HMG-CoA reductase kinase: measurement of activity by methods that preclude interference by inhibitors of HMG-CoA reductase activity or by mevalonate kinase

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Abstract Assay of HMG-CoA reductase kinase activity requires HMG-CoA reductase (reductase, E.C. 1.1.1.34) free of associated reductase kinase. Microsomal reductase insensitive to inactivation by Mg-nucleotides alone may be prepared by heating microsomes at 50°C for 15 min. The reductase in these microsomes may subsequently be inactivated by Mg-nucleotides only after addition of reductase kinase. Inactivation is a linear function of time and of cytosol protein concentration and may be reversed by treatment with a phosphoprotein phosphatase. The extent of inactivation observed under standard conditions provides an assay for reductase kinase activity. Factors present in cytosol that hinder measurement of either reductase or reductase kinase activity must be removed or inhibited. Reductase phosphatase is inhibited by 50 mM NaF. Reductase kinase kinase activity is not expressed under the assay conditions used. Mg-Nucleotide-independent inhibitors of reductase activity are removed by chromatography on DEAE-Sephacel or Blue Sepharose. Mevalonate kinase and reductase kinase are separable by chromatography on DEAE-Sephacel or Sephadex G-200. We describe a rapid chromatographic procedure for separating reductase kinase of crude fractions from mevalonate kinase and from Mg-nucleotide-independent inhibitors of reductase activity. The 1.0 M KCl eluate from DEAE-Sephacel contains all of the cytosol reductase kinase activity. This method is applicable to measurement of reductase kinase activity in cytosol or more purified fractions.—**Harwood, H. J. Jr., and V. W. Rodwell.** HMG-CoA reductase kinase: measurement of activity by methods that preclude interference by inhibitors of HMG-CoA reductase activity or by mevalonate kinase. *J. Lipid Res.* 1982. **23:** 754–761.

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The activity of HMG-CoA reductase (reductase, E.C. 1.1.1.34), the catalyst for the first committed step in polyisoprenoid biogenesis (1), is decreased by phosphorylation (2–6) and is modulated both in vitro (2, 3, 5–7) and in vivo (4) by covalent modification by MgATP. Phosphorylation lowers and dephosphorylation elevates reductase activity (6, 8). The converter proteins that catalyze the interconversion of reductase between phospho and dephospho forms are designated reductase kinase and reductase phosphatase.

Assay of reductase kinase activity by measuring the rate of incorporation of ^{32}P from γ-[^{32}P]ATP into reductase protein, feasible for selected experiments, is ill-suited to routine analyses. The counts incorporated under conditions suitable for routine work are too low for precise quantitation. Furthermore, the reductase used as substrate must be available in quantity and free of other phosphorylatable proteins, conditions difficult to realize in most laboratories. An alternate approach is to exploit the decrease in reductase activity that accompanies its phosphorylation to monitor reductase kinase activity (3, 7, 9–12). A prerequisite for this approach is kinase-free active reductase (non-phosphorylated reductase uncontaminated with reductase kinase, an enzyme present both in cytosol and microsomes (7, 9, 10, 13)).

Preparation of kinase-free reductase has previously involved repetitive washing of microsomes (7, 10, 12, 13), incubation of microsomes at 37°C (12–14), or solubilization of microsomal proteins with subsequent resolution of reductase kinase from reductase (2, 4, 5, 13, 15). These procedures exhibit major drawbacks. Repetitive washing or incubation of microsomes at 37°C extracts or inactivates variable quantities of reductase kinase (13), while solubilizing reductase is time-consuming.

Assay of reductase kinase activity in cytosol is further complicated by the presence of both Mg-nucleotide-independent inhibitors of reductase activity and of mevalonate kinase (E.C. 2.7.1.36). Mevalonate kinase phosphorylates mevalonate and produces results that mimic reductase kinase activity by decreasing the apparent amount of product formed. We report a reproducible method for preparing microsomal reductase free of reductase kinase, methods for removal of interfering substances from reductase kinase, and methods for precise measurement of reductase kinase activity in crude cytosol. We also present methods for assuring that mevalonate

Abbreviation: HMG, 3-hydroxy-3-methylglutaryl.

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is not converted to phosphomevalonate during assay of fractions that may contain mevalonate kinase.

