Purification of 3-hydroxy-3-methylglutaryl coenzyme A reductase from human liver

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Abstract The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase from human liver was purified to homogeneity. Electrophoresis of the purified human enzyme on non-denaturing gels revealed a single protein-staining band that co-migrated with reductase activity and could be selectively removed by treatment with cross-reacting antibody prepared to the purified reductase from rat liver. On SDS polyacrylamide gel electrophoresis, the purified reductase showed only one protein-staining band corresponding to a molecular weight of approximately 52,000. Based on the sedimentation rate during sucrose density gradient centrifugation, most of the enzyme from crude solubilized preparations had an apparent molecular weight of approximately 104,000. The human reductase, like the rat liver enzyme, was dimeric and composed of subunits of approximately 50,000 molecular weight.—**Tanaka R. D., P. A. Edwards, S. H. Lan, E. M. Knöppel, and A. M. Fogelman**. Purification of 3-hydroxy-3-methylglutaryl coenzyme A reductase from human liver. *J. Lipid Res.* 1982. **23:** 523–530.

Supplementary key words cholesterogenesis • immunotitration

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase catalyzes the rate-controlling step in the biosynthesis of cholesterol (1). Several methods have been reported for the purification of HMG-CoA reductase from rat (2–5) and chicken (6) liver, but so far no reports have dealt with the purification of this enzyme from human tissue. HMG-CoA reductase activity can be modulated in cell cultures of human fibroblasts, leukocytes, and smooth muscle cells by modification of the lipoprotein and sterol content of the culture media (7–9). However, the mechanisms involved in these regulatory events cannot be elucidated with certainty until some of the physical properties of the human enzyme have been determined. In this report we describe the purification of HMG-CoA reductase from human liver and compare the properties of the human and rat hepatic enzymes.

EXPERIMENTAL PROCEDURE

Materials

Chemicals were obtained from the sources indicated. Dithiothreitol, NADP, CoASH, HMG-CoA, Protein-A-agarose, glucose-6-phosphate dehydrogenase, and aldolase were from Sigma Chemical Co.; agarose–hexane–HMG-CoA was from P-L Biochemicals; bovine serum albumin was from Pentex; DL-3-hydroxy-3-methyl [3-^{14}C]glutaryl CoA and [5-^{3}H]mevalonic acid were from New England Nuclear; Dowex AG1-X8 formate, sodium dodecyl sulfate, and acrylamide were from Bio-Rad. The sources of all other materials and equipment have been previously reported (5).

Tissue samples

Liver samples were obtained from brain-death patients kept preoperatively on respirators for periods ranging from 4 to 72 hr. The ages of the donors were 23, 26, 26, and 45 years. Tissue samples were transported in ice, quick-frozen, and stored in liquid nitrogen. The time between excision and storage in liquid nitrogen varied from 1 to 4 hr. All tissue samples were procured with the approval of, and by the guidelines set forth by, the University Human Subjects Protection Committee (UCLA).

HMG-CoA reductase assay

HMG-CoA reductase activity was measured using a minor modification of the radioassay previously described by Edwards, Lemongello, and Fogelman (10). The total volume of each assay was 200 μl and the reaction mixture contained 10 μl of a glucose-6-phosphate dehydrogenase solution (1.1. units), 25 μl of a solution containing 2.8 M NaCl, 0.37 M D-glucose-6-phosphate, 0.28 M EDTA, and 90 mM NADP, and 80 μl of phosphate buffer (0.4 M KCl, 0.32 M KH_2PO_4, 8.0 mM EDTA, and 10 mM dithiothreitol, pH 6.8). All samples were preincubated 20 min at 37°C before addition of 35 μl of [^{14}C]HMG-CoA (4.4 nmol, 5 Ci/mol). The reaction was stopped

Abbreviations: CoASH, coenzyme A; EDTA, ethylenediamine tetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; NADP, nicotinamide adenine dinucleotide phosphate.

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by addition of 50 μl of 33% KOH. [^{3}H]Mevalonic acid (MVA) was added as an internal standard. The mevalonic acid was converted to its lactone derivative by addition of 5 N HCl and the mevalonolactone was separated on Dowex AG1-X8 formate columns as previously described (10).

