In glutathione redox buffers, rat liver, microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase rapidly equilibrates between a reduced, active form and an oxidized, inactive form. At pH 7.0, 37 ºC, the second order rate constant for inactivation of the reduced enzyme by GSSG is 1700 ± 200 m⁻¹ min⁻¹, approximately 20-fold faster than the reaction of GSSG with a typical, unhindered thiol of pKₗ 7.7. High concentrations of GSH or lower concentrations of dithiothreitol restore the activity of the oxidized enzyme. The oxidation of the enzyme by GSSG is only 30-fold slower in the presence of saturating levels of both substrates. The incomplete inhibition of thiol/disulfide exchange by substrates can lead to significant changes in the activity of the enzyme during the assay when glutathione is present. At redox equilibrium, both in the absence and presence of substrates, the activity of the enzyme depends on the quantity [GSH]²/[GSSG], suggesting that the redox transition involves the formation of a protein-SS-protein disulfide. The equilibrium constant for the reaction HMG₄reductaseₐ + GSSG ←→ HMG₄reductaseₐ + 2 GSH is 0.55 ± 0.07 m in the absence of substrates and 0.20 ± 0.02 M in the presence of saturating levels of both substrates. Thus, HMG-CoA reductase is very sensitive to dithiol oxidation both kinetically and thermodynamically. Significant changes in the oxidation state and activity of this enzyme could be expected to result from redox changes in the thiol/disulfide oxidation state of the cellular glutathione redox buffer.

The reversible conversion of a protein thiol to a disulfide via an exchange reaction with protein or nonprotein disulfides provides an attractive mechanism for coupling the activity (1–3) or stability (4, 5) of certain enzymes to the thiol/disulfide redox status of the cell. The modulation of enzyme activity by reversible thiol/disulfide redox state changes has been convincingly demonstrated to play a role in the regulation of several chloroplast enzymes in response to light (6). Experiments with mammalian cells have provided some circumstantial evidence suggesting that this mechanism of enzyme regulation could be operative, at least under conditions of induced oxidative stress (7–10). However, the normally high concentration of glutathione (GSH) (11) in most cells coupled with a high ratio of glutathione to glutathione disulfide (GSH)/(GSSG) (12) would impose some significant constraints on the accumulation of protein disulfides in an intracellular environment (13).

The catalytic or structural properties of a large number of intracellular proteins with “essential” sulfhydryl groups can be affected in vitro by oxidizing agents such as DTT, GSSG, or cystamine. Often the effects of oxidants on the properties of the protein are reversed by the addition of reducing agents such as DTT or GSH. The presence of a high concentration of GSH (2–10 mM) (11) and a 10–400-fold excess of GSH over GSSG requires a consideration of both oxidation and reduction processes. If the disulfide state of a particular protein is rapidly reduced in a thermodynamically favorable reaction with GSH, very little disulfide bond formation (and minimal change in enzyme activity) would occur in response to physiological changes in the thiol/disulfide status of the cell (14), and regulation of enzyme activity by this mechanism would be untenable.

The relationship between the reversible oxidation and reduction of protein sulfhydryls/disulfides can be expressed as the equilibrium constant (Kₑ) for the reaction:

$$E_{red} + a \cdot GSSG \rightleftharpoons E_{ox} + b \cdot GSH$$ (1)

$$K_e = \frac{[E_{ox}][GSH]^b}{[E_{red}][GSSG]^a}$$ (2)

where the coefficients a and b depend on the number of glutathione-mixed disulfides and protein-SS-protein disulfides present in the oxidized protein. For a significant fraction of a protein to exist in an oxidized state in equilibrium with the thiol/disulfide status of the medium, the oxidation potential (defined here by Kₑ) must be close to the thiol/disulfide status of the medium as defined by the quantity [GSH]²/[GSSG]².

The enzyme HMG-CoA reductase (HMGR), which catalyzes the reductive cleavage of HMG-CoA to mevalonate and CoASH, regulates the production of a number of important metabolites including cholesterol and dolichol (15). This enzyme is regulated by a number of mechanisms including protein synthesis and degradation (16–18), feedback control...
by sterols (19-22), endocrine regulation by a variety of hormones (23, 24-27), and by phosphorylation (28-30).

HMG requires the presence of thiols for activity, either DTT or high concentrations of GSH. Low concentrations of a number of different disulfides have been shown to inactivate the enzyme (32). Roitelman and Schechter (33) have described a thiol-disulfide reductant that reduces HMGR between a form showing positive cooperativity in NADPH binding and a form which exhibits hyperbolic kinetics with respect to NADPH (23, 31-36). Ness, et al. (35) have demonstrated the occurrence of a disulfide-linked dimer (200 kDa) of the enzyme in microsomes isolated in the absence of DTT from the livers of rats fed a diet containing cholestrol, but not in microsomes isolated from rats fed a diet containing both cholestrol and mevinolin. In the former case, the fraction of dimeric enzyme increased when animals were fasted for 24-48 h (low activity), and the S0.5 for NADPH increased as the fraction of the enzyme in the dimeric form increased. Recently, Dotan and Schechter (37) have reported that a soluble rat-liver protein can activate HMGR, in the presence of NADPH, presumably by reduction.

