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REVERSIBLE INACTIVATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE: REDUCTASE KINASE AND MEVALONATE KINASE ARE SEPARATE ENZYMES

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Reductase kinase and mevalonate kinase are separated by: a) ammonium sulfate fractionation; b) chromatography on agarose-Procion Red HE3B; and c) chromatography on DEAE-Sephacel. Fractions containing only reductase kinase reversibly inactivated microsomal or homogeneous HMG-CoA reductase. Fractions containing only mevalonate kinase revealed artifactual reductase kinase activity in the absence of EDTA or mevalonic acid; however, addition of EDTA or mevalonate before reductase assay completely blocked any apparent decline in HMG-CoA reductase activity. Under these conditions no dephosphorylation (reactivation) was observed by phosphatase. The combined results demonstrate unequivocally that reductase kinase and mevalonate kinase are two different enzymes and inactivation of HMG-CoA reductase is catalyzed by ATP-Mg-dependent reductase kinase.

INTRODUCTION

It has previously been reported that the enzymic activity of rat liver 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is modulated in vitro by reversible phosphorylation (1,2). The ATP-dependent inactivation (phosphorylation) of HMG-CoA reductase is catalyzed by reductase kinase, whereas reactivation (dephosphorylation) is mediated by reductase phosphatase. Reductase kinase activity has been shown to be associated with both microsomes and cytosol (1,2). Recently Ness et al (3-5) reported that the decline in HMG-CoA reductase activity in the presence of ATP-Mg was due to the conversion of mevalonate (the product of the HMG-CoA reductase reaction) to phosphomevalonate and proposed that mevalonate kinase and reductase kinase were the same enzyme. Based on their results Ness et al (3-5) also concluded that mevalonate kinase activity could account for the ATP-Mg-dependent inhibition of HMG-CoA reductase activity previously attributed

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to reductase kinase (1,2). In this report we present definitive evidence that mevalonate kinase and reductase kinase are two different enzymes. We also report a reproducible method for the assay of reductase kinase activity in preparations containing mevalonate kinase.

MATERIALS AND METHODS

HMG-CoA, agarose-hexane-HMG-CoA, ATP, and NADPH were purchased from P-L Biochemicals; [3-^{14}C]-HMG-CoA (54.4 mCi/nmol), DL-[2-^{14}C]mevalonic acid DBED salt (50.1 mCi/nmol) were obtained from New England Nuclear; DL-mevalonic acid lactone was from Sigma; DEAE Sephacel was procured from Pharmacia. Mevalonic acid was prepared by alkaline hydrolysis of mevalonolactone.

Purification of Microsomal HMG-CoA Reductase. Rats fed 3% cholestyramine (5 days) were killed at the mid-dark period. Microsomes were isolated and used for solubilization and purification of HMG-CoA reductase as previously reported (6).

Isolation of Reductase Kinase-free Microsomal HMG-CoA Reductase. Unwashed microsomes were prepared according to the procedure described earlier (6). The microsomal pellet was suspended in a buffer containing 50 mM potassium phosphate, pH 7.2, 250 mM KCl, 2 mM DTT, and 10% glycerol and incubated at 37°C for 30 min and stored frozen at -20°C. Microsomal HMG-CoA reductase was not inactivated by ATP-Mg when subjected to the above treatment. The preparation was completely devoid of reductase kinase and reductase kinase kinase. HMG-CoA reductase was stable for at least one year if stored frozen.

Isolation of Cytosolic Reductase Kinase. Livers were homogenized in 50 mM KH_2PO_4, pH 7.4, 250 mM NaCl, 1 mM EDTA and 5 mM DTT (ESDP buffer) containing 50 mM NaF and 100 um phenylmethylsulfonyl fluoride (PMSF) as described (2). The high speed (100,000 x g) supernatant was carefully removed and stored at -20°C. Phosphoprotein phosphatase was purified from the cytosolic fraction which was isolated in 50 mM imidazole, pH 7.4, 250 mM NaCl, 1 mM EDTA and 5 mM DTT (ESDI buffer) (7).

