An Alternate Method of Purification and Properties of Rat Liver β-Hydroxy-β-methylglutaryl Coenzyme A Reductase*

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Rat liver microsomal β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) reductase was previously solubilized and then purified by a combination of standard fractionation techniques and coenzyme A affinity chromatography. The enzyme obtained by this procedure was homogeneous (Kleinsek, D. A., Ranganathan, S., and Porter, J. W. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1431-1435).

However, HMG-CoA reductase may have a varied elution profile and purification-fold on gel filtration which is dependent on the batch of agarose gel used and the ionic strength of the eluant buffer. Accordingly, estimations of the apparent molecular weight of the enzyme by this technique may range from 1,000 to 200,000.

An alternate procedure, sucrose density gradient ultracentrifugation, has been substituted for the gel filtration step in the purification of HMG-CoA reductase. This procedure, followed by coenzyme A affinity chromatography, results in enzyme with a specific activity and a purification-fold similar to the values obtained employing agarose gel filtration. The enzyme purified by this procedure is homogeneous by standard criteria.

The enzyme has a molecular weight of 200,000 and it has four subunits of 51,000 each. Several properties of the enzyme have been determined and are reported in this paper. Among the more interesting of these is its reversible cold lability in low ionic strength phosphate buffer. At low protein concentrations and exposure to a temperature of 4°C, the soluble enzyme loses 90% of its activity within 90 s. The recovery of lost enzyme activity is complete within 7½ min on warming to 37°C. Since the molecular weight of the enzyme does not change during inactivation and reactivation, the loss of enzyme activity is not due to dissociation of the enzyme into subunits. Instead, loss of enzyme activity appears to be due to discrete conformational changes that affect the active site of HMG-CoA reductase.

In mammalian systems HMG-CoA reductase (mevalonate: NADP⁺ oxidoreductase (CoA acylating); EC 1.1.1.34) is considered the rate-controlling enzyme in hepatic cholesterol biosynthesis under most physiological states (1-3). Changes in the dietary or hormonal status (4, 5) of the animal result in an alteration of HMG-CoA reductase activity. However, the molecular mechanisms by which these factors control the activity of this enzyme have not been elucidated. As a part of the initial experimentation on this problem, investigations have been carried out by us on the isolation, purification, structural, and physical properties of the enzyme. It is these studies that are the subject of this paper.

Mild and harsh solubilization procedures (6-9) have been used to liberate HMG-CoA reductase from the endoplasmic reticulum. Some of the various physical properties of the enzyme, such as susceptibility to cold inactivation, may be altered, though, as a function of the solubilization method employed. Hence, we have re-examined the apparent Kₘ values of the substrates and the pH optimum for the soluble enzyme obtained by a mild freeze-thaw treatment of microsomes (10). Physical properties not previously determined, such as the isoelectric point and lability of the enzyme as a function of time, temperature, and buffer composition, have also been determined.

Cold inactivation studies on HMG-CoA reductase have been reported by three other laboratories (8, 9, 11). Ackerman et al. (9) noted that enzyme extracted from lyophilized microsomes with buffer is not cold inactivated. In contrast, Brown et al. (8) reported that enzyme solubilized by snake venom digestion, prolonged treatment with KCl, or a fast freeze-thaw glycerol extraction of microsomes is irreversibly inactivated when cooled to 4°C unless 4 M KCl is added to the buffer. However, Heller (11), using enzyme solubilized by a slow freeze-thaw treatment of the microsomes, showed the enzyme to be reversibly cold labile. Enzyme activity lost at 0°C was completely recovered by rewarming at 37°C. Both Brown and Heller reported a lack of cold sensitivity for microsomal bound HMG-CoA reductase. In the present studies we have investigated the kinetics of cold inactivation at 4°C and subsequent reactivation at 37°C of both soluble and microsomal enzyme. Also, cold inactivation is examined in terms of enzyme structure.