Immunotitrations

In the immunotitration experiments, the antibody was added in the reaction mixture and the samples were preincubated 30 min at 37°C before addition of the ^{14}C-labeled substrate. The monospecific antibody was prepared in rabbits against purified HMG-CoA reductase from rat liver (5). Pure IgG fractions from both the monospecific antisera and preimmune sera were purified using Protein-A covalently coupled to agarose beads; sera or crude IgG fractions (5) were applied to Protein A-agarose affinity columns, the columns were washed with phosphate-buffered saline until the A_{280} was zero and the pure IgG was eluted in two column volumes of 0.2 M acetic acid (pH 2.8) (11). The acetic acid fractions were collected in tubes containing 2.0 M phosphate buffer (pH 8.0) so that the pH was raised immediately to pH 7.0. Protein concentrations of the monospecific and preimmune IgG solutions were 1.03 μg/μl and 2.05 μg/μl, respectively. Protein concentrations were determined by the method of Bradford (12) as modified by Edwards et al. (10).

Enzyme purification

Preparation of the human liver microsomes. Liver samples were thawed and minced in approximately 1.5 volumes of ice-cold buffer A (0.1 M sucrose, 0.05 M KCl, 0.04 M KH_2PO_4, 0.03 M EDTA, pH 7.2). In all subsequent steps the sample was kept at 0–5°C unless otherwise stated. The liver was homogenized with a Polytron homogenizer (Brinkmann) using four 15-sec pulses at a setting of 4, diluted with a small volume of buffer A to lower the viscosity, and centrifuged at 9,000 *g* for 10 min. The supernatant was collected, recentrifuged under identical conditions, and the upper three-fourths of the supernatant was carefully collected. Solid dithiothreitol was added to a final concentration of 10 mM and the solution was centrifuged at 100,000 *g* for 70 min. The supernatant was discarded and the microsomal portion above the translucent glycogen pellet was removed. Liver microsomes were suspended in a small volume of buffer A containing 10 mM dithiothreitol and the solution was centrifuged at 100,000 *g* for 45 min. The supernatant was decanted and the microsomal pellets were quick frozen in an ethanol-dry ice bath and stored at −20°C.

Solubilization of the enzyme. The microsomal pellets were thawed at 37°C and an equal volume of buffer B (0.1 M KCl, 0.08 M KH_2PO_4, 2.0 mM EDTA, 10 mM dithiothreitol, pH 7.2) diluted 1:1 with glycerol was added. The solution was homogenized with a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle and the homogenate was incubated at 37°C for 60 min. At 30-min intervals during the incubation, the homogenate was rehomogenized. The homogenate was diluted with two volumes of buffer B and centrifuged at 100,000 *g* for 70 min at 25°C. The upper three-fourths of the supernatant was collected and solid ammonium sulfate was added to a final concentration of 45%. The solution was stirred at room temperature for 40 min, centrifuged at 30,000 *g* for 20 min at 25°C and, after discarding the supernatant, the pellet was dissolved in buffer B containing 30% glycerol (v/v) and KCl. The KCl concentration in the buffer-glycerol solution was 1.07 M. The solutions were quick-frozen and stored in liquid nitrogen. The enzyme activity was stable for at least 14 days under these conditions.

Affinity chromatography

The enzyme solution was thawed at 37°C and incubated at 65°C for 15 min. Following dilution with an equal volume of buffer B (pH 6.8), the sample was centrifuged at 30,000 *g* for 20 min at 25°C. Solid ammonium sulfate was added to the supernatant to a final concentration of 45%, and, after stirring at room temperature for 40 min, the solution was recentrifuged at 30,000 *g* for 20 min at 25°C. The pellet was dissolved in buffer C (50 mM KCl, 40 mM KH_2PO_4, 1.0 mM EDTA, 10 mM dithiothreitol, pH 6.8) and the enzyme solution was incubated at 37°C for 30 min prior to loading onto the affinity column.

