The Effects of Mevinolin on the Thiol/Disulfide Exchange between
3-Hydroxy-3-methylglutaryl-coenzyme A Reductase and Glutathione*

Roseann E. Cappel and Hiram F. Gilbert
From the Verna and Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

The feeding of mevinolin plus cholestyramine to rats results in the production of a form of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR-CM) having thiol/disulfide redox properties different from those of 3-hydroxy-3-methylglutaryl-CoA reductase isolated from animals which had been given only cholestyramine (HMGR-C). The second-order rate constant for the inactivation of HMGR-CM by GSSG is 7-fold slower than for HMGR-C, while the second-order rate constant for the reactivation of oxidized enzyme by GSH is 100-fold slower. However, in the presence of saturating concentrations of both substrates, the rate constants for thiol/disulfide exchange are similar for both forms of the enzyme. HMGR-CM behaves as if a protein-glutathione mixed disulfide having a $R_o$ of 27 ± 4 is formed at equilibrium. In contrast, HMGR-C has previously been shown to form a protein-protein disulfide (Cappel, R. E., and Gilbert, H. F. (1988) J. Biol. Chem. 263, 12204–12212). Both forms of the enzyme are more difficult to oxidize thermodynamically in the presence of saturating levels of both substrates. For HMGR-CM, NADPH alone has no effect on the equilibrium constant for oxidation, but hydroxymethylglutaryl-CoA alone makes the enzyme approximately twice as difficult to oxidize. Under physiological conditions, HMGR-CM is thermodynamically more difficult to oxidize than HMGR-C. HMGR-C can be converted to HMGR-CM by in vitro treatment with mevinolin. A direct or indirect interaction of mevinolin with HMGR-C results in some persistent, as yet undefined, structural alteration which inhibits the formation of a protein–SS–protein disulfide upon oxidation by glutathione disulfide.

3-Hydroxy-3-methylglutaryl-CoA reductase (HMGR) catalyzes the reductive cleavage of HMG-CoA to mevalonate and free CoA, the first step in the metabolic pathway leading to the synthesis of a number of important biological products including cholesterol, dolichol, and ubiquinone (1–3). The enzyme is a glycosylated integral membrane protein located in the endoplasmic reticulum (4–11). Control of this metabolic pathway is mediated both by alterations in the amount of HMGR present and by changes in the catalytic activity of the enzyme (12). This topic has been extensively reviewed (13).

HMGR is present in low concentration in the cell, less than 0.01% of total cell protein (14). Addition of sterol-sequestering resins such as cholestyramine (Questran) or Amberlite XAD-2 to the diet of mice or rats increases the concentration of HMGR by 3–8-fold (15–17). The resin remains in the intestine of the animal and acts by absorbing dietary cholesterol and bile acids, thus depleting the animal of exogenous sterols and inducing the synthesis of HMGR. The discovery of two chemically related fungal metabolites, compactin (18) and mevinolin (19, 20), which potently inhibit the activity of HMGR, provided a means by which the level of the enzyme could be further increased. Treatment of rats with both cholestyramine and mevinolin results in the production of significantly higher levels of HMGR than does either agent alone (15, 21).

Recent reports in the literature have presented evidence that HMGR isolated from the livers of rats fed both cholestyramine and mevinolin (HMGR-CM) exhibits different properties than the enzyme isolated from the livers of rats fed only cholestyramine (HMGR-C) (4, 15, 21, 22). Both Ness et al. (21) and Roitelman and Schechter (15) have observed that HMGR-C exhibits sigmoidal kinetics with respect to NADPH when assayed in the presence of 5 mM GSH, while HMGR-CM demonstrates normal hyperbolic kinetics under the same conditions. Ness et al. (4) have demonstrated the existence of a disulfide-linked dimeric form of HMGR-C in the absence of thiols. This species could be converted to the monomeric form by the addition of DTT. For HMGR-CM, however, only the monomeric species was observed under both reducing and oxidizing conditions (4). Rogers and Rudney (23) have reported that microsomal HMGR-C incubated in vitro with mevinolin is less reactive with antibody produced against soluble HMGR than is the untreated enzyme. This effect occurs in the absence of a significant loss of enzymatic activity and is not reversed by dialysis.