The purification of HMG-CoA reductase from rat liver microsomes has been reported by a number of laboratories (6, 10, 12, 13). Analyses of these preparations by regular or SDS-polyacrylamide disc gel electrophoresis or immunodiffusion have yielded a single band. Specific activities of the enzyme, however, varied from 20 (6) to 20,000 nmol (10) of NADPH oxidized/min/mg of protein. When two of the above lower specific activity preparations were subjected to regular polyacrylamide disc gel electrophoresis, one preparation (14) (specific activity of 960) did not show coincidence of enzyme activity with the majority of protein, whereas the preparation with a specific activity of 1,382 demonstrated coincidence of
activity with protein. However, a smaller band of protein was also present on the gel (13). We have subjected our preparation (specific activity of 20,000) to this test of purity. In addition, an alternate purification method to gel filtration is introduced and the structural characteristics of this purified enzyme are examined in this paper.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals were obtained from the following sources: 3-hydroxy-3-methyl[1-14C]glutaric acid (51.9 mCi/mmol), New England Nuclear; thioester-linked agarose-hexane-coenzyme A, diethiothreitol, and coenzyme A (lithium salt), P-L Biochemicals, Inc.; glucose 6-phosphate, glucose-6-phosphate dehydrogenase, NADP+, NADPH, phosphorylase a, lactic dehydrogenase, alcohol dehydrogenase (liver), catalase, persulfate, Bio-Rad Laboratories; ampholines, pH 4 to 6, 5 to 7, and 7 to 8, Bio-Gel A-0.5m and A-1.5m, 200 to 400 mesh, acrylamides, Coomassie brilliant blue R-250, Temed, and ammonium persulfate, Bio-Rad Laboratories; amphotolines, pH 4 to 6, 5 to 7, and 7 to 10, KLB; bromphenol blue, Matheson Scientific, Inc.; blue dextran 2000 and aldolase, Pharmacia, P-L Biochemicals, Inc.; glucose phosphate, Worthington Biochemical Corp.; and collodion ultrafiltration bags, Schleicher and Schuell. All other chemicals and reagents were of analytical grade.

**Animals**

Male albino Holtzman rats weighing 180 to 200 g were used for all experiments. The animals were housed in a light-controlled room and maintained on an alternate 12-h light-dark schedule. The animals were fed ad libitum a 2% cholestyramine Wayne Lab-Blox powdered diet for a minimum of 4 days to effect maximum liver HMG-CoA reductase activity. Animals were sacrificed by decapitation at the diurnal high point of I1M-G-CoA reductase activity.

**Methods**

**Purification of HMG-CoA Reductase**

**Preparation of Microsomes**—Excised livers were placed in ice-cold homogenization medium containing 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM sucrose, and 2 mM dithiothreitol (Buffer I). All of the remaining operations were carried out at 4°C. Livers were homogenized in this medium (2 ml/g of liver) for 15 s in a Potter-Elvehjem type glass homogenizer. The homogenate solution was centrifuged at 100,000 x g for 10 min and the supernatant decanted and centrifuged at 100,000 x g for 75 min. The microsomal pellet was resuspended in Buffer I containing 50 mM EDTA and centrifuged at 100,000 x g for 60 min. The pellet was slow frozen at -20°C in the centrifuge tube and stored at this temperature until needed.

**Solubilization of Enzyme**—Microsomal pellets were kept frozen at -20°C for at least 2 h before they were thawed at room temperature. Microsomes from an average of 1 rat liver occupied each ultracentrifuge tube. The thawed pellet was homogenized in 3 ml of solubilization buffer that contained 50 mM potassium phosphate buffer of pH 7.0, 0.1 M sucrose, 2 mM dithiothreitol, 50 mM KCl, and 30 mM EDTA. This homogenization was performed in a Potter-Elvehjem homogenizer with a tight fitting Teflon pestle. Then, 7 ml of buffer was added and the sample was again homogenized with three strokes of the pestle. After remaining at room temperature for a minimum of 15 min, the suspension was centrifuged at 100,000 x g for 60 min at 20°C. The supernatant solution was collected, assayed for protein and enzyme activity, and used for the purification of the enzyme. All further operations were carried out at room temperature unless specified otherwise.

**Purification of the Solubilized Enzyme**—Soluble enzyme was precipitated at 36 to 50% saturation with (NH₄)₂SO₄. The protein pellet was then dissolved in heat treatment buffer containing 50 mM potassium phosphate (pH 7.0), 0.3 mM dithiothreitol, 30% (v/v) glycerol, and 1 M KCl at a concentration of 3 to 4 mg/ml of protein. The enzyme solution was heated at 65°C for 6 min by immersing it in a thin walled (10 mm) Pyrex test tube (1.5 x 16 cm) containing 2 to 3 ml of buffer in a 65°C water bath. The solution was cooled immediately after heating by placing the test tube in an ice bucket. The denatured protein was removed by centrifugation at 100,000 x g for 30 min at 20°C. The heat-treated extract was diluted 1:1 with solubilization buffer and then concentrated by precipitation of protein at 50% of saturation with (NH₄)₂SO₄. The protein pellet was dissolved in 50 mM potassium phosphate (pH 7.0), 50 mM KCl, 30 mM EDTA, 2 mM dithiothreitol, and 2% sucrose. The enzyme was then applied to a 5 to 90% sucrose density gradient under the conditions given below. After the ultracentrifugation step was performed, the fractions were warmed to 37°C for 20 min and then assayed for activity. Peak tubes of protein (Nos. 16 to 20, Fig. 2) were collected and concentrated on collodion bags to a protein concentration of 1 mg/ml. A description of the subsequent CoA affinity step for the purification of the enzyme is provided in Ref. 10.