The agarose-hexane-HMG-CoA affinity column was washed with 2.0 M KCl before equilibrating with buffer C. The column (0.9 cm × 14 cm) contained 0.7 ml of packed agarose beads. After the sample was applied twice to the column, the beads were washed with at least 20 column volumes of buffer C before the enzyme was eluted with buffer C containing 1.0 mM CoASH. In some instances the buffers used in the affinity chromatography and sample preparation contained either glycerol (concentrations of 30% or 50%) or bovine serum albumin (concentration of 1 mg/ml). The pure enzyme was stored in buffers containing either 30% or 50% glycerol, quick-frozen, and kept in liquid nitrogen.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was as described by Laemmli (13). The thickness of the slab gels was 1.5 mm, and the percent acrylamide in the stacking and separating gels was 2.5% and 7.5%, respectively. Gels were stained using the silver-staining procedure of Oakley, Kirsch, and Morris (14).

For comparative purposes the purified rat liver HMG-CoA reductase was also analyzed on the same gels. The rat enzyme was purified as described (5).

Non-denaturing polyacrylamide gels were run as described by Maurer (15). Maurer gel system No. 6 was used with 1.5-mm thick slab gels. The gels were polymerized using riboflavin instead of ammonium persulfate, and the running buffers contained 25 mM β-mercaptoethanol. The acrylamide concentrations in the stacking and separating gels were 2.5% and 4.9%, respectively. Gels were stained as described above. To assay reductase activity in the gels, duplicate samples were run in tandem. The gel was cut and one half was stained for protein and the other half was cut into 4-mm segments. Each segment was placed in the enzyme reaction mixture containing the [^{14}C]HMG-CoA substrate and incubated at 37°C for 24 hr. All other procedures were as previously described for the reductase assay.

To assess the homogeneity of the purified HMG-CoA reductase, enzyme samples were also incubated with either monospecific or preimmune IgG overnight at 5°C. Free IgG and antigen-antibody complexes were removed following addition of Protein-A covalently coupled to agarose beads. The samples were incubated overnight at 5°C, centrifuged, and the supernatants were analyzed on non-denaturing gel using the procedures described above.

Sucrose density gradient centrifugation

Determination of the sedimentation rate of the enzyme in 5% to 20% linear sucrose density gradients was as described (5, 16). Crude solubilized preparations of the enzyme were used in all experiments because of the instability of the purified enzyme. Samples were centrifuged at 210,000 *g* for 14 hr at 20°C. The bottoms of the tubes were punctured and 125-μl fractions were collected. HMG-CoA reductase was measured using the previously described radioassay. The molecular weight standards (bovine serum albumin, aldolase, glucose-6-phosphate dehydrogenase) were analyzed by determining the protein concentration in each fraction. The molecular weight of the enzyme was calculated using the equations described by Martin and Ames (16).

RESULTS

HMG-CoA reductase activity in human liver was low when compared to the activity present in normal rat livers (2). The average specific activity present in the microsomal preparations obtained from three different human livers was 52 pmol MVA/min per mg protein. This activity was equivalent to about 95 units of enzyme activity (1 unit = 1 nmol MVA/min) per 100 g wet weight of human liver.

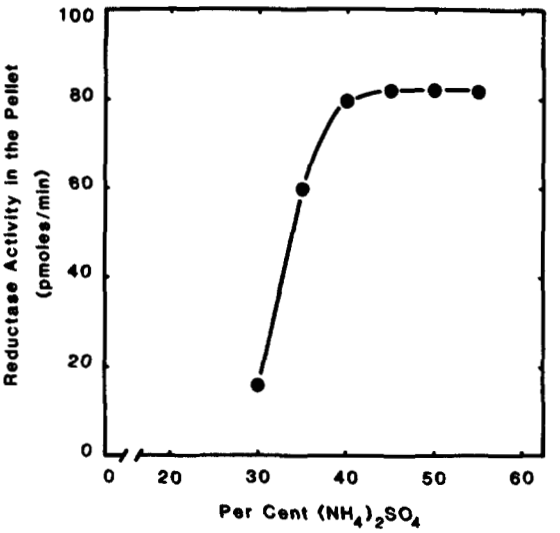

Fig. 1. Ammonium sulfate precipitation of HMG-CoA reductase activity. Increasing amounts of solid ammonium sulfate were added to crude solubilized preparations of the enzyme and, after centrifugation, the reductase activity was assayed in the pellet. The total percent recovery of enzyme activity in the pellet was approximately 30%.