HMGR is absolutely dependent on the presence of a thiol for activity (21, 22, 24) and is readily inactivated by disulfides (4, 25, 26). Because oxidized HMGR is inactive, the possibility that thiol/disulfide exchange, mediated either by GSSG or another cellular disulfide, could serve as a means of metabolic regulation should be considered. The intracellular environment is known to be highly reducing (27–33). Unless the enzyme sulphydryl group in question can undergo oxidation in this environment and remain oxidized for a significant period of time, regulation of enzymatic activity by thiol/disulfide exchange would be improbable. Thus, both thermodynamic and kinetic factors must be examined in determining
the susceptibility of a particular enzyme to regulation by thiol/disulfide exchange. This topic has been recently reviewed (34–36).

The thiol/disulfide redox properties of HMGR-C have been previously reported (26). This enzyme is thermodynamically easier to oxidize than any other mammalian enzyme which has been studied to date. The activity of the enzyme could vary between 35 and 92% in response to normal variations in the thiol/disulfide redox status of the major intracellular redox buffer, glutathione (26). The oxidized species of HMGR-C behaves as a protein–S–S–protein disulfide, rather than a protein–S–glutathione mixed disulfide (26). Because significant differences in the redox properties of HMGR-C and HMGR-CM might serve to explain some or all of the observed differences in the behavior of the two forms of the enzyme, experiments were designed to measure the redox properties of the HMGR-CM form of the enzyme.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

**Kinetics of Thiol/Disulfide Exchange between Glutathione and HMGR-CM in the Absence of Substrate**—In the absence of either substrate, rat liver HMGR-CM is totally inactivated by low concentrations of GSSG. As is shown in Fig. 1 (Mini-print), the reaction of the enzyme with GSSG is rapid, monophasic, and first-order in GSSG. The oxidative inactivation is reversible. Incubation of the GSSG-oxidized enzyme with a thiol (DTT or GSH) results in restoration of enzymatic activity. With DTT, the extent of reactivation is 80% of the initial activity. When GSH is used to reduce the enzyme, the extent of reactivation is, within the limits of experimental error, the equilibrium position predicted by the [GSH]/[GSSG] ratio present in the reaction and the $K_{eq}$ measured for this form of the enzyme (see below). The rate of reactivation by GSH is slow enough to be measured over a GSH concentration range of 2.5–40 mM (Fig. 2, Miniprint). Rate constants are summarized in Table I.

**Kinetics of Thiol/Disulfide Exchange between Glutathione and HMGR-CM under Turnover Conditions**—The thiol/disulfide exchange reaction between glutathione and rat liver HMGR-CM occurs even in the presence of saturating levels of both substrates. The rate at which the enzyme is inactivated during the assay could be measured by adding the GSH-reduced enzyme to an assay solution containing NADPH, HMG-CoA, and various concentrations of GSSG. When no additional GSSG was added at the time the assay was initiated, the rate of lactone production was linear with time over the 40-min assay period; however, when GSSG was present, the rate of lactone production decreased with time, indicating that the enzyme was being inactivated during the assay (Fig. 3, Miniprint). The apparent first-order rate constant for the loss of enzymatic activity under these conditions was determined by fitting the time course for the formation of lactone to an integrated rate equation describing the effect of enzyme inactivation on the appearance of product (26).

Reactivation of oxidized HMGR-CM by GSH can also occur under turnover conditions. Addition of oxidatively inactivated enzyme to an assay mixture containing various concentrations of GSH resulted in a significant increase in the rate of lactone formation with time (Fig. 4, Miniprint). A control in which 22 mM DTT was substituted for GSH, was used to define the $V_{max}$. For the DTT control, the rate of lactone production was linear with time, indicating that the time required to fully reduce the enzyme was insignificant compared to the intervals at which time points were taken. When GSH was used to reduce the enzyme, the rate of lactone production increased with time, resulting in a curved assay. Thus, the enzyme was being activated by GSH during the course of the assay. The apparent first-order rate constants for GSH-dependent activation of the enzyme were obtained in a manner analogous to that used for the inactivation in the presence of substrates.

