Improved methods for the assay and activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase

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Abstract A simple and rapid mixed-phase method for the quantitative assay of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and a procedure for the efficient reactivation of Mg-ATP-inactivated microsomal HMG-CoA reductase by potato acid phosphatase are described. The mixed-phase assay entails the direct addition of the acidified, deproteinized incubation mixture to a toluene-based scintillation fluor. The enzymatic reaction product [^{3}H]-mevalonolactone partitions into the toluene while unreacted ^{3}H-labeled HMG-CoA substrate remains in the aqueous phase and is not detected on scintillation counting. The accuracy and reproducibility of this method are compared to a thin-layer chromatographic assay for HMG-CoA reductase. Microsomal and solubilized HMG-CoA reductase inactivated by incubation with Mg-ATP is reactivated by purified potato acid phosphatase. Under appropriate conditions quantitative reactivation of HMG-CoA reductase is achieved, indicating that endogenous inhibitory and activating proteins regulate HMG-CoA reductase via a kinase–phosphatase system.—**Philipp, B. W., and D. J. Shapiro.** Improved methods for the assay and activation of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* 1979. **20:** 588–593.

Supplementary key words cholesterol biosynthesis · kinase · phosphatase

The hepatic synthesis of cholesterol is regulated primarily at the reductive conversion of HMG-CoA to mevalonic acid (1–3). This reaction is catalyzed by the microsomal enzyme HMG-CoA reductase.

Most assays for HMG-CoA reductase are based on the conversion of isotopically labeled HMG-CoA to mevalonic acid and the separation of the biosynthesized mevalonic acid (MVA) from the more polar HMG-CoA. While many approaches to this separation have been described (4–14), most assays have employed thin-layer chromatography to separate MVA and HMG-CoA. Extraction of MVA into an organic solvent is a prerequisite for most, but not all (5), of these assays. In this paper we describe a simple, rapid, one-step procedure for the separation of the [^{3}H]mevalonolactone synthesized by HMG-CoA reductase from the more polar HMG-CoA. Addition of an aliquot of the acidified and deproteinized reaction mixture to a toluene-based scintillation fluor results in partition of the [^{3}H]mevalonolactone into the low-polarity toluene phase in which it can be counted. The ^{3}H-labeled HMG-CoA and the HMG-CoA breakdown products formed during incubations are sufficiently polar so that they are quantitatively retained in the aqueous phase and are therefore not detected on scintillation counting.

We describe the application of this method to quantitation of the reactivation of Mg-ATP-inactivated reductase by potato acid phosphatase. Reactivation of HMG-CoA reductase by a well-characterized purified phosphatase suggests that liver proteins which activate and inactivate HMG-CoA reductase (5, 15–17) function through a kinase–phosphatase mechanism.

MATERIALS AND METHODS

Materials

DL-Hydroxymethyl-[^{3}H]glutaryl CoA was obtained from New England Nuclear Corp. We have observed that ^{3}H-labeled HMG-CoA is relatively unstable and it should therefore be stored in aliquots at −70°C. Repeated freezing and thawing results in breakdown of small amounts of HMG-CoA to a low-polarity compound which increases the background on both the TLC assay of Shapiro et al. (5) and the mixed-phase assay described here. Purification of the substrate by paper chromatography or cellulose TLC in *n*-butanol–acetic acid–water, 5:2:3 as described by the

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; TLC, thin-layer chromatography; EDTA, (ethylenedinitrilo)-tetraacetic acid; PIPES, piperazine-N-N′-bis(2-ethanesulfonic acid); DTT, dithiothreitol.

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manufacturer removes most of the breakdown product. ^{14}C-Labeled HMG-CoA was synthesized essentially as described by Goldfarb and Pitot (6). All other isotopes were from New England Nuclear. Biochemicals were obtained from Sigma Chemical Co. and Polygram Sil-G TLC sheets were from Machery and Nagel Co.