All of these observations point to a critical role for thiol/disulfide exchange processes in modulating the activity of the enzyme in vitro. On the basis of these reports, experiments were carried to determine the mechanism of, and the equilibrium constant for, the thiol/disulfide exchange between HMGR and glutathione reductase buffers. Consideration of these results, along with the range of physiologically attainable intracellular GSH/GSSG ratios, is used to evaluate the possibility that thiol/disulfide exchange between glutathione and HMGR could be a kinetically and thermodynamically competent control mechanism under thiol/disulfide reoxidation conditions likely to be found in vivo.

EXPERIMENTAL PROCEDURES

Materials—Questran (cholestyramine) was purchased from Mead Johnson. [4-14C]HMG-CoA (40-68 mCi/mmol) and [3H]mevalonate (sodium salt) (2-100 mCi/mmol) were obtained from Du Pont-New England Nuclear. Unlabeled HMG-CoA was from PL Biochemicals or from Sigma. Reduced DTT was purchased from Boehringer Mannheim. GSH, GSSG, NADPH, glucose-6-phosphate, and unlabeled mevalonate were obtained from Sigma. Glutathione reductase, type III from bakers' yeast, and glucose-6-phosphate dehydrogenase, type XV from bakers' yeast were from Sigma. Oxidized DTT was purchased from Aldrich and purified according to the method of Creighton (38). The premixed dye reagent for Bradford protein assays was obtained from Bio-Rad. Pre-poured Silica gel G plates, manufactured by Kodak (13181), were purchased from Fisher. The postcolumn reactor used for the detection of glutathione was a generous gift of Dr. Andrew J. Alpert of Poly LC (Columbia, MD). All other reagents were purchased from a variety of sources and were of the highest grade commercially available.

Animals—Male Sprague-Dawley rats, approximately 150 g, were obtained from Harlan Industries (Indianapolis). The animals were maintained on a diet of standard laboratory rat chow (Wayne Lab Blocks) and a light cycle consisting of equal 12-h periods of light and dark for at least 2 weeks prior to the beginning of the special diet described below. The animals were then continued on the same light cycle and fed the cholestyramine diet for 5-9 days prior to killing. Cholestyramine (Questran) (0.05 g/rat chow) was fed ad libitum in a solid, cubed matrix formulated from powdered Wayne Lab Blocks (810 g), water (1.9 liters), and unflavored gelatin (186 g).

Preparation of Microsomal HMG-CoA Reductase—Animals were killed by asphyxiation with carbon dioxide approximately 5 h into the dark cycle. The livers were removed immediately and placed in ice-cold, 0.25 M sucrose, 5 mM EGTA buffer, pH 7.2, containing 50 μM leupeptin, and 1 mM DTT. The enzyme was prepared according to the procedure of Ness et al. (39). Protein was determined by the method of Bradford, using bovine serum albumin as a standard (40).

The redox equilibrium in the experiments described in this study varied between 2.5 and 3.5 units/mg protein.

Assay of HMG-CoA Reductase Activity—HMG-CoA reductase was routinely assayed according to the procedure of Ness et al. (41). Assay incubation mixtures contained 100 mM potassium phosphate, pH 7.1, 200 mM potassium chloride, 1 mM disodium EDTA (standard assay buffer), 30 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, 60 μM (R, S)-[3H]HMG-CoA, and 3 mM NADPH. The radiospecific activity of HMG-CoA was adjusted between 4 and 0.25 cpm required to produce 1 pmol of mevalonate and a given experiment. All assays were incubated at 37 °C. The assay time was varied between 5 and 30 min, depending upon the requirements of each experiment, and is reported in the legend accompanying each figure. The total incubation volume for each assay was either 300 or 150 μl. HCl (2.4 N at 10% of the assay volume) was added to stop the reaction, and [3H]mevalonate (15,000 cpm) was added to correct for recovery from the thin layer chromatography plate. The samples were counted on a Beckman model LS 6800 Liquid Scintillation Counter. Recovery of the tritiated mevalonolactone averaged 70%, and each assay was corrected to 100% recovery. One unit of enzyme activity is defined as the amount of enzyme required to produce 1 nmol of mevalonate from HMG-CoA at 37 °C, pH 7.1.

Determination of the Concentration of DTT, GSH, and GSSG—The concentration of DTT and GSH in stock solutions was determined by the method of Ellman (42). The concentrations of GSH and GSSG in assay mixtures was determined by the method described by Alpert and Gilbert (12). The only deviation from the published procedure was the use of 20 ± 5 mM potassium phosphate, 1.6 ± 0.4 mM disodium EDTA, pH 6.0, as the elution buffer. This substitution decreased the separation between GSH and GSSG and allowed a more rapid determination of the glutathione concentrations. Samples could be stored at -20 °C for several days without any change in the concentration of GSH or GSSG as long as the sample contained 1 mM EDTA and was acidified to pH 2 with HCl.

Rate of Inactivation by GSSG in the Absence of Substrate—Microsomal HMGR, stored at 0.8 mg protein/ml, was diluted 5-fold into standard assay buffer containing GSSG between 0 and 200 μM. At the designated times, a 15-μl aliquot of each inactivation mixture was removed and diluted 10-fold into the assay mixture as described above. All procedures were carried out at 37 °C, pH 7.1. The incubation time was 5 min.