**Redox Equilibrium in the Absence of Substrates**—The equilibrium constant ($K_{eq}$) for the reaction

$$\text{HMGR-CM} + \text{GSSG} \rightleftharpoons \text{HMGR-CM} + \text{GSH}$$

was measured by incubating rat liver HMGR-CM in standard assay buffer containing various [GSH]/[GSSG] ratios and GSH concentrations. Each sample was assayed for enzymatic activity, [GSH], and [GSSG] after equilibrium had been established (>60 min). The rate at which the protein thiols reequilibrate with the redox buffer during the assay was minimized by using a large dilution of the preincubation solution in the assay and a short assay time. In order to determine that the preincubation time was sufficient to allow the system to come to equilibrium, redox curves were constructed by beginning with undialyzed enzyme, which is mostly active, and also with enzyme which has been dialyzed overnight against standard assay buffer and had <5% activity. The redox curves generated by these two methods were indistinguishable from each other (Fig. 5).

**Redox Equilibrium in the Presence of One or Both Substrates**—The $K_{eq}$ for rat liver HMGR-CM under turnover conditions was determined in a manner similar to that described above. The enzyme was preincubated with a series of glutathione redox buffers containing various [GSH]/[GSSG] ratios and concentrations of GSH. In order to maximize the rate at which the enzyme could reequilibrate with the glutathione redox system during the assay, the substrates were added to the preincubation mixture in a minimal volume (1.1-fold dilution of the preincubate), the assay time was increased to 60 min, and the concentration of enzyme in the preincubation had to be lowered to ensure that HMG-CoA was not depleted during the assay incubation. Identical results were obtained either starting the assay with fully active HMGR-CM or with HMGR-CM which was brought to redox equilibrium in the preincubation. The $K_{eq}$ for HMGR-CM is significantly lower (enzyme more difficult to oxidize) in the presence of saturating concentrations of both substrates than in
FIG. 5. Redox equilibrium between HMGR-CM and glutathione in the absence of substrates. HMGR-CM (0.29 mg of protein/ml) was preincubated at 37 °C in 0.1 M phosphate buffer, pH 7.1, containing various concentrations of GSH and GSSG until equilibrium was reached (>75 min). The preincubate was diluted 20-fold into standard assay mixtures, and the activity of the enzyme was measured using a 5-min assay incubation time. $V_{\text{max}}$ was determined from a control containing 60 mM DTT in addition to the indicated concentration of GSH. Experiments in which the initial activity of the enzyme was either >70% or less than 5% yielded identical redox curves. The data is plotted as a function of $R$. The curve is drawn according to Equation 7, with a $K_{\text{a}}$ of 27.

The redox behavior of HMGR-CM in the presence of a single substrate was also determined. HMGR-CM, briefly preincubated with either 60 μM HMG-CoA or with 3 mM NADPH and the NADPH-regenerating system, was equilibrated with a series of glutathione redox buffers. After equilibration with the glutathione system, an aliquot of the enzyme solution was diluted 20-fold into the usual assay mixture to minimize redox state changes during the 5-min assay. NADPH had no significant effect on the $K_{\text{a}}$ for enzyme inactivation; however, HMG-CoA reduced the $K_{\text{a}}$ by about 50% compared to the enzyme in the absence of substrates (Fig. 7).

Treatment of NADPH-C with Mevinolin in Vitro—The possibility of converting HMGR-C to a form having the redox properties of HMGR-C was tested by incubating reduced HMGR-C with the sodium salt of mevinolinic acid and measuring the redox properties of this form of the enzyme in the absence of substrates. Fig. 8 shows that the redox behavior of HMGR-C treated with mevinolin in vitro was identical to that of HMGR-CM, having lost the dependence on the absolute concentration of GSH which is characteristic of HMGR-C (26). The specific activity of fully reduced enzyme was significantly lower than that of the untreated HMGR-C form of the enzyme from which it was generated. The $V_{\text{max}}$ of fully reduced enzyme was decreased to 65 and 27% of its original the absence of substrate (Fig. 6, Table I).

FIG. 6. Redox equilibrium between HMGR-CM and glutathione in the presence of substrates. HMGR-CM was preincubated at 37 °C in 0.1 M phosphate buffer, pH 7.1, containing various concentrations of GSH and GSSG until equilibrium was reached (>75 min) or enzyme was prereduced with 5 mM DTT for at least 60 min. The preincubate was diluted 1.1-fold into standard assay mixtures, and the activity of the enzyme was measured using a 60-min assay incubation time. Both assays contained 18 μg of protein/ml. $V_{\text{max}}$ was determined from a control containing 20 mM DTT in addition to the indicated concentration of GSH. Only those samples whose half-life for the approach to equilibrium (calculated according to Equation 1) is less than 6 min were included in the data set. The data is plotted as a function of $R$. The solid curve is drawn according to Equation 7, with a $K_{\text{a}}$ of 2.0. The dotted line is drawn with the $K_{\text{a}}$ = 27 for the enzyme in the absence of substrates.

