Partial purification from rat liver microsomes of three native protein phosphatases with activity towards HMG-CoA reductase

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Abstract A procedure for the isolation and partial purification of three hydroxymethylglutaryl coenzyme A reductase phosphatases in their native high molecular weight form from rat liver microsomes is described for the first time. Reductase phosphatase Ex (M_r 90,000), I_M (M_r 75,000), and II_M (M_r 180,000) were purified 132-, 55-, and 98-fold, respectively. Treatment with 80% ethanol irreversibly inactivated the three enzymes contrary to what is found for cytosolic reductase phosphatases. The three microsomal reductase phosphatases differ among themselves and with respect to the cytosolic reductase phosphatases in molecular weight, response to inhibitors, thermal stability, and optimum pH. Indirect evidence that these three proteins are phosphatases includes their inhibition by inhibitors of phosphatase activity, such as KF, Pi, and PPi. Direct evidence includes their ability to release ^{32}P from highly radioactive homogeneous ^{32}P-labeled HMG-CoA reductase, this dephosphorylation being concomitant with activation of HMG-CoA reductase. The three phosphatases dephosphorylate ^{32}P-labeled phosphorylase a, but only reductase phosphatase II_M shows glycogen synthase phosphatase activity.—**Sitges, M., G. Gil, and F. G. Hegardt.** Partial purification from rat liver microsomes of three native protein phosphatases with activity towards HMG-CoA reductase. *J. Lipid Res.* **25:** 497–506.

Supplementary key words cholesterol biosynthesis • HMG-CoA reductase phosphatases • dephosphorylation

The rate limiting step in cholesterol biosynthesis in the liver of a number of vertebrates (1, 2) is catalyzed by HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase (NADP) EC 1.1.1.34). Recent studies support the notion that the activity of reductase may be regulated by covalent modification. Microsomal (3–6) and proteolytically solubilized reductase are inactivated in vitro in the presence of ATP-Mg^{2+} and microsomal reductase kinase[2] with the covalent binding of phosphate in two sites to the reductase molecule (7). Microsomal reductase from rat liver (8) or several cell cultures (9) is activated when homogenized tissues are incubated at 37°C. Moreover, highly radioactive homogeneous ^{32}P-labeled HMG-CoA reductase (subunit 52,000 daltons) is dephosphorylated with loss of ^{32}P by cytosolic phosphatases with concomitant increase of activity (10).

The purification of two cytosolic reductase phosphatases has been previously reported by us (11). Chromatography on DEAE cellulose separated two distinct peaks with reductase phosphatase activity that were further purified and designated reductase phosphatases Ia and IIa. These data have been recently confirmed by Brown and Rodwell (12). When both phosphatases of high molecular weight were independently treated with ethanol, they each produced a species with a lower molecular weight. All four forms removed ^{32}P from homogeneous ^{32}P-labeled reductase, producing an increase in HMG-CoA reductase activity.

The occurrence of a microsomal reductase phosphatase activity has been recently reported by Feingold et al. (13). This protein phosphatase activates HMG-CoA reductase very likely by a mechanism similar to that demonstrated in other phosphatases, i.e., by removing phosphates from HMG-CoA reductase. The activating effect of microsomal reductase phosphatase was halted by fluoride and phosphate. Previous to this paper, only one report had been published describing the existence of a microsomal phosphatase capable of activating HMG-CoA reductase provided that sodium sulfite, magnesium chloride, and EDTA were added to the incubation mixture (14).

We now report the development of a procedure for the isolation and partial purification of different microsomal protein phosphatases that activate the HMG-CoA reductase molecule. We show evidence that there are three high molecular weight forms of microsomal protein phosphatase that dephosphorylate homogeneous ^{32}P-labeled HMG-CoA reductase. When these three forms are independently treated with ethanol, all three lose their activity and no low molecular weight phosphatases are

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; reductase, HMG-CoA reductase; PMSF, phenyl methyl sulfonyl fluoride.

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obtained. In addition, some molecular properties of the microsomal reductase phosphatases are presented.

MATERIALS AND METHODS

Chemicals

Most of the reagents used in this study have been reported previously (11). Aminohexyl-Sepharose 4B was a product of PL Biochemicals. Phosphocellulose P-11 was from Whatman. Triton X-100 was from Sigma. [γ-^{32}P]ATP was prepared from ADP (Sigma) and [^{32}P]phosphate (Amersham) according to the method of Walseth and Johnson (15), modified by Palmer and Avruch (16).

Buffered solutions

Buffer I contained 500 mM sucrose, 2 mM EDTA, 2 mM EGTA, 0.5 mM PMSF, 40 mM mercaptoethanol, 40 mM Tris-HCl, pH 7.2. Buffer II was buffer I but with 300 mM sucrose instead of 500 mM sucrose. Buffer II-T was buffer II plus 0.1% (v/v) Triton X-100. Buffer III contained 1 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, 40 mM Bis-Tris, pH 6.0.

Assay of HMG-CoA reductase

HMG-CoA reductase activity in pure preparations was determined by the method of Philipp and Shapiro (17). In those cases where the enzyme preparations contained other enzymes that compete with the HMG-CoA reductase for HMG-CoA (18) and in those experiments using ^{32}P-labeled HMG-CoA reductase, [^{14}C]mevalonate was determined by the method of Bové and Hegardt (3). The determination of ^{32}P bound to protein was done following the method of Huang and Robinson (19).