Assay of Reductase Kinase Activity. Purified HMG-CoA reductase or microsomes that retained HMG-CoA reductase activity but no reductase kinase activity were used to determine the activity of cytosolic reductase kinase. The first step in the assay involved preincubation of HMG-CoA reductase in duplicate with reductase kinase at 28°C (30 min) in a medium containing 50 mM ß-glycerophosphate (pH 7.0), 5 mM DTT, 50 mM NaF, 10 mM magnesium acetate, 4 mM ATP, and 20 mg/ml BSA in a total volume of 200 ul. Control tubes contained all the ingredients except ATP. At the end of the preincubation time, the reaction was stopped by the addition of EDTA (pH 7.0, 30-fold excess in relationship to Mg^{2+} concentration). The second step involved the assay of residual HMG-CoA reductase activity in a total volume of 400 ul at 37°C for 10-15 min. The reaction was started by the addition of 0.15 M KH_2PO_4 (pH 6.9) buffer containing 0.2 M KCl, 10 mM mevalonic acid, 0.75 mM NADPH and .0375 mM DL-[3-^{14}C]-HMG-CoA (5 uci/umole). The reaction was terminated by the addition of 50 ul of 10 N HCl. Tubes were centrifuged and 200 ul of aliquots were applied to 1 ml columns of Bio-Rex 5 resin. ^{14}C-mevalonolactone was eluted with 2 ml of water, 15 ml of aquasol was added and the resulting mixture was assayed for radioactivity. One unit of reductase kinase is defined as the amount which causes a decline of 1 unit of HMG-CoA reductase during a 30 min preincubation. One unit of HMG-CoA reductase catalyzes the formation of one pmol of mevalonate in 1 min under standard assay conditions.

Protein was determined by the method of Bradford (9).

Assay of Mevalonate Kinase Activity. Cytosol or fractions of cytosol containing mevalonate kinase activity were assayed as described by Ness et al (5).

TABLE I

Effect of EDTA and Mevalonic Acid on Preparations of Reductase Kinase Containing Mevalonate Kinase Activity

Enzyme Fractions	Additions		Reductase Kinase Activity	Mevalonate Kinase Activity
	EDTA	MVA	units/mg	units/mg
A. Cytosol	-	-	69	4.05
	+	-	29	N.D.
	-	+	23	N.D.
B. DEAE Sephacel (fraction #41)	-	-	66	N.D.
	+	-	58	N.D.
	-	+	60	N.D.
C. DEAE Sephacel (fraction #8)	-	-	63	7.03
	+	-	N.D.	N.D.
	-	+	N.D.	N.D.

Cytosol (672 ug), fraction 41 (Fig. 1, 250 ug) or fraction 8 (Fig. 1, 636 ug) and reductase kinase free microsomal HMG-CoA reductase (70 ug) were preincubated with or without ATP (30 min, 28°C), as described for the reductase kinase activity. Following the preincubation, buffer or a 30-fold excess of EDTA or 10 mM mevalonate was added. HMG-CoA reductase activity was assayed by the addition of NADPH and ^{14}C-HMG-CoA. Mevalonate kinase activity in different fractions were determined as described in the text.

N.D. = nondetectable

Reaction mixtures contained 50 mM 4-(2-hydroxy-ethyl)-1-piperazineethane sulfonic acid (pH 7.4), 2 mM ATP, 4 mM $MgCl_2$, 2 mM DTT, 0.67 mM [^{14}C]-RS-mevalonate (.121 uCi/umole), and varying aliquots of enzyme. The reaction was initiated by the addition of [^{14}C]-RS-mevalonate. Following incubation at 37° for 10-15 min, the reaction was terminated by the addition of 10 ul of 2.4 N HCl. The reaction mixture was maintained at 37°C for 30 min to ensure complete lactonization. The [^{14}C]-phosphomevalonate was separated on Pasteur pipette columns (1.5 ml) containing Bio-Rad AG1-X8 resin. The columns were washed sequentially with 6 ml of 0.01 N HCl, and 4 ml of 2.4 N HCl which eluted the phosphomevalonate. One unit of mevalonate kinase catalyzes the formation of one nmol of phosphomevalonate in 1 min.