**Assay Systems**

Both biochemical and spectrophotometric assays were used to measure the activity of HMG-CoA reductase. Microsomal enzyme and soluble enzyme activities in disc gel slices were assayed by the chemical method (19). The conversion of radioactive HMG-CoA to mevalonate in an incubation mixture containing saturating levels of NADPH was also used to determine the kinetics of the cold lability of the enzyme. All other experiments involving soluble enzyme used a spectrophotometric method of assay for measuring the initial velocity of NADPH oxidation (10).

**Definition of Unit**

One unit of I1M-G-CoA reductase activity is that activity which will oxidize 1 nmol of NADPH to NADP+ in 1 min at 37°C.

**Protein Determination**

All protein solutions were treated with 10% (v/v) trichloroacetic acid. The precipitate obtained from microsomes was assayed by a modification of the biuret method (15) employing deoxycholate. Solubilized enzyme preparations were assayed for protein by the method of Lowry (16).

**Polyacrylamide Gel Electrophoresis**

Polyacrylamide disc gels (5% and pH 6.9) contained 30% glycerol. This permitted the partial recovery of HMG-CoA reductase activity from the gel (17). Electrophoresis of the gel was carried out in 5 mM Tris and 35 mM glycine at pH 8.3. At the end of the run, the gels were removed and sliced longitudinally into two equal halves. In one half, protein was stained with 0.25% Coomassie brilliant blue. The other half was sectioned into 0.4- to 1.0-cm slices, macerated in 500 μl of standard radiochemical incubation mixture (10), and incubated for 15 min at 37°C. Assays for mevalonic acid were then carried out as previously reported.

The procedure of Weber and Osborn (18) was followed for SDS-polyacrylamide disc gel electrophoresis. The SDS-5% polyacrylamide disc gels were fixed, and 20% sulfosalicylic acid (19) was added to leach out the SDS prior to staining with 0.25% Coomassie brilliant blue.

**Isoelectric Focusing**

Isoelectric focusing was carried out by a modification of the procedure described by Righetti and Drysdale (18). A narrow range of four to seven amphotolines was used as a carrier ampholine. An alternate broad range of three to ten amphotolines was fixed, and 20% sulfosalicylic acid (19) was added to make up the SDS prior to staining with 0.25% Coomassie brilliant blue.

**Radiochemical Assays**

Radiochemical assays were performed by macerating the slices in the standard radiochemical incubation mixture (10), and incubated for 15 min at 37°C. Assays for mevalonic acid were then carried out as previously reported.

**Ultracentrifugation**

Ultracentrifugation was carried out at 14°C in a Beckman model L-3-50 ultracentrifuge with a SW-27 swinging bucket rotor. The 5 to 20% sucrose density gradients contained 50 mM potassium phosphate,
Rat Liver HMG-CoA Reductase

pH 7.0, 50 mM KCl, 30 mM EDTA, and 2 mM dithiothreitol in a total volume of 38 ml. Heat-treated (1.5 mg of protein/ml) enzyme suspended in the above buffer containing 2% sucrose in a volume of 1 ml was layered on top of the gradient. Ultracentrifugation was carried out for 41 h at 25,000 rpm. The gradient was collected in 1-ml fractions with an Isco model 194 tube holder and piercing mechanism.

Enzyme Neutralization Studies

Antiserum against HMG-CoA reductase was prepared as described previously (10). Immunotitration studies were then carried out in the following buffer: 40 mM potassium phosphate (pH 7.0), 10 mM EDTA, 35 mM KCl, 20 mM NaCl, and 1 mM dithiothreitol in a reaction volume of 380 μl. Enzyme was preincubated with a 1:128 dilution of a 0 to 50% (NH₄)₂SO₄ fraction of immune rabbit serum for 30 min at 37°C. The radiochemical reaction mixture was then added to a final volume of 500 μl and incubation was carried out for 30 min. Assays for radioactive mevalonic acid were carried out as previously reported (10). When microsomal enzyme was used for immunochemical titrations it was prepared as described previously (10), except that 50 mM EDTA was included in the homogenizing buffer.