Enzyme purification

Approximately 30% of the total protein present in the liver microsomes was solubilized in buffer B containing 50% glycerol (v/v). Solubilization of HMG-CoA reductase activity varied from 10% to 97% with the average being 50% (n = 20). The cause of this variation is unknown. Treatment with buffers, pH 9.0, containing the non-ionic detergent Triton X-100 yielded about the same amount of solubilized enzyme (data not shown). However, these latter methods also solubilized approximately 70% of the microsomal proteins.

The solubilized enzyme was maximally precipitated by addition of ammonium sulfate at concentrations higher than 45% (**Fig. 1**). There was a 25% decrease in reductase activity following the heat treatment at 65°C (**Fig. 2**, circles). The initial 25% loss of activity between 0–5 min of incubation may be due to entrapment of the enzyme in the large precipitate of denatured protein. The remaining enzyme activity was relatively stable from 5 to 30 min. After the heat treatment, the specific activity in the supernatant increased approximately 4-fold (Fig. 2, triangles).

The total increase in specific activity prior to the agarose-hexane-HMG-CoA affinity column was approximately 77-fold (**Table 1**, steps 1–5). The largest increase in specific activity occurred during the solubilization and the heat treatment. The percent recovery of the initial total reductase activity was 20% prior to the affinity chromatography.

After passing the enzyme sample through the affinity column twice, approximately 70% of the enzyme was

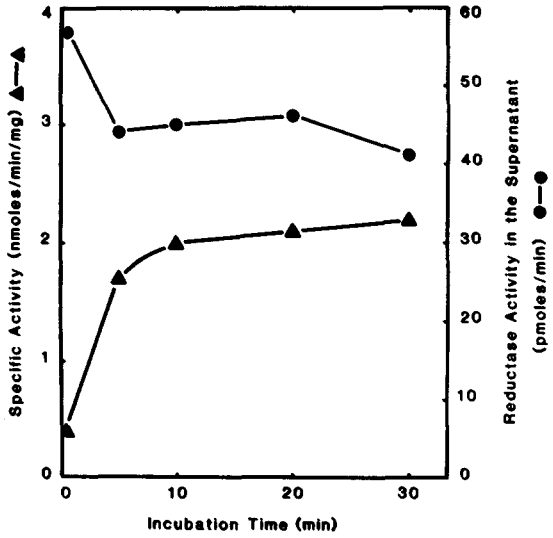

Fig. 2. The effect of incubation at 65°C on HMG-CoA reductase activity and the resulting changes in specific activity. Samples were incubated in buffer B containing 1.07 M KCl and 30% glycerol (v/v) for different lengths of time at 65°C. After centrifugation the reductase activity (● —— ●) and the specific activity (▲ - - - ▲) of the enzyme were determined in the supernatant.

bound to the beads. The inability of the remaining 30% of the enzyme activity to bind to the column was not due to saturation of the beads since this active enzyme was also not bound when it was applied to a second affinity column. Lowering the ionic strength in the buffers did not increase the amount of enzyme bound to the column (data not shown). Only approximately 1.0% of the total bound reductase activity could be eluted off the column in buffer C containing 1.0 mM CoASH. Increasing the CoASH concentration to 4 mM produced no change in the recovery. Approximately 10% of the total reductase activity bound to the column could be recovered by washing the beads with buffers of high ionic strength. However, the eluted enzyme in these latter buffers was not pure (data not shown).

There was no increase in the specific activity of the HMG-CoA reductase eluted from the affinity column with the buffer containing 1.0 mM CoASH (Table 1). However, if these data were corrected for the inhibition of reductase activity by the CoASH in the elution buffer, there was a small increase in the specific activity from 0.81 to 1.35 nmoles MVA/min per mg protein (**Fig. 3**, Table 1). The total protein in the fractions eluted with the CoASH was less than 0.4% of the initial amount of protein applied to the column. Addition of either bovine serum albumin (to a concentration of 1.0 mg/ml) or glycerol (to concentrations of 30% or 50%) to all the buffers did not significantly increase the amount of reductase activity recovered from the column (data not shown).