Assay of HMG-CoA reductase phosphatase

HMG-CoA reductase phosphatase activity was determined by measuring the increase in activity (reactivation) of a homogeneous HMG-CoA reductase which had been inactivated by phosphorylation. This was compared to a control without phosphatase. This indirect assay for the activity of reductase phosphatase was preferred to the more direct assay of determining the ^{32}P removal from the ^{32}P-labeled HMG-CoA reductase because it easily allowed for the performance of a large number of experiments. We had previously shown (4, 11) that there was a close relationship between activation of HMG-CoA reductase caused by HMG-CoA reductase phosphatase and ^{32}P removal from the ^{32}P-labeled enzyme. The assay was a modification of that reported previously (20) and was conducted as follows. Samples to be assayed (30 μl) were mixed with homogeneous HMG-CoA reductase (20 μl, 0.15 μg) and incubated at 37°C for 30 min. After that, 60 μl of the substrate mixture of the HMG-CoA reductase assay was added and incubated for 30 min more. The reductase assay was conducted as described above. The amounts of reductase phosphatase assayed were such that less than 15% of the substrate was activated in a 30-min incubation period. Reaction rates were linear with time and were proportional to the amount of enzyme under the above conditions. A control without phosphatase was tested in every series of experiments in which an effector was introduced in the assay. It was previously shown that when an effector does not modify the activity of ^{32}P-labeled HMG-CoA reductase, its content in ^{32}P is equally unmodified (10).

Other enzymatic assays

Glucose-6-phosphate dehydrogenase was determined according to the method of Rudack, Chisholm, and Holten (21). Microsomal protein (0.5 mg) (100 μl) was added to a cuvette containing a mixture of 120 mM Tris-HCl, pH 8.2, 2 mM glucose-6-phosphate, 0.9 mM NADP, and 10.4 mM $MgCl_2$. The formation of NADPH at 25°C was monitored at 340 nm in a Beckman Spectrophotometer.

5′ Nucleotidase activity was measured following the Heppel and Hilmoe method (22). Two hundred μg of microsomal protein (50 μl) was added to a tube containing 240 μl of a 415 mM glycine-NaOH buffer, pH 8.5, 42 mM $MgCl_2$, and 12.5 mM 5′-AMP. The mixture was incubated at 37°C for 15 min and the reaction was arrested by the addition of trichloroacetic acid at a final concentration of 5%. The mixture was then centrifuged and an aliquot (265 μl) of the supernatant was analyzed for inorganic phosphate (23) by incubation for 1 hr at 37°C with a mixture of 100 μl of 10% ascorbic acid and 600 μl of 0.24% ammonium molybdate in 1 N H_2SO_4. The absorbance at 820 nm was determined with a Beckman Spectrophotometer.

Phosphorylase phosphatase activity was determined by the release of ^{32}P from ^{32}phosphorylase a (0.5 mg/ml) at 30°C as described by Khandelwal, Vandenheede, and Krebs (24).

Glycogen synthase phosphatase activity was determined as described by Hiraga et al. (25).

Glycogen synthase activity was determined by the incorporation of ^{14}C glucose from UDP ^{14}C glucose into glycogen according to the method of Thomas, Schlender, and Larner (26).

Purification of HMG-CoA reductase

HMG-CoA reductase was solubilized from the 100,000 *g* pellet of livers from rats fed 5% cholestyramine for 5 days. After solubilization by freeze-thawing and glycerol extraction (27), the enzyme was inactivated with 4 mM ATP and purified to homogeneity by affinity chroma-

tography on Affi-Gel Blue and HMG-CoA agarose (10). Under these conditions, the fraction of HMG-CoA reductase that remained in the active form was 8% (305 U/mg protein) and therefore was a good substrate for the action of phosphatases.

Purification of ^{32}P-labeled HMG-CoA reductase

Radiopure ^{32}P-labeled HMG-CoA reductase (subunit M_r 52,000) was purified to homogeneity according to the method of Gil, Sitges, and Hegardt (10).

Molecular weight determinations

Molecular weights of the microsomal reductase phosphatases were calculated by gel filtration, by means of Bio-Gel A-0.5 m. A sample of each of the three reductase phosphatases together with 3 mg of each marker (catalase 240,000, lactate dehydrogenase 136,000, bovine serum albumin 66,000, and myoglobin 17,500) were applied to the column of Bio-Gel in a total volume of 2 ml. Albumin, lactate dehydrogenase, and catalase were identified at 208 nm. Myoglobin was identified at 407 nm.

Protein determination

Protein was determined by the method of Lowry et al. (28) following precipitation with trichloroacetic acid. Bovine serum albumin was used as standard.

Units

One unit of HMG-CoA reductase was defined as the amount of enzyme catalyzing the conversion of 1 nmol of HMG-CoA into mevalonate per min at 37°C. One unit of HMG-CoA reductase phosphatase activity was taken as the amount of enzyme that gives rise to an increase of 1 milliunit of HMG-CoA reductase per min at 37°C under the standard assay conditions. One unit of glycogen synthase was the amount of enzyme that catalyzes the incorporation of 1 μmol of glucose into glycogen in 1 min under the assay conditions. One unit of glycogen synthase phosphatase is the amount of enzyme that increases by 1 milliunit the glycogen synthase activity per min. One unit of phosphorylase phosphatase is the amount of enzyme that catalyzes the release of 1 nmol of phosphate in 1 min. One unit of 5′ nucleotidase is the amount of enzyme that catalyzes the release of 1 μmol of phosphate in 1 min.

