PURIFICATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE[1]

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SUMMARY: This paper describes a rapid purification procedure for 3-hydroxy-3-methylglutaryl coenzyme A reductase, the major regulatory enzyme in hepatic cholesterol biosynthesis. A freeze-thaw technique is used for solubilizing the enzyme from rat liver microsomal membranes. No detergents or other stringent conditions are required. The purification procedure employs Blue Dextran-Sepharose-4B affinity chromatography, and purification can be carried out from microsomal membranes to purified enzyme in 8 to 10 hours. The purified enzyme has a specific activity of 517 nmoles/min/mg protein, and it is 975-fold purified with respect to the original microsomal membrane suspension. SDS polyacrylamide gel electrophoresis of the purified enzyme shows only trace impurities; the subunit molecular weight for the enzyme measured by this technique is 47,000.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase, EC 1.1.1.34) catalyzes the reduction of HMG-CoA[2] to mevalonic acid. This enzyme has been shown to be the major regulatory enzyme in hepatic cholesterol biosynthesis (1-10).

Purification and investigation of the properties of liver HMG-CoA reductase have been difficult because the enzyme is bound to the microsomal membranes (4). The enzyme has been solubilized by a variety of procedures in several laboratories (11-17). The procedure recently developed by Heller and Gould (14,17) has been utilized for the studies reported here. This procedure exposes the microsomal membranes to a slow freeze-thaw treatment. Buffer extraction of this microsomal preparation yields a crude solubilized enzyme extract which possesses a high specific activity (14,17). This

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2. Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; DTE, dithioerythritol; SDS, sodium dodecylsulfate.

technique has the further advantage that the microsomes are not exposed to detergents, organic solvents or high salt, conditions which would tend to disrupt the subunit interactions essential for optimal catalytic activity of HMG-CoA reductase.

Using this crude soluble extract we have developed a simple rapid purification procedure which employs affinity chromatography and which yields HMG-CoA reductase with a specific activity that is 14- to 66-fold higher than previously reported (12,13,15).

MATERIALS AND METHODS

Chemicals: DL-[3-^{14}C]-3-Hydroxy-3-methylglutaryl coenzyme A (specific activity of 18.5 μCi per μmole) was purchased from New England Nuclear. Unlabeled HMG-CoA was obtained from P and L Laboratories. DL-[4-^{3}H]-mevalonic acid (specific activity 116 μCi per μmole) was from Amersham Searle. Unlabeled mevalonic acid was purchased from Nutritional Biochemicals. Blue Dextran, NADPH and DTE were from Sigma. CNBr activated Sepharose-4B was obtained from Pharmacia.

Animals: Male Sprague-Dawley rats (150 g) were used. For at least 10 days prior to sacrifice, these animals were subjected to a 3 AM to 3 PM dark cycle. The animals were sacrificed at 9 AM, the peak of HMG-CoA reductase activity.

Preparation of Microsomes: Liver microsomal preparations were made in a buffer containing 0.1 M sucrose, 0.05 M KCl, 0.04 M potassium phosphate, 0.03 M EDTA and 10 mM DTE at pH 7.2 (buffer A) according to the procedure described by Heller and Gould (14,17). HMG-CoA reductase was solubilized from the microsomes by the freeze-thaw method developed by Heller and Gould (17).

Incubation and Assay Conditions: The sample to be tested for enzyme activity (0.2 ml) was first preincubated at 37° with NADPH (2.8 mM) for 20 minutes. Then DL-[3-^{14}C]-HMG-CoA (66,500 dpm) was added giving a final concentration of 300 μM. Incubation was carried out in an atmosphere of

nitrogen at 37°. Incubation times ranged from 2 to 20 min. Mevalonic acid formation was linear for 60 min.

The reaction was stopped by the addition of 2 N HCl (1 ml), and DL-[4-^{3}H]-mevalonic acid (0.04 μCi) was added as an internal reference. Each sample was transferred to a counting vial, using buffer A (2 ml) as a wash. Unlabeled mevalonic acid (10 mg) was added to each sample, followed by the addition of a saturating quantity (2.3 g) of an equal molar mixture of dry mono- and dibasic potassium phosphate. After incubation in an atmosphere of air for 30 min at 37° in a Dubnoff shaker, chloroform (6 ml) was added. The chloroform was separated from the aqueous phase on Whatman phase separating paper, the chloroform extract was evaporated to dryness under a stream of nitrogen, scintillator was added, and radioactivity was assayed using a 3-channel liquid scintillation counter (Packard 3375).

