on sodium dodecyl sulfate-gel electrophoresis (29, 31). Thus, the conclusion was drawn that the freeze-thaw solubilized enzyme is a proteolytic product of a higher molecular weight protein (27, 29). Recently, Chin et al. (30) presented evidence that the $M_r = 62,000$ can be produced from a 90,000 $M_r$ protein. The data presented here show clearly that the soluble proteolytic product shows different enzyme kinetics than the kinetics displayed by the membrane-bound enzyme (Fig. 6). This freeze-thaw solubilized enzyme no longer displays cooperative kinetics towards NADPH, and a constant Hill coefficient of 1 is observed regardless of the thiol concentration used for its activation. Thus, the partial proteolysis which takes place during solubilization may result in enzyme subunits that do not possess cooperative interaction(s) any longer. Alternatively, it is possible that the membrane-bound enzyme contains, in addition, a regulatory protein subunit which is lost during solubilization.

While the full physiological significance of the observations above is yet to be assessed, there is a general line of evidence which indicates that thiols, and possibly disulfides, may play a role in the regulation of HMG-CoA reductase. The reported physiological concentrations of GSH range between 0.5 and 10 mM (32–36) and it is interesting to note that the largest variations in the Hill coefficient for NADPH occur within this range of concentrations (see Fig. 2). This supports the possibility that the variation in hepatic glutathione concentrations is a predominant factor in the regulation of HMG-CoA reductase. Viña et al. (36) have shown that, in rat liver, at different metabolic states, the ratio [GSH]/[GSSG] in vivo may vary between 5 and 20 while other investigators reported GSSG concentrations that are somewhat different from those measured by Viña et al. (37–39). Isaacs and Binkley (40, 41) showed that the ratio [GSH]/[GSSG] varies diurnally and that compounds that stimulate formation of cAMP reduce significantly the hepatic thiol/disulfide ratio. This metabolite has also been shown to be involved in the short term regulation of HMG-CoA reductase activity via the protein phosphorylation-dephosphorylation mechanism (for review see Ref. 42). In a recent report, Gilbert (43) suggested, based on the work of Isaacs and Binkley (40), that the thiol/disulfide ratio, as dictated by the second messenger cAMP, could serve as a “third messenger” to modulate enzymatic activities (43). The data presented above indicate that the activity of hepatic HMG-CoA reductase may be viewed in the context of the cellular Thiol-Status, as was also suggested previously (9, 10).

The reduction of GSSG to GSH by the enzyme glutathione reductase utilizes NADPH which is also the cosubstrate for HMG-CoA reductase reaction. The highest reported concentrations of free hepatic NADPH are 300–380 μM NADPH (36, 44). Thus, our studies were conducted well within the physiological concentrations of this substance. Since earlier reports indicated that disulfides, including GSSG, inhibit HMG-CoA reductase and thiols activate it (7–10), it is clear that the same reductant (NADPH) is necessary for both activation and activity of this enzyme. The data presented here show for the first time that activation of the microsomal reductase by thiols is not an all-or-none process. Rather, the enzyme is “sensitive” enough to detect variations in the cellular GSH level and to modulate its activity accordingly. This model suggests that at low cellular Thiol-Status, cholesterol synthesis, as determined by the activity of HMG-CoA reductase, is finely tuned to the cellular NADPH concentrations. At high Thiol-Status, where it is also expected that the cellular NADPH concentration will be high, the catalytic activity of HMG-CoA reductase is enhanced and is no longer modulated. Such metabolic control ensures that hepatic cholesterologenesis is constantly and tightly coupled to the “reductive state” of the cell.

It is clear that the conditions under which HMG-CoA reductase was usually assayed (high DTT) and the constant search for a purified enzyme prevented a previous observation that this enzyme’s activity is thiol-dependent, allosterically modulated. Future studies of the metabolic regulation of HMG-CoA reductase will have to take into account the different properties of the membrane-bound and the solubilized enzyme.

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