MATERIALS

Chemicals

Chemicals were from the following sources: phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO); Blue Sepharose, DEAE-Sephacel, and Sephadex G-200 (Pharmacia Fine Chemicals, Upsala, Sweden). All other chemicals were from previously listed sources (16–18). [3-^{14}C]HMG-CoA was snythesized and purified by the method of Williamson and Rodwell (18). Silica gel (EK 13179) and cellulose (EK 13254) TLC sheets were from Eastman Kodak, Rochester, NY. Tenfold purified low molecular weight rat liver phosphoprotein phosphatase (16) was a gift of Dr. Willis Brown.

Buffered solutions

TEDK contains 40 mM Tris (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl. TEDSF contains 50 mM Tris (pH 7.7) 1.0 mM EDTA, 5.0 mM dithiothreitol, 250 mM sucrose, and 50 mM NaF. PEDK contains 40 mM K_xPO_4 (pH 7.5), 1.0 mM EDTA, 5.0 mM dithiothreitol, and 70 mM KCl.

Animals

Female Wistar strain rats (150–200 g) from our department colony were fed commercial lab chow and water ad libitum and housed in a windowless room darkened from 1500–0300 hr and illuminated from 0300–1500 hr. Rats were killed by a blow at the base of the skull.

Microsomal reductase

Rats were killed at mid-dark period. All subsequent operations described below are at 0–4°C. Mince the livers from six rats (approx. 60 g) in a Harvard press into TEDK (1.0 ml per g of liver) that contains 1 mM phenylmethylsulfonyl fluoride. Homogenize with 15 strokes of a Dounce homogenizer. Centrifuge the homogenate (10 min, 27,000 *g*) and discard the precipitate. Decant and centrifuge the supernatant liquid (90 min, 105,000 *g*). Discard the high-speed supernatant liquid (cytosol). Suspend the precipitate in TEDK (0.38 ml per g of wet liver) by gentle homogenization with a motor-driven Potter-Elvehjem pestle. Divide the suspension into 2.0-ml portions and freeze under liquid N_2 for future use. Reductase in this preparation averages 90% fully active as judged by assay of activity before and after full activation by treatment with phosphoprotein phosphatase. If frozen in liquid N_2, reductase activity is stable for at least 4 months.

Heated microsomal reductase

The preparation derives from the observation of George, Menon, and Ramasarma (19) that the reductase of microsomes heated briefly at 50°C can no longer be inactivated by Mg-nucleotides alone. Just prior to use, microsomal reductase is thawed, heated to and maintained at 50°C for 15 min, then cooled rapidly to 4°C.

Liver cytosol

Liver cytosol is prepared as described for microsomal reductase with the following exceptions: TEDSF containing 1 mM phenylmethylsulfonyl fluoride replaces TEDK containing 1 mM phenylmethylsulfonyl fluoride, and the 105,000 *g* supernatant liquid (cytosol) rather than the microsomal pellet is retained. Fluoride is present to prevent phosphatase-catalyzed inactivation of reductase kinase (9).

Solutions for assay of reductase kinase activity

Stage 1 control solution. Just prior to use, mix 100 μl of 2.3 M NaF, 100 μl of 360 mM $MgCl_2$, and 400 μl of TEDSF.

Stage 1 nucleotide solution. Just prior to use, mix 100 μl of 2.3 M NaF, 100 μl of 360 mM $MgCl_2$, 300 μl of TEDSF, and 100 μl of a solution containing 90 mM ATP and 90 mM ADP (pH 7.0).

Stage 2 solution (for 20 assays). To a lyophilized powder of 13 mg $NADP^+$ and 4.0 mg of glucose-6-phosphate (pH 7.0), add 130 μl of 1.0 M EDTA in TEDSF, 100 μl of 5.0 mM [^{14}C]HMG-CoA (sp act 2.0 cpm/pmol), 20 μl (180,000 cpm) of R,S-[5-^{3}H]mevalonate (sp act 629 Ci/mol), and 20 μl (4 units) of glucose-6-phosphate dehydrogenase.

METHODS

Protein

Protein was measured by the method of Bradford (20) using bovine serum albumin as standard.

Liquid scintillation techniques

Portions of TLC sheets to be counted were cut in small pieces, added to 4.0 ml of Beckman Redi-Solve scintillation fluor and counted in a Beckman CPM 100 scintillation spectrometer.