Fig. 7. Redox equilibrium between HMGR-CM and glutathione in the presence of a single substrate. HMGR-CM (0.29 mg of protein/ml) was preincubated with either 3 mM NADPH (●) or 60 μM HMG-CoA (■) in 0.1 M phosphate buffer, pH 7.1, containing various concentrations of GSH and GSSG until equilibrium was reached (>75 min at 37 °C). The preincubate was diluted 20-fold into standard assay mixtures, and the activity of the enzyme was measured using a 5-min assay incubation time. $V_{\text{max}}$ was determined from a control containing 60 mM DTT in addition to the indicated concentration of GSH. Experiments in which the initial activity of the enzyme was either >70% or <5% yielded identical redox curves. The data are plotted as a function of $R$. Solid curves are drawn according to Equation 7, with a $K_{\text{a}}$ of 26 and 16.
reactions by which the P-SS-G and the P-SS-P species are formed are described in Equations 2, 3, 4, 5, 6, 7, and 8. The oxidized enzyme product of the reaction described in Equation 2 might be either a protein–SS–G mixed disulfide (P–SS–G) or a protein–SS–protein disulfide (P–SS–P). The reactions by which the P–SS–G and the P–SS–P species are formed are described in Equations 3 and 4, respectively.

\[
E_{SS}^G + GSSG \rightleftharpoons E_{SS}^P + GSH \tag{3}
\]

\[
E_{SS}^P = E_{SS} + GSH \tag{4}
\]

If P–SS–G is the only oxidized species formed, the equilibrium constant (\(K_{eq}\)) for Equation 3 is defined by Equation 5

\[
K_{eq} = \frac{[PSSG][GSH]}{[PSSH][GSSG]} \tag{5}
\]

For convenience, the quantity \([GSH]/[GSSG]\) will be referred to as \(R\). If P–SS–P is formed, \(K_{eq}\) is defined by Equation 6.

\[
K_{eq} = \frac{[PSSP][GSH]^2}{[PSSH][GSSG]} \tag{6}
\]

The quantity \([GSH]^2/[GSSG]\) will be referred to a \(R\cdot[GSH]\) for convenience.

The fraction of the enzyme in the reduced (active) form in the case of the formation of a P–SS–G disulfide and in the case of the formation of a P–SS–P disulfide are described by Equations 7 and 8, respectively

\[
\frac{[PSS]}{P_t} = \frac{R}{K_{eq} + R} \tag{7}
\]

\[
\frac{[PSS]}{P_t} = \frac{R \cdot [GSH]}{K_{eq} + R \cdot [GSH]} \tag{8}
\]

where \(P_t\) is total protein. In the case of the formation of P–SS–G, the activity is a function of \(R\) alone and is independent of \([GSH]\). Thus, a plot of activity against \(R\) will take the form of a single rectangular hyperbola. If a P–SS–P is formed, the activity will be a function of \(R\cdot[GSH]\), and a plot of activity as a function of \(R\) will take the form of a series of rectangular hyperbolas, one for each fixed concentration of GSH (if the data is replotted as activity versus \(R\cdot[GSH]\), a single rectangular hyperbola will result). For HMGR-CM in the absence of substrates or in the presence of either or both substrates (Table I), the data clearly indicate that the formation of a P–SS–G mixed disulfide can fully account for the behavior of HMGR-CM in glutathione redox buffers (Figs. 5–7).

The presence of NADPH during redox equilibration has no significant effect on the \(K_{eq}\), and HMGR-CoA alone only makes the enzyme about 2-fold more difficult to oxidize (lower \(K_{eq}\)). However, the presence of both substrates under turnover conditions makes the enzyme approximately 10-fold less sensitive to oxidation (Table I). Based on a study of the kinetic mechanism of proteolyzed rat liver HMGR, Tanzawa and Endo (43) have concluded that the binding of the substrates is sequential with HMGR-CoA binding before NADPH. Thus, the inability of NADPH alone to alter the \(K_{eq}\) of the enzyme is not unexpected. However, Qureshi et al. (44) have suggested a random kinetic mechanism for the yeast enzyme.