Rate of Reactivation by GSSG in the Presence of Both Substrates—Microsomal HMGR, at a concentration of 0.9 mg protein/ml, was preincubated with 0.5 mM GSSG in standard assay buffer at 37 °C for 30 min. To initiate the reactivation reaction, an aliquot of the above enzyme mixture was diluted 40-fold into assay solutions containing GSH at concentrations varying between 4 and 37 mM, glutathione reductase (10 units/ml), 3 mM NADPH, and the NADPH-regenerating system described above. A 30-μl aliquot of each DTT was used to determine the rate of mevalonate production by fully reduced HMGR. At the indicated times, a 150-μl aliquot of the reactivation mixture was removed and added to 15 μl of 2.4 N HCl to stop the reaction. The concentration of GSH and GSSG in the assay mixtures was determined by the method described above. Neither GSH or GSSG concentrations changed significantly (<5%) during the course of the experiment.

Rate of Reactivation by GSH in the Presence of Both Substrates—Microsomal HMGR, at a concentration of 0.9 mg protein/ml, was preincubated with 0.5 mM GSSG in standard assay buffer at 37 °C for 30 min. To initiate the reactivation reaction, an aliquot of the above enzyme mixture was diluted 40-fold into assay solutions containing GSH at concentrations varying between 0 and 100 and at glutathione concentrations between 5 and 50 mM. Each experiment contained a reaction system described above. A 30-μl aliquot of each DTT was used to determine the rate of mevalonate production by fully reduced HMGR. At the indicated times, a 150-μl aliquot of the reactivation mixture was removed and added to 15 μl of 2.4 N HCl to stop the reaction and the mevalonate formed was determined as described above.

Redox Equilibrium in the Absence of Substrates—HMGR was incubated under standard conditions with a series of glutathione reductase buffers containing GSH and GSSG at ratios varying between 0 and 100 and at glutathione concentrations between 0 and 50 mM. Each experiment contained a reaction system described above. The rate of reactant concentration was measured by monitoring the rate of mevalonate production. The results indicate that the rate at which the enzyme re-equilibrates with the redox buffer...
during the assay. The assay incubation time was 5 min. Based on the measured concentrations of GSH and GSSG in the assay, and the rate constants for the reduction and oxidation of the enzyme in the presence of substrates, the rate constant for the approach to equilibrium in the assay was calculated. Only those samples having a half-life in the assay which was at least five times greater than the assay incubation period were included in the data.

Redox Equilibrium in the Presence of Both Substrates—A series of glutathione redox buffers containing microsomal HMGR was prepared as described in the preceding section except that the concentration of enzyme was varied between 0.05 and 0.7 mg protein/ml. In order to maximize the rate at which the enzyme and glutathione equilibrate during the assay, the assay time was increased to 10 or 30 min and the dilution of GSH and GSSG into the assay was minimized (1.1-fold). The amount of HMGR present in the assay was decreased to prevent substrate depletion. Based on the measured GSH and GSSG concentrations and the rate constants for reduction and oxidation of the enzyme in the presence of substrates, the rate constant for the approach to equilibrium was calculated for each sample. Only experiments in which the half-life for approach to equilibrium was less than 0.3 times the assay time were included.

In order to keep the volume in which the substrates was added as small as possible, the glucose 6-phosphate required for the recycling of the NADPH during the assay had to be added to the initial incubation buffer. To ensure that the presence of the glucose 6-phosphate in the preincubate had no effect on the result, a redox experiment at 20 mM glutathione in the absence of glucose 6-phosphate was run as a control. (Glucose 6-phosphate was also omitted from the assay). This curve was not significantly different from a curve in which glucose 6-phosphate was included in the preincubation buffer.

Data Analysis—Equilibrium data of % activity as a function of R or RfR (GS/H) were fit to a rectangular hyperbola (Equations 5 and 6) using an unweighted, nonlinear least squares approach (see ref. 13). Errors are reported ± the standard deviation of the estimate. Since Equations 5 and 6 are hyperbolic functions, it is possible to rearrange them to double-reciprocal form so that a plot of 1/5 against 1/RfR will yield a straight line with y-intercept of 1/km and negative x-intercept of -1/Km. Pseudo-first order rate constants were determined by unweighted, nonlinear least squares fits to a simple monoequilibrium function.

The rate constants for the activation and inactivation of HMGR in the presence of substrates were obtained by analyzing the time course of product formation during the assay. The velocity of product formation in the assay were fit to Equation 4. In order to maximize the rate at which the enzyme and glutathione equilibrate during the assay, the assay time was increased to 10 or 30 min and the dilution of GSH and GSSG into the assay was minimized (1.1-fold). The amount of HMGR present in the assay was decreased to prevent substrate depletion. Based on the measured GSH and GSSG concentrations and the rate constants for reduction and oxidation of the enzyme in the presence of substrates, the rate constant for the approach to equilibrium was calculated for each sample. Only experiments in which the half-life for approach to equilibrium was less than 0.3 times the assay time were included.

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The rate constants for the activation and inactivation of HMGR in the presence of substrates were obtained by analyzing the time course of product formation during the assay. The velocity of product formation (v) was assumed to change from an initial value at time 0 (v0) representing the activity of the enzyme in the preincubation to a final activity (vf) representing the activity that the enzyme finally achieves as a result of activation or inactivation during the assay. Assuming that this change in enzyme activity occurs in a first order process with rate constant k:

\[ \frac{dP}{dt} = v_t = v_f + (v_0 - v_f) e^{-kt} \]  

Integration of Equation 3 between the limits of \( t = 0 \) and \( t \) yields

\[ P_t = v_0 t + (v_0 - v_f) (e^{-kt} - 1)/k \]  

where \( P_t \) is the amount of product formed at any time \( t \). Experimental measurements of the amount of product formed as a function of time in the assay were fit to Equation 4 by a nonlinear least squares algorithm. For experiments designed to follow the activation of the enzyme in the assay, the value of \( v_f \) was fixed at the velocity observed in the presence of DTT. For experiments designed to follow the inactivation of the enzyme, the final velocity \( v_f \) was set to zero.