Analyses of the purified enzyme

The purified HMG-CoA reductase was shown to be essentially homogenous when analyzed by polyacrylamide gel electrophoresis. One major protein-staining band was observed after sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified enzyme (**Fig. 4**). The apparent molecular weight of the protein was 52,000. For comparative purposes, the purified rat liver enzyme was also analyzed. The apparent molecular weight for the rat enzyme was 54,000 which is similar to the molecular weight previously reported by Edwards et al. (5). In order to reveal the presence of minor components, the gel shown in Fig. 4 was intentionally overloaded. Some of the faint lines seen on the gel are artifacts created by the silver staining procedure as described by Oakley et al. (14).

Further evidence of homogeneity was obtained when the purified HMG-CoA reductase from human liver was analyzed by electrophoresis under non-denaturing conditions. In **Fig. 5**, the purified human and rat enzymes were both electrophoresed on the same gel. The human and rat enzyme each gave a single staining band although the R_f values of 0.14 and 0.52, respectively, were different. However, analyses of the same sample of human

TABLE 1. Purification of HMG-CoA reductase from human liver

Purification Step	Total Protein	Total Units	Specific Activity	Percent Recovery	Fold Purification
	mg	*nmol MVA/min*	*nmol MVA/min/ mg protein*	*%*	
Microsomal suspension	3230	36.8	0.011	100	1
Solubilization in 50% glycerol	468.4	35.8	0.074	97	6.7
45% Ammonium sulfate precipitate	95.7	11.0	0.115	30	10.5
65°C Heat treatment	12.9	7.5	0.584	20	53.1
45% Ammonium sulfate precipitate	9.5	8.0	0.845	20	76.8
HMG-CoA affinity column	0.041[a]	0.136[a]	0.813	0.14	73.9

The values shown are from one experiment in which HMG-CoA reductase was purified from 163 g of human liver.
[a] Total from combined CoASH eluted fractions. In one fraction the protein concentration was too low to be accurately measured, however the total enzyme activity was included in the calculations.

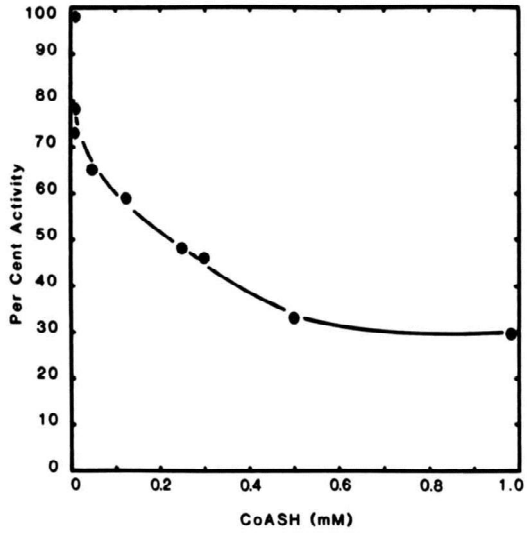

Fig. 3. CoASH inhibition of HMG-CoA reductase activity. Increasing amounts of CoASH were added to solubilized preparations of the enzyme. Samples were preincubated in the presence of CoASH for 30 min at 37°C before addition of the ^{14}C-labeled substrate. Assay conditions were those stated in the text.

enzyme on different occasions periodically revealed minor protein bands with R_f values of 0.52, 0.14, and less than 0.14, suggesting that the reductase may form aggregates under the conditions of electrophoresis. Similar alterations in the band pattern have been observed with the pure rat enzyme.

When purified enzyme was run on non-denaturing gels, and the gels were cut into segments and assayed for enzyme activity, HMG-CoA reductase activity was associated with the protein-staining band with an R_f of 0.14 (**Fig. 6**). Enzyme activity in the gel segments also occurred in the segment that preceded the major portion of the protein band. This may result from the fact that the larger enzyme aggregates may retain reductase activity better than smaller aggregates.

Binding of human reductase to anti-rat-reductase IgG

Monospecific antibody prepared to the purified rat liver enzyme cross-reacted and inactivated the human HMG-CoA reductase (**Fig. 7**). Approximately 80% of the reductase activity could be inhibited by the monospecific antibody, while preimmune antibody had no inhibitory activity.