Purification of HMG-CoA reductase phosphatases from rat liver microsomes

Step 1: Preparation of microsomes. Female Sprague-Dawley rats (200–250 g) fed ad libitum with laboratory chow (Panlab S.A.) were killed by decapitation and the livers were excised and homogenized 1:3 (w/v) in buffer I in a Potter-Elvejhem homogenizer with a tight-fitting Teflon pestle. The homogenate was centrifuged at 30,000 *g* for 30 min and the supernatant was immediately recentrifuged at 100,000 *g* for 90 min at 4°C; the supernatant was then discarded. The microsomal fraction was separated from the glycogen pellet by vortexing the tubes gently, and then allowing the viscous wet fraction to flow smoothly down from the tubes by gravity. This microsomal fraction was resuspended in buffer II and the suspension was recentrifuged at 100,000 *g* for 90 min at 4°C; the supernatant was discarded. The microsomes were separated from the small glycogen pellet by the procedure described above.

Step 2: Solubilization of microsomes. The microsomal suspension was homogenized at 4°C in buffer II-T in a hand glass homogenizer in the proportion 1:1 (v/w) with respect to the weight of original liver, and was incubated in an ice bath for 1 hr. Thereafter, the suspension was centrifuged at 100,000 *g* for 90 min at 4°C. The resulting supernatant solution was carefully removed with a Pasteur pipet, and the pellet was extracted two more times. The three extracts were combined.

Step 3: DEAE-cellulose chromatography. The solubilized solution was loaded on to a DEAE-cellulose column (4.5 × 6 cm) which had been equilibrated previously with buffer II; then the column was washed with buffer II until the absorbance of the fractions at 280 nm returned to the base-line (approx. 150 ml). The column was then eluted with 200 ml of a linear gradient 0–600 mM KCl in buffer II. Five-ml fractions were collected at a flow rate of 60 ml/hr. Two different peaks of protein phosphatase which activate homogeneous HMG-CoA reductase, ATP-inactivated, were obtained and designated fraction Ex (excluded) and Rt (retained), respectively. Active fractions of protein phosphatase Rt were concentrated by precipitation with ammonium sulfate at 70% saturation. The precipitate obtained after centrifugation at 30,000 *g* for 15 min was dissolved in a minimum volume of buffer II, and dialyzed overnight against the same buffer.

Step 4A: Chromatography on phosphocellulose on phosphatase Ex. The preparation of reductase phosphatase Ex (all pooled active fractions of the DEAE-cellulose chromatography previous to the application of the gradient) was loaded on to a phosphocellulose column (4.5 × 6 cm) previously equilibrated with buffer II. The column was washed with the same buffer until the absorbance at 280 nm returned to the base-line, and thereafter a linear gradient of 200 ml of 0–800 mM KCl in buffer II was applied. Five-ml fractions were collected at a flow rate of 60 ml/hr. Fractions with reductase phosphatase activity were concentrated by precipitation with ammonium sulfate at 70%. The precipitate that was collected by centrifugation at 30,000 *g* for 15 min was resuspended in buffer II and dialyzed extensively against the same buffer.

Step 5A: Aminohexyl-Sepharose 4B chromatography. The concentrated enzyme from step 4A was applied to an aminohexyl-Sepharose chromatography column (1.9 × 3.4 cm) equilibrated with buffer II. The column was washed until the A_{280} returned to the base-line (approx. 100 ml) and the reductase phosphatase activity was eluted with a linear gradient of 300 ml of 0–500 mM KCl. Fractions with reductase phosphatase activity were concentrated by precipitation with ammonium sulfate at 70% saturation. The precipitate was collected by centrifugation at 30,000 *g* for 15 min, resuspended in a minimum volume of buffer II, and dialyzed repeatedly against the same buffer.

Step 6A: Bio-Gel filtration. The concentrated solution of step 5A was introduced in a Bio-Gel A 0.5 m column (1.7 × 90 cm) previously equilibrated with buffer II plus 100 mM KCl. Fractions with activity were pooled and concentrated with an Amicon ultrafiltration cell equipped with a PM-10 filter. The KCl was removed by repeated ultrafiltration and addition of buffer II. The resulting preparation was designated reductase phosphatase Ex.

Step 4B: Aminohexyl-Sepharose 4B chromatography of fraction Rt. The concentrated dialyzed fractions of step 3 corresponding to the reductase phosphatase activity retained by the DEAE-cellulose chromatography were applied to an aminohexyl-Sepharose 4B column (1.9 × 3.5 cm) equilibrated with buffer II. The column was washed with buffer II until the A_{280} disappeared and thereafter a linear gradient of 500 ml of 0–500 mM KCl was applied. Five-ml fractions at a rate of 60 ml/hr were collected. Two different peaks of protein phosphatase that activate homogeneous reductase which was in the inactive form, were obtained and designated, respectively, fractions I_M and II_M (M from microsomes). Each of the two pooled fractions was brought to 70% saturation with ammonium sulfate. After 30 min the precipitates were collected by centrifugation (30,000 *g*, 15 min) and the supernatants were discarded. The pellets were dissolved in a minimum volume of buffer II and dialyzed overnight with at least two changes of buffer.

Step 5B: Bio-Gel filtration. The dialyzed preparations I_M and II_M were independently applied to Bio-Gel A 0.5 m columns (1.7 × 90 cm) previously equilibrated with buffer II plus 100 mM KCl. Fractions with activity were independently concentrated by ultrafiltration using an Amicon cell equipped with a PM-10 filter. The resulting preparations were designated reductase phosphatases I_M and II_M.