Duplicate samples varied by less than 7% of the mean value for reductase kianse and mevalonate kinase determinations.

RESULTS AND DISCUSSION

Reductase kinase is a cytosolic enzyme which inactivates (phosphorylates) HMG-CoA reductase in the presence of ATP-Mg^{++}. The inactivation was completely reversed by phosphoprotein phosphatase (Tables I and II, 1-2). Cytosol or its fractions or extracts of unwashed microsomes used for reductase kinase will also contain mevalonate kinase. Incomplete complexation of ATP-Mg or removal of mevalonate kinase following inactivation of HMG-CoA reductase by ATP-dependent

TABLE II

Inactivation and Reactivation of Purified HMG-CoA Reductase

	Fractions	Addition		HMG-CoA Reductase Activity (units)	
		ATP (mM)	Mg^{++}	No Phosphatase	Added Phosphatase
A.	Cytosol	-	10	2.59	2.35
		4	10	0.51	2.39
B.	0-25% Ammonium Sulfate Fraction	-	10	3.49	2.60
		4	10	0.89	2.59
C.	DEAE Sephacel Column (fraction #41)	-	10	11.14	12.94
		4	10	6.77	12.77
D.	DEAE Sephacel Column (fraction #8)	-	10	7.54	8.06
		4	10	8.14	8.40

Inactivation: Cytosol (804 ug), the 0-25% pellet (1340 ug), fraction 41 (Fig. 1, 250 ug) or fraction 8 (Fig. 1, 636 ug) was incubated in the presence of purified reductase (.11 ug in A and B; .22 ug in C and D), with or without 4 mM ATP as described for the RK assay in the text. Inactivation was terminated by the addition of 30-fold excess of EDTA.

Reactivation: Quadruplicate 35 ul aliquots taken from each tube and diluted to 100 ul with ESDI buffer. 100 ul of flouride-inactivated or active phosphatase (68 ug) were added, and the mixture was incubated at 37°C for 30 min. Dephosphorylation was terminated by the addition of 20 ul of 1 M NaF containing all the ingredients for reductase assay (see Methods).

reductase kinase would cause mevalonate kinase to consume the [^{14}C]-mevalonate formed from [^{14}C]-HMG-CoA during the HMG-CoA reductase assay (Table I, 3-5). The loss of [^{14}C]-mevalonate can be completely blocked by the addition of either a 30-fold excess of EDTA (in relationship to Mg^{++}) or 10 mM mevalonate before starting the HMG-CoA reductase assay (Table I).

In the absence of EDTA or mevalonate, cytosol revealed artifactual reductase kinase activity due to the interaction of [^{14}C]-mevalonate with mevalonate kinase. Addition of excess EDTA or mevalonate was associated with authentic reductase kinase activity (Table I). Furthermore, cytosolic mevalonate kinase activity was detected only in the absence of EDTA or mevalonate (Table I). These results support the conclusion that reductase kinase and mevalonate kinase are two different enzymes. In order to definitively establish that reductase kinase and mevalonate kinase are separate enzymes, two kinases have been separated by three purification schemes. Cytosol or fractions of cytosol were fractionated by: a) chromatography on DEAE sephacel; b) ammonium sulfate; and c) chromatography on 5% agarose-Procion