RESULTS

Kinetics of Thiol/Disulfide Exchange in the Absence of Substrates—Rat liver HMGR is rapidly and completely inactivated by preincubation with low concentrations of GSSG (Fig. 1), consistent with the previously observed sensitivity of this enzyme to thiol oxidation (31, 32). Controls containing no GSSG lose activity slowly, presumably due to oxidation by dissolved oxygen. Addition of reduced DTT to the inactivated enzyme totally restores (>90%) both the activity lost by the reaction with GSSG and the activity lost by air oxidation. High concentrations of GSH (>20 mM) will at least partially restore the enzyme activity lost by treatment with GSSG (see below). Inactivation is first order in GSSG concentration (Fig. 1, inset). The second order rate constant for the inactivation by GSSG is shown in Table I. Under the conditions of this experiment there is no significant change in the activity of the enzyme during the assay. In addition to GSSG, HMGR is also inactivated by a number of other low molecular weight disulfides including CoASSG and oxidized DTT (data not shown).

The previously observed effect of glutathione concentration on the activity of the enzyme (33) is a consequence of contaminating GSSG present in GSH solutions. In freshly prepared GSH solutions, GSSG is invariably present at levels representing 1-2% of the total glutathione concentration ([GSH]/[GSSG] = 50–100). This level can be substantially increased by air oxidation during storage at neutral pH, even in the absence of EDTA. In the presence of excess DTT (9 mM) to fully reduce the GSSG to GSH, HMGR activity is independent of the GSH concentration between 0–50 mM (data not shown).

An attempt was made to measure the rate constant for the GSH-dependent reactivation of GSSG-oxidized HMGR in the absence of substrates by diluting the GSSG-oxidized enzyme into excess GSH and removing aliquots for enzyme assay as a function of time. At low GSH concentrations where the rate

![Fig. 1. Time course for the inactivation of HMGR in the absence of substrates by GSSG. Microsomal HMGR (1.2 mg of protein/ml) was preincubated with the indicated concentration of GSSG at 37 °C, pH 7.1, in 0.1 M potassium phosphate buffer for the indicated time and assayed for residual enzyme activity. , no addition; , 7.3 μM GSSG; ▲, 15 μM GSSG; ○, 34 μM GSSG. The solid curves were drawn using a monoequilibrium function with rate constants of 0.0123, 0.032, 0.046, and 0.084 min⁻¹ respectively. A plot of \( k_{\text{obs}} \) against the GSSG concentration is shown in the inset. The line in the inset was drawn with a second order rate constant of \( 1.7 \times 10^5 \) M⁻¹ min⁻¹ and a rate constant of 0.014 min⁻¹ at zero GSSG concentration.](image-url)

**TABLE I**

Summary of observed rate and equilibrium constants for the thiol/disulfide exchange involving glutathione and HMGR

<table>
<thead>
<tr>
<th>Reaction</th>
<th>( k )</th>
<th>( K_{\text{eq}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate Absent</td>
<td>HMGR(_{\text{rat}}) + GSSG</td>
<td>1700 ± 200 M⁻¹ min⁻¹</td>
</tr>
<tr>
<td></td>
<td>HMGR(_{\text{rat}}) + GSH</td>
<td>±10 M⁻¹ min⁻¹</td>
</tr>
<tr>
<td>Substrate Present</td>
<td>HMGR(_{\text{rat}}) + GSSG</td>
<td>60 ± 4 M⁻¹ min⁻¹</td>
</tr>
<tr>
<td></td>
<td>HMGR(_{\text{rat}}) + GSH</td>
<td>0.6 ± 0.06 M⁻¹ min⁻¹</td>
</tr>
</tbody>
</table>
Kinetics of Thiol/Disulfide Exchange in the Presence of Substrates—The presence of both substrates at saturating concentrations does not totally prevent either the oxidation of the enzyme by GSSG or the reduction of the oxidized enzyme by GSH. The thiol/disulfide exchange is sufficiently rapid, even in the presence of substrates, that significant activity changes can occur during the assay for enzyme activity. The direction and magnitude of the activity changes during the assay depend on the GSH and GSSG concentrations present before and after the addition of substrates as well as the duration of the assay. Unless suitable precautions are taken, conventional fixed time assays for enzyme activity can seriously over or underestimate the activity of the enzyme in the preincubation mixture.

In order to observe the effects of GSSG on the activity of HMGR during the assay, microsomal HMGR was preincubated with 20 mM GSH (containing approximately 2% GSSG) in the absence of substrates, conditions under which the enzyme is mostly active (>65%). The enzyme in the preincubation was then diluted (40-fold) into a solution containing various levels of GSSG and substrate. Control assays containing DTT (5–20 mM) are linear over the assay time used. Assays containing GSSG, however, lose significant activity during the assay as indicated by a decrease in the rate of product formation with time (Fig. 2). The final glutathione status in the assay ([GSH]0/[GSSG] < 1 mM) was such that complete enzyme inactivation occurred at equilibrium. The apparent first order rate constant for the loss of enzyme activity in the assay can be determined by fitting the observed data (nmol product formed as a function of time) to an integrated rate expression describing a time-dependent change in enzyme activity during the course of the assay (see “Experimental Procedures”). The rate of inactivation increases with increasing GSSG concentration (Fig. 2, inset). The second order rate constant for GSSG inactivation of HMGR in the presence of substrates is shown in Table I.