Mevalonate kinase activity

Conversion of 5-phosphomevalonate to 5-pyrophosphomevalonate by phosphomevalonate kinase (E.C. 2.7.4.2) can lower the apparent activity of mevalonate kinase (both enzymes are present in cytosol). To quantify mevalonate kinase activity, we therefore determined the

TABLE 1. Inactivation of the reductase activity of heated microsomes requires both Mg-nucleotides and cytosol[a]

Microsomes	Additions	Reductase Activity: Less Mg-Nucleotides	Reductase Activity: Plus Mg-Nucleotides	Difference
			pU	
Unheated	None	120 ± 5	50 ± 2	70
Heated	None	115 ± 20	118 ± 15	0
Heated	Cytosol	100 ± 5	22 ± 3	78

[a] Heated or unheated microsomes plus cytosol where indicated were incubated at 37°C for 30 min in the presence or absence of Mg-nucleotides (8.0 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM ADP). Reductase activity was then determined in a second 30-min incubation at 37°C. Data are for duplicate assays ± the difference from the mean.

incorporation of isotope from R,S[5-^{3}H]mevalonate into total phosphomevalonates.

Assay mixtures contain, in a final volume of 75 μl, 560 nmol ATP, 375 nmol $MgCl_2$, 330 nmol R,S[5-^{3}H]mevalonate (sp act 3.2 cpm/pmol), 70 nmol EDTA, 350 nmol dithiothreitol, 18.8 μmol sucrose, 3.5 μmol NaF, 3.5 μmol of Tris (pH 7.7), and up to 1.5 nU of mevalonate kinase. Control incubations lack added ATP. Following incubation at 37°C for 30 min, immerse tubes in boiling water for 5 min, then centrifuge (2 min; Beckman Mirofuge) to sediment denatured protein. Apply 10-μl portions of the supernatant liquids to cellulose TLC sheets ruled into sixteen 1.5 × 20 cm channels and develop in n-butanol–formic acid–H_2O::77:10:13 (v/v/v) (21–24) to separate mevalonate from its phosphorylated derivatives. R_f values are: mevalonate 0.97, 5-phosphomevalonate 0.33, 5-pyrophosphomevalonate 0.13. Cut the region R_f 0–0.5 from the sheet and count in 4.0 ml of Beckman Redi-Solv fluor. Data are corrected for isotope present in the region R_f 0–0.5 due to "tailing" of mevalonate. One nU of mevalonate kinase is that which catalyzes formation of 1 nmol of phosphomevalonates in 1 min at 37°C.

RESULTS

Preparation of microsomal reductase free of reductase kinase

Unwashed microsomes contain both reductase and associated reductase kinase. As noted by George et al. (19), the reductase of microsomes heated at 50°C for 15 min (heated microsomes) is no longer inactivated by Mg-nucleotides alone. Reductase in heated microsomes is, however, inactivated by addition of Mg-nucleotides plus cytosol (**Table 1**). This observation forms the basis for assay of reductase kinase activity. This heat treatment has no adverse effect on reductase activity as reductase is stable to 65°C for 10 min, a major step in its purification (5, 16).

Assay of reductase kinase activity

The procedure is conducted in two stages. In Stage 1, microsomal reductase, reductase kinase, and Mg-nucleotides are combined and reductase kinase catalyzes inactivation of reductase. In Stage 2, [^{14}C]HMG-CoA, [^{3}H]mevalonate, and EDTA are added and the reductase activity remaining is assayed. A control incubation (receives Stage 1 control solution) is included for each experimental incubation (receives Stage 1 nucleotide solution). Reductase kinase activity, calculated from the difference between control and experimental assays, is expressed as the pU decrease in reductase activity. One pU of reductase is that which catalyzes formation of 1 pmol of mevalonate in 1 min at 37°C. One pU of reductase kinase is thus defined here as that which produces a decrease in reductase activity of 1 pU during the 30-min incubation of Stage 1.

Stage 1. Heated microsomes, 10–20 μl (about 150 pU of reductase activity), and 10–40 μl (0–100 pU) of reductase kinase are mixed with 10 μl of a) control solution, or b) nucleotide solution. Both mixtures are diluted to 75 μl with TEDSF and incubated at 30°C for 30 min. Final reactant concentrations in both control and experimental incubations are 8.0 mM $MgCl_2$, 1.0 mM EDTA, 50 mM Tris (pH 7.7), 85 mM NaF, 5.0 mM dithiothreitol, 180 mM sucrose, and 23 mM KCl. Experimental incubations contain, in addition, 2.0 mM ATP and 2.0 mM ADP.