The purified human enzyme was treated with the monospecific antibody and the IgG and the enzyme–antibody complexes were removed with Protein-A-agarose beads. Identical samples were treated with preimmune antibody and the supernatants from both preparations were analyzed by electrophoresis on non-denaturing gels. No protein-staining bands were present in the samples treated with the monospecific antibody, while the protein

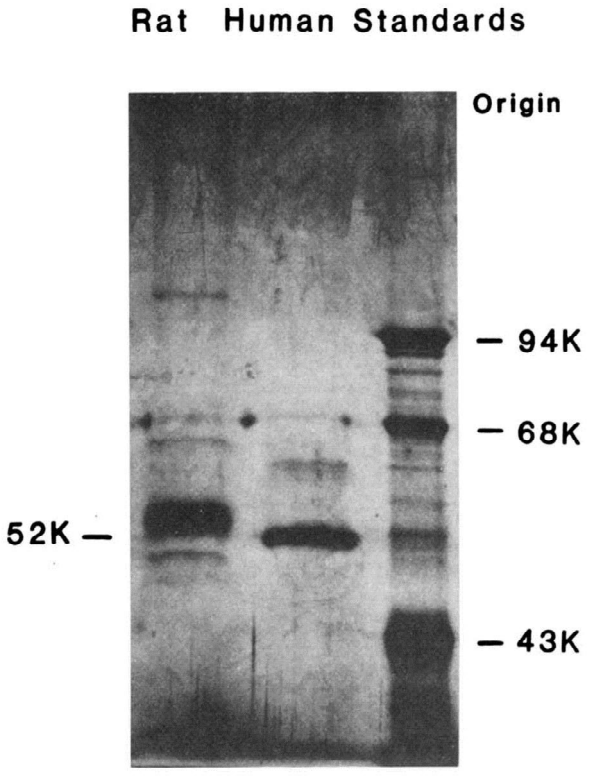

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified human and rat liver HMG-CoA reductase. Approximately 60 ng of both the human and rat enzymes were applied to the polyacrylamide gel. Gels were stained for protein using a silver staining procedure as described by Oakley et al. (14). The relative positions of the protein standards are indicated on the right of the figure. The calculated position of a polypeptide of 52,000 daltons is also shown.

band with the R_f of 0.14 was still present in the samples treated with the preimmune antibody (**Fig. 8**).

Sucrose density gradients

To determine the apparent molecular weight of the enzyme, preparations of the crude solubilized enzyme were analyzed on sucrose density gradients (**Fig. 9**). Impure preparations of the enzyme were used in these analyses because of the instability of the purified enzyme. Two peaks of reductase activity were detected after centrifugation and the positions of the major and minor

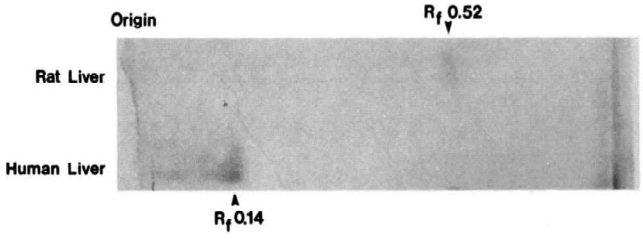

Fig. 5. Electrophoresis of the purified human and rat liver HMG-CoA reductase on non-denaturing polyacrylamide gels. Approximately 60 ng of each enzyme was loaded onto the gel. Gels were stained as stated in Fig. 4. The bromophenol blue front is not shown.

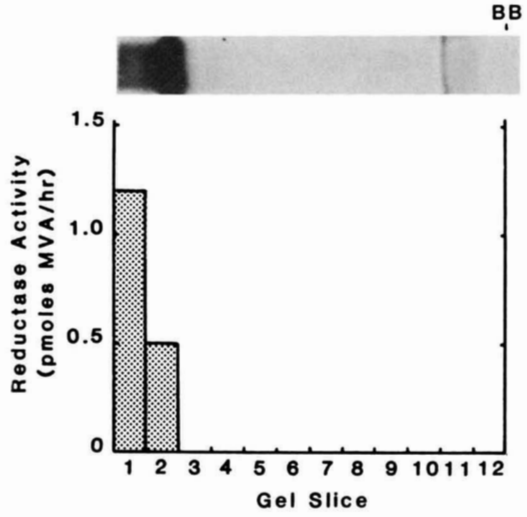

Fig. 6. Electrophoresis of the purified HMG-CoA reductase from human liver and the association of reductase activity with the protein-staining band. Approximately 100 ng of the enzyme was loaded onto the polyacrylamide gel and tandem samples were run. One sample was stained for protein and the other was cut into 4-mm segments. Each segment was assayed for reductase activity as described in the text. Enzyme activity was absent in gel slices 3 through 12.

peaks corresponded to apparent molecular weights of 102,000 and 163,000, respectively. The $S_{20,w}$ for the major enzyme population was 6.145. The minor peak contained less than 20% of the total reductase activity and may result from the enzyme binding to other membrane proteins or from enzyme–enzyme aggregation.