RESULTS

Criteria for Homogeneity of Enzyme—As reported earlier (10), the enzyme prepared in our laboratory appears homogeneous by a number of physical tests which include the migration as a single band of protein on regular and SDS-polyacrylamide disc gel electrophoresis, the production of a

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein (mg)</th>
<th>Protein (mg/ml)</th>
<th>Total units</th>
<th>Units/mg protein</th>
<th>% yield</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microsomes</td>
<td>740.</td>
<td>8300.</td>
<td>11.2</td>
<td>38,200</td>
<td>4.6</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Soluble extract</td>
<td>244.</td>
<td>295.</td>
<td>1.2</td>
<td>13,000</td>
<td>44</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, 36 to 50% of saturation</td>
<td>22.</td>
<td>86.</td>
<td>3.8</td>
<td>10,250</td>
<td>121</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>11.</td>
<td>16.</td>
<td>1.5</td>
<td>8,490</td>
<td>530</td>
<td>22</td>
<td>113</td>
</tr>
<tr>
<td>(NH₄)₂SO₄, 0 to 50% of saturation</td>
<td>1.7</td>
<td>8.</td>
<td>4.7</td>
<td>5,810</td>
<td>709</td>
<td>15</td>
<td>151</td>
</tr>
<tr>
<td>Sucrose density gradient fractionation</td>
<td>13.</td>
<td>1.2</td>
<td>0.092</td>
<td>3,880</td>
<td>3,230</td>
<td>10</td>
<td>687</td>
</tr>
<tr>
<td>CoA affinity column</td>
<td>2.3</td>
<td>0.078</td>
<td>0.034</td>
<td>1,320</td>
<td>16,900</td>
<td>3.4</td>
<td>3,600</td>
</tr>
</tbody>
</table>
The protein standards used were characterized in a previous study (18). SDS-5% polyacrylamide disc gel electrophoresis was performed on pure enzyme and standards were run on the same or separate gels.

Structural Organization of the Enzyme—Analyses of the structural organization of the enzyme were carried out by molecular sieve chromatography and SDS-polyacrylamide disc gel electrophoresis. An independent method from Bio-Gel agarose molecular sieve chromatography is required to establish an accurate molecular weight for HMG-CoA reductase, inasmuch as the elution behavior of this enzyme on Bio-Gel agarose columns varies as a function of the particular batch of gel used. This finding is discussed later in relationship to Fig. 6.

The molecular weight of HMG-CoA reductase was determined on two different gel matrices, with Bio-Gel A-1.5m and Sephadex G-200 columns (Fig. 4). A molecular weight of 200,000 was obtained from both columns.

The monomeric polypeptide molecular weight of pure enzyme was determined by SDS disc gel electrophoresis with the molecular weight standards shown in Fig. 5. A single band of protein corresponding to a molecular weight of 51,000 was obtained. Thus, HMG-CoA reductase appears to be a tetrameric enzyme composed of four identical subunits.

Enzyme Behavior on Bio-Gel Agarose Chromatography—
batch of gel. The particle size of the gel was 200 to 400 mesh and to coincidence with the broad protein contaminant peak. At that point a homogeneous enzyme preparation is no longer possible, even after passage through the coenzyme A affinity step. High salt washes of the gel fail to reopen the "binding sites" for IIMG-CoA reductase. Presumably, this is due to an irreversible interaction of the enzyme or other proteins with the gel residues. The use of an agarose gel that does not interact with HMG-CoA reductase or the use of a high ionic strength buffer shifts the enzyme activity into a symmetrical peak that coincides with the bulk of eluted protein with a molecular weight approximating 200,000. The recovery of enzyme activity (under these conditions) is 80 to 90%, but it represents only a 2-fold purification with a specific activity of 1,000 units/min/mg of protein.

**Enzyme Orientation in the Endoplasmic Reticulum—** HMG-CoA reductase is an extrinsic protein of the endoplasmic reticulum in which the active site is positioned toward the cytosol. This conclusion is supported by the physical and immunological properties of the enzyme. The enzyme is liberated from the membranes by mild treatments, i.e. low ionic strength and neutral pH buffers, and remains soluble. Table II shows that the membrane-bound and soluble enzymes have similar enzymic activity neutralization endpoints when reacted with antisera produced against solubilized HMG-CoA reductase. Control serum (nonimmune serum) or antiserum with antibodies to HMG-CoA reductase removed neutralizing effects when titrated against these enzyme preparations. Thus, the antigenic determinants on HMG-CoA reductase that affect the catalytic site appear to be quantitatively similar in the soluble and membrane-associated states. The positioning of the active site of the microsomal enzyme is consistent with the cytosolic location of HMG-CoA synthase and mevalonate phosphorylation.

**Physicochemical Properties of Solubilized Enzyme—** Data on the physicochemical properties of HMG-CoA reductase solubilized by a slow freeze-thaw treatment of microsomes are given in Figs. 7 to 10. Michaelis-Menten constants for HMG-CoA reductase were determined by a spectrophotometric analysis of the initial velocity of the oxidation of NADPH to NADP⁺. The apparent $K_m$ values for both substrates were calculated from double reciprocal plots in which one substrate concentration was present in saturating amounts and the other in a concentration range sufficient to provide Lineweaver-Burk plots.