Taken together, the above experiments suggest that the oxidized enzyme product of the thiol/disulfide interchange reaction between HMGR-CM and glutathione is a P–SS–G mixed disulfide. The observation by Ness et al. (4) that the oxidation of HMGR-C yields a disulfide-linked dimer, while oxidation of HMGR-CM does not, is consistent with the above result.

**Comparison of the Redox Properties of HMGR-CM and HMGR-C**—The most striking difference between the redox properties of HMGR-CM and HMGR-C forms of the enzyme is found in a comparison of the oxidized enzyme product of the thiol/disulfide exchange reaction with glutathione. HMGR-C forms a P–SS–P disulfide (26), while HMGR-CM forms a P–SS–G mixed disulfide. Thus, the equilibrium redox behavior of HMGR-C is dependent both on the \([GSH]/[GSSG]\) ratio and on the absolute concentration of GSH, while the equilibrium redox properties of HMGR-CM are dependent only on the \([GSH]/[GSSG]\) ratio.

Which form of the enzyme is more easily oxidized at equilibrium, HMGR-C or HMGR-CM? In order to compare two
enzymes, one of which is oxidized to a P-SS-G and the other of which is oxidized to a P-SS-P disulfide, a standard GSH concentration must be adopted. The term $K_{\text{eq}}$ will be used to refer to the apparent $K_{\text{eq}}$ at a given GSH concentration. For a P-SS-G, $K_{\text{eq}} = K_{\text{eq}}$. For a P-SS-P, $K_{\text{eq}} = K_{\text{eq}}/[\text{GSH}]$. At equilibrium in 1 mM GSH, HMGR-C is 20-fold easier to oxidize than HMGR-CM. At 100 mM GSH, however, the situation is reversed; HMGR-CM is 5-fold more easily oxidized than HMGR-C. Near 20 mM GSH, the $K_{\text{eq}}$ values for the two forms of the enzyme are nearly equal.

In Vitro Conversion of HMGR-C to a Form Having the Redox Properties of HMGR-CM—The structural reason for the difference in redox behavior between HMGR-C and HMGR-CM is not yet known. Mevinolin, in the lactone or the diacid form, may either bind directly to HMGR or interact with one or more cellular systems to alter the structure or environment of the enzyme in a manner which does not significantly change its molecular weight. Alterations in the glycosylation state, proteolytic cleavage of a small fragment, or changes in the phospholipid composition of the membrane in the immediate area of the enzyme are possible examples of the latter. However, the observation that in vitro incubation of HMGR-C with mevinolinic acid altered the redox behavior to that shown by the HMGR-CM form of the enzyme favors the argument that the direct binding of mevinolin to a site on the enzyme results in altered enzyme specific activity and changes redox behavior. Similarly, Rogers and Rudney (23) observed that in vitro incubation of HMGR-C with the carboxylic acid form of mevinolin caused a 3-fold decrease in the ability of the enzyme to react with antibody raised against the soluble proteolyzed form of HMGR.

The effect of mevinolin on the redox behavior of HMGR can be produced in a cell-free system and is likely due to a direct interaction of mevinolin with the protein or to some covalent modification which can occur in isolated microsomes. Maximal changes in the reactivity toward antibody (23) and the thiol/disulfide redox properties of the enzyme occur when microsomes are treated with ≤10 nM mevinolin. At this concentration of mevinolin, the enzyme is <40% inhibited; however, changes in antibody recognition (23) and thiol/disulfide redox behavior are complete. Thus, the effects of mevinolin on the antibody recognition and the thiol/disulfide redox behavior of the enzyme are at least partially separable from the effect on activity, suggesting that mevinolin is capable of binding to more than one site on the enzyme.

With the yeast HMG-CoA reductase, comprehensive kinetic studies of the binding of compactin to the enzyme (45) suggested an interaction which was competitive with HMG-CoA. The rate constant for dissociation of the bound inhibitor from the enzyme was measured to be $6.5 \times 10^{-3}$ s$^{-1}$ (half-life of 1.8 min). If the rat liver enzyme behaves similarly, extensive dialysis would be expected to remove the inhibitor. However, even after prolonged dialysis or repeated washing of the microsomal enzyme, the effect of mevinolin on the redox behavior still persists. Thus, there appears to be a site which binds mevinolin very tightly and alters the redox and immunological properties of the protein. The occupancy of this site, however, cannot completely inhibit the enzyme or no activity would be observed.

