SOLUBILIZATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE FROM LYOPHILIZED RAT LIVER MICROSOMES: LACK OF EVIDENCE FOR COLD LABILITY IN THIS SOLUBLE ENZYME PREPARATION[1]

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SUMMARY: This paper describes a simple and reliable method for the solubilization of rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase by extracting lyophilized microsomes with buffer. This procedure is suitable for the preparation of large quantities of soluble enzyme and does not require the use of organic solvents, detergents or other stringent conditions which might alter the properties of the enzyme. A test of cold lability in 3-hydroxy-3-methylglutaryl coenzyme A reductase solutions is described. No substantial cold lability was observed. These results indicate that the sensitivity to cold reported by other investigators probably is not an inherent property of the enzyme.

3-Hydroxy-3-methylglutaryl coenzyme A reductase (E.C. 1.1.1.34) has been well established as the major regulatory enzyme for hepatic cholesterol biosynthesis (1-11). This enzyme which catalyzes the conversion of HMG-CoA[2] to MVA is particulate in nature and microsomal in origin (12). In 1967 Linn (13) reported the solubilization of HMG-CoA reductase from an acetone powder of rat liver microsomes. Subsequently, Kawachi and Rudney (14) reported that they could not solubilize the enzyme by Linn's procedure. Consequently, they developed a solubilization procedure employing relatively high concentrations of sodium deoxycholate. However, the specific activity of the soluble enzyme prepared with this detergent was low. In previous work (15), using a modification of Linn's method, we have been successful on certain

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2. Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid.

occasions in completely solubilizing HMG-CoA reductase from an acetone powder of rat liver microsomes prepared in this laboratory and from an acetone powder of whole pig liver prepared in a commercial laboratory. However, only one commercial pig liver preparation contained significant reductase activity and the activity of our rat liver preparations was quite variable. (In retrospect, this variability probably resulted from several factors foremost of which was a lack of appreciation for the significance of the diurnal rhythm in HMG-CoA reductase activity.) Therefore, we decided to develop a method for solubilizing the enzyme that would not employ organic solvents or detergents and which would be suitable for the preparation of large quantities of soluble enzyme. We now report that HMG-CoA reductase can be solubilized by extracting lyophilyzed rat liver microsomes with buffer.

During the course of this work, two other solubilization procedures have been developed by Heller and Gould (16) and Brown, Dana, Dietschy and Siperstein (17). Brown et al. observed extreme cold lability in their soluble enzyme preparations and reported that HMG-CoA reductase was the first solubilized microsomal enzyme found to be cold sensitive. In view of this report, we decided to ascertain if a similar sensitivity to cold could be observed in our HMG-CoA reductase solutions.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats, weighing 250-300 g, were used. For at least 10 days prior to sacrifice, these animals were subjected to a 3 AM to 3 PM dark cycle. Under these conditions, HMG-CoA reductase is at the peak of its diurnal rhythm at the normal time of sacrifice, 8 to 9 AM (5-8).

Assay for HMG-CoA Reductase Activity: Incubations were carried out after the addition of substrate (see Tables 1 and 2) at 37°C in an atmosphere of nitrogen for one hour in a Dubnoff shaker. The reaction was stopped by the addition of 2 N HCl (1 ml) and then 4-^{3}H-MVA (0.055 μCi) was added as an internal reference. The reaction mixture was allowed to stand 20 minutes to assure

lactonization and equilibration of the mevalonate. The protein which had precipitated was removed by centrifugation and the supernatant was evaporated to dryness under a stream of dry nitrogen. The dry residue was extracted once with 2 ml of hot benzene containing 50 ul of 12 N HCl then twice with 2 ml of hot unacidified benzene. The combined benzene extracts were evaporated to dryness. The residue was dissolved in 30 ul of chloroform and applied to a 9.5 x 2 cm mylar backed silica gel strip which had been impregnated with ammonium carbonate. After development with 5:1 chloroform/methanol the strip was serially segmented into 0.5 cm sections. Each section was placed in a counting vial and 2 ml of 0.5 M acetic acid was added. Following the addition of 10 ml of Aquasol (New England Nuclear), each sample was assayed with a three channel liquid scintillation counter (Packard 3375).