The chloroform extract contains only mevalonolactone. This has been verified over a wide range of enzyme activities and at all biological levels, i.e., microsomes, soluble extract, and purified enzyme (18). The verification procedure was a thin layer chromatographic method (16) using 25:1 chloroform-methanol as the eluting solvent.

One unit of enzyme activity is defined as the formation of one nanomole of mevalonic acid per min at 37°.

Preparation of Blue Dextran-Sepharose-4B: The procedure employed was similar to that described by Ryan and Vestling (19). Pharmacia CNBr activated Sepharose-4B (15 g) was swollen and washed in a total of 3 liters of HCl (1 mM) at room temperature for 15 min. The HCl solution was removed by suction, followed by washing with 500 ml of cold 0.1 M $NaHCO_3$ (pH 8.3). To a thick slurry of this CNBr activated Sepharose-4B a solution of Blue Dextran (1 g dissolved in 50 ml of 0.4 M Na_2CO_3, pH 10.0) was added. The mixture was stirred gently for 18 hours at 4°. It was filtered on a suction funnel, and then resuspended in 0.1 M tris-HCl buffer (400 ml,

pH 8.0). Gentle stirring was conducted for 1 hour at room temperature, and then the tris buffer was removed by suction. The gel was washed with 1 M KCl (150 ml), followed by 0.4 M Na_2CO_3 (100 ml). Washing was continued using alternating aliquots of 1 M KCl and 0.1 M $NaHCO_3$ until a total of 3 liters of each solution were used. The gel was washed with 2 liters of distilled water and finally with buffer A.

Protein Determination: Protein was determined by the method of Lowry et al. (20) after precipitating the protein with 5% trichloroacetic acid and redissolving the precipitate in 1 N NaOH as described previously by Heller and Gould (14).

Polyacrylamide Gel Electrophoresis: SDS polyacrylamide gel electrophoresis was performed using 7.5% gels at pH 6.6 (tris/acetate/SDS) in a manner similar to that described by Bio-Rad Laboratories (21). The gels were stained using coomassie blue.

RESULTS

Purification of HMG-CoA Reductase: Liver microsomes were prepared in buffer A as previously described by Heller and Gould (14,17). The microsomal pellets were subjected to the slow-freeze procedure described by these authors (17). The microsomal pellets were then stored at -20° until needed. Five pellets were then thawed, 16 ml of buffer A was added to each pellet, followed by homogenization for 30 seconds with a tight-fitting Teflon pestle. Each suspension was centrifuged at 105,000 x g for 60 min. The 105,000 x g supernatant (78 ml) was carefully removed and NADPH (138 mg) was added to this crude soluble extract. This preparation was then heated at 37° for 20 min, followed by heating at 60° for 10 min. The heated extract was centrifuged at 27,000 x g for 15 min at room temperature.

The heated soluble extract (75 ml) was then applied to a Blue Dextran-Sepharose-4B column (45 g, 4 x 10 cm) which had been equilibrated and washed with buffer A. Chromatography was performed at room temperature (20°). A total of 315 ml of buffer A was used to collect the break-

through fractions. Elution was next conducted with buffer A adjusted to a KCl concentration of 0.2 M. Two fractions (100 ml each) were collected. Elution was then conducted with buffer A adjusted to a KCl concentration of 0.5 M. The first fraction (75 ml) contained no enzyme. Then 20 ml fractions were collected. Fractions 3 and 4 of the 20 ml fractions contained the major portion of enzyme activity. Fractions 3 and 4 were pooled and concentrated using pressure dialysis (Amicon PM-30 membrane which had been washed with buffer A containing 2 mM NADPH). The volume of the solution containing the purified enzyme was 2.1 ml.

The quantitative aspects of the purification procedure are shown in Table 1. A 12-fold purification was achieved in the solubilization procedure from the microsomes with a yield of 71%. Next the enzyme extract

TABLE 1

PURIFICATION OF HMG-CoA REDUCTASE

	Volume (ml)	Enzyme activity (units/ml)	Total enzyme activity (units[a])	Protein (mg/ml)	Specific activity (nmoles/min/ mg protein)	Purification (-fold)	Yield (%)
Microsomal suspension	80.0	6.07	485.6	11.53	0.53	1	100
Soluble extract	78.0	4.42	344.8	0.69	6.41	12	71
Soluble extract + NADPH (60° for 10 min)	75.0	3.22	241.5	0.10	32.20	61	50
Blue Dextran-Sepharose-4B column[b]	2.1	45.45	95.5	0.088	516.5	975	20

[a] One unit of enzyme activity is defined as the formation of one nanomole of mevalonic acid per min at 37°.