Stage 2. Immediately after the conclusion of Stage 1, 13 μl of Stage 2 solution is added to all tubes. The tubes are transferred to a second water bath and incubated at 37°C for 30 min to determine the reductase activity remaining (25). Final reactant concentrations in both control and experimental incubations are 6.8 mM $MgCl_2$, 76 mM EDTA, 46 mM Tris (pH 7.7), 76 mM NaF, 4.6 mM dithiothreitol, 176 mM sucrose, 20 mM KCl, 0.32 mM [3-^{14}C]HMG-CoA (sp act 2.0 cpm/pmol), 2 mU/ml glucose-6-phosphate dehydrogenase, and 150,000 cpm/ml [5-^{3}H]mevalonate (sp act 630 mCi/nmol). Experimental incubations contain, in addition, 1.7 mM ATP and 1.7 mM ADP.

Assay parameters

Kinase-dependent inactivation of reductase is a function of time and cytosol protein concentration (**Fig. 1**), requires Mg-nucleotides (Fig. 1, right), and is phosphatase-reversible (**Table 2**). Time courses were linear (Fig. 1) in the presence or absence of 250 mM KCl during Stage 1. Reductase kinase inactivated 66% by pretreatment with phosphoprotein phosphatase gave similar results with maximal reductase kinase activity reaching

only 30% that of untreated fractions. Reductase kinase kinase is thus unable to act during Stage 1. Since reductase kinase phosphatase is inhibited by 50 mM NaF (8), the reductase kinase activity measured by the assay represents the fraction of reductase kinase present in the active form.

Separation of reductase kinase from factors that hinder measurement of its activity

Cytosol contains factors that hinder measurement of reductase kinase activity. Phosphoprotein phosphatase activity, which both inactivates reductase kinase (9) and reactivates reductase that has been inactivated by reductase kinase (26), is inhibited by 50 mM NaF (8). Mg-Nucleotide-independent inhibitors of reductase activity also are present in cytosol (26–29). Assay of small amounts of cytosol minimizes, but does not eliminate, the effects of the above factors. Furthermore, dilution decreases the sensitivity of the reductase kinase assay. Mg-Nucleotide-independent inhibitors of reductase activity may be removed by ion-exchange chromatography on DEAE-Sephacel (**Fig. 2**, left) or by dye ligand chromatography on Blue Sepharose (Fig. 2, right). Recovery of reductase kinase activity by these procedures is reproducible and exceeds by about 15% that measured by the dilution technique.

Resolution of reductase kinase and mevalonate kinase by ion exchange chromatography

Recent reports from one laboratory (30, 31) suggest that loss of reductase activity in the presence of MgATP is due solely to conversion of mevalonate to phosphomevalonate and that reductase kinase and mevalonate kinase

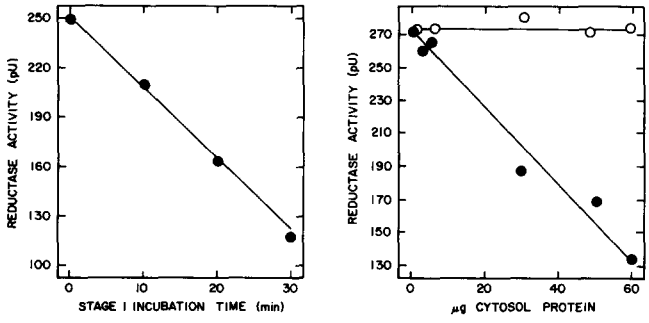

Fig. 1. Inactivation of microsomal reductase as a function of incubation time and of protein concentration. Left: Heated microsomes were mixed with 60 μg of cytosol protein and Mg-nucleotides (8.0 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM ADP) and incubated at 30°C for the indicated times (Stage 1). Residual reductase activity was then determined in a second 30-min incubation at 37°C (Stage 2). Right: Heated microsomes were mixed with the indicated quantities of cytosol protein in the presence (●) or absence (○) of Mg-nucleotides (8.0 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM ADP) and incubated at 30°C for 30 min (Stage 1). Reductase activity was then determined in a second 30-min incubation at 37°C (Stage 2).