### TABLE II

<table>
<thead>
<tr>
<th>HMG-CoA reductase preparation</th>
<th>Antiserum endpoint/ enzyme activity units neutralized</th>
<th>Antiserum/ equivalent enzyme unit fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble</td>
<td>$\mu$ antisemur/mmol metabolite/min</td>
<td>-fold change</td>
</tr>
<tr>
<td>Soluble</td>
<td>42.8/25.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Microsomal</td>
<td>38.5/19.9</td>
<td>1.1</td>
</tr>
</tbody>
</table>

The elution of HMG-CoA reductase was retarded on several different lots of Bio-Gel A-0.5m. This aberrant behavior, which appears to be due to ionic interactions between residues on the gel and HMG-CoA reductase, results in a highly resolved enzymic peak but a low recovery of activity. Depending on the batch of Bio-Gel used, calibration with molecular weight standards indicated an enzymic molecular weight of approximately 200,000 or <1,000, or the enzyme was not eluted. This peculiar behavior of the enzyme is not a function of the preparation (Fig. 6). Increasing the ionic strength of the buffer by the addition of KCl or a change in the Bio-Gel lot alters the elution pattern of HMG-CoA reductase. A value of approximately 230,000 daltons was obtained when a high ionic strength buffer was used (Fig. 6B). Enzyme correlating to an apparent molecular weight of 1,000 (Fig. 6A) exhibits a skewed, front leading edge of enzyme activity that is resolved from the majority of contaminating proteins. This results in a 7- to 8-fold purification of heat-treated enzyme and a specific activity of 3,700 units/min/mg of protein. Yields of activity, however, are only 30 to 40%. SDS-polyacrylamide disc gel electrophoresis of the enzyme reveals a single major staining band of protein and appears to represent a mixture of active and inactive HMG-CoA reductase which can then be separated by coenzyme A affinity chromatography (10). A major disadvantage of utilizing this type of pseudo-"affinity" gel filtration is found when the agarose gel is used repeatedly. This results in a gradual shift of the enzyme activity fractions to coincidence with the broad protein contaminant peak. At that point a homogeneous enzyme preparation is no longer possible, even after passage through the coenzyme A affinity step. High salt washes of the gel fail to reopen the "binding sites" for IIMG-CoA reductase. Presumably, this is due to an irreversible interaction of the enzyme or other proteins with the gel residues. The use of an agarose gel that does not interact with HMG-CoA reductase or the use of a high ionic strength buffer shifts the enzyme activity into a symmetrical peak that coincides with the bulk of eluted protein with a molecular weight approximating 200,000. The recovery of enzyme activity (under these conditions) is 80 to 90%, but it represents only a 2-fold purification with a specific activity of 1,000 units/min/mg of protein.
FIG. 7. Lineweaver-Burk plots of reciprocals of substrate concentration and initial velocities of mevalonic acid formation by HMG-CoA reductase. Enzyme was purified through the sucrose density gradient ultracentrifugation step. Saturating concentrations of DL-HMG-CoA and NADPH used were $3.6 \times 10^{-4}$ M and $7.8 \times 10^{-4}$ M, respectively. A, the concentration of DL-HMG-CoA was determined by light absorbance at 260 nm and by sulfhydryl analysis with the Ellman reagent after complete hydrolysis of HMG-CoA with base. The HMG-CoA concentration of the d isomer is given. B, the concentration of NADPH was determined from light absorbance at 340 nm. The slopes of the lines in plots A and B were subjected to linear regression analysis.

FIG. 8. A, isoelectric focusing of HMG-CoA reductase on a 5% polyacrylamide disc gel. The gel contained 2% of carrier ampholines. The determinations of pH and enzyme activity of the gel slices are described under "Experimental Procedures." pH values (O-O) and enzyme activities expressed as radioactivity in counts per min (O-O) are plotted. B, determination of the pH optimum of soluble HMG-CoA reductase. The activity of HMG-CoA reductase as a function of pH was determined under the reaction conditions described for the spectrophotometric assay under "Experimental Procedures." Incubation mixtures were buffered with 0.1 M potassium phosphate (C-C) or 0.1 M glycine-NaOH (O-O). C, effect of temperature on enzyme activity. A 0 to 50% (NH$_4$)$_2$SO$_4$, fraction of heat-treated enzyme was suspended at a protein concentration of 1.0 mg/ml in a 0.1 M potassium phosphate, pH 7.0, buffer containing 2 mM dithiothreitol. An aliquot (40 µl) was added to an incubation mixture and HMG-CoA reductase activity was determined at the stated temperature for 90 s. At the end of this time the conversion of [14C]HMG-CoA into [14C]mevalonate was determined. D, stability of HMG-CoA reductase during heat treatment. A 35 to 50% (NH$_4$)$_2$SO$_4$, fraction of soluble enzyme was suspended in a buffer of 0.05 M potassium phosphate, pH 7.0, 3 mM dithiothreitol, 30% (v/v) glycerol, and 1.0 M KCl at a protein concentration of 9.4 mg/ml. This mixture was incubated at the indicated temperatures and times. Heat-denatured protein was then centrifuged down and the supernatant solution was analyzed for protein and enzyme activity.
other substrate concentration was varied (Fig. 7). The concentration of substrate was in the range recommended by Cleland (21) of 1/5 to 4 times the \( K_v \) value for NADPH and 1/5 to 5 times that for \( \nu \)-HMG-CoA. The apparent \( K_v \) for \( \nu \)-HMG-CoA is \( 1.7 \times 10^{-5} \) M and for NADPH \( 3.0 \times 10^{-5} \) M.