Similar experiments can be performed starting with oxidized enzyme (<5% activity) produced by preincubation of microsomal HMGR with 0.5 mM GSSG and diluting the enzyme into an assay medium containing substrates, additional GSH, glutathione reductase, and an NADPH-regenerating system. Using this method the GSH/GSSG ratio can be driven to unmeasurably large values (>1000). Under these conditions the enzyme activity increases during the assay as indicated by an upward curvature of the product formed as a function of assay time (Fig. 3). At the higher concentrations of GSH, the enzyme regains almost full activity as shown by comparing the final velocity reached in GSH with that obtained in DTT. The apparent first order rate constant for the
between HMGR and Glutathione Redox Buffers—The equilibrium constant \( K_{eq} \) for the equilibration of HMGR activity with a glutathione redox buffer (Equation 1) in the absence of substrate was measured by incubating the enzyme in a series of GSH/GSSG redox buffers until equilibrium was established and then measuring both the activity of the enzyme and the concentrations of GSH and GSSG in each sample. Based on the measured rate constants in the absence of substrates, the length of time required to reach redox equilibrium could be estimated. For concentrations of total glutathione greater than or equal to 20 mM, equilibrium was reached within 30 min (>7 half-lives for the approach to equilibrium). For glutathione concentrations between 5 and 20 mM, 60 min were required to assure that all samples reached equilibrium. Experiments starting with active enzyme obtained in the presence of a low concentration of DTT or with inactive enzyme produced by incubation with low concentrations of GSSG resulted in the same final activity.

In order to minimize activity changes during the assay, short assay times (5 min), increased radiospecific activity of the substrate, and large (10-fold) dilutions into the assay mixture were employed (details are included under “Experimental Procedures”). The rate at which each sample should approach equilibrium in the assay was calculated from the GSH and GSSG measurements made on each sample at the completion of the experiment and the rate constants for the reaction of HMGR with GSH and GSSG in the presence of both substrates. Only those data points where the half-life for re-equilibration in the assay was at least five times the assay time were accepted. Thus, the error in the activity measurement due to re-equilibration in the assay is 13% or less for every point. The dependence of the HMGR activity at redox equilibrium on [GSH]/[GSSG] or on [GSH]'/[GSSG] is shown in Fig. 4. The \( K_{eq} \) for HMGR in the presence of saturating levels of both substrates was determined using a procedure which maximized the activity changes occurring during the assay. The conditions for the preincubation of the enzyme with the glutathione redox buffers were identical to those described above. In this case, however, it was necessary to keep the concentrations of GSH and GSSG high during the assay so that the enzyme would reequilibrate faster than the time of the assay. Thus, the length of the assay time was increased, and the substrates were added in a small volume to minimize dilution of the glutathione. For each experimental point the rate constants for approach to equilibrium with GSH and GSSG in the presence of substrate were used to estimate the time required to reach redox equilibrium during the assay. In all cases, 100% activity corresponds to the velocity of a control containing DTT.

**FIG. 5.** Redox equilibrium between HMGR and glutathione in the presence of substrates. HMGR (0.35-0.7 mg of protein/ml) was preincubated in 0.1 M phosphate, pH 7.1, 37°C with various concentrations of GSH and GSSG until equilibrium was reached. The assay was initiated by the addition of substrates in a small volume and continued for 10 or 30 min. Only those samples which should have reached redox equilibrium (3-6 half-lives) during the assay are included. A, data plotted as a function of R [GSH] according to Equation 5. The curve is drawn with a \( K_{eq} \) of 200 mM. The dotted curve is drawn with a \( K_{eq} \) of 550 mM, the value determined in the absence of substrates. B, data plotted as a function of R at constant [GSH]. A, 47 mM GSH, \( \triangle \), 16 mM GSH; \( \bullet \), 10 mM GSH; and \( \bigcirc \), 4 mM GSH. Curves are drawn using a \( K_{eq} \) of 200 mM according to Equation 5. In all cases, 100% activity corresponds to the velocity of a control containing DTT.

**Discussion**

**Mechanism of Thiol/Disulfide Exchange**—The susceptibility of HMGR to inactivation by sulphydryl oxidation has been
well documented for both the microsomal and proteolytically modified forms of the rat liver enzyme as well as the enzyme from yeast (31–36). In the absence of substrate, rat liver HMGR is rapidly inactivated by a process which is first order in GSSG concentration (Fig. 1). The presence of saturating concentrations of both substrates only partially inhibits enzyme inactivation by GSSG. The site of disulfide bond formation is not completely blocked by the presence of substrates.

The activity of the GSSG-oxidized enzyme can be completely restored by reduction with excess DTT or high concentrations of GSH, suggesting the occurrence of a reversible thiol/disulfide exchange between the enzyme and low molecular weight thiols and disulfides. The activity of the fully reduced (active) enzyme is not affected by even high concentrations of GSH if excess DTT is included in the medium to reduce the GSSG present as a contaminant of the GSH. The previously reported effects of GSH concentration on enzyme activity (41, 43) are thus likely to be associated through GSSG oxidation of protein sulphydral groups rather than by allosteric binding of GSH to the enzyme.