Preparation of microsomes

Female 100–200 g Sprague-Dawley rats were killed by cervical dislocation at or near the peak of the daily circadian rhythm in reductase activity. In most experiments rats were fed a diet containing 2% cholestyramine for 3 days prior to the preparation of microsomes. Livers were excised, rinsed in ice-cold KESD buffer containing potassium phosphate, 10 mM, pH 6.8; EDTA, 2 mM; sucrose, 250 mM; and DTT, 1 mM. The livers were then minced through a tissue press and homogenized in three volumes of the above buffer per g of liver by four strokes with the loose pestle of a Dounce homogenizer and one stroke with the tight pestle. Mitochondria and cell debris were sedimented by centrifugation two times at 12,000 *g* for 15 min at 4°C (18). Crude microsomes were prepared from the two 12,000 *g* supernatants by sedimentation at 105,000 *g* for 90 min at 4°C. The pellet was resuspended in the above buffer (1 ml/g liver) and resedimented at 105,000 *g* for 90 min at 4°C. The washed microsomal pellets were quick-frozen in an acetone–dry ice bath and stored at −60°C prior to use.

Solubilization of HMG-CoA reductase

Frozen microsomes were thawed slowly to room temperature and resuspended in buffer (1 ml/5g liver) containing Tris, 25 mM, pH 7.5; EDTA, 10 mM; and DTT, 10 mM (TED buffer). The microsomes were incubated for 30 min at 37°C, diluted with the above buffer to 1 ml/g liver and sedimented at 105,000 *g* for 90 min at 23°C. The 105,000 *g* supernatant contained 70–80% of the original reductase activity. The pellet was resuspended, incubated, diluted, and sedimented as described above. This procedure solubilized an additional 30–40% of the microsomal reductase activity. The two 105,000 *g* supernatants were combined, made 2.6 M in NaCl, and 50 mM in potassium phosphate, pH 7.5, and heated to 60°C for 10 min. Denatured proteins were removed by sedimentation at 105,000 *g* for 30 min at 23°C. The supernatant was concentrated in an Amicon ultrafiltration cell with a PM10 membrane to approximately 0.4 mg protein/ml, quick-frozen in an acetone–dry ice bath and stored at −70°C for up to several months with retention of 90–100% of the original activity.

Assay of HMG-CoA reductase

Incubations were performed in 400-μl Beckman microfuge tubes in a total volume of 100 μl essentially as described by Shapiro et al. (5). Microsomal or solubilized reductase in the appropriate buffer was preincubated for 20 min at 37°C. Cofactors mix was then added so that the final incubation contained: $5.2 \cdot 10^{-2}$ M glucose-6-phosphate, $5.3 \cdot 10^{-3}$ M NADP, 1 unit of glucose-6-phosphate dehydrogenase, $3.8 \cdot 10^{-2}$ M MVA lactone, and $5 \cdot 10^{-4}$ M DL-HMG-CoA (sp act, 2631 dpm/nmol or 3926 dpm/nmol for ^{3}H-labeled HMG-CoA and 1250 dpm/nmol for ^{14}C-labeled HMG-CoA). For the mixed-phase assay 3500 dpm of [^{14}C]MVA lactone was used as standard. In those TLC assays using ^{14}C-labeled HMG-CoA as substrate, 35,000 dpm of [^{3}H]MVA lactone was used as an internal standard. After incubation for 20 min at 37°C the reaction was terminated by addition of 25 μl of 1 N HCl. The samples were shaken vigorously and incubated for 15 min at 37°C to allow complete lactonization of the mevalonic acid and then were deproteinized by centrifugation for 2 min in a Beckman Microfuge B. TLC assays were carried out on 75-μl aliquots of each sample essentially as described by Shapiro et al. (5). For mixed-phase assay, 50 μl of the sample (or the indicated volume) was added to 5 ml of toluene scintillation fluor (toluene + 0.8% PPO). The mixture was shaken briefly but vigorously and counted immediately or after the indicated times for 10 min on each channel.