The isoelectric point of the enzyme was determined by isoelectric focusing on polyacrylamide disc gels. A single symmetrical peak of HMG-CoA reductase activity was obtained on a linear pH gradient of 4 to 7 (Fig. 8A). The pI of the enzyme is 6.2 ± 0.1.

The pH optimum of the enzyme ranges from 6.25 to 7.25 (Fig. 8B). A gradual decrease in enzyme activity on either side of the pH optimum is observed.

Initial velocity assays in phosphate buffer showed that \( 41^\circ \)C is optimal for HMG-CoA reductase activity (Fig. 8C).

Heat stability curves for HMG-CoA reductase in 1 m KCl and 30% glycerol at different temperatures showed that the enzyme is stable at 62.5°C for 8 min or more. A decrease in enzyme activity occurs at higher temperatures. After 10 min at 70°C, only 30% of the original HMG-CoA reductase activity remained. The specific activity of the enzyme preparation was greatest after heat treatment at 65°C for 6 min. Hence, this was used as a purification step even though a 10% loss of enzyme activity is incurred.

**Cold Lability Properties of the Enzyme**—The kinetic data on the reversible cold lability of the enzyme were determined by initial velocity measurements of enzyme activity after cold exposure and subsequent rewarming (Fig. 9A). The rate of cold inactivation is a function of the enzyme concentration (Fig. 9A). At the lower protein concentration, 80% and 100% of activity were lost after 10 min and 70 min of cold exposure, respectively. In contrast, 50% of the enzyme activity remained after 10-min incubation at the higher protein concentration and incomplete cold inactivation was observed after 70 min of incubation. The rate of reactivation of cold inactivated enzyme by rewarming to 37°C was rapid. After 7 min the reaction was complete.

The cold inactivation of enzyme is greatly reduced when the buffer is changed to a higher ionic strength (Fig. 9B). Enzyme suspended in heat treatment buffer (1 M KCl and 30% glycerol) exhibits only a 20% loss of activity when subjected to 4°C for 70 min, and it is completely reactivated when placed at 37°C in the protein ranges tested (0.23 to 2.30 mg/ml). Thus, a high salt glycerol buffer confers both heat (Fig. 8D) and cold (Fig. 9B) stability on the enzyme, thereby implicating a hydrophobic bonding requirement for the enzyme to be in an active state. However, a delicate balance of charge is essential to maintain enzyme activity. The deletion of 1 M KCl or 30% glycerol or the inclusion of 4 M KCl results in a decline of enzyme activity at high temperatures. The molecular basis for this maintenance of enzyme activity is discussed later in relationship to Fig. 10.

It was demonstrated in enzyme assays with an incubation period of 1 min that HMG-CoA reductase in the endoplasmic reticulum also exhibits a reversible cold lability. The kinetic data of this cold inactivation and subsequent reactivation (Fig. 9C) are similar to those observed for soluble enzyme. The reactivation of cold inactivated enzyme is not due to a phosphoprotein phosphatase-mediated reaction (22) since in the buffer is changed to a higher ionic strength (Fig. 9B). Enzyme suspended in heat treatment buffer (1 M KCl and 30% glycerol) exhibits only a 20% loss of activity when subjected to 4°C for 70 min, and it is completely reactivated when placed at 37°C in the protein ranges tested (0.23 to 2.30 mg/ml). Thus, a high salt glycerol buffer confers both heat (Fig. 8D) and cold (Fig. 9B) stability on the enzyme, thereby implicating a hydrophobic bonding requirement for the enzyme to be in an active state. However, a delicate balance of charge is essential to maintain enzyme activity. The deletion of 1 M KCl or 30% glycerol or the inclusion of 4 M KCl results in a decline of enzyme activity at high temperatures. The molecular basis for this maintenance of enzyme activity is discussed later in relationship to Fig. 10.