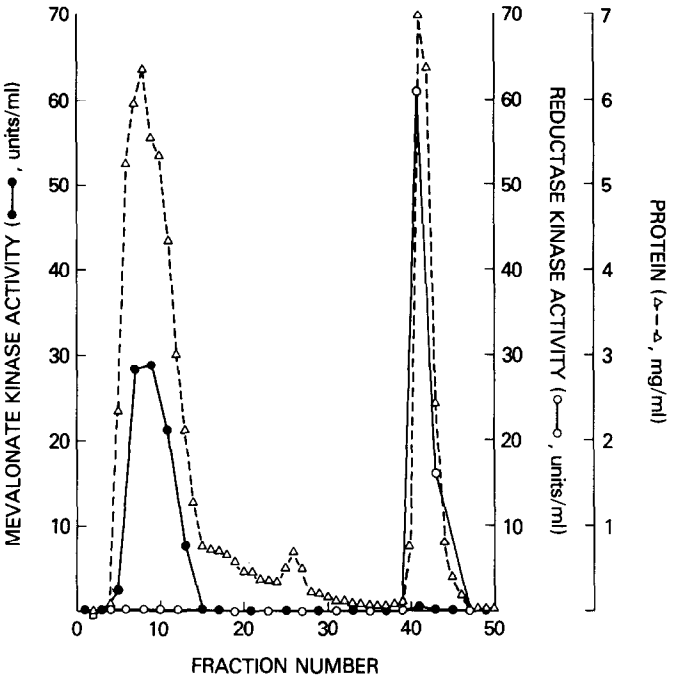

Fig. 1 Resolution of cytosolic MK and RK by chromatography on DEAE Sephacel. A 2.5 x 48 cm DEAE Sephacel column was equilibrated in 50 mM Tris, 50 mM KCl, 2 mM EDTA, pH 7.5, 2 mM DTT and .25 M sucrose (buffer A) at 4°C. Cytosol (48 ml; 1.15 g) diluted to 82 with water (5.2 mmho) was applied to the column. Fractions (17 ml) were collected by washing with buffer A. Beginning at fraction 27 the column was washed with buffer A containing 65 mM KCl. At fraction 34 column was eluted with buffer A containing .5 M KCl. Fractions were assayed for protein (Δ), reductase kinase (O), and mevalonate kinase (●) activities.

Red HE3B. It is evident from Fig. 1 that mevalonate kinase eluted in the void volume (fractions 8-10) whereas reductase kinase was bound to DEAE sephacel and eluted with 0.5 M KCl. The washed (x4) 0-25% ammonium sulfate fraction of cytosol was associated with reductase kinase activity and less than 1% of mevalonate kinase. Most of the mevalonate kinase activity remained in the post 25% supernatant (Table III). Reductase kinase at pH 6.5 did not bind to a 5% agarose-Procion Red HE3B column, whereas mevalonate kinase bound to the column and was eluted in ESDP buffer (Table IV). Less than 5% of cytosolic mevalonate kinase activity was present in the void volume.

Fraction 8 (Fig. 1) contained only mevalonate kinase activity (Table I). Artifactual reductase kinase activity due to phosphorylation of the [^{14}C]-mevalonate formed during HMG-CoA reductase assay was present in the absence of EDTA or mevalonate. Artifactual reductase kinase activity was completely abolished

TABLE III

Separation of Reductase Kinase and Mevalonate Kinase by Ammonium Sulfate Fractionation

Fractions	Reductase Kinase Activity		Mevalonate Kinase Activity	
	total units ($\times 10^{-3}$)	units/mg	total units	units/mg
A. Cytosol	160	66	7620	3.15
B. 0-25% precipitate (washed x4)	19	99	28	.149
C. Supernatant	43	23	4196	2.28

120 ml of cytosol was fractionated with ammonium sulfate (0-25%) and centrifuged (40,000 x g, 10 min). The precipitate was washed 4 times in 24 ml of buffer A saturated with 30% ammonium sulfate and dissolved in 14 ml of 50 mM Tris-HCl, 1 mM EDTA (pH 7.5), 5 mM DTT, .25 M sucrose and 50 mM NaF. Cytosol (804 ug), 0-25% precipitate (1340 ug) and supernatant (1460 ug) were assayed for reductase kinase and mevalonate kinase activities as described under Methods. Reductase activity (units/mg) in control samples used in RK assay was 147, 129, 75 for A, B, and C respectively.