Inactivation and reactivation of HMG-CoA reductase

The microsomal pellets that had been quick-frozen and stored at −60°C were thawed, resuspended (0.5 ml/g liver) in the buffers indicated under each experiment, and inactivated essentially as described by Nordstrom, Rodwell, and Mitschelen (15). Inactivated microsomes were used immediately or stored at −60°C for up to 3 months without undergoing detectable changes.

Crude activator protein was isolated from rat liver as described by Nordstrom et al. (15) through the ammonium sulfate precipitation. In order to avoid inhibition of potato acid phosphatase by inorganic phosphate (19), reactivations by both potato acid phosphatase and the crude rat liver activator were carried out in PIPES, 50 mM, pH 6.0 or 6.5; NaCl, 100 mM; DTT, 10 mM; EDTA, 10 mM or in the TED buffer described above but at pH 6.5. Unless otherwise specified, reactivation was for 2 hr at 37°C for the reactivations with potato acid phosphatase and for 40 min at 37°C for reactivations with the endogenous activator protein.

TABLE 1. Effect of aqueous sample volume on the mixed-phase assay for HMG-CoA reductase

μl of Sample Added to Fluor	nm MVA/min/mg
25	16.2[a]
50	15.3
75	14.6
100	14.8
200	15.1

[a] Solubilized HMG-CoA reductase (0.39 mg/ml) was assayed as described under Methods; the indicated sample volumes were added to 5 ml of toluene scintillation fluor, shaken vigorously, and counted after 20 min. The data represent an average of three independent experiments and are presented as nmol MVA synthesized per min per mg in each assay.

Protein determination

Protein concentrations were determined by the method of Lowry et al. (20) using a bovine serum albumin standard. Samples were first dialyzed against NaCl solutions to prevent interference by thiols.

RESULTS

Optimization of conditions for the mixed-phase assay

In our initial studies we added a 50-μl aliquot to the toluene scintillation fluor. Since incubation volumes used for HMG-CoA reductase assays vary widely from laboratory to laboratory, we examined the effect of increasing sample volume on the efficiency of the mixed-phase assay. The data in **Table 1** indicate that at all sample volumes tested similar counting efficiencies were obtained using the mixed-phase assay. The mixed-phase assay can therefore be applied without modification to larger volume assays of low activity reductase preparations.

The partition of mevalonic acid lactone into the toluene phase is virtually instantaneous. Identical results are obtained when the sample is counted immediately or at all other times investigated including 11.5 hr after sample addition. Incubation of the sample in toluene at 37°C did not alter the counting efficiency. Since partition of the mevalonic acid lactone into the toluene phase is rapid and quantitative (data not shown), the internal standard of [^{14}C]mevalonic acid is added solely to quantitate the size of the aliquot added to the toluene. ^{14}C-Labeled compounds are counted (with low efficiency) when they are in the aqueous phase and the use of ^{14}C-labeled HMG-CoA as substrate should therefore be avoided as it increases the assay blank several-fold.

Comparison of TLC and mixed-phase assays

In order to demonstrate that the mixed-phase assay of HMG-CoA reductase is accurate and reproducible, we performed experiments comparing the mixed-phase and TLC assays for HMG-CoA reductase under a variety of conditions. Incubations of the soluble and microsomal reductase were linear for at least 30 min with both assays (**Fig. 1**). Mevalonate synthesis exhibited a linear increase with increasing concentrations of solubilized HMG-CoA reductase (Fig. 1). The results were identical when the mixed-phase and TLC assays were compared in parallel experiments.