RESULTS

Purification of microsomal reductase phosphatases

When the Triton X-100-solubilized fractions of rat liver microsomes were chromatographed on a DEAE-cellulose column, the HMG-CoA reductase phosphatase activity was resolved in two peaks (**Fig. 1**). These were probably not preparative artifacts since the same two fractions of reductase phosphatase activity were consistently observed when reasonably mild conditions of liver homogenization were used. Fraction Ex was not retained by the column and was excluded in the first fractions. Other reductase phosphatase activity was retained in the column and eluted by applying the linear gradient at approximately 230 mM KCl. Fraction Ex is clearly different from the cytosolic reductase phosphatases previously reported by us (11) since all of these phosphatases were retained by the DEAE-cellulose column.

Reductase phosphatase Ex shows chromatographic properties clearly distinct from those of any other protein phosphatase thus far published as it is excluded from many columns; we have tested this property in carboxymethylcellulose, Affi Gel Blue, Histone Sepharose, and polylysine Sepharose. As a result, the purification process was done in phosphocellulose in which reductase phosphatase Ex was retained and, after the application of a linear gradient, was eluted in a single peak at approximately 370 mM KCl (**Fig. 2**). This activity was further chromatographed in aminohexyl-Sepharose 4B, eluting at 180 mM concentration of KCl in buffer II, when the gradient was applied (**Fig. 3**). The chromatography on Bio-Gel A-0.5 m of active fractions of the Sepharose column produced a single peak of reductase phosphatase activity (**Fig. 4**).

Chromatography on aminohexyl-Sepharose of the reductase activity Rt eluted from the DEAE-cellulose column after application of the gradient separates two reductase phosphatases, I_M and II_M, of different molecular weight (**Fig. 5**). The first eluted at 240 mM KCl and the

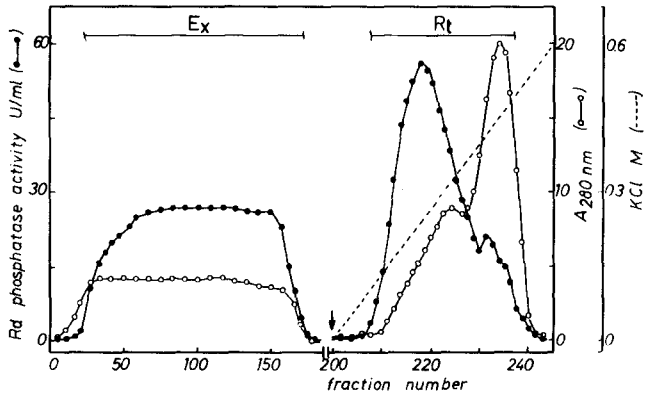

Fig. 1. Chromatography of the Triton X-100-solubilized fractions of rat liver microsomes on DEAE-cellulose. Conditions are described in the text. The point at which the linear gradient 0–600 mM KCl in buffer II was applied is indicated by the arrow. Absorbance (O) and HMG-CoA reductase phosphatase activity (●) values are represented on the right and left ordinate respectively. Concentrations of KCl are represented to the right.

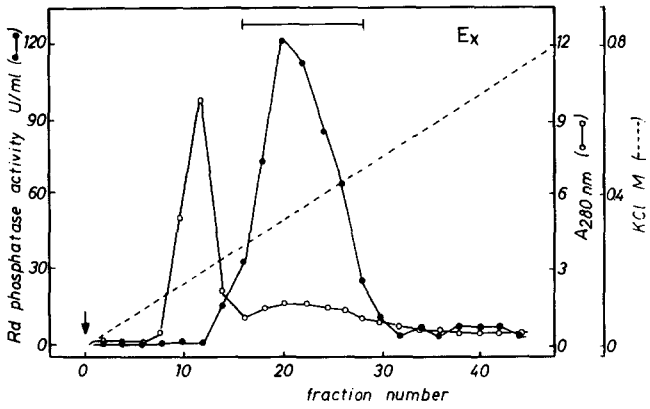

Fig. 2. Chromatography on phosphocellulose of reductase phosphatase Ex. The pooled fractions of peak Ex from DEAE-cellulose chromatography were chromatographed on a phosphocellulose column. Conditions are described in Methods. The figure represents the profile obtained when the linear gradient 0–800 mM KCl in buffer II was applied. Symbols as in Fig. 1.

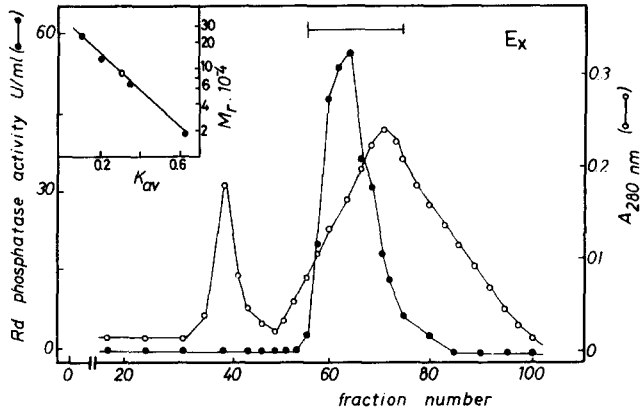

Fig. 4. Gel filtration on Bio-Gel A-0.5 m of reductase phosphatase Ex. Active fractions from chromatography in aminohexyl-Sepharose 4B, pooled and concentrated, were applied to a Bio-Gel A 0.5 m column (1.7 × 90 cm) equilibrated with buffer II plus 100 mM KCl. Fractions (1.8 ml) were obtained at a flow rate of 18 ml/h. The molecular weight of phosphatase Ex was calculated by its elution volume (open symbol) as compared with those of marker proteins catalase, lactate dehydrogenase, bovine serum albumin, and myoglobin (closed symbols) as shown in the insert.

second at 380 mM KCl. The enzyme preparations I_M and II_M were further purified by chromatography on Bio-Gel A-0.5 m. As shown in **Fig. 6,** both reductase phosphatases I_M and II_M exhibit only one molecular form.