The molecular basis for the dissimilarity in the properties of the two forms of HMGR remains obscure. The inability of HMGR-CM to form a P-SS-P disulfide could be explained by either a direct blocking of a single sulfhydryl group on the enzyme by bound mevinolin or even a covalent chemical reaction between the lactone form of mevinolin and a protein thiol. The differences in reactivity toward antibody, however, would seem to suggest that the interaction of mevinolin with HMGR results in a more generalized alteration in the tertiary or quaternary structure of the protein. Conformational changes involving the surface of the protein or perturbation of a monomer-dimer equilibrium could provide a plausible explanation for both phenomena.

In Vitro Consequences of the Redox Properties of HMGR-CM on the Assay of HMGR Activity—Even freshly prepared stock solutions of GSH at neutral pH contain from 0.5–2% GSSG which is formed by autoxidation of GSH. Thus, every glutathione solution actually constitutes a redox buffer. Because the reaction between the lactone form of mevinolin and a protein or changes in the phospholipid composition of the membrane can occur at a significant rate in the presence of saturating levels of both substrates suggests that this process might be a viable method for regulation of HMGR activity in vivo. The active site of HMGR faces the cytosolic side of the endoplasmic reticulum. Neither the absolute concentration of GSH nor the [GSH]/[GSSG] ratio of the cytosolic compartment is known with certainty (32, 35, 46-48). At physiological levels of GSH (2-3 mM and GSSG = 3 μM), HMGR-CM will be thermodynamically easier to oxidize than HMGR-C. For example, under conditions of GSH = 3 mM and GSSG = 30 μM (typical of a fasted rat liver), HMGR-CM would be 79% reduced at equilibrium while HMGR-C would be only 35% reduced. Based on the in vitro data, HMGR activity in liver cells of cholestyramine- plus mevinolin-fed rats could be expected to be more resistant to inhibition by sulfhydryl oxidation than the enzyme from animals which had been fed only cholestyramine.

Acknowledgments—Mevinolin was graciously provided by Merck. Dr. Andrew J. Alpert (PolyLC, Columbia, MD) provided the postcolumn reactor used in the determination of GSH and GSSG concentrations.

REFERENCES

1. Panini, S. R., Rogers, D. H., and Rudney, H. (1985) in Regulation of HMG-CoA Reductase (Price, B., ed) pp. 149-181, Academic Press, Orlando, FL
2. Sabine, J. R. (1983) in 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (Sabine, J. R., ed) pp. 3-18, CRC Press, Inc., Boca Raton, FL
3. James, M. J., Potter, J. E. R., and Kandutsch, A. A. (1983) in 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase (Sabine, J. R., ed) pp. 19-28, CRC Press, Inc., Boca Raton, FL
4. Ness, G. C., McCrery, M. J., Sample, C. E., Smith, M., and Pendleton, L. C. (1985) J. Biol. Chem. 260, 16385-16399
HMG-CR-Thiol/Disulfide Exchange

5. Clarke, C. F., Edwards, P. A., Lan, S.-F., Tan, N. D., and Fogelman, A. M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 3305–3308

6. Edwards, P. A., Lan, S.-F., Tan, N. D., and Fogelman, A. M. (1983) *J. Biol. Chem.* 258, 7272–7275

7. Hardeman, E. C., Jenke, H. S., and Simon, R. D. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 1516–1520

8. Liscum, L., Luecky, K. L., Chin, D. J., Ho, Y. K., Goldstein, J. L., and Brown, M. S. (1983) *J. Biol. Chem.* 258, 8450–8455

9. Ness, G. C., Phillips, C. E., and Eichler, D. C. (1983) *J. Lipid Res.* 24, 1441–1449

10. Liscum, L., Cummings, R. D., Anderson, R. G. W., DeMartino, G. N., Goldstein, J. L., and Brown, M. S. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 7165–7169

11. Edwards, P. A., Kempner, E. S., Lan, S.-F., and Erickson, S. K. (1985) *J. Biol. Chem.* 260, 10278–10282

12. Edwards, P. A., and Fogelman, A. M. (1985) in Regulation of HMG-CoA Reductase (Preiss, B., ed) pp. 133–148, Academic Press, Orlando, FL