Reactivation of HMG-CoA reductase by potato acid phosphatase

HMG-CoA reductase exhibits a protein-mediated inactivation in the presence of Mg-ATP and is reactivated by a second protein present in rat liver homogenates (15, 17). Nordstrom et al. (15) recently showed that microsomal HMG-CoA reductase derived from rats under a variety of physiologic conditions exhibited only about 25% of its maximum activity. They also observed a fluoride-sensitive partial reactivation of HMG-CoA reductase during many isolation procedures. In an effort to eliminate this potential source of variability in observed reductase activity and to obtain some insight into the mechanism of in vivo

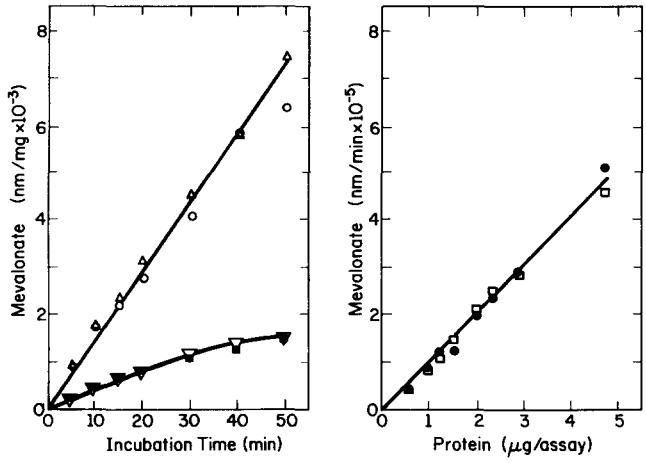

Fig. 1. Mevalonate synthesis as a function of incubation time and protein concentration. Microsomes, prepared from rats fed a diet containing 2% cholestyramine for 3 days and killed at the peak of the cyclic rhythm, were incubated in duplicate in PIPES buffer, pH 6.5, and assayed as described in Materials and Methods. In the *left panel* microsomal reductase (97.5 μg/assay) and solubilized reductase (10 μg/assay) were assayed by both the mixed-phase and TLC assays (microsomal reductase: mixed phase (▽) and TLC (■) assays; solubilized reductase: mixed phase (△) and TLC (○) assays). In the *right panel* increasing amounts of solubilized reductase in TED buffer were incubated in duplicate as described in Materials and Methods. The HMG-CoA reductase activity of the paired samples was determined by the mixed phase (□) and TLC (●) assays.

reactivation, we developed conditions for the reactivation of HMG-CoA reductase by commercially available potato acid phosphatase.

Maximal reactivation of reductase was achieved at 20–25 μg of phosphatase per 0.79 mg of microsomal protein (**Fig. 2**) and an incubation time of 2 hr.

Reactivation was dependent on the phosphatase activity of the enzyme as incubation in phosphate, which effectively inhibits the enzyme (19), quantitatively inhibits reactivation (**Table 2,** PEDS buffer). Reactivation was most effective at an acid pH close to the pH optimum of the phosphatase (19). The best combination of enzyme stability and efficient reactivation was obtained by incubation in PIPES buffer at pH 6.0. Significant inactivation of reductase was observed in imidazole, TED, and acetate buffer at acid pH.

The reactivation of Mg-ATP-inactivated HMG-CoA reductase by the endogenous rat liver cytosol activator and potato acid phosphatase was examined using both the mixed-phase and TLC assays for reductase activity. When low concentrations of phosphatase and activator protein are used reactivation is nonquantitative and additive (data not shown). Quantitative reactivation of HMG-CoA reductase is achieved by addition of higher concentrations of either protein (**Table 3**). Addition of saturating levels of both proteins does not increase reductase activity above that observed with either protein alone (Table 3). These data suggest that the endogenous rat liver activator protein and the potato acid phosphatase act via a common mechanism to reactivate HMG-CoA reductase.