The overall purification of microsomal reductase phosphatase is presented in **Table 1.** Microsomal HMG-CoA reductase phosphatases are very unstable when they are not frozen at −40°C. Consequently throughout the purification process there are great decreases in activity especially in the DEAE-cellulose and phosphocellulose chromatographies. However, the three enzymes maintained their level of activity for several months frozen at −40°C. The high percentage of activity solubilized from microsomes can probably be explained because the enzymes do not fully express their activity in the membranes. Specific activities of 1375, 575, and 1030 units/mg protein, respectively, were attained corresponding to 130-, 55-, and 98-fold purification for reductase phosphatases Ex, I_M, and II_M. The reproducibility of the procedure may be assessed by the following data collected over the course of a series of experiments. Specific activities at the end of the purification process during ten purifications averaged 1310 ± 93, 523 ± 72, and 980 ± 110 units/mg protein (mean ± SD). None of the preparations was homogeneous.

The reductase phosphatases obtained following this procedure were microsomal in origin since the microsomal

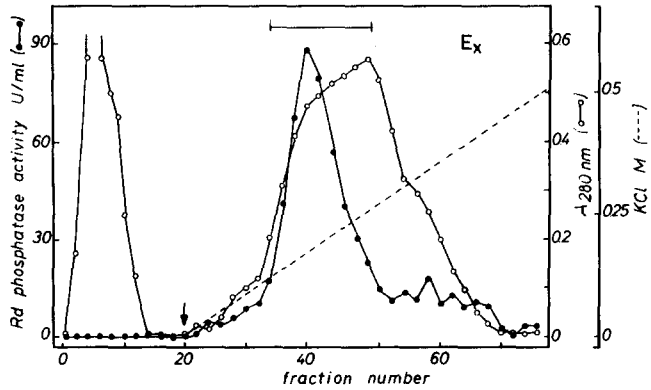

Fig. 3. Chromatography on aminohexyl-Sepharose 4B of reductase phosphatase Ex. The active fractions from chromatography in phosphocellulose, pooled and concentrated as described in Methods, were applied to an aminohexyl-Sepharose 4B column. The point at which the linear gradient 0–500 mM KCl in buffer II was applied is indicated by the arrow. Symbols as in Fig. 1.

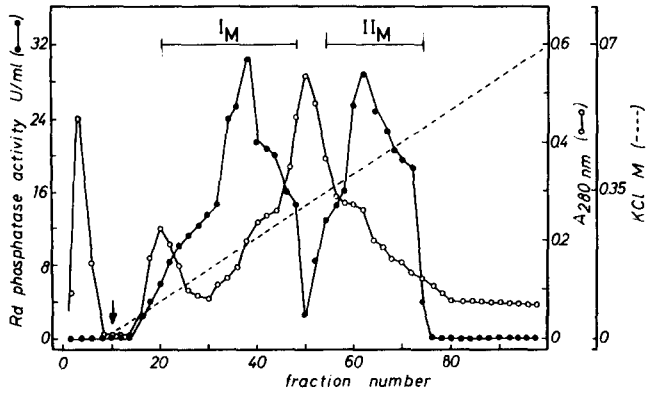

Fig. 5. Chromatography on aminohexyl-Sepharose 4B of the peak Rt of the DEAE cellulose chromatography (Fig. 1). The pooled concentrated fractions of peak Rt of DEAE-cellulose chromatography were loaded onto an aminohexyl-Sepharose 4B column. Conditions are given in the text. The point at which the linear gradient 0–600 mM KCl in buffer II was applied is indicated by the arrow. Absorbance (O) and concentrations of KCl (——) are represented on the right. HMG-CoA reductase phosphatase activity is represented on the left. Peaks with activity I_M and II_M were independently pooled and processed as described in the text.

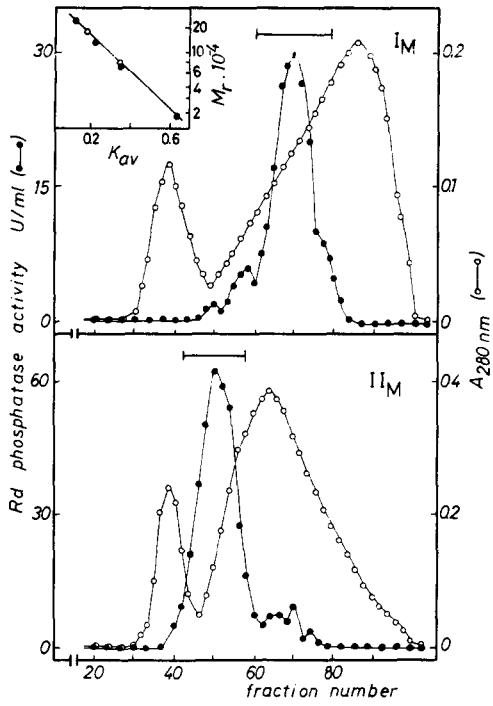

Fig. 6. Gel filtration on Bio-Gel A-0.5 m of microsomal reductase phosphatases I_M and II_M. Aliquots of 2 ml of preparations I_M and II_M from the aminohexyl-Sepharose 4B chromatography were applied to a Bio-Gel A-0.5 m column (1.7 × 90 cm) equilibrated with buffer II plus 100 mM KCl. Two-ml fractions were collected at a flow rate of 18 ml/hr. The molecular weights of phosphatases I_M and II_M were calculated by their elution volume (open symbols) as compared with those of marker proteins, catalase, lactate dehydrogenase, bovine serum albumin, and myoglobin (closed symbols) as shown in the insert.