TABLE 2. Phosphoprotein phosphatase restores reductase activity to heated microsomes treated with cytosol plus Mg-nucleotides[a]

Additions	Reductase Activity	
	Direct	After Phosphatase
	pU	
None	174	179
2.0 mM ATP + 2.0 mM ADP	41	184

[a] Blue Sepharose Fraction (see Fig. 2), 9.0 mg protein, was applied to a 6.0 ml (3.5 × 1.4 cm) column of DEAE-Sephacel in TEDSF, washed in with 22 ml of TEDSF, and eluted with a 50-ml gradient of 0–1.0 M KCl in TEDSF. A 30-μl portion of the 0.15–0.2 M KCl eluate (9.0 μg protein) was mixed with 30 μl of heated microsomes, 360 nmol of $MgCl_2$, and the indicated additions (final volume 90 μl) and incubated at 30°C for 30 min. A 20-μl portion was then mixed with 30 μl of PEDK ± 80 μg of phosphoprotein phosphatase and incubated at 37°C for 30 min. The phosphate in PEDK precipitates Mg^{2+} as magnesium fluorophosphate, terminates the kinase reaction, and removes NaF (which inhibits phosphatase activity). Stage 2 solution was then added and reductase activity was measured in a third 30-min incubation at 37°C.

are one and the same enzyme. Mevalonate kinase can produce false positives for reductase kinase activity by catalyzing phosphorylation of the [^{14}C]mevalonate generated by reductase. This may be accompanied by depletion of up to 50% (100% of the S-isomer) of the added R,S-[^{3}H]mevalonate internal standard. However, mevalonate kinase and reductase kinase are separable by ion exchange chromatography. Mevalonate kinase fails to bind to DEAE-Sephacel at pH 7.7, while reductase kinase binds and is eluted with 1.0 M KCl (**Fig. 3**).

Resolution of reductase kinase and mevalonate kinase by molecular exclusion chromatography

Upon chromatography on Sephadex G-200, reductase kinase (molecular weight 380,000 daltons (3)) elutes in the void volume while mevalonate kinase (molecular weight 98,000 (32) to 90,000 daltons[2]) elutes with an apparent molecular weight of about 90,000 daltons (**Fig. 4**).

Blocking mevalonate kinase activity in crude extracts

Reductase kinase may be separated from mevalonate kinase by ion exchange or molecular exclusion chromatography. However, the ability to block mevalonate kinase during Stage 2 of the assay is essential when preparations also contain mevalonate kinase. To obtain a preparation that contains both kinases but is free of inhibitors of reductase activity, we chromatographed cytosol on Blue Sepharose. Both kinases bind and are eluted with 1.0 M KCl (**Fig. 5**).

[2] Ness, G. Personal communication.

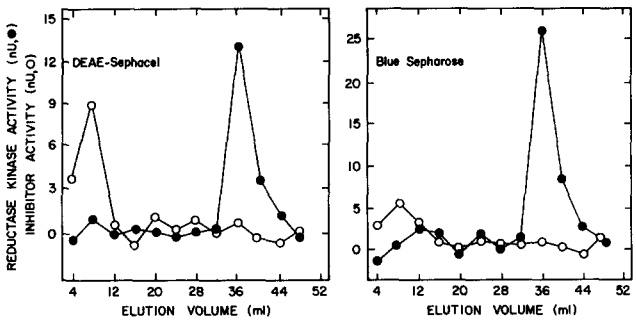

Fig. 2. Separation of reductase kinase from inhibitors of reductase activity by chromatography on DEAE-Sephacel or Blue Sepharose. DEAE-Sephacel and Blue Sepharose columns, 1.1 × 9.2 cm (7.0 ml), were equilibrated with TEDSF. In both instances the sample applied was 1.0 ml of cytosol (22.5 mg protein). Both columns were eluted first with 28 ml of TEDSF, and then with 25 ml of 1.0 M KCl in TEDSF. Fractions, 4.0 ml, were collected and assayed for reductase kinase activity (●) and for inhibition of reductase activity in the absence of added Mg-nucleotides (○). One pU of inhibitor activity is that which will inhibit 1 pU of reductase activity. Left: DEAE-Sephacel. Right: Blue Sepharose. Fractions 9 and 10 (32–36 and 36–40 ml of eluate, respectively) from each column, which contain all of the eluted protein and reductase activity, were combined to give the DEAE fraction and the Blue Sepharose fraction, respectively.