by the inclusion of excess EDTA or mevalonate in the incubation prior to HMG-CoA reductase assay (Table I). Fraction 41 (Fig. 1) contained authentic reductase kinase activity with no mevalonate kinase activity either in the

TABLE IV

Resolution of Reductase Kinase and Mevalonate Kinase by Agarose Procion Red HE3B Column Chromatography

Fractions	Reductase Kinase Activity		Mevalonate Kinase Activity	
	total units	units/mg	total units	units/mg
A. 0-45% $(NH_4)SO_4$ precipitate	3020	20.8	71	.49
B. Void Volume	1302	27.4	3.78	.079
C. MES Wash	N.D.	N.D.	.27	.064
D. ESDP Wash	N.D.	N.D.	43.4	1.63

Agarose-Procion Red HE3B column (2.5 x 4.3 cm) was equilibrated in 10 mM [2-(N-Morpholino) Ethane Sulfonic Acid], (MES buffer, pH 6.5), 2 mM DTT, 20% glycerol. 10 ml of desalted 0-45% ammonium sulfate fraction of cytosol (145 mg protein) was applied to the column, 14 ml collected as void. Column was washed with 120 ml of MES buffer and then with ESDP buffer. MES and ESDP washes were fractionated with 0-65% ammonium sulfate and the precipitate was dissolved in 14 ml of MES buffer. 0-45% precipitate (725 ug), void volume (340 ug), MES wash (30 ug) and ESDP wash (191 ug) were used for reductase kinase and mevalonate kinase activities determinations. Initial reductase specific activity (units/mg) were used in reductase kinase assay as control was 99 and 59 for A and B fractions respectively.

N.D. = nondetectable

presence or absence of EDTA or mevalonate (Table I). The prerequisite for the expression of mevalonate kinase activity during HMG-CoA reductase assay is the availability of ATP-Mg in the medium. Addition of excess mevalonic acid (10 mM) before HMG-CoA reductase assay consumed all the mevalonate kinase (A and C, Table I).

Cytsol containing reductase kinase activty was able to inactivate (phosphorylate) purified HMG-CoA reductase, provided excess EDTA or mevalonic acid was added in the incubation. This inactivation was completely reversed by phosphatase (Table II). The 0-25% ammonium sulfate pellet (4 x washed, Table III) or fraction 41 (Fig. 1) containing no mevalonate kinase were able to inactivate HMG-CoA reductase equally either in the presence or absence of EDTA and mevalonate (Tables I and II). Incubation of inactivated enzyme with phosphatase was associated with complete restoration of enzymic activity (Table II). Fraction 8 containing only mevalonate kinase activity failed to inactivate (phosphorylate) purified HMG-CoA reductase, therefore no dephosphorylation (reactivation) was observed following incubation with phosphatase (Table II). Unlike the findings of Ness et al (3-5), the inactivation of HMG-CoA reductase was time, ATP, and reductase kinase concentration dependent (data not shown). In addition, the inhibition was not reversed by dilution (Table II), dialysis or by addition of EDTA (Table I-IV).

The combined results demonstrate unequivocally that mevalonate kinase and reductase kinase are two separate enzymes and furthermore that reversible inactivation (phosphorylation) of HMG-CoA reductase is catalyzed by reductase kinase. The artifactual effect of mevalonate kinase can and was totally eliminated either by using preparations free from mevalonate kinase or by the inclusion of excess EDTA or mevalonate before the assay of HMG-CoA reductase. Since reductase kinase is highly labile[1] (Table III and IV), it is possible that the preparation and amounts used by Ness et al (3-5) had little or no reductase kinase activity. In the absence of active reductase kinase no reversible phosphorylation of HMG-CoA reductase could possibly be demonstrated (3-5).

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[1] Beg, Z. H., and Stonik, J. A. (unpublished observation)

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