Protein Assay: Protein was measured by the Biuret method in a manner similar to that described by Heller and Gould (16).

RESULTS AND DISCUSSION

Preparation of Soluble HMG-CoA Reductase: A liver microsomal suspension was prepared using the buffer (0.07 M NaCl, 0.03 M EDTA, 0.01 M 2-mercaptoethanol, pH 6.0) and centrifuge techniques described by Linn (13). This suspension was rapidly frozen, using liquid nitrogen, into a shell about the walls of a flask and then lyophilized for 17 to 18 hours under vacuum (50 microns Hg or less). The dry cake of microsomes obtained was powdered by very gentle grinding in a mortar and then stored over Drierite at -80°C. This process customarily resulted in the loss of no more than one-third of the enzymatic activity present in the fresh microsomal suspension. The lyophilized microsomes were suspended by homogenization in a buffer (pH 8) containing potassium phosphate (0.02 M), EDTA (0.03 M) and 2-mercaptoethanol (0.01 M); 60 ml of buffer was utilized per gram of powder. The mixture was allowed to stand at 4°C for 1 to 2 hours and then it was centrifuged (218,000 x g) for one hour. The supernatant obtained was centrifuged (218,000 x g) for an additional hour before the soluble reductase activity was assayed. Since the microsomes

TABLE 1

SOLUBILIZATION OF HMG-CoA REDUCTASE

Each incubation mixture contained 40 μmoles potassium phosphate, 60 μmoles EDTA and 20 μmoles 2-mercaptoethanol (excluding the salts present in the lyophilized microsomes) in a total volume of 2 ml. After the addition of 9 μmoles NADPH, each mixture was preincubated for 20 minutes and then 0.29 μmoles R,S-3-^{14}C-HMG-CoA (specific activity 0.10 μCi/μmole) was added; incubation was conducted for 1 hour. This level of substrate has been shown to be saturating.

Enzyme system	Protein (mg/ml)	Total MVA formed (nmoles)	Specific activity (nmoles MVA formed/ mg protein/hour)	Relative specific activity
Suspension of lyophilized microsomes	6.75	69.1	5.10[a]	1.00
Suspension of insoluble material remaining after extraction	5.50	37.5	3.41	0.67
Extract of lyophilized microsomes	0.68	17.5	13.00	2.55
Frozen extract of lyphilized microsomes	0.68	19.9	14.74	2.89

[a] The standard deviation of the assay procedure performed on duplicate samples is equal to ±0.07 nmoles per mg protein per hour or a standard error of 1.4%.

were prepared in an acidic buffer, the lyophilized microsomes contained these salts which were also extracted; consequently, the pH of the extract was 6.8. In addition to the evidence for solubility obtained by centrifugation, the extract has behaved as a true aqueous solution during purification by ion exchange and adsorption chromatography.

The results of a typical extraction performed on 300 mg of lyophilized microsomes are shown in Table 1. As may be seen, the reductase activity in the soluble fraction represents 25% of the activity present in the original lyophilized microsomal suspension and 32% of the total enzymatic activity recovered after extraction. The solubilization procedure also results in a 2.5-3 fold purification of the reductase activity. It is important to emphasize that these results represent a single buffer extraction of our least active micro-

TABLE 2

STABILITY OF SOLUBLE HMG-CoA REDUCTASE AT 4°C

Each incubation mixture contained 40 μmoles potassium phosphate, 60 μmoles EDTA, 20 μmoles 2-mercaptoethanol (excluding the salts extracted from the lyophilized microsomes present in the solutions which were not dialyzed) and 1.40 mg protein in a total volume of 2 ml. After the addition of .9 μmoles NADPH, each mixture was preincubated for 20 minutes and then 0.29 μmoles R,S-^{14}C-HMG-CoA (specific activity 0.10 μCi/μmole) was added; incubation was conducted for 1 hour.