The loss of mevalonate that occurs during Stage 2 of the assay (96% loss of the S-isomer) is blocked by inclusion of 78 mM EDTA (3% loss of S-isomer) or by 78 mM EDTA plus a 114 mM mevalonate trapping pool (1.5% loss of S-isomer), and is almost completely blocked by inclusion of mevalonate alone (7% loss of S-isomer). While this 78 mM EDTA inhibits mevalonate kinase activity (**Fig. 6**), it has little or no effect on reductase kinase activity since reductase kinase acts during Stage 1. Mevalonate kinase therefore cannot act during either stage of the reductase kinase assay. Neither mevalonate nor substrates for its formation are present during Stage 1, and EDTA blocks 99.6% of the mevalonate kinase activity during Stage 2 (**Table 3**).

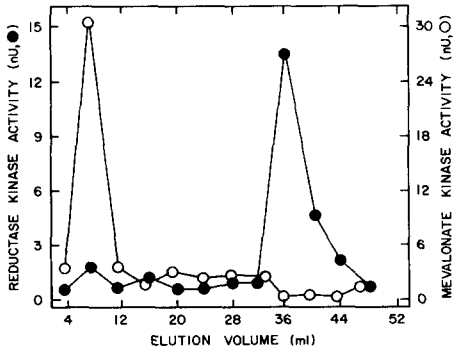

Fig. 3. Resolution of mevalonate kinase and reductase kinase by ion exchange chromatography on DEAE-Sephacel. Cytosol, 1.0 ml (22.5 mg protein) was added to a 1.1 × 9.2 cm (7.0 ml) DEAE-Sephacel column and eluted as described in Fig. 2. Symbols are: (●), reductase kinase activity and (○), mevalonate kinase activity.

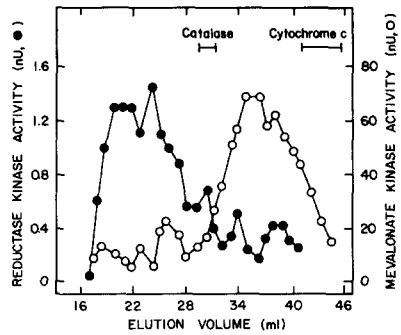

Fig. 4. Partial resolution of mevalonate kinase and reductase kinase by molecular exclusion chromatography on Sephadex G-200. Solid $(NH_4)_2SO_4$, 8.7 g, was added to 36 ml of cytosol (40% saturation). The mixture was stirred at 4°C for 10 min after all solid had dissolved, then centrifuged (10 min, 27,000 *g*). The supernatant liquid was discarded. The precipitate was suspended in TEDSF (final volume of 5.2 ml) to give the A.S. fraction. A.S. fraction, 3.3 ml, was applied to a 1.9 × 45 cm (78 ml) column of Sephadex G-200 in TEDSF. The column was eluted with TEDSF (1.0-ml fractions). Protein first appeared in fraction 18. Fractions were assayed for mevalonate kinase (○), reductase kinase (●), catalase, and cytochrome c. Catalase was detected by evolution of O_2 from H_2O_2 and cytochrome c was measured by absorbance at 410 nm. The apparent molecular weight of mevalonate kinase, estimated by reference to the mobilities of catalase and cytochrome c, is approximately 90,000 daltons.

Even in the absence of 78 mM EDTA, the conditions under which mevalonate kinase must act are highly unfavorable. Maximum mevalonate kinase activity occurs at a ratio of ATP to $MgCl_2$ of 1.5–2.0 (24). This ratio is 0.25 during Stage 2. In addition, 4.0 mM free Mg^{2+} is present, a concentration inhibitory to mevalonate kinase (24). Under these conditions (absence of added EDTA), less than 7% of the mevalonate kinase activity is expressed (Table 3).

MgADP is a cosubstrate for reductase kinase, but not for mevalonate kinase

MgADP is a cosubstrate for reductase kinase, but not for mevalonate kinase. Substitution of MgADP for Mg-nucleotides permits expression of 46% of the reductase kinase activity measured using Mg-nucleotides, or MgATP alone while simultaneously preventing expression of over 97% of the mevalonate kinase activity measured using MgATP—even in the absence of added EDTA (**Table 4**). However, since reductase kinase activity is approximately 50% of that measured using Mg-nucleotides, we do not recommend use of MgADP alone for routine work.