preparation prior to solubilization was devoid of contamination by lysosomes, cytosol, and plasma membranes. Glucose-6-phosphate dehydrogenase, a typical cytosolic enzyme, was absent in washed microsomes before and after solubilization. Since 5'-nucleotidase activity in the purified microsomes was very low (0.00274 units/mg protein · min), we can conclude that plasma membranes were absent in microsomal preparation. Lysosomes were separated in the first centrifugation (30,000 *g*) of the rat liver homogenate.

Ethanol treatment

Many cytosolic preparations of protein phosphatases change their molecular weight under an unusual procedure involving 80% ethanol precipitation at room temperature (29). This procedure denatures many contaminating proteins and dissociates the catalytic subunit from other proteins of the holoenzyme. This procedure has been adopted by many authors to isolate protein phosphatases from different sources (30–32). In a previous study we applied the method to obtain the low molecular weight reductase phosphatases from rat liver cytosol (11). We tried to apply the method of the ethanol treatment to the microsomal reductase phosphatase preparations Ex, I_M, and II_M, but we failed in the isolation of active fractions. There was no reductase phosphatase activity in any of the three microsomal reductase phosphatase preparations Ex, I_M, and II_M after the ethanol treatment, from which it can be concluded that ethanol irreversibly inactivates the microsomal reductase phosphatases.

Optimum pH of phosphatases

The optimum pH when homogeneous HMG-CoA reductase is used as the substrate is similar for all three reductase phosphatases (**Fig. 7**). Preparations I_M and II_M exhibit an almost identical profile, with 6.0 being the

TABLE 1. Purification of HMG-CoA reductase phosphatases Ex, I_M, and II_M from rat liver[a]

		HMG-CoA Reductase Phosphatase	Protein	Specific Activity	Purification	Yield
		units	*mg*	*units/mg protein*	*-fold*	%
Microsomes		33501	3194	10.5	1	100
Solubilized fraction		187485	1290	145.3	13.8	559
DEAE-cellulose	Ex	144023	973	148	14.1	429
	Rt	34330	162	212	20.2	102
Phosphocellulose	Ex	39984	126.5	316	30.1	119
Aminohexyl-Sepharose 4B	Ex	26745	27.8	962	91.6	80
Bio-Gel A-0.5 m	Ex	19085	13.9	1373	130.7	57
Aminohexyl-Sepharose 4B	I_M	14685	27.5	534	50.8	44
	II_M	12458	33.2	375	35.7	37
Bio-Gel A-0.5 m	I_M	11155	19.4	575	55	33
	II_M	12053	11.7	1030	98	36

[a] The amount of liver was 190 g.

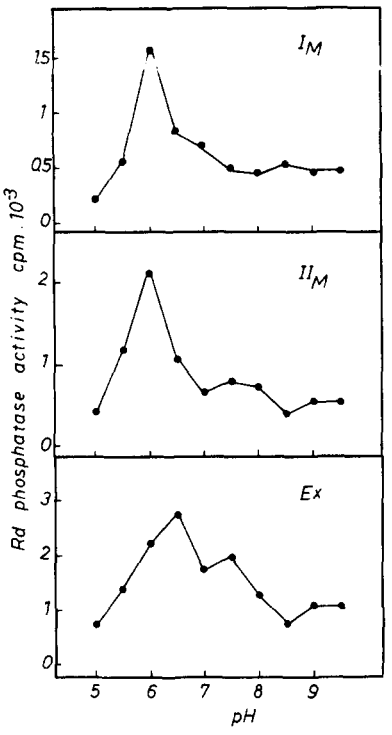

Fig. 7. The optimum pH of HMG-CoA reductase phosphatases Ex, I_M, and II_M. Activities were measured with homogeneous rat liver HMG-CoA reductase as substrate. The assay system (50 μl) contained 0.15 μg of reductase and values of reductase phosphatases ranging between 0.87 and 1.04 units depending on phosphatases, in buffer III. The reaction mixtures were incubated at 37°C for 20 min. At this time, the reductase assay was started by adding 60 μl of the substrate and the cofactors mixture and was further incubated for 30 min. Controls were performed without HMG-CoA reductase phosphatase. The differences between the cpm of mevalonate in assays and controls at every pH are represented on the ordinate.

optimum pH in buffer III. Preparation Ex exhibits a similar but not identical profile with the optimum value corresponding to pH 6.5.

Molecular weight

Microsomal reductase phosphatases Ex, I_M, and II_M had M_r values of 90,000, 75,000, and 180,000, respectively as judged by chromatography on Bio-Gel A-0.5 m (Figs. 4 and 6).

Thermal stability

Reductase phosphatases Ex, I_M, and II_M held in buffer III at the same concentration (1.26 mg/ml) were warmed at 50°C for different periods to test their respective thermal stability. Aliquot portions were withdrawn and tested for reductase phosphatase activity. The times at which each of the three reductase phosphatases diminished their initial activity to a level of 50% was 20, 5, and 60 min for phosphatases Ex, I_M, and II_M, respectively, indicating that phosphatase II_M was somewhat stable at this temperature with phosphatases Ex and I_M exhibiting decreasing stability.