13. Priess, B. (ed) (1985) *Regulation of HMG-CoA Reductase*, pp. 1–300, Academic Press, Orlando, FL

14. Hardeman, E. C., Endo, A., and Simon, R. D. (1984) *Arch. Biochem. Biophys.* 232, 549–561

15. Roitelman, J., and Shechter, I. (1986) *J. Biol. Chem.* 261, 5061–5066

16. Goldfarb, S., and Pitot, H. C. (1972) *J. Lipid Res.* 13, 797–801

17. Yezerksy, M., Shetter, L. I., Konv, N., and Langer, O. (1980) *J. Lab. Clin. Med.* 96, 965–973

18. Endo, A., Kuroda, A. M., and Tawakazu, K. (1986) *FEBS Lett.* 73, 323–326

19. Endo, A. (1979) *J. Antibiot.* (Tokyo) 32, 852–864

20. Albers, A. W., Chen, W. J., Kuron, G., Hunt, V., Huff, J., Hoffman, C. L., Jollis, J., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensons, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77, 3857–3861

21. Ness, G. C., Fallet, S. J., Pendleton, L. C., and Smith, M. S. (1984) *J. Biol. Chem.* 260, 12931–12936

22. Roitelman, J., and Shechter, I. (1984) *J. Biol. Chem.* 259, 870–877

23. Rogers, D. H., and Rudney, H. (1982) *J. Biol. Chem.* 257, 10650–10659

24. Tormaran, C. D., and Scallen, T. J. (1981) *Circulation* 64, IV–270 (Abstr. 1031)

25. Gilbert, H. F., and Stewart, M. D. (1981) *J. Biol. Chem.* 256, 1782–1785

26. Cappel, R. E., and Gilbert, H. F. (1988) *J. Biol. Chem.* 263, 2204–2212

27. Sies, H., Brigelius, R., and Ackermann, T. P. M. (1983) in *Functions of Glutathione: Biochemical, Physiological, Toxicological, and Clinical Aspects* (Larsson, A., Orrenius, S., Holmgren, A., and Mannervik, B., eds) pp. 231–240, Raven Press, New York