DISCUSSION

Assay of reductase kinase activity, which involves measuring a decrease in reductase activity, is conducted in two stages. In Stage 1, reductase, reductase kinase,

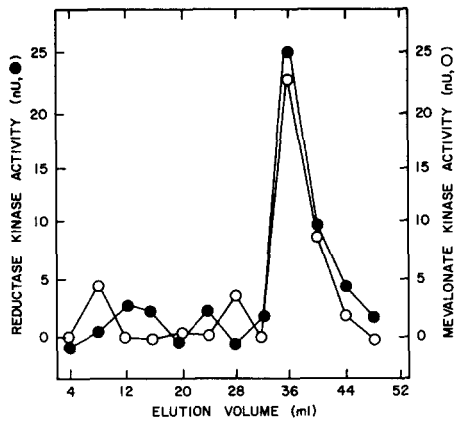

Fig. 5. Coelution of mevalonate kinase and reductase kinase from Blue Sepharose. The sample and chromatographic conditions are identical to that of Fig. 2, right. Fractions were assayed for reductase kinase (●) and for mevalonate kinase activity (○).

and Mg-nucleotides (ATP + ADP) are combined and reductase is partially inactivated. In Stage 2, reductase substrates are added and the reductase activity remaining after Stage 1 is measured.

Factors present in cytosol that may interfere with measurement of reductase kinase activity include phosphoprotein phosphatases (reactivate reductase and inactivate reductase kinase), mevalonate kinase (metabolizes mevalonate), Mg-nucleotide-independent inhibitors of reductase activity, and reductase kinase kinase (activates reductase kinase). These all may be removed or inhibited by techniques that are both rapid and reliable.

Phosphoprotein phosphatase activity is inhibited by 50 mM NaF (16). Mevalonate kinase activity is best

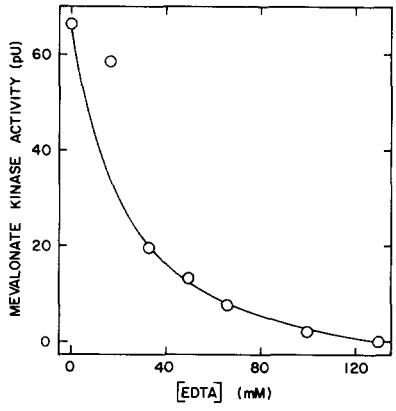

Fig. 6. EDTA specifically inhibits mevalonate kinase activity. A 50-μl portion of the A.S. fraction (see Fig. 4) was assayed in a mevalonate kinase assay which contained 10 μl of reductase kinase Stage 1 nucleotide solution rather than 560 nmol ATP and 375 nmol $MgCl_2$ and had included increasing amounts of EDTA from 0–133 mM. After 30 min at 37°C, the reaction was quenched by immersion in a boiling water bath for 5 min and the amount of phosphomevalonate produced was determined. Similar concentrations of EDTA, added during Stage 2, had no effects on the amount of reductase kinase activity measured.

TABLE 3. The concentration of Mg-nucleotides present in Stage 2 is unfavorable for expression of mevalonate kinase activity[a]

Additions				Mevalonate Kinase Activity	
$MgCl_2$	ATP	ADP	EDTA		
mM				*pU*	%
5.0	7.5	0	0	1019	(100)
8.0	2.0	2.0	0	67	6.6
8.0	2.0	2.0	78	4.0	0.4

[a] A.S. Fraction (see Fig. 4), 50 μl, was assayed for mevalonate kinase activity under the following conditions: 1) 7.5 mM ATP, 5.0 mM $MgCl_2$; 2) 8.0 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM ADP (added as 13 μl of Stage 1 nucleotide solution); 3) 8.0 mM $MgCl_2$, 2.0 mM ATP, 2.0 mM ADP, 78 mM EDTA. Control incubations lacked only nucleotides. Data are corrected for ^3H chromatographing in the region 0–0.5 present in control incubations (about 200 cpm, due to trailing of mevalonate).

blocked by inclusion during Stage 2 of 78 mM EDTA. Somewhat lower EDTA concentrations (ca. 30 mM) presently are used by most investigators (2, 4, 6, 10, 12). Substitution of MgADP for Mg-nucleotides during Stage 1 or inclusion of a 10 mM mevalonate trapping pool (with or without inclusion of 78 mM EDTA) during Stage 2 offer no advantages over the sole use of 78 mM EDTA. Reductase kinase activity measured using MgADP is only 50% of that measured using Mg-Nucleotides, and high concentrations of mevalonate both inhibit reductase activity and cause band spreading of radiolabeled mevalonate on silica gel chromatographs.