Inhibition of HMG-CoA reductase phosphatases by fluoride, phosphate, and pyrophosphate

When homogeneous HMG-CoA reductase (0.04 μg) was incubated with 4.5 units of each of the three purified microsomal reductase phosphatases in the presence of different concentrations of KF, a decrease in reductase phosphatase activity was observed, starting at 1 mM KF (**Fig. 8**). The behavior of the three reductase phosphatases was somewhat similar. Phosphatases Ex, I_M, and II_M were inhibited by 50% by 2 mM KF. Complete inhibition of reductase phosphatases occurred at concentrations of 10 and 20 mM KF for phosphatases I_M and II_M. Phosphatase Ex retained 20% activity even at 100 mM KF. These values are clearly different from those of cytosolic reductase phosphatases (33). Inorganic phosphate was a less potent inhibitor, as concentrations of 10 mM were necessary to inhibit reductase phosphatase I_M and Ex by 50%, and even higher concentrations were necessary with phosphatase II_M. Concentrations of 100 mM phosphate

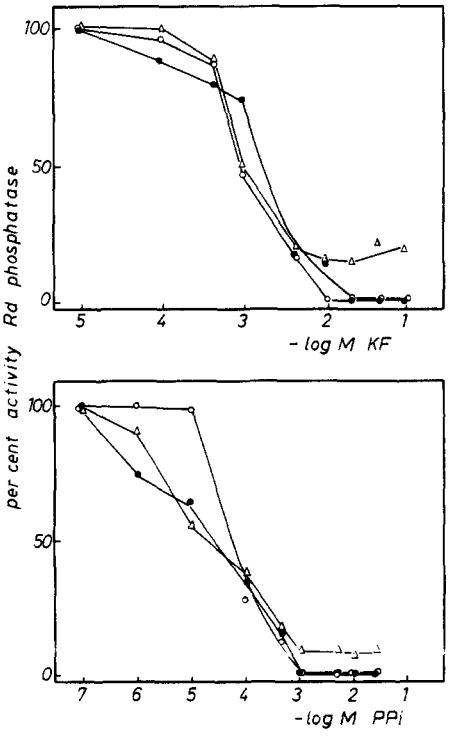

Fig. 8. Effect of varying concentrations of KF (upper panel) and pyrophosphate (lower panel) on activity of purified microsomal HMG-CoA reductase phosphatases. Reductase phosphatases Ex (Δ), I_M (○), and II_M (●) (4.5 units each, 10 μl) were incubated with homogeneous HMG-CoA reductase (0.04 μg, 30 μl) and variable concentrations of KF or PPi (10 μl) at 37°C for 30 min. At the end of this time, the mixtures were assayed for HMG-CoA reductase. Phosphatase activity in the absence of effectors was taken as 100%.

fully inhibited phosphatase II_M, but 20–30% activity was left in reductase phosphatase I_M and Ex.

Pyrophosphate appears to be a potent inhibitor of several proteins phosphatases in experiments in vitro. In fact, we have previously shown the inhibitory action of PPi on ^{32}P release catalyzed by cytosolic reductase phosphatases with ^{32}P-labeled HMG-CoA reductase as substrate (10, 34). The effect of various concentrations of inorganic pyrophosphate on inhibition of microsomal rat liver reductase phosphatases was tested. Reductase phosphatases Ex, I_M, and II_M were inhibited 50% by concentrations between 0.02 and 0.05 mM. Full inhibitions for phosphatases I_M and II_M were observed at 1 mM PPi, leaving 10% activity on phosphatase Ex even at concentrations of 20 mM PPi.

Substrate specificity of microsomal reductase phosphatases

The three microsomal reductase phosphatases were assayed for activity using two additional substrates, glycogen synthase and glycogen phosphorylase. Phosphorylase phosphatase activity was 162, 458, and 773 units/mg protein for phosphatases I_M, II_M, and Ex, respectively. When glycogen synthase D was used as the substrate, only preparation II_M showed measurable phosphatase activity (0.0108 units/mg of protein). The other two showed activity values of about 1/20 of the activity of II_M and, as a consequence, should not be considered to contain glycogen synthase phosphatase activity.

Kinetics of ^{32}P release and activation of ^{32}P-labeled HMG-CoA reductase

It was of some interest to us to know whether our microsomal HMG-CoA reductase phosphatases were actually protein phosphatases with dephosphorylating action on HMG-CoA reductase. This precaution was necessary since it has not yet been established that activation of HMG-CoA reductase by microsomal reductase phosphatase was due to removal of phosphate from the molecule. To assess that our preparations were actually protein phosphatases, aliquots of the three preparations were incubated with highly radioactive ^{32}P-labeled homogeneous HMG-CoA reductase. Results are presented in **Fig. 9.** A release of ^{32}P from labeled HMG-CoA reductase concomitant with an increase in HMG-CoA reductase activity was observed. The release of ^{32}P, as well as the increase of HMG-CoA reductase activity, in the case of both phosphatases I_M and II_M, was halted when 100 mM KF was added to the incubation mixture (data not shown).