In glutathione redox buffers, the inactive (oxidized) form of the enzyme comes to equilibrium with active (reduced) form. For a system in which the thiol/disulfide exchange equilibrium involves a protein dithiol in equilibrium with a protein-SS-protein disulfide, the percentage of reduced protein is dependent on the quantity $R \cdot [\text{GSH}]$ as described in Equation 5 where $R$ is defined as the quantity [GSH]/[GSSG]. A plot of % activity as a function of $R \cdot [\text{GSH}]$, can, thus, be defined by a simple rectangular hyperbola (13).

\[
\% \text{ Reduced} = \frac{100 \cdot R \cdot [\text{GSH}]}{K_\text{a} + R \cdot [\text{GSH}]}
\]  

(5)

However, if the equilibrium involves the formation of only protein-SSG mixed disulfides, the fraction of reduced (active) protein will depend only on the quantity $R$ and be independent of the absolute concentration of GSH (Equation 6) (44).

\[
\% \text{ Reduced} = \frac{100 \cdot R}{K_\text{a} + R}
\]  

(6)

where $R$ is defined as [GSH]/[GSSG] and $K_\text{a}$ is the equilibrium constant for the reaction described in Equation 1.

If the oxidized protein represents a protein-SS-protein disulfide (Equation 5) and the [GSH] is held constant while R is varied, the resulting activity at equilibrium will be described by a rectangular hyperbola; however, the apparent $K_\text{a}$ will depend on [GSH] ($K_\text{a,exp} = K_\text{a}/[\text{GSH}]$). Such behavior is clearly demonstrated by the data in Figs. 4 and 5, confirming that the oxidized, inactive HMGR represents a protein-SS-protein disulfide. The data in Figs. 4 and 5 also illustrate an important feature of the response of systems forming protein-SS-disulfides to changes in glutathione status. For these systems changing [GSH] even at a constant R will result in increase protein oxidation (this would correspond to a simple dilution of a redox buffer). In addition changing GSH concentration without changing the GSSG concentration will change the quantity $R \cdot [\text{GSH}]$ by a factor which is proportional to [GSH]$^2$. At constant [GSSG] reduction of [GSH] by 2-fold would oxidize the enzyme in a 4-fold more oxidizing redox buffer.

The observed equilibrium behavior of HMGR in a glutathione redox buffer suggests the formation of an inactive protein-SS-protein species. Oxidation of HMGR by GSSG is accompanied by the formation of an intersubunit disulfide cross-link (35, 41). This would suggest that the observed oxidation potential might be a function of protein concentration. However, in this microsomal preparation, HMGR is an integral membrane protein so that dilution of the total microsomal protein will not affect the microscopic concentration of protein subunits. Variation of the microsomal protein concentration between 0.44–1.2 mg/ml had no observable effect on the oxidation potential in the absence of substrates. Lower protein concentrations could not be used due to restrictions imposed by the sensitivity of the assay.

**Effects of Thiol/Disulfide Exchange on HMGR Activity**

The rapid approach of HMGR to thermodynamic equilibrium with glutathione redox buffers even in the presence of the substrate, significantly complicates the measurement of enzyme activity in the presence of glutathione. One consequence of the formation of a protein-SS-protein disulfide in equilibrium with a protein dithiol is that the amount of oxidized (inactive) enzyme will decrease as the concentration of GSH increases at a fixed ratio of [GSH]/[GSSG] (Equation 6). Freshly prepared solutions of GSH invariably contain some GSSG (generally 1–2%). All GSH solutions will, therefore, comprise a [GSH]/[GSSG] redox buffer. Dilution of the enzyme and redox buffer into an assay will lead to increased oxidation (due to the decrease in [GSH]/[GSSG]) while the presence of substrates will lead to decreased oxidation (due to the decrease in the apparent $K_\text{a}$). The redox state and activity of the enzyme will tend to change between the redox state established in the preincubation and the one dictated by the conditions of the assay. These reasonably rapid redox state changes must be considered when the activity of HMGR is measured in glutathione solutions in the absence of DTT. Rottem and Shechter (33, 49) have shown that the $V_{\text{max}}$ (as well as other kinetic properties described below) of HMGR depends on the glutathione concentration present in the assay. The $V_{\text{max}}$ activity was shown to increase in a sigmoidal fashion with increasing GSH concentration ($S_0.5 = 7$ mM GSH), approaching a limiting value at high GSH. Their observations are consistent with the activity of the enzyme coming to equilibrium with the glutathione redox buffer.

**Oxidation Potential of HMGR in Relation to Other Proteins**—Comparison between proteins which form protein-SSG-mixed disulfides and those that form protein-SS-protein disulfides cannot be made without establishing a standard state of GSH concentration at which such comparisons are made. (Standard reduction potentials, $E'_0$, arbitrarily choose a standard state of 1 mM). Since the physiological concentration of GSH is closer to 1 mM than to 1 M, an arbitrary standard state of 1 mM GSH will be used here. At 1 mM GSH, the ratio of [GSH]/[GSSG] required to keep a protein half-reduced at equilibrium will be defined as $R_{0.5}$. For a protein forming a protein-SS-protein disulfide, the $R_{0.5}$ will depend on the standard state GSH concentration according to the relation $R_{0.5} = K_\text{a} / [\text{GSH}]$. For proteins forming only protein-SSG mixed disulfides, $R_{0.5}$ will be independent of the concentration of GSH. Using a 1 mM GSH standard state, the $R_{0.5}$ for HMGR is 550. In contrast, the oxidation potential of rabbit muscle phosphofructokinase (which forms only protein-SSG-mixed disulfides) is such that the $R_{0.5}$ is 7 (44), regardless of the concentration of glutathione.