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FIG. 10. Molecular weight determination of HMG-CoA reductase on Sephadex G-200 at 4°C. Enzyme prepared as in Fig. 9A was subjected to a temperature of 4°C for 3 h at a protein concentration of 0.55 mg/ml and then applied to the column. The eluant buffer was the same as when the chromatography was performed at room temperature (Fig. 4B) and the column dimensions were 1.6 x 87 cm. Enzyme activity was measured after the 1-ml eluate fractions were placed in a 37°C water bath for 30 min. Protein standards were assayed for enzyme activity.

Inclusion of 50 mm fluoride ion does not prevent the elevation of microsomal enzyme activity at 37°C.

As mentioned above, hydrophobic bonding forces appear to play an important role in maintaining the enzyme in an active form in a high salt-glycerol buffer at 4°C. The molecular basis for cold inactivation in the absence of this buffer may be the disruption of hydrophobic forces and subsequent dissociation of the enzyme to subunits, intramolecular conformational changes of the enzyme, or a temperature-induced phase transition of lipid moieties attached to the enzyme. To determine which physical process was occurring, enzyme was preincubated at 4°C and then applied to molecular sieve chromatography on Sephadex G-200 at this temperature. After rewarming of eluant fractions at 37°C, a single symmetrical peak of HMG-CoA reductase activity corresponding to a molecular weight of 197,000 was obtained (Fig. 10). Thus, dissociation of enzyme into 51,000 dalton subunits is not the mechanism of cold inactivation.

DISCUSSION

The major aims of this study were the purification of rat liver HMG-CoA reductase to homogeneity and a determination of its structural, kinetic, and physical properties. The enzyme has now been purified to homogeneity by two procedures which differ by one fractionation step. In these procedures either a pseudo-"affinity" agarose gel filtration or a sucrose density gradient ultracentrifugation may be used.

A combination of physical criteria in conjunction with specific activity values is needed to evaluate enzyme purity. Previous reports of the specific activity of pure enzyme from rat liver have been, in terms of units per min per mg of protein, 20 (6), 960 (12), and 1,382 or 5,048 (13), with purification-folds from the microsomes of 323, 350, and 2,747 or 1,242, respectively. In contrast, enzyme purified by our procedure has a specific activity of 17,000 to 19,000 and represents a purification-fold of 3,600 to 4,100 from the microsomes. This activity of rat liver HMG-CoA reductase is similar to that of the purified yeast enzyme (23).

Differences in the method of solubilization of the enzyme, in assays for enzyme activity, in the buffers used, and in the stability of the enzyme may partially account for the wide range of specific activities reported previously. However, most of the differences in activity can be attributed to the presence of protein impurities, a mixture of active and inactive species, or to the presence of an inhibitor in earlier preparations. The enzyme that is prepared by our procedure, in contrast, is homogeneous, according to assays for several of its physical properties. These include assays of size, charge, and immunological identity of the enzyme (10). In addition, we observed co-migration of enzyme activity with the single band of protein on polyacrylamide disc gel electrophoresis. This method proved to be a valuable criterion of enzyme purity, inasmuch as enzyme preparations of lower specific activity that were reported to be pure by size or immunological tests have been shown to be impure by this method (14).

The procedure of enzyme purification reported in this paper results in yields of approximately 10% from the solubilized enzyme state or 3 to 4% from the microsomes. About 2 µg of pure enzyme is obtained from 10 g of rat liver. Thus, per 1 g of liver tissue, the total amount of active microsomal HMG-CoA reductase is approximately 6 µg of protein.

Difficulties with Bio-Gel agarose chromatography in the purification of HMG-CoA reductase necessitated the employment of sucrose density gradient ultracentrifugation as an alternate procedure of purification. This method results in an enzyme activity peak that is well resolved from the majority of contaminating proteins. The behavior of HMG-CoA reductase on Bio-Gel agarose gel filtration is a function of the particular batch of gel and the ionic strength of the buffer used. The majority of contaminating proteins appear to elute from a column unaffected by these two parameters, and as a result a broad protein peak of constant elution position is obtained. HMG-CoA reductase, when devoid of interactions with the agarose residues, will elute as a 200,000 molecular weight species coincident with the major protein peak. This results in a low fold purification of enzyme and an incomplete purification in the subsequent step of affinity chromatography. Multiple molecular weight values have been obtained for HMG-CoA reductase on agarose gel filtration (12). However, the reason for these various molecular weights was not defined. In this paper it is shown that the interaction of HMG-CoA reductase with certain batches of Bio-Gel agarose appears to be very strong, inasmuch as no enzyme activity is recovered unless the ionic strength of the eluant is raised. Agarose is purified from agar containing carboxyl and sulfate groups attached to the galactose ring. It is also known from previous studies (24) that agar retards the elution of basic proteins such as cytochrome c from a column. This result suggests that Bio-Gel agarose provides the ionic sites for the binding of basic residues or regions on HMG-CoA reductase.