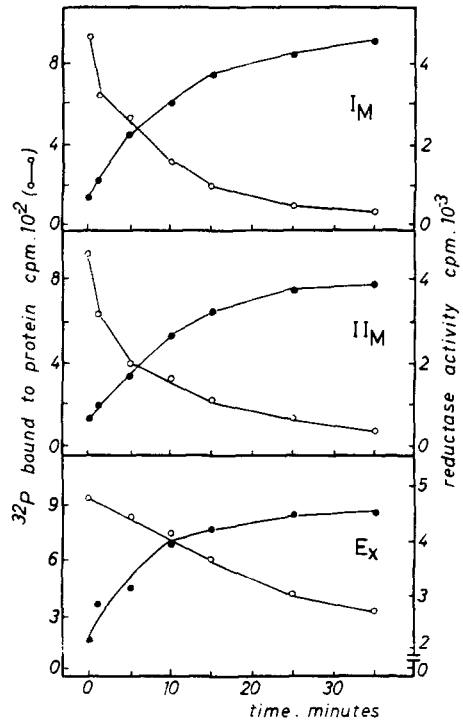

Fig. 9. Kinetics of dephosphorylation of microsomal phosphatases. ^{32}P-Labeled HMG-CoA reductase (40 μl, 3.4 μg, 9600 cpm) was incubated at 37°C in buffer III plus 5 mM $MnCl_2$ with 1270, 936, and 862 units of reductase phosphatases I_M, II_M, and Ex, respectively, in a total volume of 490 μl. At the indicated times, aliquots of 15 μl were assayed for HMG-CoA reductase activity (●) and aliquots of 50 μl were assayed for ^{32}P-bound to protein (○), and the values are represented on the right and left ordinate, respectively.

DISCUSSION

Present results indicate for the first time the existence of three forms of microsomal HMG-CoA reductase phosphatase with the ability to activate HMG-CoA reductase. While our own studies were in progress, Feingold et al. (13) reported the existence of a protein phosphatase activity in microsomes which produced the activation of microsomal HMG-CoA reductase. These authors demonstrated that incubation at 37°C for 40 min increased the reductase activity twofold and that this activation was blocked by KF and phosphate. We have isolated, characterized, and purified three reductase phosphatases from rat liver microsomes, different among themselves by criteria of different chromatographic behavior, different M_r, and somewhat different optimum pH and thermal stability at 50°C.

These phosphatases are actually of microsomal origin and not associated with the glycogen pellets, as the purification process is started from the microsomal portion separated from the glycogen pellet by a decantation procedure described in Methods. Moreover, the low 5′-nucleotidase activity of the washed microsomes ($^1/_{365}$ of the specific activity in plasma membranes (35)) and the absence of glucose-6-phosphate dehydrogenase show that neither plasma membranes nor cytosol are the source of this phosphatase activity.

Direct evidence supports the idea that the preparations used are protein phosphatases with dephosphorylating and activating action on HMG-CoA reductase. When highly radioactive homogeneous ^{32}P-labeled HMG-CoA reductase was incubated with each of the three reductase phosphatases, ^{32}P was released with a concomitant increase in HMG-CoA reductase activity. These data are interesting since the occurrence of reductase phosphatase in microsomal fractions, as is the case with HMG-CoA reductase, supports the notion that these protein phosphatases may be more involved than the cytosolic reductase phosphatases in the activation of the enzyme in vivo.

The comparison of the properties of these microsomal phosphatases with those of cytosolic origin, reported by Gil et al. (11), allows us to suggest that they are probably different enzymes. Reductase phosphatase Ex shows a clear distinct property as it is not retained in the DEAE-cellulose column. In addition, although they are retained in the DEAE-cellulose column, the behavior of reductase phosphatases I_M and II_M, in this column and in aminohexyl-Sepharose 4B, is very different from that described by Gil et al. (11). Moreover, there is no coincidence with any of the described molecular weights of the cytosolic reductase phosphatases and the results of the ethanol treatment provide an additional conclusive difference from the cytosolic phosphatases.

The three microsomal reductase phosphatases become inhibited by the common inhibitors of protein phosphatases, namely F^-, Pi, and PPi. However, some differences can be pointed out in the inhibitory process. Phosphatase Ex is not fully inhibited by any of the three inhibitors even at concentrations at which most reductase phosphatases are fully inhibited. On the other hand, the effect of KF on these microsomal phosphatases is clearly distinct from the cytosolic high molecular weight, as reductase phosphatases (33) are much more inactivated in our experiments. Pyrophosphate appears as the most potent inhibitor of the three, just as do other cytosolic phosphatases; these results reinforce the idea that these phosphatases are different from cytosol phosphatases.

All the reductase phosphatases I_M, II_M, and Ex show phosphorylase phosphatase activity, while only reductase phosphatase II_M shows glycogen synthase phosphatase activity. Thus, I_M, II_M, and Ex probably represent protein phosphatases with broader specificity than reductase phosphatase.

The comparison of our phosphatases with those purified by Ingebritsen, Stewart, and Cohen (36) is not an easy one. These authors report the occurrence of residual phosphatase activity (2, 5, 6, and 20%, respectively, for protein phosphatases 2C, 2B, 2A, and 1) in rabbit liver microsomes. We cannot compare our microsomal phosphatases with the most abundant phosphatase given by these authors (PrP-1, 20%) as the M_r of the holoenzymatic preparation is not given in the articles by Ingebritsen and co-workers (37–40).

On the other hand, these authors argue (38) that protein phosphatase 2C is mostly that which regulates HMG-CoA reductase as it is most abundant in the liver. The small amount of PrP-2C present in microsomes as shown by these authors (2%) minimizes its relevancy in HMG-CoA reductase dephosphorylation. More studies on microsomal protein phosphatases will help to clarify its relevance to dephosphorylation of reductase in vivo and also dephosphorylation of proteins other than HMG-CoA reductase.

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