The variation in thiol oxidation potentials among various proteins is quite large. Measured $R_{0.5}$ values in glutathione redox buffers at a 1 mM standard state include 14 for fatty acid synthase (13), 2.0 $\times 10^{-5}$ for glycogen phosphorylase (45), 10$^6$ for chloroplast fructose-1,6-bisphosphatase (46), and 2000 for Escherichia coli thioredoxin (47). On this scale, NADPH ($E'_0 = -0.32$ V) would be half reduced at a [GSH]/[GSSG] ratio of 10$^4$ (47). Protein thiol oxidation potentials can span a very large range (a factor of 10$^{11}$ or 15 kcal/mol free energy difference). This large range of oxidation potentials undoubt-
edly reflects contributions from protein conformational energetics since the enthalpic contribution of differences in dihedral angle alone to the energy of disulfide bond stability in a variety of proteins has been estimated to be 0.5–4.5 kcal/mol (48).

Relationhip between HMGR Oxidation and in Vivo Conditions—Several authors (31, 33, 35) have speculated that the interconversion between thiol and disulfide forms of HMGR may play some role in metabolic regulation. In evaluating this intriguing possibility, both the kinetics and the thermodynamics of the reaction of HMGR with biological disulfides and the reduction of the oxidized enzyme by biological thiols must be considered.

A significant change in enzyme activity in response to changes in the intracellular glutathione redox status requires the presence of a significant amount of the enzyme in the oxidized state under some metabolic condition. Measurements of total GSH and GSSG in normal liver have shown that GSSG levels are 0.25–0.5% that of GSH ([GSH]/[GSSG] = 200–400 at a [GSH] of 8–10 mM so that [GSSG] = 0.02–0.5 mM) (12, 49). There is a significant drop in both [GSH]/[GSSG] (to 100–150) and [GSH] (to 3–4 mM) in the liver from a fasted animal (12). Conditions of glutathione depletion (50) or drug-induced oxidative stress (7, 9, 14) are accompanied by even more substantial drops in [GSH]/[GSSG] (to <10) and in GSH concentration (to <20% of the original concentration). Assuming uniform distribution of GSH and GSSG throughout the liver (a precarious assumption), the thiol/disulfide status ([GSH]/[GSSG]) would be expected to vary between 300–4000 mM under normal conditions.

For significant regulation of enzyme activity by this mechanism, protein disulfides must be capable of existence in an environment where 90–99.8% of the glutathione is reduced. This imposes both kinetic and thermodynamic constraints on this mechanism of regulation. Kinetically, the formation of an oxidized, disulfide state must be sufficiently fast to occur in vivo (half-life of a few minutes to a few hours) and sufficiently thermodynamically favorable to accumulate in the presence of a 100–400-fold excess of GSH over GSSG.

The rate constants for thiol/disulfide exchange between unhindered, low molecular weight thiols and disulfides are certainly rapid enough to be kinetically competent. For example, the second order rate constant for reaction of a thiol of pK, 7.7 with GSSG at pH 7.0, 30 °C is 90 M⁻¹ min⁻¹. Using this estimate it is possible to predict that the uncatalyzed rate constant for thiol/disulfide exchange between glutathione and a dithiol of Kox of 0.55 M would yield a rate constant of 90 M⁻¹ min⁻¹ for the reaction between GSSG and the protein dithiol and a rate constant of 160 M⁻² min⁻¹ for the reaction between the protein disulfide and GSH (51). At a concentration of 50 μM GSSG and 8 mM GSH, the rate constant for approach to equilibrium for this hypothetical di-thiol/disulfide can be estimated to be about 0.02 min⁻¹ or a half-life of about 30 min. For HMGR under the same conditions, the half-life for approach to equilibrium is 7 min in the absence of substrates and 90 min in the presence of substrates. Even in the absence of enzymatic catalysis, and in the presence of saturating levels of substrate, this rate is sufficiently fast to allow significant changes in the activity of HMGR over a relatively short period of time.

Thermodynamically, the thiol/disulfide oxidation potential (Kox) of HMGR, even in the presence of saturating concentrations of substrate, is high enough that an appreciable fraction of enzyme could be present in a latent disulfide form. Table II shows the percentage of HMGR, fatty acid synthase, chloroplast fructose-1,6-bisphosphatase, and phosphofructokinase which would be expected to be reduced in different glutathione redox buffers. The glutathione status has been chosen to approximate measured total levels of GSH and GSSG in liver under various conditions. Both fatty acid synthase and phosphofructokinase (muscle) would be expected to be totally reduced under most of these conditions, except under severe oxidative stress. However, HMGR would be expected to undergo significant changes in redox state in response to changes in the concentrations of GSH and GSSG.

There have been few demonstrations of redox state changes for specific proteins in vivo. Ness et al. (41) have observed changes in the extent of HMGR disulfide cross-linking in rat liver in response to dietary manipulation. The activity and So.5 for NADPH was found to correlate with the extent of disulfide cross-linking. With HMGR from the livers of fasted animals, where R/[GSH] would be expected to be lower, the extent of enzyme cross-linking (oxidation) was significantly higher than in control experiments (12). Thomas (9) has demonstrated, at least under conditions of induced oxidative stress, that heart cells can accumulate significant concentrations of specific protein-SSG-mixed disulfides.