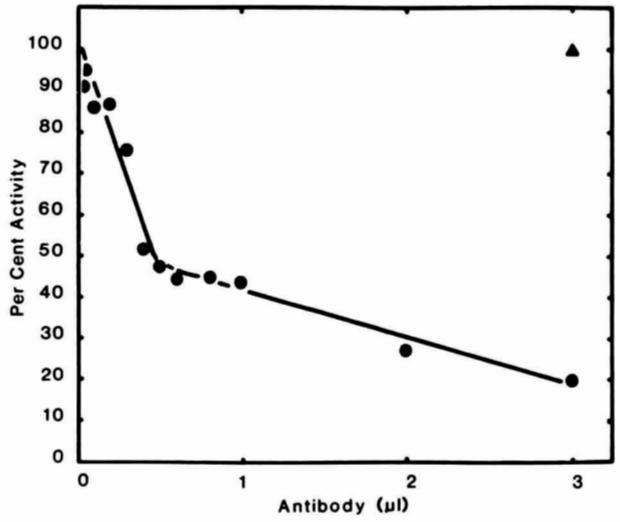

Fig. 7. Inactivation of the human HMG-CoA reductase by cross-reacting antibody prepared to the rat liver reductase. Increasing amounts of monospecific or preimmune antibody were added to solubilized preparations of the human enzyme. The samples were preincubated with the antibody for 30 min at 37°C before addition to the ^{14}C-labeled substrate. The protein concentrations of the monospecific (●) and preimmune (▲) antibody were 1.03 µg/µl and 2.05 µg/µl, respectively.

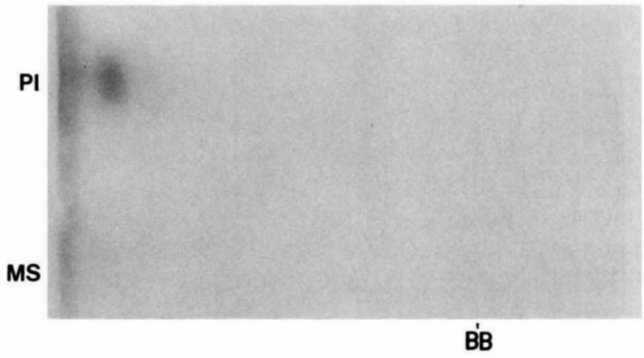

Fig. 8. Electrophoresis on non-denaturing polyacrylamide gels of HMG-CoA reductase samples that were pretreated with either monospecific (MS) or preimmune (PI) antibody prepared to the rat liver reductase. Approximately 100 ng of the human enzyme was treated with either 20 µl of the monospecific or 10 µl of the preimmune antibody. The protein concentrations of the monospecific and preimmune antibody were 1.03 µg/µl and 2.05 µg/µl, respectively. IgG and antigen–antibody complexes were removed following addition of Protein A covalently coupled to agarose beads. After centrifugation, the supernatants were analyzed by electrophoresis.

DISCUSSION

In the human liver the average specific activity of HMG-CoA reductase was low (52 pmol MVA/min per mg protein) and was similar to the values reported by Nicolau et al. (17) and by Ahlberg et al. (18).