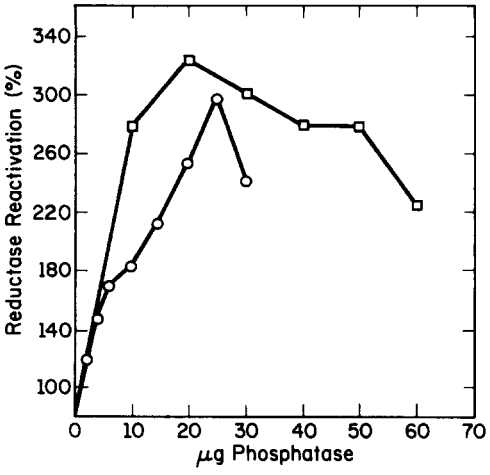

Fig. 2. Reactivation of microsomal reductase as a function of potato acid phosphatase concentration. Microsomes were prepared from rats killed at the peak of the cyclic rhythm (sp act 1.00 nmol/min per mg) and inactivated by incubation with Mg-ATP as described in Materials and Methods (sp act after inactivation was 0.208 nmol/min per mg.) Each incubation contained 0.79 mg of Mg-ATP-inactivated microsomal protein and the indicated amounts of potato acid phosphatase. Incubations were carried out in TED buffer at pH 6.5 for 1 hr (○) or 2 hr (□) at 37°C. At either 1 or 2 hr 50-μl and 75-μl aliquots were removed from each tube and assayed for reductase activity by the TLC method. Data represent the average of these duplicate incubations. In the absence of added phosphatase the activity of Mg-ATP-inactivated microsomes was essentially constant and no reactivation was observed. The specific activities of the samples incubated for 2 hr with phosphatase were: 10 μg, 0.791 nmol/min per mg; 20 μg, 0.886 nmol/min per mg; 30 μg, 0.848 nmol/min per mg; 40 μg, 0.762 nmol/min per mg; 50 μg, 0.727 nmol/min per mg; 60 μg, 0.677 nmol/min per mg. Thus the incubation containing 20 μg of added potato acid phosphatase was reactivated 325% (4.3-fold) relative to inactivated microsomes incubated without added phosphatase and reached 88% of the activity of the initial microsome preparation whose activity was constant over the time course of the experiment.

TABLE 2. Extent of reactivation of Mg-ATP-inactivated microsomes by acid phosphatase in various buffers

Buffer		Fold Reactivation[a]
PEDS[b]	pH 6.5	0.0[c]
TED	pH 6.5	3.2
	pH 7.0	1.1
Acetate	pH 5.0	2.4
	pH 5.5	4.9
	pH 6.0	3.3
Imidazole	pH 6.0	4.6
	pH 6.5	1.3
PIPES	pH 6.0	4.0[d]
	pH 6.5	1.5

[a] Fold reactivation represents the increase in HMG-CoA reductase activity over that in a control sample of inactivated microsomes incubated under identical conditions without added phosphatase.

[b] PEDS buffer contains potassium phosphate, 50 mM; EDTA, 1 mM; DTT, 10 mM; sucrose, 250 mM. Imidazole buffer contains imidazole, 50 mM; NaCl, 100 mM; EDTA, 10 mM; DTT, 10 mM. Acetate buffer contains acetate, 100 mM; EDTA, 10 mM; DTT, 10 mM.

[c] Each incubation was carried out in a total volume of 225 μl containing 0.96 μg of Mg-ATP-inactivated microsomal protein in the specified buffers and 25 μg of potato acid phosphatase. Incubation was for 2 hr at 37°C. Assays were performed by the TLC method using ^{14}C-labeled HMG-CoA as a substrate.

[d] Specific activities for the microsomes incubated in PIPES buffer at pH 6.0 were: active microsomes, 1.14 nmol/min per mg; inactivated microsomes, (immediately after inactivation), 0.268 nmol/min per mg; inactivated microsomes (incubated for 2 hr without added phosphatase), 0.276 nmol/min per mg; reactivated microsomes 1.17 nmol/min per mg.