Certain batches of agarose gel retard the elution of HMG-CoA reductase markedly. This interaction between gel residues and enzyme results in lower yields of enzyme and an apparent molecular weight of <1,000. The position of elution of the enzyme can be shifted, however, to an earlier eluate position corresponding to a molecular weight in the range of 200,000 when KCl is included in the eluting buffer. This type of aberrant behavior of HMG-CoA reductase on agarose gel filtration is required for the preparation of homogeneous enzyme consisting of active and inactive species that are separable by CoA affinity chromatography (10).

In view of the inconsistent elution profiles obtained with agarose gel filtration, a different gel matrix consisting of a cross-linked dextran polymer was used to determine the molecular weight of HMG-CoA reductase. A value of 200,000
Daltons was obtained from a calibrated Sephadex G-200 column. Analysis of the enzyme for subunit structure by SDS-polyacrylamide disc gel electrophoresis revealed a monomeric molecular weight of 51,000. Hence, the enzyme is composed of four identical size subunits. This result is consistent with the tetrameric structure of the yeast HMG-CoA reductase molecule (23), which has a molecular weight of 264,000. The subunit molecular weight is not in agreement, however, with previous reports of 65,000 (6) and 120,000 (12). It is similar though to the 47,000 molecular weight reported by Srikantaiah et al. (13).

Apparent \( K_m \) values of 1.7 \( \times 10^{-5} \) M for D-HMG-CoA and 3.0 \( \times 10^{-5} \) M for NADPH have been determined from double reciprocal plots of initial velocity measurements of enzyme activity and substrate concentration. These results are consistent with previous values obtained for D-HMG-CoA with soluble enzyme (6, 11, 17). In contrast, \( K_m \) values for NADPH for enzyme solubilized with deoxycholate, or by a fast freeze-thaw glycerol extraction, or by a modified slow freeze-thaw treatment of the microsomes yielded estimations of 8.7 \( \times 10^{-5} \) M (6), 1.1 \( \times 10^{-4} \) M (17), or 3.2 \( \times 10^{-4} \) M (13), respectively. Under fasting and fed conditions, the cytosolic concentration of NADPH is reported to be in the \( 10^{-7} \) range (25), and thus HMG-CoA reductase is exposed in the liver cell to saturating levels of NADPH. Hence, the low \( K_m \) value we obtain for NADPH probably negates \textit{in vivo} regulation of enzyme activity through an alteration of cytosolic NADPH concentration. This conclusion is based on two assumptions. First, compartmentalization of the reductant does not exist near the periphery of the endoplasmic reticulum which is the location site of HMG-CoA reductase, and second, the soluble enzyme retains the kinetic properties of microsomal HMG-CoA reductase.

Deg (20) recently reported a pI value of 6.7 for chicken liver HMG-CoA reductase. Rat liver HMG-CoA reductase with a pI of 6.2 also appears to have a significant proportion of basic amino acid residues, and this may account for its interaction with agarose gel binding sites. The pH optimum of 6.3 to 7.3 of HMG-CoA reductase solubilized by the slow freeze-thaw method is comparable to enzyme obtained by deoxycholate extraction (6) or snake venom digestion, high salt treatment, or fast freeze-thaw glycerol extraction (8).

Previous studies (8, 11) have reported that only soluble enzyme and not microsomal enzyme displays cold sensitivity. In addition, high concentrations (10 \( ^{-2} \) M) of NADPH can prevent the cold lability of the enzyme (27, 28). We have found that microsomal enzyme exhibits reversible cold lability with kinetics similar to that observed with soluble enzyme if assays are carried out with short incubation periods. Thus, the membrane matrix does not protect the enzyme from cold inactivation. Since the enzyme is associated with the endoplasmic reticulum, it is possible that structural changes in the membrane \textit{i.e.} protein-lipid interactions, due to temperature-induced phase transitions, may regulate enzyme activity. Evidence has been produced in this study that suggests hydrophobic forces are important in maintaining the enzyme in an active conformation in that a KCl-glycerol buffer prevents both cold and heat lability of the enzyme. It has also been shown through gel filtration studies that the enzyme is not dissociated into 51,000 dalton subunits during cold inactivation.

Recently it has been shown (99) that lipids incorporated as liposomes alter enzyme activity. The possibility remains that lipid attached to the microsomal enzyme is an integral part of the HMG-CoA reductase molecule, and that upon solubilization it remains bound to the enzyme and stabilizes the enzyme in an active conformation. Extraction procedures which result in soluble enzyme displaying a lack of cold sensitivity could represent enzyme containing an altered lipid content. An alternate explanation for cold inactivation could be the disruption of hydrophobic forces of a larger aggregate of the 200,000 dalton species that may exist at 37°C.

REFERENCES