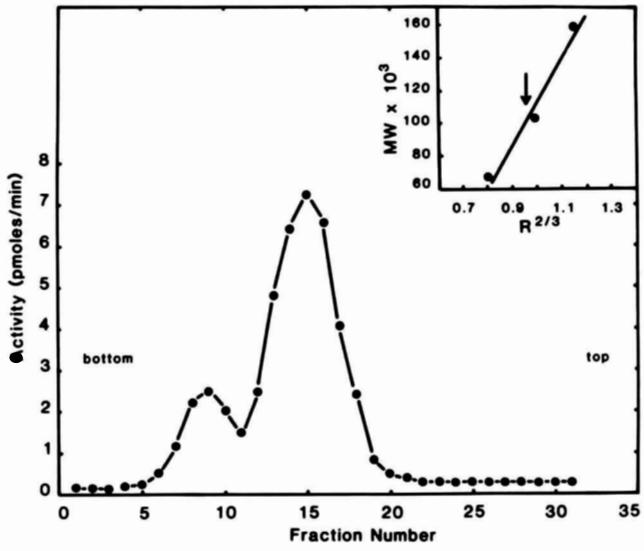

Fig. 9. Sucrose density gradient centrifugation of HMG-CoA reductase from human liver. Solubilized preparations of the enzyme were loaded onto 5% to 20% linear sucrose density gradients. Samples were centrifuged at 210,000 *g* for 14 hr at 20°C. After centrifugation, the tubes were punctured and 125-µl fractions were collected. HMG-CoA reductase activity was measured using the radioassay stated in the text. The standards (bovine serum albumin, aldolase, glucose-6-phosphate dehydrogenase) were analyzed by determining the protein concentration. The molecular weight of the enzyme eluting in fraction 15 was calculated using the equations described by Martin and Ames (16) and using glucose-6-phosphate dehydrogenase as the standard with an $R^{2/3}$ arbitrarily equal to 1.0.

The methods used to purify HMG-CoA reductase from liver were similar, but not identical, to those previously used to purify the enzyme from rat liver (5). A major step in the purification of the rat liver enzyme involved the affinity chromatography (5). However, following purification of the human enzyme on the agarose-hexane-HMG-CoA affinity column, only about 1.0% of the total activity could be recovered in the specific CoASH elution and there was almost no increase in the specific activity. We concluded that the protein eluted from the affinity column by CoASH was essentially homogeneous but was composed of relatively inactive enzyme. The purified HMG-CoA reductase was homogeneous based on: *1*) the presence of a single protein-staining band after electrophoresis on either denaturing (Fig. 4) or non-denaturing (Fig. 5) gels; *2*) the comigration of enzyme activity with the single protein-staining band observed after electrophoresis on non-denaturing gels; and *3*) the selective removal of this band by monospecific antibody which cross-reacted and inactivated the human enzyme. Therefore, these data support the conclusion that the purified enzyme lost most of its activity after elution from the affinity column. The low recovery of enzyme activity did not result from failure to efficiently elute the enzyme since increasing the CoASH concentration by 4-fold did not increase the recovery. Also, only approximately 10% of the activity could be recovered if the column was washed subsequently with a buffer of eight times higher ionic strength. The loss of enzyme activity may be due to the very low protein concentration (10–20 μg/ml) present in the specific CoASH eluant. However, addition of either bovine serum albumin or glycerol to the buffers did not stabilize enzyme activity. Hence the purification of the active enzyme preparations may require large amounts of enzyme.

The physical properties of the human HMG-CoA reductase were similar to those of the rat liver enzyme. Both enzymes have a dimeric structure (mol wt, 102–104,000) composed of two similar subunits of approximately 52,000 molecular weight. Based on analyses using sucrose density gradient centrifugation, a small portion (<20%) of the human enzyme may exist as a trimer (mol wt, 163,000). However, only relatively crude preparations of the enzyme were used in these studies and these data may result from the reductase binding to other solubilized membrane proteins or to self aggregation as exhibited with the purified enzyme.

Other similarities between the human and rat liver enzymes include the K_m (7, 10, 19), the resistance to heat inactivation at 65°C (Fig. 2) (5), the solubilization of enzyme activity in buffers containing 50% glycerol, and the cross-reactivity of monospecific antibody prepared to the purified rat liver enzyme. The latter point may be of great importance since it may facilitate the study of the regulation of HMG-CoA reductase in human tissues by use of immunologic techniques. The use of the cross-reacting antibody would also preclude the arduous task of purifying large amounts of the human enzyme in order to prepare monospecific antibody. We are currently investigating the regulation of the human enzyme in cultured cells using the cross-reacting antibody raised to the pure rat reductase.

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