[b] After affinity chromatography the fractions containing the major portion of enzyme activity were concentrated by pressure dialysis.

was activated (22) by incubation with NADPH. NADPH then stabilizes HMG-CoA reductase for heat treatment (60° for 10 min). The heat step yields an additional 5-fold purification. The final purification step is the Blue Dextran-Sepharose-4B affinity column which yields an additional 16-fold purification. The overall purification factor achieved is 975-fold in 20% yield. The specific activity for the purified enzyme is 517 nmoles/min/mg protein.

An experiment (Fig. 1) employing SDS polyacrylamide gel electrophoresis was performed upon the crude solubilized extract (Fig. 1a) and purified HMG-CoA reductase (Fig. 1b). Only trace impurities are observed for the purified enzyme (Fig. 1b). The subunit molecular weight for purified HMG-CoA reductase determined by this technique is 47,000.

The half-life for purified HMG-CoA reductase at 4° was approximately 9 days.

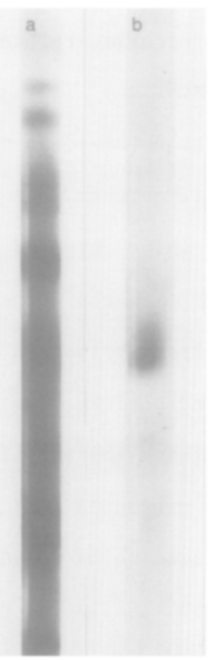

Figure 1. SDS polyacrylamide gel electrophoresis of (a) the crude solubilized extract and (b) purified HMG-CoA reductase.

DISCUSSION

HMG-CoA reductase was first solubilized from liver microsomes by Linn (11), using buffer extraction of an acetone powder of rat liver microsomes. Kawachi and Rudney (12) reported solubilization and purification of HMG-CoA reductase using deoxycholate as a solubilizing agent. The specific activity of the purified enzyme was quite low (7.8 nmoles/min/mg protein), although substantial evidence has been presented supporting the purity of the preparation (13). It is likely that deoxycholate is a potent inhibitor of the enzyme and that it would tend to disrupt the subunit interactions essential for optimal catalytic activity of HMG-CoA reductase. Brown et al. (15) described the partial purification of HMG-CoA reductase from rat liver. The specific activity reported for this preparation (15) was 37.8 nmoles/min/mg protein. Heller and Gould (14) have reported partial purification of HMG-CoA reductase from rat liver. The reported specific activity was 13.6 nmoles/min/mg protein. A later report (17) mentions a partially purified enzyme with a specific activity of 81 nmoles/min/mg protein. No experimental details were given concerning the more highly purified preparation.

Thus the specific activity obtained in the present article (517 nmoles/min/mg protein) is substantially higher than previously reported values. It is 66-fold higher than the value obtained by Higgins, Brady and Rudney (13), and it is 14-fold higher than the value reported by Brown et al. (15).

These findings suggest strongly that in addition to enzyme purity the three dimensional structure and conformation of HMG-CoA reductase are of critical importance in determining the specific activity. Evidence supporting this idea has been presented (13). In the present purification procedure techniques are utilized which would favor the preservation of a highly active enzyme: i) the solubilization procedure (17) consistently yields crude extracts of high specific activity; ii) the enzyme is activated and stabilized by preincubation with NADPH (22); iii) no dialysis

procedures are required during purification; iv) a rapid affinity column procedure is employed.

The total time required for purification from microsomal membranes to purified enzyme is approximately 8 to 10 hours.

Thompson, Cass and Stellwagen (23) have shown that Blue Dextran-Sepharose-4B has a strong affinity for proteins which possess the super-secondary structure called the dinucleotide fold. In the case of HMG-CoA reductase the dinucleotide fold would be important in binding NADPH, the coenzyme for the reaction. Corey-Pauling-Koltin models of NAD^+ and of the blue chromophore of Blue Dextran show that the blue chromophore can assume a conformation that mimics the orientation of the aromatic rings and anionic groups characteristic of NAD^+ as it would bind to the dinucleotide fold (23). The same reasoning would be true also for NADPH.

SDS polyacrylamide gel electrophoresis shows a subunit molecular weight for purified HMG-CoA reductase of 47,000. In earlier studies Higgins, Brady and Rudney (13) and Brown, Dana and Siperstein (24) reported a molecular weight for solubilized HMG-CoA reductase of 200,000. Based upon these results it is reasonable to conclude that the enzyme probably is a tetramer containing four subunits.

Since the procedure described in the present article yields HMG-CoA reductase with a substantially higher specific activity than previously published reports, this procedure should provide an important tool for studying the molecular properties, possible allosteric modifications and the regulation of HMG-CoA reductase, the rate-limiting enzyme in hepatic cholesterol biosynthesis.

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