TABLE 4. Recovery of [^3H]mevalonate is essentially quantitative, even in the absence of added EDTA, when MgADP is substituted for Mg-nucleotides[a]

Additions to		Recovery of [^3H]Mevalonate	
Stage 1	Stage 2		
		cpm	%
None	EDTA	2348	98.6
None	None	2415	101.4
ATP + ADP	EDTA	2532	106.3
ATP + ADP	None	1728	72.6
ATP	EDTA	2338	98.2
ATP	None	1727	72.5
ADP	EDTA	2428	102.6
ADP	None	2349	98.6

[a] Blue Sepharose fraction, 80 μl (40 μg), was mixed (in a final volume of 140 μl) with 450 pU of heated microsomal reductase and 20 μl of Stage 1 control solution plus the indicated nucleotide additions and incubated at 30°C for 30 min. Where present, the total nucleotide concentration was 4.0 mM. Where both ATP and ADP were added, both were present at equal (2.0 mM) concentrations. Each incubation mixture was then divided in two equal portions. One portion received 13 μl of Stage 2 solution which lacked EDTA. The other received 13 μl of Stage 2 solution that contained 481 mM EDTA (final concentration 71 mM). Both were incubated at 37°C for 30 min. Loss of [^3H]mevalonate in the absence of EDTA during Stage 2 is attributed to mevalonate kinase.

Assaying small quantities of cytosol reduces, but does not eliminate, the effects of Mg-nucleotide-independent inhibitors of reductase activity, and significantly reduces the dynamic range of the assay. Separation of reductase kinase from these inhibitors and from mevalonate kinase is best achieved by chromatography of cytosol on DEAE-Sephacel with elution by 1.0 M KCl. The eluate contains 112 ± 2% of the reductase kinase activity measurable in diluted cytosol. While chromatography of cytosol on Blue Sepharose also separates these inhibitors from reductase kinase, it does not resolve reductase kinase from mevalonate kinase. Providing that 78 mM EDTA is present during Stage 2, chromatography on Blue Sepharose nevertheless provides an acceptable alternative to chromatography on DEAE-Sephacel.

Finally, we asked whether reductase kinase, itself an interconvertible enzyme (3, 8, 9, 10, 12), undergoes changes in its activity during analysis. Although activation of reductase kinase by reductase kinase kinase in the presence of the MgATP present throughout Stage 1 might be anticipated, we find no evidence that this in fact occurs. Reductase kinase kinase activity thus appears not to be expressed under the conditions of our assay. The data therefore represent solely the preexisting catalytically active form of reductase kinase.

The ability of reductase kinase (14, 16) and the inability of mevalonate kinase to utilize MgADP (22, 33, 34) has been previously noted. Reductase kinase activity expressed in the presence of MgADP may result from formation of traces of MgATP sufficient to catalyze reductase inactivation but insufficient to catalyze mevalonate phosphorylation. For example, while incorporation of 1 pmol of phosphate from MgATP into reductase results in a decrease of 1,000 pmol of mevalonate produced per min, the same amount of MgATP will phosphorylate only 1 pmol of mevalonate. Both ADP and ATP are required for inactivation of reductase (11, 14, 16). This is difficult to reconcile with the idea that reductase kinase consists of a single protein that catalyzes transfer of the γ-phosphate of ATP to a seryl residue of reductase. Incorporation of the γ-phosphate of ATP into reductase protein has, however, been amply documented (2–6, 35) and incorporation is indeed into a seryl residue (35). In addition, Beg, Stonik, and Brewer (3) have purified reductase kinase to apparent homogeneity, which argues against the presence of a second protein of as yet unknown function. The absolute requirement for ADP and its ability to replace ATP for inactivation of reductase will require detailed mechanistic investigation before a convincing explanation may be advanced.

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