Experiment number	Enzyme system	Time of exposure to 4°C (hours)	Total MVA formed (nmoles)	Specific activity (nmoles MVA formed/mg protein/hour	Relative specific activity
One	Extract	0	20.7	14.8	1.00
	Extract	16.5	14.3	10.2	0.69
	Dialyzed extract	16.5	20.1	14.4	0.97
	Dialyzed extract	40.5	14.5	10.4	0.70
Two	Dialyzed extract	16.8	21.8	15.6	1.00
	Dialyzed extract	40.2	19.8	14.1	0.90
	Dialyzed extract + KCl	0	0	0	0
	Dialyzed extract + KCl	23.4	0	0	0
	KCl removed from dialyzed extract	23.4	16.0[a]	11.4	0.73

[a]The sample was not completely homogeneous. This is the maximum MVA formed assuming all the ^{14}C-activity detected was present as MVA.

somal preparation. Frequently, 50% of the recoverable enzymatic activity is present in the soluble fraction. The reductase has been solubilized on 19 different occasions from four separate microsomal preparations. The extract is stable to freezing and retains enzymatic activity for months when stored at -80°C as do the lyophilized microsomes. Consequently, this procedure can consistantly provide substantial quantities of crude soluble HMG-CoA reductase

for subsequent purification without the use of organic solvents or detergents which might alter the properties of the enzyme.

One additional aspect of these results is of importance. The enzyme preparations have been preincubated with NADPH prior to the addition of substrate. For reasons which have not yet been determined, preincubation with this cofactor results in a two-fold increase in the amount of MVA formed over that obtained by the same extract without NADPH preincubation.

Test of Cold Lability in HMG-CoA Reductase Solution: An extract of lyophilized microsomes was prepared as previously described. One aliquot of this extract was assayed immediately; another was allowed to stand at 4°C for 16.5 hours before assay. The remaining extract was dialyzed at 4°C for 16.5 hours against buffer (pH 6.8) containing potassium phosphate (0.02 M), EDTA (0.03 M) and 2-mercaptoethanol (0.01 M) which removed the buffer salts extracted from the lyophilized microsomes. One aliquot of this dialyzed extract was assayed immediately; another was allowed to stand for 24 hours at 4°C before assay. In another study, a dialyzed extract was prepared as before; aliquots were assayed immediately and after standing 23.4 hours at 4°C. Solid KCl was added to the remaining extract to a final concentration of 4 M. After 15 minutes, one aliquot was assayed immediately; another was assayed after standing 23.4 hours at 4°C; and a third was assayed after the KCl was removed by dialysis at 4°C for 23.4 hours. The results of these experiments are shown in Table 2.

As may be seen, the extract exhibits no remarkable cold lability. These results also indicate that high salt concentrations tend to destabilize rather than stabilize soluble HMG-CoA reductase. These findings are in contrast to those reported by Brown, Dana, Dietschy and Siperstein (17). These workers have solubilized HMG-CoA reductase from rat liver microsomes by incubation with snake venom and by exposure to high salt (4 M KCl) or high glycerol (50% v/v) concentrations. The enzyme solubilized with venom was purified by ammonium sulfate precipitation followed by heating in a solution containing 4 M KCl at 63° for 10 minutes. The reductase remaining in solution exhibited

extreme cold lability except in the presence of 4 M KCl. When the salt concentration was reduced to 1 M, 67% of the enzymatic activity was lost within 10 minutes at 4°C.

Linn (13) and Kawachi and Rudney (14) reported no cold lability in their soluble HMG-CoA reductase preparations. Heller and Gould (16) observed no instability in their enzyme solutions except after ammonium sulfate precipitation. Thus reductase solubilized by four different methods has exhibited no significant cold lability. Consequently, the sensitivity to cold observed by Brown et al. appears to result from the solubilization and purification procedures used by these investigators, and it probably is not an inherent property of the enzyme. Alternatively, HMG-CoA reductase may exist in two forms, one of which is cold labile. However, Brown et al. reported the solubilization of 90% of the reductase activity present in their original microsomal preparation. Consequently, if the reductase has two forms, the procedure used by these investigators to prepare the microsomes must have fortuitously inactivated the cold stable form.

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