28. Orrenius, S., and Moldeus, P. (1984) *Trends Pharmacol. Sci.* 5, 432–435

29. Kasai, J. (1987) *Biochim. Biophys. Acta* 928, 121–123

30. Lauterburg, B. H., Smith, C. V. Hughes, H., and Mitchell, J. R. (1984) *J. Clin. Invest.* 73, 124–133

31. Meister, A., and Anderson, M. E. (1983) *Annu. Rev. Biochem.* 52, 711–760

32. Alpert, A. J., and Gilbert, H. F. (1985) *Anal. Biochem.* 144, 553–562

33. Kosower, N. S., and Kosower, E. M. (1978) *Int. Rev. Cytol.* 54, 109–160

34. Ziegler, D. M. (1985) *Annu. Rev. Biochem.* 54, 306–329

35. Gilbert, H. F. (1984) *Methods Enzymol.* 107, 330–351

36. Gilbert, H. F. (1989) *Adv. Enzymol. Relat. Areas Mol. Biol.*, in press

37. Ness, G. C., Sample, C. E., Smith, M., Pendleton, L. C., and Eichler, D. C. (1986) *Biochem. J.* 233, 167–172

38. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254

39. Mokin, G. L. (1985) *Arch. Biochem. Biophys.* 243, 70–77

40. Kita, R., Brown, M. S., and Goldstein, J. L. (1980) *J. Clin. Invest.* 66, 1094–1107

41. Stubbs, R. J., Schwartz, M., and Bayne, W. F. (1986) *J. Chromatogr.* 383, 438–443

42. Walters, D. W., and Gilbert, H. F. (1986) *J. Biol. Chem.* 261, 13135–13143

43. Raus, K., and Endo, A. (1979) *J. Biochem. (Tokyo)* 98, 195–201

44. Qureshi, N., Dugan, R. E., Cleland, W. L., and Porter, J. W. (1976) *Biochemistry* 1519–14197

45. Nakamura, C. E., and Ables, R. H. (1985) *Biocemistry* 24, 1364–1376

46. Tateishi, N., Higashi, T., Shinya, S., Naruse, A., and Sakamoto, Y. (1974) *J. Biochem.* (Tokyo) 75, 93–103

47. Issacs, J. T., and Binkley, F. (1977) *Biochem. Biophys. Acta* 498, 29–38

48. Sies, H., Brigelius, R., and Graf, P. (1987) *Adv. Enzyme Regul.* 26, 175–189
Radical Equilibrium in the Absence of Substrate. HMGR-CM was pre-incubated in standard assay buffer with a series of glutathione redox buffers containing GSH and GSSG at ratios varying between 0 and 100% at concentrations ranging between 2 and 110 μM. The enzyme was diluted into the buffer and assayed with a 60 mM DTT in addition to the indicated concentration of GSH. The concentration of π-intermediate in the pre-incubation solution was 0.26 mm. After equilibration at 37°C, the reaction was initiated by the addition of the indicated enzyme concentration and the activity was determined using the indicated incubation period. Another aliquot was assayed by determining the activity in the pre-incubation solution with 30 mm DTT-glucose-6-phosphate and 0.4 μM of glucose-6-phosphate dehydrogenase. The activities were corrected by subtracting the activity in the absence of the enzyme. The standard assay was assayed with standard assay buffer with the indicated concentration of GSH and GSSG. The activity was determined using the indicated incubation period. The enzyme was pre-incubated with NADPH, 30 mm DTT-glucose-6-phosphate and 0.4 μM of glucose-6-phosphate dehydrogenase. The activity was determined using the indicated incubation period.

Only those samples having a half-life in the assay of >30 min were included in the data set. In order to determine that the pre-incubation was sufficient to allow the system to come to equilibrium, reaction curves were constructed by beginning with unblocked enzyme, which is mostly active, and also with enzyme which had been delayed overnight against standard assay buffer and had < 0.4% activity. The same equilibrium position was achieved regardless of the initial reaction state of the enzyme.

Radial Equilibrium in the Presence of Both Substrates. All experiments were carried out as described in the previous section, except that either 60 μM NADPH or 30 μM NADPH was included in the pre-incubation mixture. The enzyme was then treated with NADPH, 30 mm DTT-glucose-6-phosphate and 0.4 μM of glucose-6-phosphate dehydrogenase. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

In Ultra-Reduction of HMGR-CM to a Form of the Enzyme Having the Redox Properties of GSH/GSSG. GSH/GSSG (GSH/GSSG) was used to reduce HMGR-CM in standard assay buffer with either 2 or 50 μM reduced homovanillic acid (0.2% vol/vol) with or without 0.1 μM homovanillic acid. The reaction was carried out in a final concentration of 10% DTT and allowed to proceed for 30 min, after which it was diluted into the assay buffer and assayed with indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.

Inactivation of the Enzyme. The enzyme was pre-incubated with 0.2 or 50 μM DTT and 0.1 μM homovanillic acid for 30 min. The enzyme was diluted into the assay buffer and assayed with the indicated concentrations of GSH and GSSG. The activity was determined using the indicated incubation period. The activity was determined using the indicated incubation period.
Fig. 4. Reactivation of HMGR-CM by GSH using the activity assay. Microsomal HMGR-CM (0.18 mg protein) was reacted by following in 0.1 M phosphate buffer, pH 7.1, with 0.5 mM GSSG for 30 min at 37°C. To initiate the reactivation, the reaction mixture was diluted forty-fold into standard assay solution containing GSH at the indicated concentrations, glutathione reductase (1 U/ml) to reduce GSSG to GSH. The ratio of [GSH]/[GSSG] was > 500:1 in all experiments. At the specified times, the concentration of reduced GSH was determined. (●), 32 mM DTT; (▲), 36 mM GSH; (□), 17 mM GSH; ( ●), 12 mM GSH; and ( ■), 4.7 mM GSH. The solid curves were generated with an integrated rate equation described previously (27). The initial velocity at time zero was 0.012 for the DTT group and was assumed to be zero for the GSH containing samples. The velocity at zero time was 0.012, 0.008, 0.012, 0.012, and 0.012 mmol/liter/min, respectively, and the corresponding forward rate constant was > 10, 0.89, 0.033, 0.007, and 0.0076 mmol/min. The curve is a plot of k_{obs} vs. [GSH]; the line is drawn using a second-order rate constant of 1.7 M^{-1} min^{-1}. 