Figs 4 and 5 demonstrate that HMGR is about 3-fold more easily oxidized thermodynamically in the absence of substrate than in its presence. The presence of substrates, however, does not prevent the occurrence of significant changes in enzyme oxidation state. Whether one of the substrates alone is capable of shifting the Kox or whether the presence of both substrates is required is not known.

The intracellular concentration of (S)-HMG-CoA has been estimated to be about 10 μM (52). Clinkenbeard et al. (53) have shown that there are separate mitochondrial and cytosolic pools of HMG-CoA, and that, in avian liver, the concent-

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Kox</th>
<th>Fed</th>
<th>Fasted</th>
<th>Oxidatively stressed</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−Substrates</td>
<td>550 mM</td>
<td>81</td>
<td>35</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>+Substrates</td>
<td>200 mM</td>
<td>92</td>
<td>60</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>14 mM</td>
<td>99.4</td>
<td>96</td>
<td>60</td>
<td>13</td>
</tr>
<tr>
<td>Chloroplast fructose-1,6-bisphosphatase</td>
<td>2 × 10^6 mM</td>
<td>0.1</td>
<td>0.01</td>
<td>0.001</td>
<td>46</td>
</tr>
<tr>
<td>Phosphofructokinase (muscle)</td>
<td>7</td>
<td>98</td>
<td>93</td>
<td>75</td>
<td>44</td>
</tr>
</tbody>
</table>
tration of HMG-CoA is dependent on ([acetyl-CoA³/ [CoASH]). Therefore, slight changes in the concentrations of either of these species will cause significantly larger changes in the concentration of HMG-CoA. Gibbons et al. (24) have suggested that changes in the concentration of cystolic HMG-CoA in response to hormonal or metabolic stimuli might serve to regulate HMGR activity. Ness et al. (39) have measured a Ke for HMG-CoA of 5 μM for rat liver microsomal HMGR. Roitelman and Shechter (43) have demonstrated that the apparent Ke of the rat liver microsomal enzyme varies with the dietary treatment of the animal. For animals whose diet was supplemented with cholestyramine alone or cholestyramine plus mevinolin, the Ke for (S)-HMG-CoA was 6.8 μM (43). For rats whose diet was not supplemented with either drug, the Ke was 21 μM (43). Thus, the cystolic concentration of HMG-CoA lies within the range of the in vitro Ke values reported for the microsomal enzyme.

Perhaps the greatest source of discussion regarding HMGR has been the behavior of the enzyme with respect to NADPH (33, 35, 39, 41, 43, 54, 55). Cellular concentrations of NADPH ranging from 300 to 500 μM have been reported for rat liver (56–58). Significant changes in the concentration of NADPH in response to feeding and fasting have not been observed (58). The fact that the apparent Ke of NADPH varies significantly with the dietary treatment of the animal has been well established (41, 43). The solubilized, proteolyzed form of the enzyme exhibits hyperbolic kinetics with a Ke of 35 μM (35, 43) which is similar to the Ke observed for microsomal HMGR isolated from rats fed a diet containing both cholestyramine and mevinolin (Ke = 30 μM) (41, 43). The microsomal enzyme isolated from rats fed an unsupplemented diet exhibits sigmoidal kinetics with an apparent So.5 of 20 μM when assayed in the presence of 5 mM GSH (39, 43). There is somewhat less agreement, however, concerning the results for microsomal HMGR isolated from rats fed a diet supplemented with cholestyramine alone. Roitelman and Shechter (43) report sigmoidal plots with a So.5 of 76 μM. Ness et al. (41) report a So.5 of 160 μM.

The role of thiols with respect to the apparent So.5 values is also controversial. Roitelman and Shechter (33, 55) have shown that increasing the concentration of glutathione from 4.5 to 27 mM decreases the sigmoidicity of the kinetic plots. On the other hand, Ness et al. (39) have reported that the addition of glutathione increases the sigmoidicity of the kinetic plots and yields a Hill coefficient which approaches 2. This discrepancy might be attributed to the use of different buffer systems and/or differences in the preincubation or assay conditions. However, the redox data presented here could provide an alternative explanation in that the measured activity of the enzyme can depend on the amount of GSSG contamination in the GSH, the concentration of GSH used in the assay, the dietary treatment of the animal from which the enzyme was isolated, the time of preincubation, and the time required for the assay.

Conclusions—Based on in vitro measurements, thiol/disulfide exchange between glutathione redox buffers and HMGR could be kinetically and thermodynamically competent as a mechanism of regulation in which enzyme activity is coupled to changes in intracellular thiol/disulfide status. There appears to be no fundamental kinetic or thermodynamic constraint which a priori precludes the occurrence of protein disulfides in the “reducing environment” found in most cells. Despite the possibilities discussed here, the establishment of thiol/disulfide exchange as an in vivo regulatory mechanism must await the demonstration that both reduced and oxidized forms of the enzyme can and do occur in the cell, that the redox state (and activity) of a specific protein changes in response to alterations in the cellular thiol/disulfide status, and that this change in activity is consistent with the metabolic demands of the organism.

REFERENCES

1. Guzman-Baron, E. S. (1965) Adv. Enzymol. 11, 201–266
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42. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70-77