DISCUSSION

Several lines of evidence indicate that it is the phosphatase activity of potato acid phosphatase that is responsible for the reactivation of Mg-ATP-inactivated microsomal HMG-CoA reductase. *a*) No reactivation is observed when Mg-ATP-inactivated microsomes are incubated in the absence of the phosphatase; *b*) the addition of the protease inhibitor phenylmethylsulfonyl fluoride to incubations has no effect on reactivation by the phosphatase; *c*) reactivation is maximal at acid pH and roughly parallels the pH profile for the phosphatase activity of potato acid

TABLE 3. Reactivation of Mg-ATP-inactivated microsomal reductase by activator protein and potato acid phosphatase

Sample	TLC			Mixed-Phase Assay		
	nmol MVA/min/mg	% of Active Microsomes	Fold Reactivation	nmol MVA/min/mg	% of Active Microsomes	Fold Reactivation
Active microsomes	4.79[a]			4.43		
Inactive microsomes	1.74[b]	36		1.85	43	
Reactivated by:						
32 μg Activator	5.76	120	3.3	5.33	117	2.7
25 μg Phosphatase	5.90	123	3.4	6.15	135	3.1
32 μg Activator plus 25 μg Phosphatase	5.51	115	3.2	6.05	133	3.1

[a] Microsomes were prepared from rats fed a 2% cholestyramine diet for 3 days and killed at the peak of the cyclic rhythm.

[b] 0.39 mg of Mg-ATP-inactivated microsomal reductase was incubated in a final volume of 200 μl in PIPES buffer, pH 6.0, with the indicated amounts of either activator protein, potato acid phosphatase, or both. Incubations with activator protein were performed at 37°C for 40 min and with phosphatase for 2 hr at 37°C. In the combined incubation the activator was added to the incubation tube 1 hr and 20 min after the phosphatase. At the end of incubation aliquots were removed and assayed in the usual manner by both the TLC and mixed-phase methods. Fold reactivation is defined as the increase in reductase activity over controls incubated under identical conditions without potato acid phosphatase or rat liver activator.

phosphatase (19); and *d*) addition of low concentrations of inorganic phosphate to incubations strongly inhibits the phosphatase activity of potato acid phosphatase (19) and quantitatively blocks reactivation of Mg-ATP-inactivated HMG-CoA reductase (Table 2).

The quantitative reactivation of Mg-ATP-inactivated microsomal reductase by purified potato acid phosphatase strongly suggests that in vivo modulation of HMG-CoA reductase occurs via phosphorylation and dephosphorylation. In an independent study reported after the completion of this work Ingebritsen et al. (17) described the reactivation of HMG-CoA reductase by rat liver phosphorylase phosphatase which has many properties in common with the endogenous rat liver activator protein. The endogenous rat liver activator protein therefore appears to exert its physiologic effect by functioning as a phosphatase. The mixed-phase assay for HMG-CoA reductase represents a simple, rapid, and reproducible assay for this important regulatory enzyme. The mixed-phase and TLC assays yield comparable blanks when zero-time or incubated control assays are performed with a given preparation of ^{3}H-labeled HMG-CoA substrate. The blanks in both the mixed-phase and TLC assays appear to be caused by unidentified low-polarity impurities in commercial preparations of ^{3}H-labeled HMG-CoA and not by HMG-CoA breakdown products formed during incubations. The impurities in ^{3}H-labeled HMG-CoA can be largely eliminated by purification using thin-layer chromatography (see Materials and Methods). The specifications on a typical lot of ^{3}H-labeled HMG-CoA indicated it was 99.6% pure. (The other lots of ^{3}H-labeled HMG-CoA we have used were 99.9% and 99.5% pure.) The assay blank in the mixed-phase assay for this substrate in both zero-time and incubated controls was 0.3% of input radioactivity. After purification of the substrate by TLC, the blank was reduced to approximately 0.1% of input radioactivity (35 cpm under our conditions). The sensitivity of the assay is illustrated by the fact that under typical assay conditions (20-min incubations with 100 μg of protein) the blank was equal to the amount of ^{3}H-labeled MVA produced by an enzyme preparation having a specific activity of only 0.03 nmol/min per mg. In all of the experiments we have performed, under a variety of conditions and enzyme activities, the mixed-phase and TLC assays gave identical results (Fig. 1; Table 3). The assay is exceedingly simple as it entails only the addition of the acidified, deproteinized sample to a toluene-based scintillation fluor; yet it results in a nonspecific background as low as the much more tedious TLC assay for HMG-CoA reductase.

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